

**Interactions of insecticides, entomopathogenic fungi, and earthworms as they relate  
to white grub IPM in turfgrass systems**

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# **Interactions of insecticides, entomopathogenic fungi, and earthworms as they relate to white grub IPM in turfgrass systems**

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## **Abstract**

White grubs (Coleoptera: Scarabaeidae) are important turfgrass pests in Virginia. Insecticides such as the neonicotinoid imidacloprid are commonly applied to turfgrass in order to control these pests. As an alternative to synthetic insecticides, entomopathogenic fungi (EPF), including *Metarhizium brunneum* (Petch) and *Beauveria bassiana* (Balsamo) Vuillemin may also be used for white grub control. The interaction of combining these two control tactics for white grubs in Virginia merits further investigation as does their effects on other soil organisms such as earthworms, which cohabitate with white grubs in turfgrass soil ecosystems. Herein, I investigate the following: 1) the efficacy of combined applications of the EPF, *M. brunneum* and *B. bassiana* with lower rates of imidacloprid or the diamide insecticide, chlorantraniliprole against white grubs; 2) interactions of earthworms with white grubs and EPF; and 3) the effect of white grub control products on earthworms. In the laboratory, a combined application of one half the recommended rate of chlorantraniliprole plus the full recommended rate of *B. bassiana* caused significantly higher mortality of third instar *Cyclocephala* spp. grubs than the untreated control. In the field, imidacloprid applied at lower rates as a single treatment or as part of a combined treatment with EPF resulted in significantly fewer grubs when applications were made in June. In the greenhouse, Japanese beetle, *Popillia japonica* Newman females laid a significantly reduced number of eggs in turf treated with lower rate of imidacloprid either applied as a single treatment or as part of a combined treatment compared with untreated control. In an earthworm-

white grub interaction study, the earthworms *Eisenia fetida* (Savigny) and *E. hortensis* (Michaelsen) were shown to transfer *B. bassiana* spores from fungus-infected soil to uninfected soil in the laboratory. However, the presence of earthworms in fungal infected soil did not enhance the mortality of *Cyclocephala* spp. grubs. In bioassays conducted in the laboratory, only two neonicotinoids, dinotefuran and imidacloprid, caused significantly higher mortality to adult *Lumbricus terrestris* L. earthworms than untreated control consistently. When applied as a drench to turfgrass in spring, summer, and fall, none of the insecticides significantly reduced the earthworm densities compared with a water control.

# **Interactions of insecticides, entomopathogenic fungi, and earthworms as they relate to white grub IPM in turfgrass systems**

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## **General Audience Abstract**

White grubs cause serious damage to turfgrass in Virginia and as a part of turf management programs, insecticides such as the neonicotinoid imidacloprid are routinely applied to eliminate these pests. However, there are environmental concerns over the excessive use of neonicotinoids. In addition, these insecticides are typically only effective when they are applied in the summer to target small grubs. Herein, I investigated if combining reduced rates of imidacloprid or another insecticide, chlorantraniliprole, with commercially-available insect-killing fungi *Beauveria bassiana* or *Metarhizium brunneum*, could enhance white grub control. Neither the insecticides alone, nor the aforementioned fungal biopesticides provided effective control of larger white grubs in lab bioassays and field experiments. Additional research was aimed at understanding the interactions of insecticides and fungi on both white grubs and earthworms, which cohabitate frequently with white grubs in turfgrass soil ecosystems. In a greenhouse experiment, Japanese beetle females laid fewer eggs in turf treated with imidacloprid, but that chlorantraniliprole or the insect disease causing fungi did not affect beetle egg laying. In another experiment, earthworms were shown to transfer insect disease causing fungi in the soil in the laboratory. However, the presence of earthworms did not increase white grub fungal infection in fungi-infested soil in the lab. Additional experiments showed that two neonicotinoids, dinotefuran and imidacloprid, killed more earthworms than other insecticides when applied to soil in the lab. However, when applied as a drench to turfgrass in spring, summer, or fall, neither of these insecticides nor several

others registered for use on turfgrass resulted in fewer earthworms compared with a water control. The complex interactions between turf-damaging white grubs, the insecticides used to control them, insect disease-causing fungi in the soil and non-target beneficial organisms such as earthworms warrant further investigation to help us move toward more sustainable pest management approaches in the future.

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## **Chapter 1**

### **Literature Review**

Some of the text in this chapter is published in:

Gyawaly, S., A. M. Koppenhöfer, S. Wu and T. P. Kuhar. 2016. Biology, ecology and management of masked chafer grubs in turfgrass. *Journal of Integrated Pest Management*.7:1–11.

#### **White grub biology and management**

White grubs are larvae of beetles in the family Scarabaeidae. Many species of white grubs feed on grass roots and damage cultivated turf grasses (Table 1.1). White grubs are the most widespread and most destructive group of insect pests of turfgrass in the northern two-thirds of the U.S., i.e., the cool-season and transition zones with respect to turfgrass adaptation zones (Brandenburg 2006). There are several important introduced white grub species in the U. S., the most common and widespread of which is the Japanese beetle, *Popillia japonica* Newman. However, among the important native scarab pests of turf grasses, masked chafers, *Cyclocephala* spp. are probably the most wide-spread (Vittum et al. 1999). Among these, the northern masked chafer, *Cyclocephala borealis* Arrow, and the southern masked chafer, *C. lurida* Bland, are the most important species. In Virginia, *P. japonica* and *Cyclocephala* spp. are the most common species of white grubs that occur in turfgrass (Dimock 2004).

#### **Biology and life cycle of annual white grubs**

The various species differ in size and color. The eggs vary in size. Freshly laid eggs are whitish, but eventually turn into a darker color. Larvae have C-shaped, white-colored bodies with sclerotized heads. The head color varies from yellowish to reddish brown. Larvae also have three pairs of well-developed thoracic legs.

Annual white grubs are holometabolous insects. Adults (beetles) are typically active in the summer months (Potter 1981). Most white grubs that infest turfgrass in the United States have either a one- or three-year life cycle (Potter 1998). Annual white grubs feed on cool season

turfgrass roots in late spring, then transform into adults, which lay eggs in early summer.

Females lay clutches of eggs in the soil or in the thatch layer of turf. Larvae progress through three instars feeding on grass roots primarily throughout the summer and fall. Larvae respond to the initiation of cold weather by burrowing deep into the soil to overwinter. In spring, third instars return to the thatch layer and resume feeding before they pupate, usually in late spring.

### **Effect of soil moisture and temperature on larval survival**

Soil moisture and temperature are very crucial for the development and survival of eggs. Female beetles prefer to lay eggs in moist, well-drained soil high in organic matter (Potter 1995). Potter (1983) reported that soil moisture levels below the wilting point cause desiccation leading to death of eggs. High soil temperature combined with low soil moisture negatively affects the survival of eggs. Potter and Gordon (1984) observed that no eggs survived a soil temperature of 40°C and < 8% soil moisture.

### **White grub injury in turfgrass**

White grubs are serious pests of cool-season turfgrass (Brandenburg 2006). The primary injury comes from larvae feeding on the roots (Tashiro 1987, Potter 1982); however, the grubs of *Cotinis nitida* L. also injure turf by horizontal tunneling. White grub root feeding results in the appearance of brown irregular patches in small areas, which increase as grubs develop and feed extensively (Potter 1982, Tashiro 1987, Potter and Braman 1991). Severely damaged turf can be pulled up easily. Damage is further aggravated by vertebrate predators that dig the turf in search of white grubs (Potter and Braman 1991). The damage caused by white grubs costs millions of dollars each year. It is estimated that *P. japonica* alone causes a loss of about \$234 million to turfgrass each year (Anonymous 2010).

### **White grub management in turfgrass**

Pest management practices are more or less similar for all species of white grubs (Potter and Potter 2013). However, species can differ in their life cycles and susceptibility to control methods, which highlights the importance of knowing which species of grubs occur in an area. In particular, biological control products such as bacteria, entomopathogenic fungi (EPF), and nematodes tend to be more species specific than synthetic insecticides. Thus, proper identification of the grub species in question becomes more important when using biological control products.

### **White grub sampling and thresholds**

White grubs are soil dwelling insects; thus their presence is often difficult to detect until damage occurs. Monitoring of white grubs can be done by taking sod/soil samples to a depth of 7.5–10 cm (3-4 in.) and examining the roots and soil for larvae (Potter 1998, Vittum et al. 1999). This can be done with a flat-blade spade by cutting three sides of a turf square and peeling back the flap. More efficiently, cores can be taken with a standard golf cup cutter (10.8 cm diam.) and examined on a tray. Due to the patchy distribution of white grubs, samples need to be taken in a grid pattern with samples taken every 2–6 m depending on the size of the sampled area; or on golf course fairway samples are taken 9–14 m apart in parallel lines or zig-zag patterns (Potter 1998, Niemczyk and Shetlar 2000). Experienced samplers can process about 20 cores per hour.

The action threshold for white grubs in turfgrass is determined by the number of grubs present per unit area (i.e., ft<sup>2</sup> or m<sup>2</sup>). Factors such as the species of grub, management, and condition of the turf affect the action threshold for grubs. For example, the action threshold of *P. japonica* is > 10 grubs/ 0.1 m<sup>2</sup>, but for *C. borealis* it is 6–8 grubs/ 0.1 m<sup>2</sup> (Tashiro 1987).

Although white grub sampling and action thresholds can be a cornerstone of turf IPM, these

techniques are often time consuming and generally not practiced (Potter 2005, Held and Potter 2012).

### **Control tactics**

**Cultural control.** Deep-rooted and vigorous turfgrass can tolerate higher grub densities and more feeding than weak and stressed plants. Cultural practices including irrigation, fertilization, mowing, and thatch management may be used to promote grass health and root recuperation from damage. For example, nitrogen fertilization of damaged areas in fall can help the grass recover from grub damage (Crutchfield et al. 1995). Appropriate irrigation in the late summer and fall, particularly when late instars feed voraciously, improves grass tolerance to feeding by alleviating root loss and promoting root regrowth (Potter 1982). However, irrigation should be minimized during the oviposition period to reduce attraction of egg-laying females and survival of eggs and young larvae (Potter et al. 1996). Also, mowing height is directly related to turf root depth and ability to regrow damaged roots (Christians 1998), and increasing cutting height may increase turf tolerance to grub feeding and reduce grub densities, possibly because taller grass harbors more natural enemies providing better grub suppression (Potter et al. 1996). Computer simulations suggested that turf aeration may kill as many as 40% of grubs with appropriate hole patterns (Blanco-Montero and Hernandez 1995). Cranshaw and Zimmerman (1989) reported 55% mortality of white grubs in turfgrass when about 2 holes/12 cm<sup>2</sup> were made by spiked sandals, which indicates that the use of such techniques has potential for white grub control in turf.

**Biological control.** A wide range of microbial agents and natural enemies infect or attack white grubs including *Cyclocephala* spp. Many of these natural enemies have been studied for

biological control of white grubs. These include entomopathogenic bacteria, fungi, and nematodes as well as insect predators and parasitoids.

***Entomopathogenic bacteria.*** *Paenibacillus popilliae* (Dutky) (formerly *Bacillus popilliae*) and *P. lentimorbus* (Dutky) are the causal agents of milky disease in many Scarabaeidae larvae in the U.S. (Klein 1992, Garczynski and Siegel 2007, Jurat-Fuentes and Jackson 2012). These bacterial species have many strains that are species-specific with little to no cross infectivity among species (White 1947, Harris 1959). Warren and Potter (1982) reported that the *Cyclocephala* strain of *P. popilliae* could be very virulent against *C. lurida*. Only the strain infecting *P. japonica* larvae has been commercialized. However, the efficacy of commercially available milky spore products for *P. japonica* grub is dubious (Redmond and Potter 1995). Factors such as quality of commercial milky spore product as well as unfavorable soil temperature for disease development in the field seem to affect efficacy of these products (Pfeifer 2012 and references therein).

Among many insecticidal *Bacillus thuringiensis* (Bt) strains, Bt subsp. *japonensis* var. *buibui* has been reported to have insecticidal properties against white grubs (Obha et al. 1992). Bixby et al. (2007) found that rates as low as 100 g of  $\delta$ -endotoxin/ha provided control of oriental beetle, *Anomala orientalis* Waterhouse, and Japanese beetle. However, *C. borealis* has been found to be less susceptible to this bacterial toxin (Mashtoly et al. 2009). In addition, this strain is not commercially available. *Bacillus thuringiensis* subsp. *galleriae* SDS-502 strain appears to be effective against *C. lurida* (Baxendale et al. 2005, Stamm et al. 2009). To be effective, both Bt strains have to be applied against early instars.

An insecticide based on the bacterium *Chromobacterium subtsugae* Martin et al., and its fermentation products has also been shown to be effective against *C. lurida* (Stamm et al. 2012,

2013). More research on the efficacy of this commercially available insecticide against white grubs is needed.

***Entomopathogenic nematodes.*** Members of the families Steinernematidae and Heterorhabditidae are the most widely studied groups of nematodes that infect white grubs (Grewal et al. 2005, Georgis et al. 2006). However, their efficacy differs among grub species (Grewal et al. 2002, Koppenhöfer et al. 2004). *Cyclocephala borealis*, *C. lurida*, and especially *C. pasadenae* are less susceptible to common entomopathogenic nematodes such as *Heterorhabditis bacteriophora* Poinar and *Steinernema glaseri* (Steiner) under laboratory and greenhouse conditions (Koppenhöfer et al. 2004, 2006). In field studies, good control of *C. borealis* has been observed with *H. zealandica* Poinar X1 strain (72–96%), *S. scarabaei* Stock and Koppenhöfer (84%), and *H. bacteriophora* GPS11 strain (47–83%) (Grewal et al. 2004, Koppenhöfer and Fuzy 2003), but not with *H. bacteriophora* TF strain (Koppenhöfer and Fuzy 2003), *S. glaseri* MB strain (0%), nor *S. kraussei* (Steiner) (50%) (Grewal et al. 2004). Baxendale et al. (2003) found *H. zealandica* effective (80 – 90%) against *C. lurida*, against *C. hirta*, *H. bacteriophora* NC1 strain (13–48%), *S. glaseri* NC strain (9%), and *S. kushidai* Mamiya (33%) were not effective, against *C. pasadenae*, *H. bacteriophora* NC1 strain (8%) (Koppenhöfer et al. 1999) and *H. bacteriophora* (10%) (Dreistadt et al. 2004) were ineffective.

***Entomopathogenic fungi.*** *Metarhizium* and *Beauveria*, two genera of entomopathogenic fungi, cause green and white muscardine diseases, respectively, in insects. They are well known for their ability to infect white grubs in natural habitats. *Beauveria bassiana* (Balsamo) Vuillemin is registered for white grub control in the United States, but its efficacy against white grubs under field conditions is highly variable (Morales-Rodriguez and Peck 2009, Bélair et al. 2010). Mortality of third instar *C. lurida* by *M. brunneum* (Petch) F52 strain [a restored species

name for some isolates of *M. anisopliae* (Metchnikoff) Sorokin including strain F52] and *B. bassiana* GHA strain was very low under laboratory, greenhouse, and field conditions, and the lack of adequate contact of the fungal conidia with the target insect might be a major factor limiting the control efficacy (Wu 2013, Wu et al. 2014).

***Predators and Parasitoids.*** In addition to entomopathogens, natural enemies including invertebrate and vertebrate predators and parasitoids may be also manipulated to provide natural suppression of white grub populations. A native parasitoid, *Tiphia pygidialis* Allen (Hymenoptera: Tiphidae), is an important natural enemy of *Cyclocephala* spp. larvae. Rogers and Potter (2004) reported 33% parasitism by *T. pygidialis* of *Cyclocephala* larvae collected in Kentucky.

Many predatory ground beetles, rove beetles, and ants prey on eggs and young grubs as food (Koppenhöfer 2007, and references therein). In particular, ants play an important role in natural control by feeding on their eggs (Zenger and Gibb 2001). In an experiment to investigate the role of ant predation, Zenger and Gibb (2001) found that the ant *Solenopsis molesta* (Say) can remove as much as 83% of eggs in a field.

These natural enemies can be conserved by modifying cultural practices, i.e., raising mowing height to provide habitat refuge, and planting wildflower beds to supplement food for predators and parasitoids. In addition, avoiding unnecessary insecticide sprays (i.e., spot treatment instead of broadcast applications), proper timing of applications, and reducing the use of broad-spectrum insecticides such as carbamates, organophosphates, and pyrethroids, would reduce the risk of either direct kill or the depletion of food resources for natural enemies (Koppenhöfer, 2007, and references therein).

**Chemical control.** Currently, chemical insecticides are the primary method for control of white grubs including masked chafers (Table 1.2). In most cases, they offer the only practical method to control white grub densities that have already reached damaging levels (Baxendale and Grant 1995). Based on the timing of an application relative to presence of different white grub developmental stages, applications can be roughly grouped as either curative or preventive (Potter and Potter 2013). Each approach has its own advantages and disadvantages.

With curative control, insecticides are typically applied in late summer, after the eggs have hatched and larvae are actively feeding, either after damage to turf has occurred (typically when 3<sup>rd</sup> instars are present) or after sampling has detected densities above the action threshold (typically before the 3<sup>rd</sup> instars occur). Most insecticides used for curative control typically have a relatively short residual activity (usually 2 to 3 weeks or less), and also tend to be more effective if applied to target younger larvae. Curative applications are intended to quickly suppress grub feeding activity and prevent further damage on turf. Insecticides that provide control of larger larvae include the carbamate carbaryl (Sevin<sup>TM</sup>), the neonicotinoid clothianidin, (Arena<sup>TM</sup>) and especially the organophosphate trichlorfon (Dylox<sup>TM</sup>). Regardless of the product, rainfall or post-treatment irrigation should be applied to leach the insecticide residues into the root zone (Potter 1998). Advantages of a curative control approach include: 1) treatments are applied only if damaging grub populations are known to be present, reducing unnecessary insecticide use; 2) because white grub infestations are usually localized, only certain portions of the turf or “hot spots” need to be treated (Potter 1998). Disadvantages of the curative approach include: 1) proper timing of treatments can be tricky; insecticides applied too early may degrade before most eggs have hatched, but if insecticides are applied too late, the grubs will be harder to kill and turf damage may have already occurred (Potter 1998); and 2) because managed



turfgrass often implies a close proximity to people, pets, homes, and businesses, pesticide applications during the summer months of peak activity may pose greater risks of human exposure than other times of the year.

Preventive insecticide applications are made before the damage by grubs has occurred to the turf or potentially damaging densities of grubs are detected by monitoring, usually before or while the adults are laying eggs. With preventive control, the insecticide is basically applied as insurance. Preventive control requires the use of insecticides with relatively long residual activity in soil. Many of the newer insecticides persist long enough to allow applications as early as about 4 (halofenozide), 6 (clothianidin, imidacloprid, thiamethoxam), or even 10 (chlorantraniliprole) weeks before peak egg-laying activity of the adults. Optimal activity for these insecticide products generally can be expected when applied around peak egg-laying activity of the adults. However, earlier applications are often chosen because rainfall (necessary to move products into soil if no irrigation is available) patterns are more reliable earlier in the season, or because it may be easier to coordinate them with other management activities. Advantages of the preventive strategy include: 1) a greater flexibility in application timing; 2) applications made when there is greater chance for rainfall to water the insecticide into the root zone; and 3) opportunity to use insecticides that have more selective activity on target insects and pose relatively less hazard to humans, pets, birds, fish, or the environment. Disadvantages of the preventive approach include: 1) preventive insecticides typically are only effective against young grubs and thus, will not control older or overwintered (3<sup>rd</sup>-instar) grubs; 2) the decision to treat is made before knowing if and where damaging grub problems will occur; thus, preventive control often results in a greater amount of area being treated unnecessarily.

Neonicotinoid insecticides, especially imidacloprid, have become the primary insecticides used for preventive white grub control. The neonicotinoids imidacloprid, clothianidin, and thiamethoxam all provide effective preventive and early curative control of masked chafer grubs (Heller and Walker 2002a,b,c,e; Heller et al. 2006a,b,c, 2008a,b; Shetlar and Andon 2013; Gyawaly et al. 2015). However, early application of these insecticides can negatively affect hymenopteran parasitoids of the grubs (Rogers and Potter 2003). In addition, commercial mixtures of these insecticides with pyrethroids such as imidacloprid + bifenthrin and clothianidin + bifenthrin also provide effective preventive and early curative control (Heller et al. 2006a, 2008a,c; Ramm et al. 2010). It should be noted that the pyrethroid component of these mixtures probably is not contributing much to the grub control as pyrethroids typically do not move very well into the soil. Moreover, these combination products have an extremely broad activity spectrum and will obviously have even more non-target effects than the neonicotinoids by themselves. On the other hand, combination products are convenient, especially for the landscape industry, as their activity against both soil-dwelling insect pests and surface-active insect pests would allow for fewer applications. However, because of the short residual activity of bifenthrin, these combinations are most effectively used when surface insect pests are active. Recently, increased concerns over non-target effects of neonicotinoids on pollinators has resulted in the U.S. Environmental Protection Agency mandating products containing imidacloprid, thiamethoxam, dinotefuran, or clothianidin to put bee protection pesticide labels on such products. This label prohibits the use of these insecticides on blooming plants that may be visited by pollinators. The potential harmful effects of neonicotinoids on bee pollinators was highlighted recently in a field study by Larson et al. (2013), who found that treating lawns with the

recommended rate of clothianidin when white clover was blooming significantly affected weight gain and queen production of bumble bees.

More IPM-friendly insecticide options include chlorantraniliprole, a diamide insecticide that has demonstrated excellent control of masked chafer grubs (Buss et al. 2006; Toda et al. 2006; Heller et al. 2006c; 2008d,e,f,g; 2009; Royer et al. 2009; Shetlar and Andon 2013; Gyawaly et al. 2015), and the insect growth regulator, halofenozide, which is highly effective when applied to target small grubs (Held et al. 2000; Muegge et al. 2000; Heller and Walker 2000, 2002d; Muegge and Quigg 2002; Heng-Moss et al. 2005).

With all insecticides, the presence of thatch, which is above the soil, can be a problem for the management of white grubs. The thatch layer prevents insecticides from making contact with grubs (Tashiro 1987, Brandenburg 2006). Immediate post application irrigation, however, may enhance insecticide movement down through the thatch layer (Potter and Braman 1991). Cultural practices such as irrigation, nutrient management, mowing height, etc. could be manipulated to aid in reducing white grub numbers. Such manipulations, however, may interfere with management practices for other biotic and abiotic stresses.

### **Entomopathogenic Fungi:**

Many fungal species are able to infect insects and are commonly known as entomopathogenic fungi (EPF) (Charnley and Collins 2007). There are at least 700 species of fungi, mostly in the phyla Ascomycota and Zygomycota, which are pathogenic to insects (Hajek and Butler 2000). Many of these fungi infect and cause epizootics on pest insects and are able to suppress pest populations under suitable field conditions (Pilz et al. 2011). EPF inhabit various natural and agricultural habitats including soil (St. Leger 2008). Of those, two species, *M.*

*anisopliae sensu lato* and *B. bassiana* in the order Hypocreales are the most common species and those two species are focus of this literature.

The pest management potential of EPF has been known for a long time. Ainsworth (1956) reported that Italian scientist Agostino Bassi first discovered that *B. bassiana* caused white muscardine disease in silkworms in 1835. However, the use of EPF has not been widely accepted. Currently, although mycoinsecticides are not a major share of worldwide pesticide market, there are more than 10 entomopathogenic fungi species developed as mycopesticides (de Faria and Wraight 2007). Of the over 150 mycopesticides products, more than 65 percent are based on two species, *B. bassiana* and *M. anisopliae sensu lato* (de Faria and Wraight 2007).

### **Infection process and dispersal**

The infection process of *B. bassiana* and *M. anisopliae sensu lato* starts with the contact between a susceptible host cuticle and virulent conidia, which are asexual reproductive structures (Hummel et al. 2002). EPF enter the host body via surface contact rather than ingestion (a common mode of entomopathogenic bacteria invasion in insect). On the host body, the conidia germinate and form the appresoria with which it penetrates the host body. The conidia use both mechanical pressure and enzymes to penetrate host cuticle and may produce toxins to suppress host defenses and pass through the cuticle (Charnley and Collins 2007). Once EPF enter the host body, they grow and mature within the insect's hemocoel and disrupt organs, produce toxic compounds, and eventually kill the insect. After the host dies, fungal hyphae cover the cadaver with new conidia (Hajek and St. Leger 1994). Unlike entomopathogenic nematodes, EPF do not actively search for host. Their dispersal, however, may be assisted by abiotic (wind, rain) and biotic factors (arthropods, earthworms) (Dromph 2001, Meyling and Eilenberg 2007, Shapiro

and Brown 2013). The fungi may persist in the cadaver for long time or may form spores in or on the cadaver thus completing its life cycle.

### **Host range and use against soil insects:**

As a species, entomopathogenic fungus may have a wide host range and a single species of fungus may infect several species of insects in different orders (Uma Devi et al. 2008). However, EPF show higher virulence against host species to which they were associated indicating that they have some degree of host preference (Hajek and Butler 2000 and references therein). EPF are generally considered safe to humans. However, there have been reports of EPF related illness in humans, but only in highly compromised immune conditions or in children (Motley et al. 2011).

EPF occur in many ecological habitats; however, soil is considered a better habitat for EPF because of the absence of some adverse factors such as UV light that are harmful to EPF survival (Keller and Zimmerman 1989). Several studies, mostly in the laboratory, have been carried out to screen virulent strains and to test the efficacy of *B. bassiana* and *M. anisopliae sensu lato* against soil insects including white grubs (Tables 1.3 and 1.4). Of the various fungal isolates/strains tested against soil insects, isolates of *B. bassiana* and *M. anisopliae sensu lato* have been found virulent against many coleopterans (weevils, wireworms, white grubs), thysanopterans (thrips), dipterans (maggots), and hymenopterans (sawflies) (Alston et al. 2005, Bruck et al. 2005, Ansari et al. 2008, Lozano-Tovar et al. 2013, Dotaona et al. 2015, Saito and Brownbridge 2016). *Metarhizium brunneum* and *B. bassiana*, have been reported to successfully control pest populations at the field level as well. Reddy et al. (2014) reported that the applications of *M. brunneum* and *B. bassiana* in wheat reduced wireworm populations, enhanced plant stand counts, and increased yield when applied as granules or drenches. However, the

efficacy of EPF is highly variable and is affected by species of target insect, strain of EPF, application rate, and climatic conditions (Tables 1.3 and 1.4).

Being one of the major soil insect pests in many systems, white grubs have been among the major subjects of EPF based pest management studies (Table 1.5). Many studies have been conducted globally to screen local isolates of *B. bassiana* and *M. anisopliae sensu lato* that are virulent to white grubs with mixed results (Beron and Diaz 2005, Lopes et al. 2013, Erler and Ates 2015). Some of these isolates are found to be highly virulent against white grubs (Flores et al. 2002, Guzman-Franco et al. 2012). Successful control of *Adoryphorus couloni* (Burmeister), a white grub pest of Australian pasture, using *M. anisopliae sensu lato* has been achieved (Rath et al. 1995 a, b) indicating that white grubs may be a good target for EPF in some systems.

However, many common species of white grubs in the U. S. in turfgrass systems show highly variable and often low susceptibility to *B. bassiana* and *M. anisopliae sensu lato* making it difficult to predict the efficacy of these EPF in the field (Morales-Rodriguez et al. 2010). Wu (2013), for example, found that *B. bassiana* and *M. anisopliae sensu lato* caused very low mortality of *Cyclocephala* spp. Similar low mortality of *P. japonica* was observed in the field when plots were treated with *B. bassiana* and *M. brunneum* (Ramoutar et al. 2010). However, Behle et al. (2015) reported a significant reduction of *P. japonica* grub densities in the field when treated with *M. brunneum* in fall indicating that EPF can be effective against white grubs.

In addition to directly infecting hosts, recent studies have shown that EPF can colonize host plants. Such endophytic associations of EPF in plants have been reported between many species of plants; mostly with *B. bassiana* and some with *M. anisopliae sensu lato* (Vidal and Jaber 2015 and references therein). Such endophytic association can cause negative effects on

many herbivores (Bing and Lewis 1991, Akello et al. 2008). However, their endophytic association with turfgrass species and effect on soil insects is not known well.

### **Combination of entomopathogenic fungi with insecticides**

Applications of EPF combined with some insecticides have been found to result in better control of insects. Such interactions might make it possible to control a pest more effectively than with a single control agent. Hiromori and Nishigaki (2001) reported that an insecticide applied at a lower rate can weaken an insect's immune system and reduce the insect's ability to defend against infection by fungal spores. Moreover, Ambethgar (2009) reported that the combination of entomopathogenic fungi and a sub-lethal rate of insecticide could play an important role in resistance management. Many studies have reported synergistic interactions between entomopathogenic fungi and insecticides in other systems (Quintela and McCoy 1998, Ramakrishnan et al. 1999, Jaramillo et al. 2005, Brito et al. 2008). However, for white grub control in turfgrass systems, few studies have been conducted to test the interaction of entomopathogenic fungi and insecticides. Morales-Rodriguez and Peck (2009) reported that combinations of *B. bassiana* or *M. anisopliae* strain F52 (currently referred to as *M. brunneum*) with neonicotinoids showed potential for the control of third instar grubs in laboratory and greenhouse studies. The effects of combining these fungi with other previously untested classes of insecticides such as diamides are unknown.

### **Earthworms**

Earthworms are ubiquitous soil organisms. They have a cylindrical body comprised of many small segments. With a mouth located at the anterior end of their body, earthworms ingest mineral soil, organic matter, microscopic organisms and nematodes (Curry and Schimdt 2007). Earthworms move using setae arising from the body segments. Mature worms have a swollen

ring or saddle-like structure towards the head, called a clitellum. Earthworms are hermaphroditic, having both male and female reproductive organs. The openings of reproductive organs are situated anteriorly. Earthworms oviposit in sac-like structures called cocoons, which are secreted by the clitellum.

### **Earthworms in the turfgrass habitat, and potential pesticide toxicity**

Many species of earthworms occur in the U. S. Of the reported 10 families of earthworms in North America (6 exotic and 4 native) two introduced families, Lumbricidae and Megascolecidae are the most common (Reynolds and Wetzel 2004). In natural habitats, including turfgrass, earthworms in the family Lumbricidae are most common (Potter et al. 1990, Hale 2013). Turfgrass covers a considerable acreage of total cultivated land in the US, where a significant amount of pesticides are applied annually (Aspelin 2003). These pesticides, however, may negatively affect earthworm populations that inhabit turfgrass. In fact, many pesticides have been reported to be toxic or to have sub-lethal effects on earthworms (Choo and Baker 1998, Casabé et al. 2007). Among turf pesticides, some insecticides (primarily carbamate and some organophosphates) and benomyl (a fungicide) have been found to be extremely toxic to earthworms (Potter et al. 1990), whereas most herbicides have been shown to be less toxic to earthworms.

### **Interactions of earthworms with other soil organisms**

In addition to influencing soil physical properties, earthworms affect many biological functions in soil where they share their habitat with large numbers of micro and macro organisms. Many studies have reported the direct and indirect impacts of earthworms on various soil organisms (Stephens et al. 1994, Bohlen et al. 2004, Groffman et al. 2004, Suarez et al. 2004). Gut content analysis of earthworms revealed that soil organisms including bacteria, fungi and nematodes, are



important sources of earthworm nutrition (Curry and Schimdt 2007). Studies have reported that fungal spores and nematodes can survive in the earthworm gut and that the presence of earthworms in soil helps spread fungal spores and nematodes quickly (Hutchinson and Kamel 1956, Shapiro et al. 1993). Earthworms are known to serve as vectors of mycorrhizal fungi (Reddell and Spain 1991, Gange 1993) and plant pathogens, e.g. *Fusarium oxysporium f. sp. raphani* (Toyota and Kimura 1993), *Synchytrium endobioticum* (Schilbersky) Percival (Hampson and Coombes 1989). Earthworms are also reported to disperse actinomycetes (Doube et al. 1994).

The interaction of earthworms with other soil organisms may result in positive or negative effects to plants. Earthworms have been reported to reduce the populations of plant pathogenic nematodes in corn fields (Boyer et al. 1999). Earthworms can reduce disease incidence in plants probably by increasing the populations of microorganisms that compete or suppress plant pathogens (Elmer 2009). Elmer (2009) found that the augmentation of soil with *Lumbricus terrestris* L. reduced soil borne diseases of asparagus, eggplant and tomato. However, the transfer of plant pathogens via earthworms has also been reported (Liu et al. 2012). The effect of earthworms on soil insect pests has not received much research attention, despite the fact that these species co-inhabit the same environment. Among arthropods, collembolans are reported to transmit entomopathogenic fungi species *B. bassiana*, *Beauveria brongniartii* (Saccardo) Petch, and *M. anisopliae sensu lato* (Dromph 2003). Shapiro and Brown (2013) demonstrated that *L. terrestris* can transfer *B. bassiana*, which can result in increased infection of *Galleria mellonella* (L.) larvae. However, it is still unclear if such transfer of EPF by earthworms actually results in increased infection of agricultural pests such as white grubs, which are susceptible to *B. bassiana*.

## Research Objectives:

Objective 1. To determine the effect of combined applications of entomopathogenic fungi and lower rates of insecticides on white grubs.

1.a. To evaluate the efficacy of combined applications of entomopathogenic fungi and lower rate of insecticides on 3<sup>rd</sup> instar *Cycocephala* spp. grubs.

1.b. To evaluate the field efficacy of combined applications of entomopathogenic fungi and lower rate of insecticides for preventive and curative white grub control.

1.c. To determine the effect of insecticides on EPF viability and virulence in the laboratory

1.d. To determine the effect of combined applications of EPF and lower rate of insecticides on oviposition of *Popillia japonica* (Coleoptera: Scarabaeidae) under greenhouse conditions.

Objective 2. To determine the interactions of insecticides, entomopathogenic fungi, and earthworms as they relate to white grub IPM in turfgrass systems.

2.a. To determine whether earthworms can transport the EPFs, *B. bassiana*, in the soil and enhance fungal infections of white grubs.

2.b. To determine the effect of earthworms on oviposition of *Popillia japonica* in greenhouse bioassays.

2.c. To determine the efficacy of selected insecticides on earthworms in laboratory and field experiments.

## References:

- Ainsworth, G. C. 1956. Agostino Bassi, 1773-1856. *Nature* 177: 255–257.  
<http://dx.doi.org/10.1038/177255a0>.
- Akello, J., T. Dubois, D. Coyne, and S. Kyamanywa. 2008. Effect of endophytic *Beauveria bassiana* on populations of the banana weevil, *Cosmopolites sordidus*, and their damage in tissue-cultured banana plants. *Entomologia Experimentalis et Applicata* 129: 157–165.
- Alston, D. G., D. E. Rangel, L. A. Lacey, H. G. Golez, J. J. Kim, and D. W. Roberts. 2005. Evaluation of novel fungal and nematode isolates for control of *Conotrachelus nenuphar* (Coleoptera: Curculionidae) larvae. *Biological Control* 35: 163–171.
- Ambethgar, V. 2009. Potential of entomopathogenic fungi in insecticide resistance management (IRM): a review. *Journal of Biopesticides* 2: 177–193.
- Anonymous. 2010. American-lawns, Grubs. <http://www.american-lawns.com/insects/grubs.html>.
- Ansari, M. A., and T. M. Butt. 2012. Evaluation of entomopathogenic fungi and a nematode against the soil-dwelling stages of the crane fly *Tipula paludosa*. *Pest Management Science* 68: 1337–1344.
- Ansari, M. A., and T. M. Butt. 2013. Influence of the application methods and doses on the susceptibility of black vine weevil larvae *Otiorhynchus sulcatus* to *Metarhizium anisopliae* in field-grown strawberries. *BioControl* 58: 257–267.
- Ansari, M. A., S. Vestergaard, L. Tirry, and M. Moens. 2004. Selection of a highly virulent fungal isolate, *Metarhizium anisopliae* CLO 53, for controlling *Hoplia philanthus*. *Journal of Invertebrate Pathology* 85: 89–96.

- Ansari, M. A., F. A. Shah, L. Tirry, and M. Moens. 2006. Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. *Biological Control* 39: 453–459.
- Ansari, M. A., M. Brownbridge, F. A. Shah, and T. M. Butt. 2008. Efficacy of entomopathogenic fungi against soil-dwelling life stages of western flower thrips, *Frankliniella occidentalis*, in plant-growing media. *Entomologia Experimentalis et Applicata* 127: 80–87.
- Ansari, M. A., M. Evans, and T. M. Butt. 2009. Identification of pathogenic strains of entomopathogenic nematodes and fungi for wireworm control. *Crop Protection* 28: 269–272.
- Aspelin, A. L. 2003. Pesticide usage in the United States: Trends during the 20th century. [http://www.pestmanagement.info/pesticide\\_history/full\\_doc.pdf](http://www.pestmanagement.info/pesticide_history/full_doc.pdf)
- Baxendale, F. P., and J. A. Grant. 1995. Principles and history of turfgrass pest management, pp. 105–109. *In* R. L. Brandenburg and M. G. Villani (eds.), *Handbook of turfgrass insect pests*. Entomological Society America, Lanham, MD.
- Baxendale, F. P., R. W. Baxendale, and S. Rochefort. 2005. Use of NemaGreen and SDS502 for control of white grubs, 2004. *Arthropod Management Tests* 30: G7.
- Baxendale, F. P., T. E. Eickhoff, and T. M. Heng-Moss. 2003. Control of white grubs with the entomopathogenic nematode, *Heterorhabditis zealandica*, 2003. *Arthropod Management Tests* 28: G9.
- Behle, R. W., D. S. Richmond, M. A. Jackson, and C. A. Dunlap. 2015. Evaluation of *Metarhizium brunneum* F52 (Hypocreales: Clavicipitaceae) for control of Japanese beetle larvae in turfgrass. *Journal of Economic Entomology* 108: 1587–1595.

- Bélaïr, G., A. M. Koppenhofer, b, J. Dionne, and L. Simard. 2010. Current and potential use of pathogens in the management of turfgrass insects as affected by new pesticide regulations in North America. *International Journal of Pest Management* 56: 51–60.
- Beris, E. I., D. P. Papachristos, A. Fytrou, S. A. Antonatos, and D. C. Kontodimas. 2013. Pathogenicity of three entomopathogenic fungi on pupae and adults of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Pest Science* 86: 275–284.
- Beron, C. M., and B. M. Diaz. 2005. Pathogenicity of hyphomycetous fungi against *Cyclocephala signaticollis*. *BioControl* 50:143–150.
- Bing, L. A., and L. C. Lewis., 1991. Suppression of *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. *Environmental Entomology* 20: 1207–1211.
- Bixby, A., S. R. Alm, K. Power, P. Grewal, and S. R. Swier. 2007. Susceptibility of four species of turfgrass-infesting scarabs (Coleoptera: Scarabaeidae) to *Bacillus thuringiensis* Serovar *japonensis* strain *Buibui*. *Journal of Economic Entomology* 100: 1604–1610.
- Blanco-Montero, C. A., and G. Hernandez. 1995. Mechanical control of white grubs (Coleoptera: Scarabaeidae) in turfgrass using aerators. *Environmental Entomology* 24: 243–245
- Bohlen, P. J., S. Scheu, C. M. Hale, M. A. McLean, S. Migge, P. M. Groffman, and D. Parkinson. 2004. Non-native invasive earthworms as agents of change in northern temperate forests. *Frontiers in Ecology and the Environment* 2: 427– 435
- Boyer, J., R. Michellon, A. Chabanne, G. Reversat, and R. Tibere. 1999. Effects of trefoil cover crop and earthworm inoculation on maize crop and soil organisms in Reunion Island. *Biology and Fertility of Soils* 28: 364–370.

- Brandenburg, R. L. 2006. White grubs still pose challenge to turfgrass research. *Turfgrass Trends* 62: 57–61.
- Brito, E. S., A. Rodrigues de Paula, L. P. Vieira, C. Dolinski, and R. I. Samuels. 2008. Combining vegetable oil and sub-lethal concentrations of imidacloprid with *Beauveria bassiana* and *Metarhizium anisopliae* against adult guava weevil *Conotrachelus psidii* (Coleoptera: Curculionidae). *Biocontrol Science and Technology* 18: 665–673.
- Bruck, D. J. 2005. Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. *Biological Control* 32: 155–162.
- Bruck, D. J., J. E. Snelling, A. J. Dreves, and S. T. Jaronski. 2005. Laboratory bioassays of entomopathogenic fungi for control of *Delia radicum* (L.) larvae. *Journal of Invertebrate Pathology* 89: 179–183.
- Buss, E., P. Ruppert, O. Warren, C. Congdon, O. Kostromytska, and J. Nichols. 2006. Preventive control of masked chafer grubs, 2005. *Arthropod Management Tests* 31: G63.
- Casabé, N., L. Piola, J. Fuchs, M. L. Oneto, L. Pamparato, S. Basack, R. Gimenez, R. Massaro, J. C. Papa, and E. Kesten. 2007. Ecotoxicological assessment of the effects of glyphosate and chlorpyrifos in an Argentine soya field. *Journal of Soils and Sediments* 7: 232–239.
- Charnley, A. K, and S.A. Collins. 2007. Entomopathogenic fungi and their role in pest control, pp. 159–187. *In* C. P. Kubicek and I. S. Druzhinina (eds.), *The mycota: Environmental and microbial relationships IV*. Springer, Berlin, Germany.
- Choo, L. P. D., and G. H. Baker. 1998. Influence of four commonly used pesticides on the survival, growth, and reproduction of the earthworm *Aporrectodea trapezoides* (Lumbricidae). *Australian Journal of Agricultural Research* 49: 1297–1303.
- Christians, N. 1998. *Fundamentals of turfgrass management*. Ann Arbor Press, Chelsea, MI.

- Cranshaw, W. S., and R. J. Zimmerman. 1989. Biological, mechanical, and chemical control of turfgrass-infesting scarabs in Colorado. *Southwestern Entomologist* 14: 351–355.
- Crutchfield, B. A., D. A. Potter, and A. J. Powell. 1995. Irrigation and fertilization effects on white grubs feeding injury to tall fescue turf. *Crop Science* 35: 1122–1126.
- Curry, J. P., and O. Schmidt. 2007. The feeding ecology of earthworms-a review. *Pedobiologia* 50: 463–477.
- Davidson, G., and D. Chandler. 2005. Laboratory evaluation of entomopathogenic fungi against larvae and adults of onion maggot (Diptera: Anthomyiidae). *Journal of Economic Entomology* 98: 1848–1855.
- Dimock, W. J. 2004. Spatial factors affecting white grub presence and abundance in golf course turf. Ph.D. dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA.
- Dotaona, R., B. A. L. Wilson, M. M. Stevens, J. Holloway, and G. J. Ash. 2015. Screening of tropical isolates of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) for virulence to the sweet potato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *International Journal of Tropical Insect Science* 35: 153–163.
- Doube, B. M., P. M. Stephens, C. Davoren, and M. H. Ryder. 1994. Interactions between earthworms, beneficial soil microorganisms and root pathogens. *Applied Soil Ecology* 1: 3–10.
- Dreistadt, S. H., M. L. Flint, and H. K. Kaya. 2004. *Heterorhabditis bacteriophora* and imidacloprid alone and combined for masked chafer control in tall fescue, 2001. *Arthropod Management Tests* 29: G32.

- Dromph, K. M. 2001. Dispersal of entomopathogenic fungi by collembolans. *Soil Biology and Biochemistry* 33: 2047–2051.
- Dromph, K. M. 2003. Collembolans as vectors of entomopathogenic fungi. *Pedobiologia*, 47: 245–256.
- Eckard, S., M. A. Ansari, S. Bacher, T. M. Butt, J. Enkerli, and G. Grabenweger. 2014. Virulence of in vivo and in vitro produced conidia of *Metarhizium brunneum* strains for control of wireworms. *Crop Protection* 64: 137–142.
- Elmer, W. H. 2009. Influence of earthworm activity on soil microbes and soilborne diseases of vegetables. *Plant Disease* 93: 175–179.
- Erler, F., and A. O. Ates. 2015. Potential of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* (Coleoptera: Scarabaeidae), as biological control agents against the June beetle. *Journal of Insect Science* 15: 44.
- Evans, B. G., K. S. Jordan, M. Brownbridge, and R. H. Hallett. 2015. Effect of temperature and host life stage on efficacy of soil entomopathogens against the swede midge (Diptera: Cecidomyiidae). *Journal of Economic Entomology* 108: 473–483.
- de Faria M. R., and S. P. Wraight. 2007. Mycoinsecticides and mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *BiolControl* 43: 237–256.
- Flores, A. G., W. D. la Rosa, J. C. Rojas, and A. E. Castro-Ramirez. 2002. Evaluation of *Beauveria bassiana* and *Metarhizium anisopliae* (Mitosporic) against species of the white grub complex in the South of Mexico. *Southwestern Entomologist* 27: 73–83.
- Gange, A.C. 1993. Translocation of mycorrhizal fungi by earthworms during early succession. *Soil Biology and Biochemistry* 25: 1021–1026.



- Garczynski, S. F., and J. P. Siegel. 2007. Bacteria, pp. 175–198. *In* L. A. Lacey and H. K. Kaya (eds.), *Field manual of techniques in invertebrate pathology*. Kluwer, Dordrecht, The Netherlands.
- Georgis, R., A. M. Koppenhöfer, L. A. Lacey, G. Bélair, L. W. Duncan, P. S. Grewal, M. Samish, L. Tan, P. Torr, and R.W.H.M. van Tol. 2006. Successes and failures in the use of parasitic nematodes for pest control. *Biological Control* 37: 103–123.
- Giroux, F., R. Lavallée, É. Bauce, and C. Guertin. 2015. Susceptibility of the Japanese beetle, *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae), to entomopathogenic Hypocreales fungi. *Phytoprotection* 95: 1–6.
- Grewal, P. S., S. K. Grewal, V. S. Malik, and M. G. Klein. 2002. Differences in susceptibility of introduced and native white grub species to entomopathogenic nematodes from various geographic localities. *Biological Control* 24: 230–237.
- Grewal, P. S., K. T. Power, S. K. Grewal, A. Suggars, and S. Haupricht. 2004. Enhanced consistency in biological control of white grubs (Coleoptera: Scarabaeidae) with new strains of entomopathogenic nematodes. *Biological Control* 30: 73–82.
- Grewal, P. S., A. M. Koppenhöfer, and H. Y. Choo. 2005. Lawn, turfgrass, and pasture applications, pp. 115–146. *In* P. S. Grewal, R. U. Ehlers and D. I. Shapiro-Ilan (eds.), *Nematodes as biocontrol agents*. CABI Publishing, Wallingford, United Kingdom.
- Groffman, P. M., P. J. Bohlen, M.C. Fisk, M, and T. J. Fahey. 2004. Exotic earthworm invasion and microbial biomass in temperate forest soils. *Ecosystems* 7: 45–54.
- Guzman-Franco, A. W., J. Hernandez-Lopez, J. N. Enriquez-Vara, R. Alatorre-Rosas, F. Tamayo-Mejia, and L. D. Ortega-Arenas. 2012. Susceptibility of *Phyllophaga polyphylla*

- and *Anomala cincta* larvae to *Beauveria bassiana* and *Metarhizium anisopliae* isolates, and the interaction with soil properties. *BioControl* 57: 553–563.
- Gyawaly, S., R. R. Youngman, C. A. Laub, and T. P. Kuhar. 2015. Evaluation of insecticide application timings against white grubs in cool season turfgrass in Virginia, 2013. *Arthropod Management Tests* 40: G10.
- Hajek, A. E., and L. Butler. 2000. Predicting the host range of entomopathogenic fungi, pp. 263–276. *In* P. A. Follett and J. J. Duan (eds.), *Non-target effects of biological control*, Springer, New York, USA.
- Hajek, A.E., and R. J. St. Leger. 1994. Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology* 39: 293–322.
- Hale, C. 2013. *Earthworms of the great lakes* 2nd ed. Kollath + Stensaas Publishing, Duluth, MN.
- Hampson, M. C., and J. W. Coombes. 1989. Pathogenesis of *Synchytrium endobioticum*. VII. Earthworms as vectors of wart disease of potato. *Plant and Soil* 116: 147–150.
- Harris, E. D. Jr. 1959. Observations on the occurrence of a milky disease among larvae of the northern masked chafer, *Cyclocephala borealis* Arrow. *Florida Entomologist* 42: 81–83
- Held, D. W., B. Kreuger, N. Mason, R. Lopez, E. Eliason, A. Walston, and D. A. Potter. 2000. Effect of post-treatment irrigation on the efficacy of CGA 293,343 (Meridian) against white grubs in turf 1998. *Arthropod Management Tests* 25: G23.
- Held, D. W., and D. A. Potter. 2012. Prospects for managing turfgrass pests with reduced chemical inputs. *Annual Review of Entomology* 57: 329–354.

- Heller, P. R., and R. Walker. 2000. Suppression of white grubs with applications of experimental CGA293343 formulations, imidacloprid, and halofenozide, 1999. *Arthropod Management Tests* 25: G32.
- Heller, P. R., and R. Walker. 2002a. Preventive suppression of white grubs with applications of experimental and conventional formulations of imidacloprid, halofenozide, and permethrin, 2001. *Arthropod Manage Tests*. 27: G17.
- Heller, P. R., and R. Walker. 2002b. Evaluation of registered formulations to curatively suppress white grubs, 2001. *Arthropod Management Tests* 27: G39.
- Heller, P. R., and R. Walker. 2002c. Early fall evaluation of registered and experimental formulations to curatively suppress white grubs, 2001. *Arthropod Management Tests* 27: G61.
- Heller, P. R., and R. Walker. 2002d. Curative suppression of white grubs with applications of conventional formulations, 2001. *Arthropod Manage Tests*. 27: G62.
- Heller, P. R., and R. Walker. 2002e. Midsummer evaluation of registered and experimental formulations to suppress white grubs, 2001. *Arthropod Management Tests* 27: G63.
- Heller, P. R., D. Kline, and P. J. Ellis. 2006a. Application of Allectus and Merit to preventively suppress white grubs at three timing intervals, 2005. *Arthropod Management Tests* 31: G34.
- Heller, P. R., D. Kline, and P. J. Ellis. 2006b. Application of Arena and 430 Merit to suppress white grubs at preventive and curative timing intervals, 2005. *Arthropod Management Tests* 31: G35.

- Heller, P. R., D. Kline, and P. J. Ellis. 2006c. Early July application of DPXE2Y45, Grubex, and Merit to preventively suppress scarab white grubs, 2005. *Arthropod Management Tests* 31: G38.
- Heller, P. R., D. Kline, and A. Houseman. 2008a. Preventive timing study with Aloft, Arena, and Merit formulations to suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G12.
- Heller, P. R., D. Kline, and A. Houseman. 2008b. Preventive timing study with Meridian and Merit formulations to suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G13.
- Heller, P. R., D. Kline, and A. Houseman. 2008c. Allectus application timing study to suppress scarab white grubs, 2007. *Arthropod Manage Tests*. 33: G15.
- Heller, P. R., D. Kline, and A. Houseman. 2008d. Mid-April application of Acelepryn and Merit formulations to preventively suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G16.
- Heller, P. R., D. Kline, and A. Houseman. 2008e. Early May application of Acelepryn, Meridian, and Merit formulations to preventively suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G17.
- Heller, P. R., D. Kline, and A. Houseman. 2008f. Mid-June application of Acelepryn, Meridian, and Merit formulations to preventively suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G18.
- Heller, P. R., D. Kline, and A. Houseman. 2008g. Mid-May application of 452 Acelepryn granular and SC formulations compared with Merit formulations to preventively suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G21.

- Heller, P. R., D. Kline, and A. Houseman. 2009. Preventive timing and irrigation study with applications of Acelepryn and Merit formulations to suppress scarab white grubs, 2008. *Arthropod Management Tests* 34: G42.
- Heng-Moss, T. M., T. E. Eickhoff, and F. P. Baxendale. 2005. Use of Merit and competitive products for control of white grubs, 2004. *Arthropod Management Tests* 30: G13
- Hiromori, H., and J. Nishigaki. 1998. Joint action of an entomopathogenic fungus (*Metarhizium anisopliae*) with synthetic insecticides against the scarab beetle, *Anomala cuprea* (Coleoptera: Scarabaeidae) larvae. *Applied Entomology and Zoology* 33: 77–84.
- Hiromori, H., and J. Nishigaki. 2001. Factor analysis of synergistic effect between the entomopathogenic fungus, *Metarhizium anisopliae* and synthetic insecticides. *Applied Entomology and Zoology* 36: 231–236.
- Hummel, R. L., J. F. Walgenbach, M. E. Barbercheck, G. G. Kennedy, G. D. Hoyt, and C. Arellano. 2002. Effects of production practices on soil-borne entomopathogens in western North Carolina vegetable systems. *Environmental Entomology* 31: 84–91.
- Hutchinson, S. A., and M. Kamel. 1956. The effects of earthworms on the dispersal of soil fungi. *Journal of Soil Science* 7: 213.
- Jaramillo, J., C. Borgemeister, L. Ebssa, A. Gaigl, R. Tobón, and G. Zimmermann 2005. Effect of combined applications of *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) strain CIAT 224 and different dosage of imidacloprid on the subterranean burrower bug *Cyrtomenus bergi* Froeschner (Hemiptera: Cydnidae). *Biological Control* 34: 12–20.

- Jurat-Fuentes, J. L., and T. A. Jackson. 2012. Bacterial entomopathogens, pp. 265–349. *In* F. E. Vega and H. K. Kaya (eds.), *Insect pathology*, 2nd edn. Elsevier Academic Press, San Diego, CA.
- Kabaluk, T. 2014. Targeting the click beetle *Agriotes obscurus* with entomopathogens as a concept for wireworm biocontrol. *BioControl* 59: 607–616.
- Keller, S., and G. Zimmermann. 1989. Mycopathogens of soil insects, pp. 239–270. *In* N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber (eds.), *Insect-fungus interactions*. Academic Press, London, United Kingdom.
- Klein, M. G. 1992. Use of *Bacillus popilliae* in Japanese beetle control, pp. 179–189. *In* T. A. Jackson and T. R. Glare (eds.), *Use of pathogens in scarab pest management*. Intercept Ltd., Andover, United Kingdom.
- Koppenhöfer, A. M. 2007. Integrated pest management of white grubs, pp. 315–334. *In* M. Pessaraki (ed.), *Handbook of turfgrass management and physiology*. CRC Press, Boca Raton, FL.
- Koppenhöfer, A. M., and E. M. Fuzy. 2003. *Steinernema scarabaei* for the control of white grubs. *Biological Control* 28: 47–59.
- Koppenhöfer A. M., H. Y. Choo, H. K. Kaya, D. W. Lee, and W. D. Gelernter. 1999. Increased field and greenhouse efficacy with a combination of entomopathogenic nematode and *Bacillus thuringiensis* against scarab grubs. *Biological Control* 14: 37–44.
- Koppenhöfer, A. M., E. M. Fuzy, R. Crocker, W. Gelernter, and S. Polavarapu. 2004. Pathogenicity of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. scarabaei*, (Rhabditida: Heterorhabditidae, Steinernematidae) against 12 white grub species (Coleoptera: Scarabaeidae). *Biocontrol Science and Technology* 14: 87–92.

- Koppenhöfer, A. M., P. S. Grewal, and E. M. Fuzy. 2006. Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *H. zealandica*, and *Steinernema scarabaei* against five white grub species (Coleoptera: Scarabaeidae) of economic importance in turfgrass in North America. *Biological Control* 38: 397–404.
- Larson, J. L., C. T. Redmond, and D. A. Potter. 2013. Assessing insecticide hazard to bumble bees foraging on flowering weeds in treated lawns. *PLoS One* 8, p.e66375.
- Liu, B., D. Roos, S. Butler, B. Richter, and F. J. Louws. 2012. Vegetable seedling diseases associated with earthworm castings contaminated with *Phytophthora capsici* and *Pythium attrantheridium*. *Plant Health Progress*.  
<https://www.plantmanagementnetwork.org/sub/php/research/2012/earthworm/earthworm.pdf>
- Lopes, R. B., D. A. Souza, C. M. Oliveira, and M. Faria. 2013. Genetic diversity and pathogenicity of *Metarhizium* spp. associated with the white grub *Phyllophaga capillata* (Blanchard) (Coleoptera: Melolonthidae) in a soybean field. *Neotropical Entomology* 42: 436–438.
- Lozano-Tovar M. D., A. Ortiz-Urquiza, I. Garrido-Jurado, A. Trapero-Casas, and E. Quesada-Moraga. 2013. Assessment of entomopathogenic fungi and their extracts against a soil-dwelling pest and soil-borne pathogens of olive. *Biological Control* 67: 409–420.
- Mashtoly, T. A., M. S. El-Zemaity, M. I. Hussien, and S. R. Alm. 2009. LC and LD50 values of *Bacillus thuringiensis* serovar *japonensis* strain *Buibui* toxin to oriental beetle and northern masked chafer larvae (Coleoptera: Scarabaeidae). *Journal of Economic Entomology* 102: 1891–1895.

- Meyling, N. V., and J. Eilenberg. 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biological Control* 43: 145–155.
- Morales-Rodriguez, A., and D. C. Peck. 2009. Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale* and *Popillia japonica*. *Biological Control* 51: 169–180.
- Morales-Rodriguez, A., A. Ospina, and D. C. Peck. 2010. Variation in the laboratory susceptibility of turf-infesting white grubs (Coleoptera: Scarabaeidae) to biological, biorational and chemical control products. *Pest Management Science* 66: 90–99.
- Motley, W. W., A. T. Melson, and J. E. Mortensen. 2011. Pediatric *Metarhizium anisopliae* keratitis. *Journal of American Association for Pediatric Ophthalmology and Strabismus* 15: 101–103.
- Muegge, M. A., C. Payne, and R. Bader. 2000. Evaluation of Mach-2 and Merit for control of white grubs in turf, 1999. *Arthropod Management Tests* 25: G74.
- Muegge, M. A., and R. Quigg. 2002. Evaluation of Mach 2 and Merit for control of white grubs in turf, 2000. *Arthropod Management Tests* 27: G9.
- Myrand, V., J. P. Buffet, and C. Guertin. 2015. Susceptibility of cabbage maggot larvae (Diptera: Anthomyiidae) to Hypocreales entomopathogenic fungi. *Journal of Economic Entomology* 108: 34–44.
- Muñiz-Reyes, E., A. W. Guzmán-Franco, J. Sánchez-Escudero, and R. Nieto-Angel. 2014. Occurrence of entomopathogenic fungi in tejocote (*Crataegus mexicana*) orchard soils and their pathogenicity against *Rhagoletis pomonella*. *Journal of Applied Microbiology* 117: 1450–1462.



- Niemczyk, H. D., and D. J. Shetlar. 2000. Destructive turf insects, 2nd ed. H.D.N. Books, Wooster, OH.
- Nong, X., C. Liu, X. Lu, Q. Wang, G. Wang, and Z. Zhang. 2011. Laboratory evaluation of entomopathogenic fungi against the white grubs, *Holotrichia oblita* and *Anomala corpulenta* (Coleoptera: Scarabaeidae) from the field of peanut, *Arachis hypogaea*. *Biocontrol Science and Technology* 21: 593–603.
- Obha, M., H. Iwahana, S. Asanok, N. Suzuki, R. Sato, and H. Hori. 1992. A unique isolate of *Bacillus thuringiensis* serovar *japonensis* with a high larvicidal activity specific for scarabaeid beetles. *Letters in Applied Microbiology* 14: 54–57.
- Oreste, M., N. Baser, G. Bubici, and E. Tarasco. 2015. Effect of *Beauveria bassiana* strains on the *Ceratitidis capitata*-*Psytalia concolor* system. *Bulletin of Insectology* 68: 265–272.
- Pfeiffer, D. G. 2012. Japanese beetle and other Coleoptera feeding on grapevines in eastern North America, pp. 403–439. In N. J. Bostanian, C. Vincent and R. Isaacs (eds.), *Arthropod Management in Vineyards: Pests, Approaches, and Future Directions*. Springer, Dordrecht, The Netherlands.
- Pilz, C., R. Wegensteiner, and S. Keller. 2007. Selection of entomopathogenic fungi for the control of the western corn rootworm *Diabrotica virgifera virgifera*. *Journal of Applied Entomology* 131: 426–431.
- Pilz, C., J. Enkerli, R. Wegensteiner, and S. Keller. 2011. Establishment and persistence of the entomopathogenic fungus *Metarhizium anisopliae* in maize fields. *Journal of Applied Entomology* 135: 393–403.
- Poprawski, T. J., and W. N. Yule. 1991. Incidence of fungi in natural populations of *Phyllophaga* spp. and susceptibility of *Phyllophaga anxia* (LeConte) (Col., Scarabaeidae) to *Beauveria*

- bassiana* and *Metarhizium anisopliae* (Deuteromycotina). *Journal of Applied Entomology* 112: 359–365.
- Potter D. A. 1981. Seasonal emergence and flight of northern and southern masked chafers in relation to air and soil temperature and rainfall patterns. *Environmental Entomology* 10: 793–797.
- Potter, D. A. 1982. Influence of feeding by grubs of the southern masked chafer on quality and yield of Kentucky bluegrass. *Journal of Economic Entomology* 75: 21–24.
- Potter, D. A. 1983. Effect of soil moisture on oviposition, water absorption, and survival of southern masked chafer (Coleoptera: Scarabaeidae) eggs. *Environmental Entomology* 12: 1223–1227.
- Potter, D. A. 1998. *Destructive turfgrass insects: biology, diagnosis, and control*. Ann Arbor Press, Chelsea, MI.
- Potter, D. A. 1995. Masked chafers, pp. 70–72. *In* R. L. Brandenburg and M. G. Villani (eds.), *Handbook of turfgrass insect pests*. Entomological Society of America, Lanham, MD.
- Potter, D. A. 2005. Prospects for managing destructive turfgrass insects without protective chemicals. *International Turfgrass Society Research Journal* 10: 42–54.
- Potter, D. A., and S. K. Braman. 1991. Ecology and management of turfgrass insects. *Annual Review of Entomology* 36: 383–406.
- Potter, D. A., and F. C. Gordon. 1984. Susceptibility of *Cyclocephala immaculata* (Coleoptera: Scarabaeidae) eggs and immatures to heat and drought in turfgrass. *Environmental Entomology* 13: 794–799.
- Potter, M. F., and D. A. Potter. 2013. Controlling white grubs in turfgrass. <http://www2.ca.uky.edu/entomology/entfacts/ent10.asp>

- Potter, D. A., M. C. Buxton, C. T. Redmond, C. G. Patterson, and A. J. Powell. 1990. Toxicity of pesticides to earthworms (Oligochaeta: Lumbricidae) and effect on thatch degradation in Kentucky bluegrass turf. *Journal of Economic Entomology* 83: 2362–2369.
- Potter, D. A., A. J. Powell, P. G. Spicer, and D. W. Williams. 1996. Cultural practices affect root-feeding white grubs (Coleoptera: Scarabaeidae) in turfgrass. *Journal of Economic Entomology* 89: 156–164.
- Quintela, E. D., and C. W. McCoy. 1998. Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *Journal of Economic Entomology* 91: 110–122.
- Ramm, C. M., R. A. Fithian, T. J. Prochaska, and F. P. Baxendale. 2010. Evaluation of Aloft effectiveness in discouraging forager behavior, and curative control of southern masked chafers, 2009. *Arthropod Management Tests* 35: G13.
- Ramakrishnan, R., E. R. Suiter, C. H. Nakatus, R. A. Humber, and G. W. Bennet. 1999. Imidacloprid-enhanced *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) susceptibility to the entomopathogen *Metarhizium anisopliae*. *Journal of Economic Entomology* 92: 1125–1132.
- Ramoutar, D., A. I. Legrand, and S. R. Alm. 2010. Field performance of *Metarhizium anisopliae* against *Popillia japonica* (Coleoptera: Scarabaeidae) and *Listronotus maculicollis* (Coleoptera: Curculionidae) larvae in turfgrass. *Journal of Entomological Science* 45: 20–26.
- Rath, A. C., T. B. Koen, G. C. Anderson, and D. Worledge. 1995 a. Field evaluation of the entomogenous fungus *Metarhizium anisopliae* (DAT F-001) as a biocontrol agent for the

- redheaded pasture cockchafer, *Adoryphorus couloni* (Coleoptera: Scarabaeidae).  
Australian Journal of Agricultural Research 46: 429–440.
- Rath, A. C., D. Worledge, T. B. Koen, and B. A. Rowe. 1995 b. Long-term field efficacy of the entomogenous fungus *Metarhizium anisopliae* against the subterranean scarab, *Adoryphorus couloni*. Biocontrol Science and Technology 5: 439–452.
- Reddell, P., and A.V. Spain. 1991. Earthworms as vectors of viable propagules of mycorrhizal fungi. Soil Biology and Biochemistry 23: 767–774.
- Reddy, G. V., K. Tangtrakulwanich, S. Wu, J. H. Miller, V. L. Ophus, J. Prewett, and S. T. Jaronski. 2014. Evaluation of the effectiveness of entomopathogens for the management of wireworms (Coleoptera: Elateridae) on spring wheat. Journal of Invertebrate Pathology 120: 43–49.
- Redmond, C. T., and D. A. Potter. 1995. Lack of efficacy of in vivo- and putatively in vitro-produced *Bacillus popilliae* against field populations of Japanese beetle (Coleoptera: Scarabaeidae) grubs in Kentucky. Journal of Economic Entomology 88: 846–854.
- Reynolds, J. W., and M. J. Wetzel. 2004. Terrestrial Oligochaeta (Annelida: Clitellata) in North America north of Mexico. Megadrilogica 9: 71–98.
- Rogers, M. E., and D. A. Potter. 2003. Effects of spring imidacloprid application for white grub control on parasitism of Japanese beetle (Coleoptera: Scarabaeidae) by *Tiphia vernalis* (Hymenoptera: Tiphidae). Journal of Economic Entomology 96: 1412–1418
- Rogers, M. E., and D. A. Potter. 2004. Biology of *Tiphia pygidialis* (Hymenoptera: Tiphidae), a parasitoid of masked chafer (Coleoptera: Scarabaeidae) grubs, with notes on the seasonal occurrence of *Tiphia vernalis* in Kentucky. Environmental Entomology 33: 523–527.

- Royer, T. A., N. R. Walker, and E. J. Rebek. 2009. Evaluation of early and late applications of DPX E2Y45 (chlorantraniliprole) for control of white grubs in Bermuda grass turf in Oklahoma, 2007. *Arthropod Management Tests* 34: G6.
- Rudeen, M. L., S. T. Jaronski, J. L. Petzold-Maxwell, and A. J. Gassmann. 2013. Entomopathogenic fungi in corn fields and their potential to manage larval western corn rootworm *Diabrotica virgifera virgifera*. *Journal of Invertebrate Pathology* 114: 329–332.
- Saito, T. and M. Brownbridge. 2016. Compatibility of soil-dwelling predators and microbial agents and their efficacy in controlling soil-dwelling stages of western flower thrips *Frankliniella occidentalis*. *Biological Control* 92: .92–100.
- Shapiro, D. I., E. C. Berry, and L. C. Lewis. 1993. Interactions between nematodes and earthworms: enhanced dispersal of *Steinernema carpocapsae*. *Journal of Nematology* 25: 89–192.
- Shapiro, D. I., and I. Brown. 2013. Earthworms as phoretic hosts for *Steinernema carpocapsae* and *Beauveria bassiana*: Implications for enhanced biological control. *Biological Control* 66: 41–48.
- Shetlar, D. J., and J. Andon. 2013. Preventive control of white grubs on a golf course rough, 2008. *Arthropod Management Tests* 38: G15.
- Stamm, M. D., R. W. Baxendale, L. M. Pierson, and F. P. Baxendale. 2009. Efficacy of SDS502 granules for curative control of southern masked chafer grubs, 2008. *Arthropod Management Tests* 34: G16.

- Stamm, M. D., R. W. Baxendale, K. G. Koch, and T. J. Prochaska. 2012. *Chromobacterium subtsugae* (MBI-203) for control of southern masked chafers, 2011. *Arthropod Management Tests* 37: G9.
- Stamm, M. D., T. J. Prochaska, N. A. Matz, and R. W. Baxendale. 2013. Efficacy of *Chromobacterium subtsugae* for control of southern masked chafers, 2012. *Arthropod Management Tests* 38: G16.
- Stephens, P. M., C. W. Davoren, M. H. Ryder, B. M. Doube, and R. L. Correll. 1994. Field evidence for reduced severity of rhizoctonia bare-patch disease of wheat, due to the presence of the earthworms *A. rosea* and *A. trapezoides*. *Soil Biology and Biochemistry* 26: 1495–1500.
- St. Leger, R. J. 2008. Studies on adaptations of *Metarhizium anisopliae* to life in the soil. *Journal of Invertebrate Pathology* 98: 27–276.
- Suarez, E. R., T. J. Fahey, P. M. Groffman, P. J. Bohlen, and M. C. Fisk. 2004. Effects of exotic earthworms on soil phosphorus cycling in two broadleaf temperate forests. *Ecosystems* 7: 28–44.
- Świergiel, W., N. V. Meyling, M. Porcel, and B. Rämert. 2015. Soil application of *Beauveria bassiana* GHA against apple sawfly, *Hoplocampa testudinea* (Hymenoptera: Tenthredinidae): Field mortality and fungal persistence. *Insect Science*.
- Tashiro, H. 1987. *Turf Insects of the United States and Canada*. Cornell University Press, Ithaca, NY.
- Thompson, S. R., and R. L. Brandenburg. 2006. Effect of combining imidacloprid and diatomaceous earth with *Beauveria bassiana* on mole cricket (Orthoptera: Gryllotalpidae) mortality. *Journal of Economic Entomology* 99: 1948–1954.

- Toda, L., F. P. Baxendale, and T. M. Heng-Moss. 2006. Use of DPX E2Y45 for control of white grubs, 2005. *Arthropod Management Tests* 31: G27.
- Toyota, K., and M. Kimura. 1993. Earthworms disseminate a soil-born plant pathogen, *Fusarium oxysporium* f. sp. *raphani*. *Biology and Fertility of Soils* 18: 32–36.
- Uma Devi, K., J. Padmavathi, C. U. M. Rao, A. A. P. Khan, and M. C. Mohan. 2008. A study of host specificity in the entomopathogenic fungus *Beauveria bassiana* (Hypocreales, Clavicipitaceae). *Biocontrol Science and Technology* 18: 975–989.
- Vidal, S., and L. R. Jaber. 2015. Entomopathogenic fungi as endophytes: plant-endophyte-herbivore interactions and prospects for use in biological control. *Current Science* 109: 46–54.
- Vittum, P. J., M. G. Villani, and H. Tashiro. 1999. Turfgrass insects of the United States and Canada. Cornell University Press, Ithaca, NY.
- Warren, G. W., and D. A. Potter. 1982. Pathogenicity of *Bacillus popilliae* (Cyclocephala strain) and other milky disease bacteria in grubs of the southern masked chafer (Coleoptera: Scarabaeidae). *Journal of Economic Entomology* 76: 69–74.
- White, R. T. 1947. Milky disease infecting *Cyclocephala* larvae in the field. *Journal of Economic Entomology* 40: 912–914.
- Wu, S. 2013. Efficacy of entomopathogenic nematodes and entomopathogenic fungi against masked chafer white grubs, *Cyclocephala* spp. (Coleoptera: Scarabaeidae). Ph.D. dissertation, Virginia Polytechnic Institute and State University, Blacksburg.
- Wu, S., R. R. Youngman, L. T. Kok, C. A. Laub, and D. G. Pfeiffer. 2014. Interaction between entomopathogenic nematodes and entomopathogenic fungi applied to third instar

- southern masked chafer white grubs, *Cyclocephala lurida* (Coleoptera: Scarabaeidae), under laboratory and greenhouse conditions. *Biological Control* 76: 65–73.
- Yokoyama, T., M. Hasegawa, A. Fujiie, M. Sawada, and K. Noguchi. 1998. Microbial control of scarab beetle larvae by a formulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) in a sweet potato field. *Applied Entomology and Zoology* 33: 215–218.
- Zenger, J. T., and T. J. Gibb. 2001. Impact of four insecticides on Japanese beetle (Coleoptera: Scarabaeidae) egg predators and white grubs in turfgrass. *Journal of Economic Entomology* 94: 145–149.



Table 1.1. Major white grub pests of turfgrass in the USA.

Sub family	Common name	Latin name	Origin, Life cycle
Aphodinae	black turfgrass ataenius	<i>Ataenius spretulus</i> (Haldeman)	Native, Annual/Biannual
Cetoniinae	green June beetle	<i>Cotinis nitida</i> L.	Native, Annual
Dynastinae	northern masked chafer	<i>Cyclocephala borealis</i> Arrow	Native, Annual
Dynastinae	southern masked chafer	<i>Cyclocephala lurida</i> Bland	Native, Annual
Melolonthinae	Asiatic garden beetle	<i>Maladera castanea</i> (Arrow)	Japan/China, Annual
Melolonthinae	European chafer	<i>Rhizotrogus majalis</i> (Razoumowsky)	Europe, Annual
Melolonthinae	May or June beetle	<i>Phyllophaga</i> spp.	Native, Annual/ Multiyear
Rutelinae	Japanese Beetle	<i>Popillia japonica</i> Newman	Japan, Annual
Rutelinae	Oriental beetle	<i>Anomala orientalis</i> Waterhouse	Philippines/Japan, Annual

Table 1.2. Insecticides currently registered for control of white grub/ masked chafer grubs in turfgrass in the USA.

Active ingredient	Insecticide class	Application timing <sup>a</sup>	Trade names
<i>Bacillus thuringiensis</i> subsp. <i>galleriae</i> strain SDS-502	Microbial (Bacterium)	Early curative	grubGONE
<i>Beauveria bassiana</i>	Microbial (Fungus)	Early curative, Curative	BotaniGard
Carbaryl	Carbamate	Early curative, Curative	Sevin
Chlorantraniliprole	Diamide	Preventive, Early curative	Acelepryn, Scotts Grub-Ex
Clothianidin	Neonicotinoid	Preventive, Early curative, Curative	Arena
Clothianidin +Bifenthrin	Neonicotinoid + Pyrethroid	Preventive, Early curative, Curative	Aloft
Dinotefuran	Neonicotinoid	Preventive, Early Curative	Zylam
<i>Heterorhabditis bacteriophora</i>	Microbial (Nematodes)	Early curative, Curative	e.g., Nemasys G, Heteromask, Terranem, nema-green
Halofenozide	Diacylhydrazine	Preventive, Early curative	Natural Guard Grub Control
Imidacloprid	Neonicotinoid	Preventive, Early curative	Merit, Bayer Advanced Lawn Season-long Grub Control
Imidacloprid+Bifenthrin	Neonicotinoid + Pyrethroid	Preventive, Early curative	Allectus
Imidacloprid + Cyfluthrin	Neonicotinoid + Pyrethroid	Preventive, Early curative	Bayer Advanced Complete Insect Killer
Thiamethoxam	Neonicotinoid	Preventive, Early curative	Meridian
Trichlorfon	Organophosphate	Early curative, Curative	Dylox, Bayer Advanced 24-hour Grub Control

<sup>a</sup> Preventive = until larvae start to appear in soil, targeting first instars; Early curative = targeting first and second instars; Curative = targeting late second and third instars.

Table 1.3. Efficacy of *Beauveria bassiana* against soil insects.

Pests/ Hosts	Efficacy	Author	Comment
<i>Ceratitis capitata</i> (Wiedemann) (Diptera: Tephritidae)	~ 88-96%	Lozano-Tovar et al. (2013)	Lab, puparium/adult mortality
<i>Ceratitis capitata</i> (Diptera: Tephritidae)	~18-88%	Beris et al. (2013)	Lab, pupa and adult mortality
<i>Delia radicum</i> (L.) (Diptera: Anthomyiidae)	<15 %	Myrand et al. (2015)	Lab, percent reduction in adult eclosion
<i>Delia radicum</i> (Diptera: Anthomyiidae)	10-46%	Bruck et al. (2005)	Lab, larval mortality
<i>Delia antiqua</i> (Meigen) Diptera: Anthomyiidae	1.8-23.3%	Davidson and Chandler (2005)	Lab, larval mortality
<i>Rhagoletis pomonella</i> Walsh (Diptera: Tephritidae)	0->70%	Muñiz-Reyes (2014)	Lab, pupa and adult infection
<i>Tipula paludosa</i> Meigen (Diptera: Tipulidae)	0%	Ansari and Butt (2012)	Lab, percent mortality
<i>Hoplocampa testudinea</i> Klug (Hymenoptera: Tenthredinidae)	49.4-62.3%	Świergiel et al. (2015)	Lab, percent mycosis
<i>Frankliniella occidentalis</i> (Pergande) (Thysanoptera: Thripidae)	8-33%	Saito and Brownbridge (2016)	Lab
<i>Frankliniella occidentalis</i> (Thysanoptera: Thripidae)	54-84 %	Ansari et al.(2008)	Lab, larval and pupal mortality
Not identified (Coleoptera: Elateridae)	~ 29%-100%	Reddy et al.(2014)	Field, reduction in number/trap
<i>Agriotes lineatus</i> (L.) (Coleoptera: Elateridae)	0 %	Ansari et al. (2009)	Lab, larval mortality
<i>Diabrotica virgifera virgifera</i> LeConte (Coleoptera: Chrysomelidae)	~2-11 %	Rudeen et al. (2013)	Lab, larval mortality
<i>Diabrotica virgifera virgifera</i> (Coleoptera: Chrysomelidae)	3-10 %	Pilz et al. (2007)	Lab, larvae and adult infection
<i>Scapteriscus borellii</i> Giglio-Tos (Orthoptera: Gryllotalpidae)	~ 25-92.5%	Thompson and Brandenburg (2006)	Lab, adult mortality

Table 1.4. Efficacy of *Metarhizium anisopliae sensu lato* against soil insects.

Pests/ Hosts	Efficacy	Author	Comment
<i>Delia radicum</i> (L.) (Diptera: Anthomyiidae)	28-85 %	Bruck et al. (2005)	Lab, mortality
<i>Delia radicum</i> (Diptera: Anthomyiidae)	18-64%	Myrand et al. (2015)	Lab, percent reduction in adult eclosion
<i>Delia antiqua</i> (Meigen) (Diptera: Anthomyiidae)	0-92.5%	Davidson and Chandler (2005)	Lab, larval mortality
<i>Ceratitis capitata</i> (Diptera: Tephritidae)	27-51%	Oreste et al. (2015)	Lab, mortality
<i>Ceratitis capitata</i> (Diptera: Tephritidae)	~ 88-97.5%	Lozano-Tovar et al. (2013)	Lab, puparium /adult mortality
<i>Ceratitis capitata</i> (Diptera: Tephritidae)	~18-46%	Beris et al. (2013)	Lab, pupa and adult mortality
<i>Contarinia nasturtii</i> Kieffer (Diptera: Cecidomyiidae)	0-82%	Evans et al. (2015)	Field, reduction in adult emergence
<i>Tipula paludosa</i> Meigen (Diptera: Tipulidae)	~11-40 %	Ansari and Butt (2012)	Lab, larval mortality
<i>Hoplocampa testudinea</i> Klug (Hymenoptera: Tenthredinidae)	65.4-68.4%	Świergiel et al. (2015)	Lab, larval mycosis
<i>Frankliniella occidentalis</i> (Pergande) (Thysanoptera: Thripidae)	34%-72%	Saito and Brownbridge (2016)	Lab, mortality
Not identified (Thysanoptera: Thripidae)	51-96 %	Ansari et al.(2008)	Lab, larval and pupal mortality
<i>Cylas formicarius</i> F. (Coleoptera: Brentidae)	80-100%	Dotaona et al. (2015)	Lab, mortality
<i>Conotrachelus nenuphar</i> (Herbst) (Coleoptera: Curculionidae)	~10-100 %	Alston et al. (2005)	Lab, corrected larval mortality
<i>Agriotes obscurus</i> (L.) (Coleoptera: Elateridae)	35-100%	Kabaluk (2014)	Field, adult mortality
<i>Agriotes</i> spp. (Coleoptera: Elateridae)	~ 5-83%	Eckard et al. (2014)	Lab, larval mortality

Pests/ Hosts	Efficacy	Author	Comment
Not identified (Coleoptera: Elateridae)	~ 29-100%	Reddy et al. (2014)	Field, reduction in number/trap
<i>Agriotes lineatus</i> (L.) (Coleoptera: Elateridae)	10-100 %	Ansari et al. (2009)	Lab, larval mortality
<i>Diabrotica virgifera virgifera</i> (Coleoptera: Chrysomelidae)	~5-28 %	Rudeen et al. (2013)	Lab, percent mortality (corrected)
<i>Diabrotica virgifera virgifera</i> (Coleoptera: Chrysomelidae)	0-90 %	Pilz et al. (2007)	Lab, larvae and adult infection
<i>Otiorhynchus sulcatus</i> F. (Coleoptera: Curculionidae)	~6-96 %	Ansari and Butt (2013)	Field, percent larval mortality measured as efficacy
<i>Listronotus maculicollis</i> Kirby (Coleoptera: Curculionidae)	31-46%	Ramoutar et al. (2010)	Field, percent control
<i>Cyrtomenus bergi</i> Froeschner (Hemiptera: Cydnidae)	2.7-39.5%	Jaramillo et al. (2005)	Lab/greenhouse, mortality

Table 1.5. Efficacy of *Beauveria bassiana* and *Metarhizium anisopliae sensu lato* against white grubs.

Pests/ Hosts	Fungi	Efficacy	Author	Comment
<i>Adoryphorus couloni</i> (Burmeister)	<i>M. anisopliae</i>	~ 80%	Rath et al. (1995 a)	Field study, mortality
<i>Adoryphorus couloni</i> (Burmeister)	<i>M. anisopliae</i>	45- 68%	Rath et al. (1995 b)	Field study, percent reduction in larval population than control
<i>Phyllophaga capillata</i> (Blanchard)	<i>M. anisopliae</i>	≤1.7%	Lopes et al. (2013)	Lab study, larval mortality
<i>Phyllophaga polyphylla</i> (Bates)	<i>M. anisopliae</i>	~4-10 %	Guzman- Franco et al. (2012)	Lab, larval mortality
<i>Phyllophaga</i> spp.	<i>B. bassiana</i>	28.3 - 61.6%	Flores et al. (2002)	Lab study
<i>Phyllophaga</i> spp.	<i>M. anisopliae</i>	21.6-90.0%	Flores et al. (2002)	Lab study
<i>Phyllophaga polyphylla</i> (Bates)	<i>B. bassiana</i>	~8-11 %	Guzman- Franco et al. (2012)	Lab, larval mortality
<i>Phyllophaga anxia</i> (LeConte)	<i>M. anisopliae</i>	2-97%	Poprawski and Yule (1991)	Lab study, different rates, routes of application and stage of grubs
<i>Phyllophaga anxia</i> (LeConte)	<i>B. bassiana</i>	0-28%	Poprawski and Yule (1991)	Lab study, different rates, routes of application and stage of grubs

Pests/ Hosts	Fungi	Efficacy	Author	Comment
<i>Anomala cincta</i> (Say)	<i>M. anisopliae</i>	~80-95%	Guzman-Franco et al. (2012)	Lab, larval mortality
<i>Anomala cincta</i>	<i>B. bassiana</i>	~2-4 %	Guzman-Franco et al. (2012)	Lab, larval mortality
<i>Anomala cuprea</i> Hope	<i>M. anisopliae</i>	50.00 %	Hiromori and Nishigaki (1998)	Lab study
<i>Anomala cuprea</i>	<i>M. anisopliae</i>	22.00 %	Hiromori and Nishigaki (1998)	Field study
<i>Anomala cuprea</i>	<i>M. anisopliae</i>	20-90 %	Yokoyama et al. (1998)	Lab study
<i>Holotrichia oblita</i> Faldermann	<i>M. anisopliae</i>	6-39 %	Nong et al. (2011)	Lab study, egg mortality
<i>Holotrichia oblita</i>	<i>B. bassiana</i>	24 %	Nong et al. (2011)	Lab study, egg mortality
<i>Anomala corpulenta</i> Motschulsky	<i>M. anisopliae</i>	31-71 %	Nong et al. (2011)	Lab study, egg mortality
<i>Anomala corpulenta</i>	<i>B. bassiana</i>	65 %	Nong et al. (2011)	Lab study, egg mortality
<i>Holotrichia oblita</i>	<i>M. anisopliae</i>	12-38 %	Nong et al. (2011)	Lab study, larval mortality
<i>Holotrichia oblita</i>	<i>B. bassiana</i>	30 %	Nong et al. (2011)	Lab study, larval mortality
<i>Anomala corpulenta</i>	<i>M. anisopliae</i>	33-100 %	Nong et al. (2011)	Lab study, larval mortality
<i>Anomala corpulenta</i>	<i>B. bassiana</i>	37 %	Nong et al. (2011)	Lab study, larval mortality

Pests/ Hosts	Fungi	Efficacy	Author	Comment
<i>Popillia japonica</i>	<i>M. anisopliae</i>	0-49%	Ramoutar et al. (2010)	Field, percent control
<i>Amphimallon majale</i>	<i>B. bassiana</i>	43.4 %	(Morales-Rodriguez et al. 2010)	Lab, percent mortality
<i>Anomala orientalis</i>	<i>B. bassiana</i>	77.0 %	(Morales-Rodriguez et al. 2010)	Lab, percent mortality
<i>Maladera castanea</i>	<i>B. bassiana</i>	36.8 %	(Morales-Rodriguez et al. 2010)	Lab, percent mortality
<i>Popillia japonica</i>	<i>B. bassiana</i>	10.4 %	(Morales-Rodriguez et al. 2010)	Lab, percent mortality
<i>Amphimallon majale</i> L.	<i>M. anisopliae</i>	69.8 %	(Morales-Rodriguez et al. 2010)	Lab, percent mortality
<i>Anomala orientalis</i>	<i>M. anisopliae</i>	77.2 %	(Morales-Rodriguez et al. 2010)	Lab, percent mortality
<i>Maladera castanea</i>	<i>M. anisopliae</i>	75.6 %	(Morales-Rodriguez et al. 2010)	Lab, percent mortality
<i>Popillia japonica</i>	<i>M. anisopliae</i>	4.2 %	Morales-Rodriguez et al. (2010)	Lab, percent mortality
<i>Popillia japonica</i>	<i>B. bassiana</i>	~37 %	Giroux et al. (2015)	Lab, percent mortality
<i>Popillia japonica</i>	<i>M. anisopliae</i>	~37 %	Giroux et al. (2015)	Lab, percent mortality
<i>Hoplia philanthus</i> (Fuesslin)	<i>M. anisopliae</i>	10-90%	Ansari et al. (2004)	Lab/glasshouse, percent mortality
<i>Hoplia philanthus</i>	<i>M. anisopliae</i>	37-65%	Ansari et al. (2006)	Field, percent control



Pests/ Hosts	Fungi	Efficacy	Author	Comment
<i>Cyclocephala signaticollis</i> Burmeister	<i>M. anisopliae</i>	No or low	Beron and Diaz (2005)	Lab, larval mortality
<i>Cyclocephala signaticollis</i>	<i>B. bassiana</i>	0-83%	Beron and Diaz (2005)	Lab, larval mortality

## Chapter 2

### **Evaluation of combined application of entomopathogenic fungi and insecticides against third instar masked chafer grubs, *Cyclocephala* spp. (Coleoptera: Scarabaeidae)**

#### **Abstract**

Masked chafer grubs, *Cyclocephala* spp. (Coleoptera: Scarabaeidae), are important pests of turfgrass in Virginia. Mature third instar grubs of *Cyclocephala* spp. are particularly difficult to control using currently-available insecticides or entomopathogenic fungi (EPF) labeled for white grub control in turf when applied alone. Laboratory, greenhouse and field experiments were carried out to determine the efficacy and interactions of combined applications of two insecticides, imidacloprid and chlorantraniliprole, and two species of EPF, *Metarhizium brunneum* (Petch) and *Beauveria bassiana* (Balsamo) Vuillemin. Experiments were conducted on both non-overwintered and overwintered third instar masked chafer grubs. Treatments included two rates of the insecticides (one quarter and one half of the recommended rate), the full recommended rates of both fungi species, and each combination of insecticide plus fungi. Laboratory results showed that, for non-overwintered grubs, the combined application of one half the recommended rate of chlorantraniliprole plus the full recommended rate of the fungus *B. bassiana* caused the highest (55%) grub mortality four weeks after treatment. However, the single and combined applications did not vary significantly. In the field, imidacloprid applied in June at either rate as a single treatment or combined with EPF resulted in significantly fewer grubs than untreated control. The efficacy and interactions of combined applications of insecticides and entomopathogenic fungi are discussed.

Key words: *Cyclocephala*, interaction, entomopathogenic fungi, imidacloprid, chlorantraniliprole

**Introduction:**

White grubs (Coleoptera: Scarabaeidae) are major pests of cool-season turfgrass in the northeastern US (Potter 1998). In VA, among the various species of grubs found, masked chafer, *Cyclocephala* spp. is one of the most important species (Dimock 2004, Gyawaly et al. 2016). Like many other annual white grubs found in the USA, the adults of *Cyclocephala* spp. are active in the summer and lay eggs in late June and July. The eggs hatch to become neonate grubs that develop in the soil by continuously feeding on grass roots. They typically reach third instar by early September. With the onset of cold weather the third instars burrow deep in the soil to overwinter and avoid freezing. In the spring, when the soil temperature starts to warm, grubs move up in the soil to the thatch layer and feed on grass roots until they pupate in late May. White grub damage to turfgrass becomes most evident in the fall and spring when the larger grubs feed and the grass is under environmental stress (Potter 1998).

Preventive applications of synthetic insecticides drenched into the turf are the most common control tactic for white grubs including *Cyclocephala* spp. In Virginia, the neonicotinoid imidacloprid and the diamide chlorantraniliprole are the most popular insecticides used on turf (Laub et al. 2016). These insecticides have low toxicity to vertebrates, long residual activities and provide very good control of white grubs when applied at the appropriate time. Normally, imidacloprid and chlorantraniliprole are most effective when the grubs are small and are at the initial stage of their development (Potter 2005, Gyawaly et al. 2015). However, neither of these insecticides provide effective control of late instar white grubs in late fall and spring when grub-feeding damage is most severe (Gyawaly et al. 2015). Combining the use of biological insecticide and synthetic chemicals is an approach that researchers have been investigating in order to enhance the efficacy against larger white grubs (Morales-Rodriguez and

Peck 2009). Entomopathogenic fungi (EPF), *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium brunneum* (Petch) [restored species name for some isolates of *M. anisopliae* (Metchnikoff) Sorokin including strain F52], are biological insecticides with very low risk to humans and are registered for turfgrass use. However, the efficacy of *B. bassiana* or *M. brunneum* against white grubs is typically low when applied alone (Morales-Rodriguez and Peck 2009, Wu et al. 2014).

Combined applications of EPF with chemical insecticides such as imidacloprid have been shown to enhance mortality of third instar grubs compared to when EPF were applied alone (Morales-Rodriguez and Peck 2009). Hiromori and Nishigaki (2001) suggested that the insecticides applied at low rates weaken the insects' immune system and ability to defend against infection by fungal spores, thus enhancing the efficacy of EPF. Moreover, Quintela and McCoy (1998) reported a synergistic interaction between EPF and insecticides for control of the root weevil, *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae). Such interactions might make it possible to control a pest effectively when two control agents are applied together rather than a single control agent. However, for white grub control in turfgrass systems, relatively few studies have examined the potential of combining EPF with insecticides. Morales-Rodriguez and Peck (2009) reported that combinations of *B. bassiana* or *M. anisopliae* F 52 (currently referred to as *M. brunneum*) with neonicotinoids showed potential for the control of third instar *Popillia japonica* Newman and *Rhizotrogus majalis* (Razoumowsky) white grubs in laboratory and greenhouse studies. Herein, I investigate combining other groups of insecticides with EPF for the control of third instar *Cyclocephala* spp. grubs. The objectives of this study were, i) To evaluate the efficacy of combined applications of *B. bassiana* or *M. brunneum* plus reduced rates of imidacloprid or chlorantraniliprole against third instar *Cyclocephala* spp. grubs, and ii) To

determine the interactions of *B. bassiana* or *M. brunneum* plus imidacloprid or chlorantraniliprole against third instar *Cyclocephala* spp. grubs.

## **Materials and Methods:**

### **White grub larvae:**

Third instar *Cyclocephala* spp. grubs used in the laboratory and greenhouse experiment were collected from the Virginia Tech Turfgrass Research Center in Blacksburg, VA. The grubs used were collected in late September to early October for the fall experiment, and in late April to early May for the spring experiment, using a 30.5 cm wide Model 544844 Ryan Jr. Sod Cutter (Textron Golf, Turf & Specialty Products, Johnson Creek, WI). After collection, grubs were stored in a cold room (at 4°C) for 1-3 d. One day (24 h) before each experiment, grubs were removed from the cold room and kept at room temperature (21 to 24°C) to acclimatize. Only grubs that were apparently healthy and active were used in the experiments. Grubs were surface sterilized using 1 % sodium hypochlorite, 70 % ethanol and water following the methods of Lacey and Brooks (1997).

### **Soil:**

Laboratory and greenhouse experiments were conducted using field top soil classified as a sandy loam with 3.2 % organic matter and 6.37 pH. The soil was dried, pulverized, and screened to remove debris or plant remains. The soil was solarized by keeping in a greenhouse under clear plastic for approximately 1 month during the summer.

### **Entomopathogenic fungi:**

In the experiments examining interaction and efficacies of combined applications of EPF and insecticides, an emulsifiable suspension of *B. bassiana* strain GHA (BotaniGard ES, Laverlam International Co., Butte, MT), and an emulsifiable concentrate of *M. brunneum* strain

F 52 (Met52, Novozymes Biologicals Inc., Salem, VA) were used. BotaniGard ES contains  $2.1 \times 10^{10}$  viable spores per ml. Met52 contains  $5.0 \times 10^9$  conidia per ml with 60 % germination rate. Unless otherwise mentioned, high application rates of BotaniGard, 25.6 L/ha, and Met 52, 9.6 L/ha were used. In the experiments examining the effect of insecticides on growth and virulence of EPF, pure cultures of *B. bassiana* and *M. brunneum* were established by inoculating a single spore of each respective fungus from commercial products (BotaniGard or Met52) into potato dextrose agar (PDA) media. Conidia that had grown for 7-10 days in PDA media were used in all the experiments. All EPF culture work was done under a laminar flow hood in sterile conditions.

### **Insecticides:**

Imidacloprid (Merit 75 WP, Bayer CropScience, Research Triangle Park, NC) and chlorantraniliprole (Acelepryn 1.6 SC, Syngenta Crop Protection, Greensboro, NC) were used in the experiments. The labeled rates for Merit and Acelepryn for white grubs in turfgrass are 448.1– 602.1 g product/ha and 584.1–1166.3 ml product/ha, respectively. For this study, we used one-half and one-quarter rates of the highest recommended rates of each insecticide as previously used in similar studies (Morales-Rodriguez and Peck 2009) . In the greenhouse, insecticides were applied using a pipette. In the combined treatments EFP were first applied evenly on the surface and then insecticide was immediately added.

### **Laboratory Bioassay:**

Laboratory experiments were conducted in 30-ml plastic containers (Solo Cup Company, Lake Forest, IL) in fall 2012 and spring 2013 (Fig. 2.1). Screened sandy-loam soil (previously described) was used. Soil was moistened to 18.0 % moisture by volume. About 0.65 g of perennial ryegrass seed was added to each cup and grown for at least 7 d prior to the experiment.

A single, healthy, actively moving grub was introduced to each cup 24 h before treatment. Any grub that did not burrow into the soil within 3 h was replaced with a new one. Experiments were conducted in controlled conditions of  $25\pm 4^{\circ}\text{C}$  and 90 % relative humidity at diurnal cycle of 13:11 (light 13: dark 11). Each treatment was applied with 1 ml water. For the treatments containing only insecticide or only EPF, 1 additional ml water was added and for the control treatment, 2 ml water was applied. The plastic cups were covered with perforated lids to prevent moisture loss and to keep grubs from escaping. For the fall 2012 experiment, the treatments included  $\frac{1}{2}$  and  $\frac{1}{4}$  rate of chlorantraniliprole (Acelepryn) and full rate of two EPF: *M. brunneum*, and *B. bassiana*, plus their combinations. All assays had 4 repetitions of 10 cups with 1 grub per cup. For the spring 2013 experiment, the treatments included:  $\frac{1}{2}$  and  $\frac{1}{4}$  rate of two insecticides [imidacloprid (Merit) and chlorantraniliprole (Acelepryn)] and full rate of two EPF: *M. brunneum*, and *B. bassiana* plus all combinations of the insecticides and EPF. All assays had 4 repetitions of 10 cups with 1 grub per cup. Mortality data were recorded weekly for four weeks after treatment application. However, cumulative mortality data after 4 wk were used for analysis.

### **Greenhouse Experiment:**

The greenhouse experiment was conducted in fall 2013 and 2014 using 1-liter plastic pots. The pots were filled to the depth of  $\frac{2}{3}$  level with previously described soil. Perennial ryegrass, *Lolium perenne* L., was seeded 2-3 wk before the start of the experiment to supply food for the grubs. Ten third instar larvae were introduced to the pot 2 d before treatment application. Larvae not burrowing into the soil within 3 h were replaced. Soil was moistened to 18.0 % moisture by volume. The average temperature in the greenhouse was  $22^{\circ}\text{C}$ . Treatments were applied in a completely randomized block design. Each treatment was applied with 10 ml water.

For the treatments containing only insecticide or only EPF, 10 additional ml water was added and for the control treatment, 20 ml water was applied. Finally, 40 ml water was applied to all containers to facilitate insecticide and EPF to reach the soil. The treatments were the same as in the spring laboratory experiment described above. Mortality data were recorded four weeks after treatment application.

### **Field Experiment:**

The field experiment was conducted at two locations: the Virginia Tech Turfgrass Research Center, in Blacksburg, VA and Tazewell Country Club in Tazewell, VA in 2013. Treatments were applied as foliar broadcast sprays on 1 May (spring), 25 Jul (summer), or 3 Oct (fall), in Blacksburg and on 24 Apr (spring), 11 Jul (summer), or 1 Oct (late fall) in Tazewell using a CO<sub>2</sub> backpack sprayer equipped with 4,8002VS stainless steel spray tips and calibrated to deliver 187.09 L per ha at  $2.76 \times 10^5$  Pa. Treatments were applied at dusk to avoid any sunlight exposure to EPF. In treatments that received both insecticide and EPF (combined treatments), insecticides and EPF were applied separately where insecticide applications followed the EPF applications. The plots were watered to a depth of 2.5 cm immediately after the application of treatments. The experiments were arranged in a randomized complete block design with 4 replicates (1.5 m × 1.5 m size plot). The grass composition in Blacksburg was composed of Tall fescue, *Schedonorus arundinaceus* (Schreb.), and Kentucky blue grass, *Poa pratensis* L., and in Tazewell was composed of these same two grass species plus perennial rye grass, *L. perenne*. The treatments used in the field study were same as the treatments in the greenhouse experiment. Grubs were sampled approximately 30 d after treatment for both spring and fall treatments, and 4 months after treatment for the summer treatments. Sampling consisted of counting all live white



grubs from the center of each plot by cutting 0.092 m<sup>2</sup> area to a depth of 3.8 cm using a commercial sod cutter and a shovel.

**Effects of insecticides on *Beauveria bassiana* or *Metarhizium brunneum* viability and infection of *Galleria mellonella*:**

Conidial suspensions of *B. bassiana* and *M. brunneum* were prepared in sterile distilled water by pouring 5 ml of sterilized water in a culture plate and lightly scraping the fungi mycelia with a sterile loop. The conidial suspension was poured back into a sterile container and shaken well. A standard spore suspension of  $1 \times 10^6$  conidia/ml was prepared by counting spores in a Neubauer chamber. A PDA plate was prepared by evenly spreading 0.01 ml of the  $1 \times 10^5$  conidia per ml suspension (preparation described above) over the agar surface.

Media plates with insecticide treatments were prepared by mixing chlorantraniliprole or imidacloprid at  $\frac{1}{2}$  or  $\frac{1}{4}$  of the recommended rate in 300 ml autoclaved PDA media at ca. 50°C (before solidification). Approximately 30 ml of media were prepared and poured in sterile 9 cm diameter Petri plates (Fisher Scientific International Inc., Pittsburgh, PA). For control treatment media plates, no insecticides were added. The plates were allowed to solidify and were sealed with Parafilm (Bemis Company Inc., Oshkosh, WI) to avoid any contamination.

For the effect of insecticides on fungal viability study, 0.01 ml of the  $1 \times 10^5$  conidia/ml suspension that was growing for 1 wk at room temperature (21 to 24°C) was poured over the agar surface of each treatment. Once poured, the suspension was evenly spread over the whole surface using a sterile plastic spreader. The number of colony forming units (CFU) formed in each PDA plate was counted for 3 d. Two separate trials were conducted for *B. bassiana* and *M. brunneum*.

For the effect of insecticides on EPF infection experiment, 0.05 ml of the  $1 \times 10^6$  conidia/ml suspension that was growing for 1 wk under room temperature was poured over the agar surface with or without insecticide treatments. After one week, a conidial suspension of *B. bassiana* or *M. brunneum* was prepared in sterile distilled water in a culture plate by lightly scraping the fungi mycelia with a sterile loop. The conidia suspension was poured into a sterile container and shaken well. A standard spore suspension of  $1 \times 10^9$  conidia/ml was prepared by counting conidia in a Neubauer chamber. About 0.025 ml of the final suspension from each treatment culture was applied to individual *Galleria mellonella* (L.) larvae in a Petri dish. The dishes with larvae were then put into an environmental chamber at  $23 \pm 4^\circ\text{C}$ , 90 % RH under complete darkness. Treatments included *B. bassiana* or *M. brunneum*, suspension harvested from PDA culture, control (PDA media without insecticide),  $\frac{1}{2}$  rates of chlorantraniliprole,  $\frac{1}{4}$  rate of chlorantraniliprole,  $\frac{1}{2}$  rate of imidacloprid, and  $\frac{1}{4}$  rate of imidacloprid. In addition, a sterile distilled water treatment was also used as a second control. Fungal infection on *G. mellonella* larvae was observed weekly for three weeks. There were 10 larvae in each Petri dish with five replications.

#### **Data analysis:**

The laboratory, greenhouse and field experiments on the effect of treatments on grub mortality, and the effects of insecticides on *B. bassiana* or *M. brunneum* viability and infection of *G. mellonella* were analyzed using ANOVA in JMP 11.0 (SAS, Cary, NC). Means were separated using Tukey's test at the 0.05 level of significance. Mortality data were subjected to square root transformation for analysis, however, actual mortality data are presented in the results. EPF-insecticides interactions were analyzed using  $\chi^2$  test following methods of McVay et al. (1977), if there was a significant effect of treatments on white grub mortality. To do that, first the expected

interaction mortality value,  $M_E$ , for combined agents was calculated using the formula  $M_E = M_F + M_I (1 - M_F/100)$  where  $M_F$  is observed percent mortalities caused by the EPF, and  $M_I$  is observed percent mortalities by insecticides. Then,  $\chi^2$  value was calculated using formula  $\chi^2 = (M_{FI} - M_E)^2 / M_E$ , where  $M_{FI}$  is the observed mortality for the EPF-Insecticides combinations and  $M_E$  is expected interaction mortality value. Finally, the calculated  $\chi^2$  value was compared with  $\chi^2$  table value at 1 df. A non-additive effect was considered to have occurred if calculated  $\chi^2$  value is greater than  $\chi^2$  table value. A synergistic interaction was believed to have occurred if  $M_{FI} - M_E$  is positive and an antagonistic interaction is believed to have occurred if  $M_{FI} - M_E$  is Negative (Antagonistic) (McVay et al. 1977).

## Results

### Laboratory Bioassay:

There was a significant effect of treatment on mortality of fall-collected white grubs ( $F = 2.82$ ,  $df = 8, 24$ ,  $P < 0.05$ ). The combined application of  $\frac{1}{2}$  rate of chlorantraniliprole plus *B. bassiana* caused the highest (55%) grub mortality four weeks after treatment (Fig. 2.2).

However, there was no significant treatment effect on grub mortality for spring-collected white grubs ( $F = 1.77$ ,  $df = 14, 42$ ,  $P > 0.05$ ). Mortality ranged from 15 to 40% among treatments, and was not different than the water control (Table 2.1).

In the fall experiment, both rates ( $\frac{1}{2}$  or  $\frac{1}{4}$ ) of chlorantraniliprole applied with *B. bassiana* resulted in an additive interaction. The  $\frac{1}{4}$  rate of chlorantraniliprole applied with *M. brunneum* resulted in an additive interaction. However, the half rate of chlorantraniliprole applied with *M. brunneum* resulted in an antagonistic interaction (Table 2.2).

### Greenhouse Experiment:

There was no significant effect of treatments on grub mortality ( $F=0.58$ ,  $df=14, 42$ ,  $P>0.05$ ) in 2013 and ( $F=1.25$ ,  $df=14, 42$ ,  $P>0.05$ ) in 2014. Among the various treatments, the highest percent mortality of 50% was observed for  $\frac{1}{2}$  or  $\frac{1}{4}$  rates of chlorantraniliprole in 2013 (Table 2.1). In 2014, the highest mortality of 57.5% was observed for  $\frac{1}{2}$  rate of imidacloprid (Table 2.1). All other treatments including combined applications of imidacloprid with both EPF resulted in numerically lower percent mortality than the single treatments of imidacloprid (Table 2.1).

### **Field Experiment:**

At the Blacksburg site (Table 2.3), white grub densities were composed primarily of masked chafer, *Cyclocephala* spp. and were moderate to moderately low (mean grub numbers of 5.0 grubs/0.092 m<sup>2</sup> in the fall, and 0.75 grubs/0.092 m<sup>2</sup> in the spring). There was no significant difference among the treatments for both the April ( $F = 0.77$ ,  $df = 14, 42$ ,  $P > 0.05$ ) and October trials ( $F = 0.78$ ,  $df = 14, 42$ ,  $P > 0.05$ ; Table 5). However, there was a significant difference among the treatments for the July applications ( $F = 2.01$ ,  $df = 14, 42$ ,  $P < 0.05$ ). Significant reductions in white grub numbers occurred for  $\frac{1}{2}$  rates of chlorantraniliprole or imidacloprid,  $\frac{1}{4}$  rates of chlorantraniliprole,  $\frac{1}{2}$  rate of chlorantraniliprole + *M. brunneum*, and  $\frac{1}{2}$  rate of imidacloprid + *B. bassiana* compared with the untreated control ( $P<0.05$ ).

At the Tazewell site (Table 2.4), there was no significant effect of treatment for the October ( $F = 1.3$ ,  $df = 14, 42$ ,  $P > 0.05$ ) application; however, there was a significant difference among treatments for both April ( $F = 2.22$ ,  $df = 14, 42$ ,  $P < 0.05$ ) and July ( $F = 2.97$ ,  $df = 14, 42$ ,  $P < 0.05$ ) trials. In April, none of the treatments resulted in significantly fewer grubs than the control ( $P>0.05$ ). In June, however,  $\frac{1}{2}$  rates of imidacloprid,  $\frac{1}{4}$  rates of imidacloprid,  $\frac{1}{2}$  or  $\frac{1}{4}$  of the imidacloprid applied in combination with *M. brunneum*,  $\frac{1}{2}$  or  $\frac{1}{4}$  rates of chlorantraniliprole

applied in combinations with either of *M. brunneum* or *B. bassiana* resulted in significantly fewer white grubs than the untreated control ( $P>0.05$ ).

**Effects of insecticides on *Beauveria bassiana* or *Metarhizium brunneum* viability and infection of *Galleria mellonella*:**

There was no significant effect of insecticides on number of colony forming units for *B. bassiana* ( $F=0.89$ ,  $df=4$ ,  $20$ ,  $P>0.5$ ) and for *M. brunneum* ( $F=0.23$ ,  $df=4$ ,  $15$ ,  $P>0.5$ ) (Table 2.5).

There was a significant effect of treatments on fungal infection of *G. mellonella* larvae by *B. bassiana* ( $F=55.9$ ,  $df=5$ ,  $24$ ,  $P<0.05$ ) or *M. brunneum* ( $F=60.54$ ,  $df=5$ ,  $24$ ,  $P<0.05$ ). However, there was no significant difference in fungal infected *G. mellonella* larvae for EPF alone or EPF plus insecticide treatments for either of the EPF species ( $P>0.5$ ). In all treatments, single or combined, *B. bassiana* and *M. brunneum* caused more than 85% fungal infection (Table 2.6). No fungal infection was observed for water treated control treatments.

**Discussion:**

None of the stand-alone or combined applications of EPF and insecticide resulted in  $>60\%$  mortality of third instar grubs in any of the laboratory or greenhouse experiments (Fig. 2.2, Tables 2.1). No synergistic interactions were observed among any of the treatment combinations of insecticides and EPF, even in the trials that resulted in relatively higher mortality of grubs (Table 2.2). The mortality of third instar grubs in response to any of the treatments was relatively low. The efficacies of all the treatments generally decreased in the greenhouse experiment relative to the laboratory experiment. In the greenhouse trials there was very low mortality of grubs for all treatments and no effort was made to determine the interactions among EPF and insecticide for those trials. In the field, some combined treatments of reduced rates of either

imidacloprid or chlorantraniliprole with *B. bassiana* or *M. brunneum* resulted in significant reduction of white grub numbers in turf (Table 2.3 and Table 2.4). In both of the field experiments, however, when each of the insecticides imidacloprid or chlorantraniliprole was applied alone at ¼ rates of recommended rates or as combined treatment with EPF in June, when beetles are active, reduced white grub densities comparable to recommended rates of both insecticides (1/2 rate). This indicated how effective these insecticides can be if applied during summer.

Combined applications of two control agents sometimes results in synergistic efficacies, efficacy greater than the sum of the efficacies of the two control agents applied as stand-alone applications. However, interactions of combined treatments appear to be affected by factors including control agents used in the study and species and life stages of insects used in the study. Synergistic interactions of imidacloprid and chlorantraniliprole with entomopathogenic nematodes have been reported against white grubs (Koppenhöfer and Kaya 1998, Koppenhöfer and Fuzzy 2008). However, combined application of two different control agents does not always result in synergistic interactions (Mannion et al. 2000). Antagonistic interactions between imidacloprid and *B. bassiana* have been reported against mole crickets (Thompson and Brandenburg 2006).

This study showed that single applications of insecticides also resulted in very low percentage mortality of grubs (Tables 2.1, Fig. 2.2,). The lower percent mortality observed for insecticides in the laboratory and greenhouse studies indicate that stand alone treatments of these insecticides are not effective against mature *Cyclocephala* spp. grubs. The insecticides used in this study have long residual effect and are applied as preventive white grub control products. Though no effort was made to compare the differences in mortality of grubs for the same

insecticide treatments among different studies, it appears that the mortality of grubs was relatively reduced in the trial with overwintered grubs (Table 2.1) compared to the trial with non-overwintered grubs (Fig. 2.2). We believe that susceptibility of overwintered grubs to insecticides that target immature grubs was reduced with the increase in age of white grubs. We also found a relatively low percent mortality of third instar *Cyclocephala* spp. grubs for *B. bassiana* or *M. brunneum* stand-alone treatments which confirm that *B. bassiana* or *M. brunneum* are not very effective against mature grubs of this species. The susceptibility of white grubs to EPF varies with the species. A laboratory study with four different species of white grubs showed that third instars of *Anomala orientalis* (Waterhouse) often were much more susceptible to either *B. bassiana* or *M. anisopliae sensu lato* than *P. japonica* larvae (Morales-Rodriguez et al. 2010). *Amphimallon majale* (Razoumowsky) and *Maladera castanea* (Arrow) were moderately susceptible to those fungi. Previous studies have also found that *B. bassiana* and *M. brunneum* do not result in very high mortality of *Cyclocephala* spp. third instars (Wu et al. 2014). It is not exactly known why *Cyclocephala* spp. third instar grubs are less susceptible to EPF. However, it is possible that they also can avoid contacting EPF conidia by avoiding area with high concentration of EPF in the soil as reported in *P. japonica* grubs (Villani et al. 1994).

We did not find significant increases in efficacies of combined application of insecticide and EPF compared to single applications of either treatment category in any field trials (Table 2.3 and Table 2.4). This lack of increase in efficacy of combined applications of insecticides and EPF or low efficacies of EPF single treatments should not be considered a determining factor for the use of EPF in the field. Unlike many other chemical insecticides, EPF may not be effective against a pest immediately, but rather, they may persist in the field for a long time by reproducing on their own, thus reducing pest populations over time. For example, Milner et al.

(2003) reported that conidia of *M. anisopliae sensu lato* applied to a sugarcane field in Australia persisted for about 3 years. Rath et al. (1995) also reported that *M. anisopliae sensu lato* persisted in a pasture for 4 years. There are examples of successful pest control programs using *M. anisopliae*-based biopesticides in some systems when a well-planned large scale application is made (FAO Media Centre 2009). Improvement of application technology could enhance the efficacy of EPF in the field. Behle et al. (2015) found that the efficacy of *M. brunneum* strain 52 against *P. japonica* grubs in the field was significantly improved when applied as microsclerotia.

The insecticides used at the rates in this study did not cause direct negative effects on *B. bassiana* or *M. brunneum* viability (Table 2.5) and virulence (Table 2.6). In fact, in the fall 2012 laboratory trial, one grub in the *B. bassiana* plus ½ rate of chlorantraniliprole treatment and one grub in the *M. brunneum* plus ¼ imidacloprid treatment were found to be infected with the respective fungi (Fig. 2.3), indicating that EPF were virulent in insecticide treated soil . This finding is important because it suggests that imidacloprid or chlorantraniliprole are not harmful to natural populations of *B. bassiana* or *M. brunneum* in the soil. *Beauveria bassiana* and *M. brunneum* have been reported to naturally occur in agricultural habitats (Bidochka et al. 1998). However, the lack of increase in grub mortality for combined EPF plus insecticide treatments compared to stand-alone treatments of either EPF or insecticide in the one laboratory and both greenhouse study indicates that third instar *Cyclocephala* spp. grubs either tolerated EPF or avoided contacting EPF.

In summary, combinations of imidacloprid or chlorantraniliprole with *B. bassiana* or *M. brunneum* were not very effective against third instar *Cyclocephala* spp. grubs. However, these insecticides appear to be compatible with *B. bassiana* or *M. brunneum*, suggesting that these insecticides can be applied in combination with *B. bassiana* or *M. brunneum* in other systems.



The lower efficacies of *B. bassiana* or *M. brunneum* when applied in combination with imidacloprid and chlorantraniliprole are possibly due to the inability of these insecticides to weaken the immune systems of mature grubs sufficiently to make them more susceptible to EPF. Using organophosphate or carbamate insecticides that are more effective against more mature grubs in combinations with EPF might result in better control of third instar grubs. Thus, future study on combined EPF plus insecticide applications for white grub control should consider using insecticides that are more effective against mature grubs.

## References:

- Behle, R. W., D. S. Richmond, M. A. Jackson, and C. A. Dunlap. 2015. Evaluation of *Metarhizium brunneum* F52 (Hypocreales: Clavicipitaceae) for control of Japanese beetle larvae in turfgrass. *Journal of Economic Entomology* 108: 1587–1595.
- Bidochka, M. J., J. E. Kasperski, and G. A. Wild. 1998. Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Canadian Journal of Botany* 76:1198–1204.
- Dimock, W. J. 2004. Spatial factors affecting white grub presence and abundance in golf course turf. Ph.D. dissertation, Virginia Polytechnic Institute and State University, Blacksburg, VA.
- FAO Media Centre. 2009. Red locust disaster in eastern Africa prevented: Biopesticides being used on a large scale. <http://www.fao.org/news/story/en/item/21084/icode/>
- Gyawaly, S., A. M. Koppenhöfer, S. Wu and T. P. Kuhar. 2016. Biology, ecology and management of masked chafer grubs in turfgrass. *Journal of Integrated Pest Management* 7: 1–11.
- Gyawaly, S., R. R. Youngman, C. A. Laub, and T. P. Kuhar. 2015. Evaluation of insecticide application timings against white grubs in cool season turfgrass in Virginia, 2013. *Arthropod Management Tests* 40: G10.
- Hiromori, H. and J. Nishigaki. 2001. Factor analysis of synergistic effect between the entomopathogenic fungus, *Metarhizium anisopliae* and synthetic insecticides. *Applied Entomology and Zoology* 36: 231–236.

- Koppenhöfer, A. M., and E. M. Fuzy. 2008. Effects of chlorantraniliprole on *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) efficacy against and reproduction in white grubs (Coleoptera: Scarabaeidae). *Biological Control* 45: 93–102.
- Koppenhöfer A. M. and H. K. Kaya. 1998. Synergism of imidacloprid and entomopathogenic nematodes: A novel approach to white grub control in turfgrass. *Journal of Economic Entomology* 91: 618–623.
- Lacey, L. A., and W. M Brooks. 1997. Initial handling and diagnosis of diseased insects, pp. 1–16. *In* L. A. Lacey (ed.), *Manual of Techniques in Insect Pathology*. Academic Press, San Diego, CA.
- Laub, C., S. Gyawaly, and T. Kuhar. 2016. Virginia turfgrass insect management survey. Virginia Cooperative Extension Publication. ENTO-219NP.  
<http://www.pubs.ext.vt.edu/ENTO/ENTO-219/ENTO-219-PDF.pdf>
- Mannion, C., H. Winkler, D. Shapiro, and T. Gibb. 2000. Interaction of halofenozide and entomopathogenic nematodes for control of Japanese beetle grubs. *Journal of Economic Entomology* 93: 48–53.
- McVay, J. R., T. T. Gudauskas, and J. D. Harper. 1977. Effect of *Bacillus thuringiensis* and chemical insecticides on *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* 77: 590–885.
- Milner, R. J., P. Samson, and R. Morton. 2003. Persistence of conidia of *Metarhizium anisopliae* in sugarcane fields: effect of isolate and formulation on persistence over 3.5 years. *Biocontrol Science and Technology* 13: 507–516.

- Morales-Rodriguez, A., and D. C. Peck. 2009. Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale* and *Popillia japonica*. *Biological Control* 51: 169–180.
- Morales-Rodriguez, A., A. Ospina, and D. C. Peck. 2010. Variation in the laboratory susceptibility of turf-infesting white grubs (Coleoptera: Scarabaeidae) to biological, biorational and chemical control products. *Pest Management Science* 66: 90–99.
- Potter, D. A. 1998. *Destructive turfgrass insects: biology, diagnosis, and control*. Ann Arbor Press, Chelsea, MI.
- Potter, D. A. 2005. Prospects for managing destructive turfgrass insects without protective chemicals. *International Turfgrass Society Research Journal* 10: 42–54.
- Quintela, E. D., and C. W. McCoy. 1998. Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *Journal of Economic Entomology* 91: 110–122.
- Rath, A. C., D. Worledge, T. B. Koen, and B. A. Rowe. 1995. Long-term field efficacy of the entomogenous fungus *Metarhizium anisopliae* against the subterranean scarab, *Adoryphorus couloni*. *Biocontrol Science and Technology* 5: 439–452.
- Thompson, S. R., and R. L. Brandenburg. 2006. Effect of combining imidacloprid and diatomaceous earth with *Beauveria bassiana* on mole cricket (Orthoptera: Gryllotalpidae) mortality. *Journal of Economic Entomology* 99: 1948–1954.
- Villani, M. G., S. R. Krueger, P. C. Schroeder, F. Consolie, N. H. Consolie, L. M. Preston-Wilsey, and D. W. Roberts. 1994. Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. *Environmental Entomology* 23: 502–513.

Wu, S., R. R. Youngman, L. T. Kok, C. A. Laub, and D. G. Pfeiffer. 2014. Interaction between entomopathogenic nematodes and entomopathogenic fungi applied to third instar southern masked chafer white grubs, *Cyclocephala lurida* (Coleoptera: Scarabaeidae), under laboratory and greenhouse conditions. *Biological Control* 76: 65–73.

Table 2.1. Effect of single or combined applications of insecticides and entomopathogenic fungi on the mortality (Mean  $\pm$ SE) of overwintered or non-overwintered third instar *Cyclocephala* spp. grubs in the laboratory or greenhouse 4 wk after treatment (One-way ANOVA).

Treatments	% mortality (mean $\pm$ SE) of white grubs		
	Overwintered grubs in the laboratory (spring 2013)	Non-overwintered grubs in the greenhouse (fall 2013)	Non-overwintered grubs in the greenhouse (fall 2014)
chlorantraniliprole ( $\frac{1}{2}$ rate) <sup>a</sup>	25.0 $\pm$ 7.5	50.0 $\pm$ 13.6	35.0 $\pm$ 2.5
chlorantraniliprole ( $\frac{1}{4}$ rate) <sup>b</sup>	12.5 $\pm$ 4.1	50.0 $\pm$ 7.9	35.0 $\pm$ 5.5
imidacloprid ( $\frac{1}{2}$ rate) <sup>a</sup>	15.0 $\pm$ 2.5	45.0 $\pm$ 7.5	57.5 $\pm$ 5.4
imidacloprid ( $\frac{1}{4}$ rate) <sup>b</sup>	17.5 $\pm$ 2.1	40.0 $\pm$ 3.5	42.5 $\pm$ 6.4
<i>M. brunneum</i> (Met 52)	37.5 $\pm$ 6.4	47.5 $\pm$ 15.5	47.5 $\pm$ 11.3
<i>B. bassiana</i> (BotaniGard)	40.0 $\pm$ 6.1	42.5 $\pm$ 11.3	47.5 $\pm$ 4.1
chlorantraniliprole ( $\frac{1}{2}$ rate) + <i>M. brunneum</i>	20.0 $\pm$ 5.0	22.5 $\pm$ 5.4	27.5 $\pm$ 7.3
chlorantraniliprole ( $\frac{1}{4}$ rate) + <i>M. brunneum</i>	15.0 $\pm$ 2.5	47.5 $\pm$ 12.4	42.5 $\pm$ 8.9
imidacloprid ( $\frac{1}{2}$ rate) + <i>M. brunneum</i>	12.5 $\pm$ 8.1	42.5 $\pm$ 10.2	47.5 $\pm$ 5.4
imidacloprid ( $\frac{1}{4}$ rate) + <i>M. brunneum</i>	15.0 $\pm$ 2.5	27.5 $\pm$ 11.9	30.0 $\pm$ 7.9
chlorantraniliprole ( $\frac{1}{2}$ rate) + <i>B. bassiana</i>	20.0 $\pm$ 7.0	40.0 $\pm$ 6.1	37.5 $\pm$ 2.1
chlorantraniliprole ( $\frac{1}{4}$ rate) + <i>B. bassiana</i>	22.5 $\pm$ 7.3	35.0 $\pm$ 10.3	35.0 $\pm$ 7.5
imidacloprid ( $\frac{1}{2}$ rate) + <i>B. bassiana</i>	15.0 $\pm$ 2.5	40.0 $\pm$ 10.6	40.0 $\pm$ 6.12
imidacloprid ( $\frac{1}{4}$ rate) + <i>B. bassiana</i>	12.5 $\pm$ 5.4	20.0 $\pm$ 12.2	22.5 $\pm$ 8.19
water treated control	15.0 $\pm$ 5.5	40.0 $\pm$ 9.3	22.5 $\pm$ 5.4
Treatment effect	non-significant ( $P>0.05$ )	non-significant ( $P>0.05$ )	non-significant ( $P>0.05$ )

<sup>a</sup>  $\frac{1}{2}$  rate of the highest labelled rate of respective insecticide.

<sup>b</sup>  $\frac{1}{4}$  rate of the highest labelled rate of respective insecticide.

Table 2.2. Interaction of insecticides and entomopathogenic fungi in combined applications of ½ or ¼ rates of chlorantraniliprole and full rates of *Beauveria bassiana* or *Metarhizium brunneum* against non-overwintered 3<sup>rd</sup> instar *Cyclocephala* spp. grubs in the laboratory (Fall 2012).

Treatments	Observed mortality	Expected mortality	$\chi^2$	Interaction
<i>B. bassiana</i> + ½ chlorantraniliprole <sup>a</sup>	55.0	52.87	0.08	additive
<i>B. bassiana</i> + ¼ chlorantraniliprole <sup>b</sup>	32.5	36.56	0.43	additive
<i>M. brunneum</i> + ½ chlorantraniliprole	27.5	46.37	7.67	antagonistic
<i>M. brunneum</i> + ¼ chlorantraniliprole	30.0	27.81	0.17	additive

<sup>a</sup> ½ rate of the highest labelled rate of respective insecticide.

<sup>b</sup> ¼ rate of the highest labelled rate of respective insecticide.

Table 2.3. Field efficacy of single or combined treatments of insecticides and entomopathogenic fungi treatments on annual white grubs in turf in Montgomery Co., VA (One-way ANOVA).

Treatments	No. white grubs per 0.092 m <sup>2</sup> (mean ± SE)		
	(April application)	(July application)	(Oct application)
chlorantraniliprole (½ rate) <sup>a</sup>	0.00 ± 0.00	0.00 ± 0.00 a	2.50 ± 1.44
chlorantraniliprole (¼ rate) <sup>b</sup>	0.75 ± 0.37	0.25 ± 0.25 a	6.00 ± 0.61
imidacloprid (½ rate) <sup>a</sup>	0.50 ± 0.25	0.25 ± 0.25 a	4.25 ± 0.65
imidacloprid (¼ rate) <sup>b</sup>	0.25 ± 0.12	0.75 ± 0.47 ab	4.75 ± 1.85
<i>M. brunneum</i> (Met 52)	0.00 ± 0.00	1.00 ± 0.40 ab	3.25 ± 1.08
<i>B. bassiana</i> (BotaniGard)	0.25 ± 0.12	0.75 ± 0.25 ab	4.50 ± 1.52
chlorantraniliprole (½ rate) + <i>M. brunneum</i>	0.5 ± 0.25	0.00 ± 0.00 a	4.25 ± 1.02
chlorantraniliprole (¼ rate) + <i>M. brunneum</i>	0.00 ± 0.00	1.50 ± 1.19 ab	3.25 ± 1.19
imidacloprid (½ rate) + <i>M. brunneum</i>	0.25 ± 0.12	0.75 ± 0.75 ab	3.75 ± 0.89
imidacloprid (¼ rate) + <i>M. brunneum</i>	0.00 ± 0.00	0.50 ± 0.50 ab	4.00 ± 1.37
chlorantraniliprole (½ rate) + <i>B. bassiana</i>	0.00 ± 0.00	0.50 ± 0.28 ab	2.50 ± 1.15
chlorantraniliprole (¼ rate) + <i>B. bassiana</i>	0.00 ± 0.00	2.25 ± 0.47 b	3.75 ± 0.54
imidacloprid (½ rate) + <i>B. bassiana</i>	0.25 ± 0.12	0.00 a	2.25 ± 0.89
imidacloprid (¼ rate) + <i>B. bassiana</i>	0.25 ± 0.12	1.25 ± 0.62 ab	6.25 ± 1.56
untreated control	0.75±0.37	1.75 ± 0.86 b	5.00 ± 0.93
Treatment effect	non-significant ( <i>P</i> >0.05)	significant ( <i>P</i> <0.05)	non-significant ( <i>P</i> >0.05)

Means within a column with same letter are not significantly different, Tukey's test (*P*< 0.05).

<sup>a</sup> ½ rate of the highest labelled rate of respective insecticide.

<sup>b</sup> ¼ rate of the highest labelled rate of respective insecticide.



Table 2.4. Field efficacy of single or combined applications of insecticides and entomopathogenic fungi on annual white grubs in turf in Tazewell Co., VA (One-way ANOVA).

Treatments	No. white grubs per 0.092 m <sup>2</sup> (mean ± SE)		
	(April application)	(July application)	(Oct application)
chlorantraniliprole (½ rate) <sup>a</sup>	10.5 ± 2.1 ab	2.0 ± 1.5 ab	5.3 ± 1.7
chlorantraniliprole (¼ rate) <sup>b</sup>	6.0 ± 2.6 ab	1.8 ± 1.5 ab	4.0 ± 1.6
imidacloprid (½ rate) <sup>a</sup>	7.5 ± 2.5 ab	0.0 ± 0.0 a	3.8 ± 0.7
imidacloprid (¼ rate) <sup>b</sup>	10.5 ± 0.8 ab	1.3 ± 0.4 a	4.3 ± 1.3
<i>M. brunneum</i> (Met 52)	11.5 ± 2.6 ab	10.0 ± 3.24c	3.5 ± 1.7
<i>B. bassiana</i> (BotaniGard)	8.8 ± 1.3 ab	4.8 ± 2.1 ab	3.8 ± 1.3
chlorantraniliprole (½ rate) + <i>M. brunneum</i>	10.3 ± 1.6 ab	0.3 ± 0.2 a	2.5 ± 1.2
chlorantraniliprole (¼ rate) + <i>M. brunneum</i>	8.5 ± 1.0 ab	0.3 ± 0.2 a	4.3 ± 0.9
imidacloprid (½ rate) + <i>M. brunneum</i>	8.3 ± 2.5 ab	1.0 ± 0.5 a	8.3 ± 1.3
imidacloprid (¼ rate) + <i>M. brunneum</i>	6.0 ± 1.4 ab	0.8 ± 0.7 a	7.0 ± 1.6
chlorantraniliprole (½ rate) + <i>B. bassiana</i>	5.8 ± 1.6 ab	0.0 ± 0.0 a	5.3 ± 0.9
chlorantraniliprole (¼ rate) + <i>B. bassiana</i>	13.8 ± 1.8 b	0.8 ± 0.7 a	8.5 ± 2.6
imidacloprid (½ rate) + <i>B. bassiana</i>	4.0 ± 0.0 a	4.3 ± 3.4 ab	5.5 ± 2.4
imidacloprid (¼ rate) + <i>B. bassiana</i>	6.5 ± 1.8 ab	2.0 ± 0.8 ab	4.0 ± 0.4
untreated control	5.0 ± 3.2 ab	6.3 ± 1.8 b	7.0 ± 1.5
Treatment effect	significant ( <i>P</i> <0.05)	significant ( <i>P</i> <0.05)	non-significant ( <i>P</i> >0.05)

Means within a column with same letter are not significantly different, Tukey's test (*P* < 0.05).

<sup>a</sup> ½ rate of the highest labelled rate of respective insecticide.

<sup>b</sup> ¼ rate of the highest labelled rate of respective insecticide.

Table 2.5. Number of colony forming units of *Metarhizium brunneum* or *Beauveria bassiana* formed in insecticide treated or untreated culture media (One-way ANOVA).

Treatments	Numbers of fungal colony forming units (mean $\pm$ SE) per Petri plate	
	<i>M. brunneum</i>	<i>B. bassiana</i>
Control	264.75 $\pm$ 23.33	328.40 $\pm$ 11.46
chlorantraniliprole (1/2 rate) <sup>a</sup>	297.25 $\pm$ 27.21	314.40 $\pm$ 17.15
chlorantraniliprole (1/4 rate) <sup>b</sup>	269.00 $\pm$ 2.89	303.80 $\pm$ 14.68
imidacloprid (1/2 rate) <sup>a</sup>	286.75 $\pm$ 6.77	301.00 $\pm$ 3.02
imidacloprid (1/4 rate) <sup>b</sup>	280.75 $\pm$ 37.27	308.00 $\pm$ 3.80
Treatment effect	non-significant ( $P > 0.05$ )	non-significant ( $P > 0.05$ )

<sup>a</sup> 1/2 rate of the highest labelled rate of respective insecticide.

<sup>b</sup> 1/4 rate of the highest labelled rate of respective insecticide.

Table 2.6. Percentage (Mean±SE) of *Galleria mellonella* larvae infected by *Metarhizium brunneum* or *Beauveria bassiana* harvested from insecticide treated or untreated culture media (One-way ANOVA).

Treatments	Percentage (mean ± SE) of <i>G. mellonella</i> larvae showing infection	
	<i>M. brunneum</i> infection	<i>B. bassiana</i> infection
Control	0.0 ± 0.0 b	0.0 ± 0.0 b
No insecticide	94.0 ± 2.4 a	90.0 ± 3.1 a
chlorantraniliprole (½ rate) <sup>a</sup>	92.0 ± 3.7 a	94.0 ± 4.0 a
chlorantraniliprole (¼ rate) <sup>b</sup>	94.0 ± 4.0 a	88.0 ± 3.7 a
imidacloprid (½ rate) <sup>a</sup>	92.0 ± 2.0 a	92.0 ± 2.0 a
imidacloprid (¼ rate) <sup>b</sup>	94.0 ± 4.0 a	88.0 ± 3.7 a
Treatment effect	significant ( $P < 0.05$ )	significant ( $P < 0.05$ )

Means within a column with same letter are not significantly different, Tukey's test ( $P < 0.05$ ).

<sup>a</sup> ½ rate of the highest labelled rate of respective insecticide.

<sup>b</sup> ¼ rate of the highest labelled rate of respective insecticide.

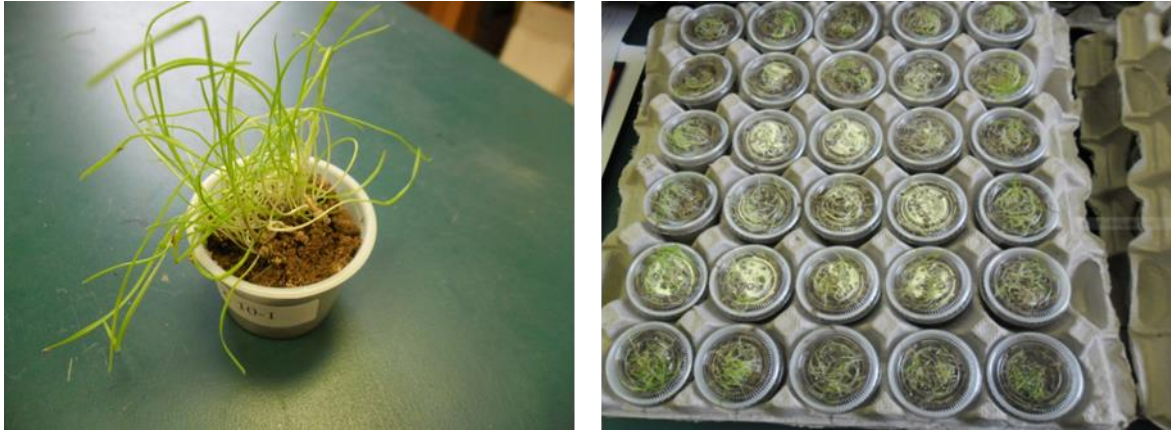


Fig. 2.1. Experimental arena for effect of combined application of entomopathogenic fungi and insecticides in laboratory experiments.

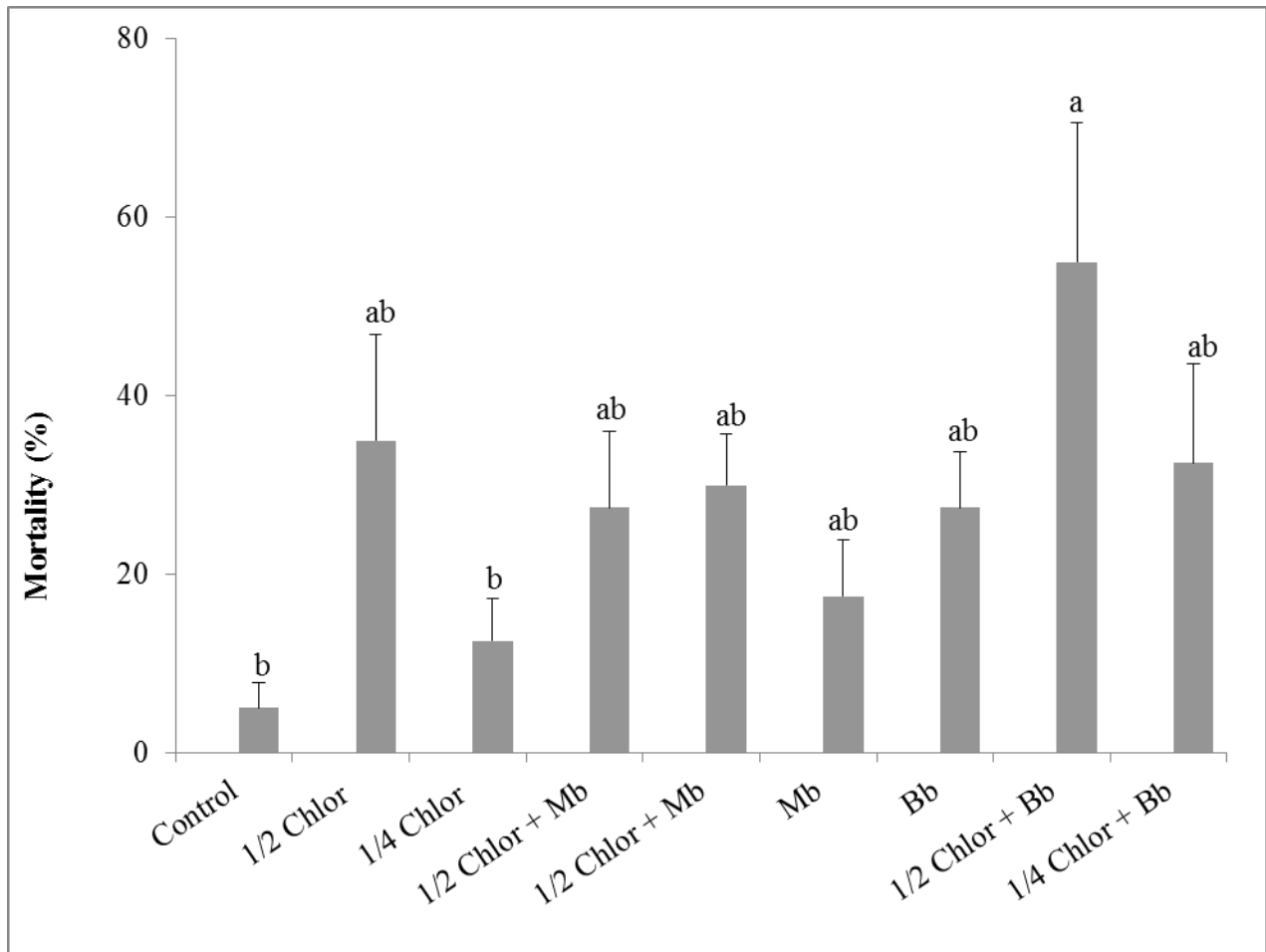


Fig.2.2. Effect of single or combined treatments of insecticide and entomopathogenic fungi on the mortality (Mean  $\pm$ SE) of non-overwintered third instar *Cyclocephala* spp. grubs 4 wk after treatment (One-way ANOVA). Means with the same letter are not significantly different (Tukey's test,  $P < 0.05$ ).

( $\frac{1}{2}$  Chlor=  $\frac{1}{2}$  rate of the highest labelled rate of chlorantraniliprole;  $\frac{1}{4}$  Chlor=  $\frac{1}{2}$  rate of the highest labelled rate of chlorantraniliprole; Mb= *M. brunneum*; Bb= *B. bassiana*)

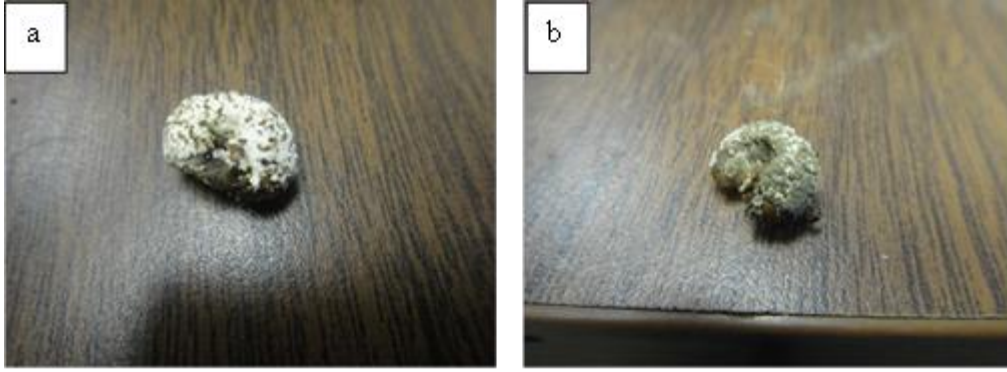


Fig. 2.3. Third instar *Cyclocephala* spp. grubs infected by, a) *B. bassiana*, b) *M. brunneum* in combined treatments with imidacloprid or chlorantraniliprole in the laboratory experiment.

## Chapter 3

### Effects of entomopathogenic fungi and insecticides on *Popillia japonica* (Coleoptera: Scarabaeidae) oviposition

#### Abstract

In the northeastern U.S. including Virginia, Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), is one of the major species of white grubs that attacks cool season turfgrass. For effective control, commercial insecticides or entomopathogenic fungi are typically applied during the summer to target the small (early instar) white grubs that are feeding near the soil surface. In addition to directly killing grubs, insecticides may affect oviposition by these beetles. Greenhouse studies were conducted to examine the effect of combined applications of two insecticides, imidacloprid and chlorantraniliprole, and two species of entomopathogenic fungi, *Metarhizium brunneum* (Petch) and *Beauveria bassiana* (Balsamo) Vuillemin, on *P. japonica* oviposition behavior. Treatments included two rates of the insecticides (one-quarter and one-half of the label recommended rate), the full label recommended rates of both fungi species, and each combination of insecticide plus fungi. For each treatment the number of eggs laid and depth of eggs in the soil was determined. Results showed that *P. japonica* females laid eggs mostly in the upper 4 cm of soil irrespective of the applied treatments. Both rates of imidacloprid either applied as a single treatment or as part of a combined treatment with EPF significantly reduced the number of eggs laid compared with untreated control ( $P < 0.05$ ).

Key words: Japanese beetle, entomopathogenic fungi, oviposition, chlorantraniliprole, imidacloprid

## **Introduction:**

Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), is a major turfgrass pest in the eastern U.S. (Potter 1998). Insecticides, both synthetic chemicals and/or biological organisms such as entomopathogenic fungi (EPF), are commonly applied for preventive control during the summer months before white grub (larval) densities can build to damaging levels.

Two of the most popular preventative control insecticides used on turfgrass in Virginia today are the neonicotinoid imidacloprid and the diamide chlorantraniliprole (Laub et al. 2016). Each of these insecticides have been shown to be effective at controlling white grub species (Heller et al. 2008a,b; Shetlar and Andon 2013; Gyawaly et al. 2015). Optimal activity for these insecticide products generally can be expected when applied near the time of peak egg-laying activity of the adults. However, earlier applications are often chosen because rainfall (necessary to move products into soil if no irrigation are available) patterns are more reliable earlier in the season, or because it may be easier to coordinate applications with other management activities.

In addition to directly killing insects, insecticides, both chemical and biologicals, applied for pest management also may affect insect behavior. Sub-lethal effects of entomopathogenic fungi on insect pests have been studied in various systems. Fargues et al. (1991) found that significantly fewer eggs were oviposited by Colorado potato beetle, *Leptinotarsa decimlineata* Say, females that survived fungal treatments as 4th instar at 22°C. Scarabaeid adult females also have been reported to respond to the presence of EPF in the soil. For example, Rath et al. (1995) reported a 75% decrease in total number of eggs laid by *Adoryphorus couloni* (Burmeister) females when exposed to  $10^6$  spores concentrations of the EPF *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae sensu lato* or *M. flavoviride* Gams & Roszypal per gram of soil. However, this effect appears to be affected by concentration of the EPF spores in the soil, as



these same authors found no effect at  $10^4$  spores per gram of soil. In contrast, *M. anisopliae sensu lato* has been found to cause *P. japonica* adults to oviposit more eggs in soil with higher spore concentration of fungi (Villani et. al. 1994).

Chemical insecticides also can affect oviposition. Imidacloprid has been found to significantly reduce total numbers of eggs deposited by the brown planthopper, *Nilaparvata lugens* Stål, compared to untreated control treatments (Bao et al. 2009). Another insecticide, chlorantraniliprole, also has been shown to reduce fecundity, egg hatch and survival of neonates of diamondback moth (Lepidoptera: Plutellidae) (Han et al. 2012).

In this study, I examined how combined applications of *B. bassiana* or *M. brunneum* (Petch) [restored species name for some isolates of *M. anisopliae* (Metchnikoff) Sorokin including strain F52] with chemical insecticides affect egg laying of *P. japonica* in potted turf bioassays in the greenhouse.

## **Materials and Methods:**

### **Entomopathogenic fungi:**

Formulations of *B. bassiana* GHA strain commercially sold as BotaniGard emulsifiable suspension (Laverlam International Co., Butte, MT) and emulsifiable concentrate of *M. brunneum* 52 strain sold as Met52 (Novozymes Biologicals Inc., Salem, VA), were used. Botanigard ES contains  $2.1 \times 10^{10}$  viable spores/ml. Met52 EC contains  $5.0 \times 10^9$  conidia per ml with 60 % germination rate. Unless otherwise mentioned, the highest application rates of BotaniGard (25.6 L/ha), and Met 52 (9.6 L/ha) were used.

### **Insecticides:**

Commercial formulations of imidacloprid (Merit 75 WP, Bayer CropScience, Research Triangle Park, NC) and chlorantraniliprole (Acelepryn 1.6 SC, Syngenta Crop Protection, Greensboro,

NC) were used in the experiments. The field rates for Merit and Acelepryn in turfgrass are 448.1– 602.1 g product/ha and 584.1–1166.3 ml product/ha, respectively. For this study, we used 1/2 and 1/4 rates of the highest recommended rate of the insecticides.

### **Greenhouse Experiment:**

In summer 2013, more than 1,000 adult *P. japonica* were collected from Kentland Research Farm near Blacksburg, VA using *P. japonica* traps baited with floral/sex dual lure (Trécé Inc., Adair, OK). The adults were kept in cages at room temperature for 3-5 d and fed fresh smartweed (*Polygonum* spp.) leaves until they were used in the experiment. Experiments were carried out in a greenhouse located at Virginia Tech in 20 cm long by 8 cm diameter PVC tubes (Fig. 3.1). The tubes were filled to approximately 15 cm height with sterilized top soil (sandy loam texture, 3.2 % organic matter, and 6.37 pH). The soil had been dried, pulverized, and screened to remove debris or plant remains, and then solarized by placing it under a plastic sheet in a greenhouse in summer for one month. Perennial ryegrass seeds were sown in each container 2 wk before the start of the experiment but were trimmed to 5 cm height to simulate a turf field for oviposition.

The experiment was arranged in a completely randomized design with 5 replicates and had 15 treatments, which included imidacloprid and chlorantraniliprole at 1/2 and 1/4 rates alone or in combination with *B. bassiana* or *M. brunneum*, each of the EPF treatments alone, and a water control (Table 1). The moisture content in the soil was maintained ca. 18% by volume. The average temperature in the greenhouse was 21°C. Insecticides were applied by pipette.

Approximately 24 h after application, five adult females were released in each container and the container was covered with fine mesh (Fig. 3.1). The tubes were sampled 10 d after treatment, and the number of eggs laid at different soil depths was recorded.

**Data analysis:**

The effect of treatment on number of *P. japonica* eggs laid was analyzed using ANOVA in JMP 11.0 (SAS, Cary, NC). Means were separated using Tukey's test at the 0.05 level of significance. All data were subjected to square root transformation to normalize variance, but untransformed means are presented.

**Results:**

For all treatments, *P. japonica* eggs were found mostly in the upper 4 cm of soil (Fig. 3.2, Table 3.1). There was a significant effect of treatment on the total number of *P. japonica* eggs recovered ( $F = 6.66$ ,  $df = 14, 56$ ,  $P < 0.05$ ). Both rates of imidacloprid as well as their combined application with either of the EPF resulted in significantly fewer eggs than the control ( $P > 0.05$ ; Table 3.1). The  $\frac{1}{2}$  rate of chlorantraniliprole combined with *B. bassiana* also resulted in fewer *P. japonica* eggs than the control. All other treatments containing chlorantraniliprole or treated with only *B. bassiana* or *M. brunneum* did not result in reduced numbers of *P. japonica* eggs compared to the control.

**Discussion:**

Insecticide applications to turfgrass do not only control the larval stage grubs, but also impact the total number of eggs deposited. In this study, applications of imidacloprid at  $\frac{1}{2}$  or  $\frac{1}{4}$  the recommended high label rate resulted in significantly fewer (80 to 90% reduction) *P. japonica* eggs deposited. These results are in contrast to George et al. (2007), who found that imidacloprid at 1 d or 7 d after application in the field at a rate comparable to the  $\frac{1}{2}$  label rate of imidacloprid used here, had no significant effect on the number of eggs deposited by *P. japonica*. The conflicting results may be a result of laboratory versus field experiments. George et al. (2007) applied the insecticide in the field and brought turf cores to the greenhouse after 1 or 8 d

exposure to use as oviposition sites. It is possible that imidacloprid degraded rapidly in the field or under these conditions. Moreover, the eggs in this study were sampled 10 d after introducing the beetles in the oviposition arena, whereas, George et al. (2007) sampled for eggs within 7 d after introducing the beetles. It is possible that more eggs would have been deposited if beetles were allowed to oviposit longer. Also, the condition and age of the *P. japonica* test subjects could also impact results.

In this study, applications of chlorantraniliprole, for the most part, did not appear to reduce egg laying in *P. japonica*. The reason for this is that chlorantraniliprole is primarily active via ingestion (Cordova et al. 2006), and it is unlikely that the *P. japonica* females did any feeding during the experiments. Imidacloprid on the other hand is active via contact as well as ingestion. Previously, significant reduction in oviposition by western cherry fruit fly, *Rhagoletis indifferens* Curran in imidacloprid treatments than in control, potentially due to mortality of the adults exposed to insecticide, has been reported (Yee 2008). In our study also, we observed that 100% beetles were killed in all treatments including imidacloprid whereas 2 live females were found in control treatments 10 days after treatment. However, a significant reduction in oviposition by *P. japonica* female only in imidacloprid treatments, in general, suggests that the contact toxicity of imidacloprid to adults might have had an effect on reduced oviposition in those treatments.

The EPF, *B. bassiana* or *M. brunneum* did not appear to reduce egg laying in *P. japonica* in this experiment. Many insects are able to detect the presence of EPF in an environment and may respond to an area with EPF presence differently (Thompson et al. 2007, George et al. 2013). Avoidance behavior of insects to entomopathogenic fungi in soil has been observed in mole crickets (Thompson et al. 2007) and white grubs (Villani et al 1994, Fry et al. 1997). In

contrast to avoidance behavior of mole crickets and *P. japonica* grubs, some insects might be attracted to EPF-infected insects or sites. Such insects include *Anopheles stephensi* Liston (Diptera: Culicidae) females, which are attracted to and become infected by *B. bassiana* spores, *B. bassiana* infected caterpillars, and *B. bassiana* wetted cloth (George et al. 2013), and *Monomorium pharaonis* L. (Hymenoptera: Formicidae) adults which are attracted to *M. brunneum* infected nests (Pontieri et al. 2014). Villani et al. (1994) also reported similar reaction of *P. japonica* adults to soil infested with *M. anisopliae sensu lato* where female beetles deposited significantly higher numbers of eggs. This study, however, did not find an increase in *P. japonica* oviposition in soil treated with either EPF; this may have been due to difference in the concentrations of EPF used in this study compared to Villani et al. (1994). Unfortunately, Villani et al. (1994) did not indicate the exact concentration of conidia concentration used in their study.

## References:

- Bao, H., S. Liu, J. Gu, X. Wang, X. Liang, and Z. Liu. 2009. Sublethal effects of four insecticides on the reproduction and wing formation of brown planthopper, *Nilaparvata lugens*. *Pest Management Science* 65: 170–174.
- Cordova, D., E. A. Benner, M. D. Sacher, J. J. Rauh, J. S. Sopa, G. P. Lahm, T.P. Selby, T.M. Stevenson, L. Flexner, S. Gutteridge, D.F. Rhoades, L. Wu, R. M. Smith, and Y. Tao. 2006. Anthranilic diamides: a new class of insecticides with a novel mode of action, ryanodine receptor activation. *Pesticide Biochemistry and Physiology* 84: 196–214.
- Fargues, J., J. C. Delmas, J. Augé, and R. A. Lebrun. 1991. Fecundity and egg fertility in the adult Colorado beetle (*Leptinotarsa decemlineata*) surviving larval infection by the fungus *Beauveria bassiana*. *Entomologia Experimentalis et Applicata* 61: 45–51.
- Fry, R. C., L. A. Fergusson-Kolmes, S. A. Kolmes, and M. G. Villani. 1997. Radiographic study of the response of Japanese beetle larvae (Coleoptera: Scarabaeidae) to soil-incorporated mycelial particles of *Metarhizium anisopliae* (Deuteromycetes). *Journal of the New York Entomological Society* 105: 113–120.
- George, J., C. T. Redmond, R. N. Royalty, and D. A. Potter. 2007. Residual effects of imidacloprid on Japanese beetle (Coleoptera: Scarabaeidae) oviposition, egg hatch, and larval viability in turfgrass. *Journal of Economic Entomology* 100: 431–439.
- George, J., N. E. Jenkins, S. Blanford, M. B. Thomas, and T. C. Baker. 2013. Malaria mosquitoes attracted by fatal fungus. *PLoS ONE* 8.
- Gyawaly, S., R. R. Youngman, C. A. Laub, and T. P. Kuhar. 2015. Evaluation of insecticide application timings against white grubs in cool season turfgrass in Virginia, 2013. *Arthropod Management Tests* 40: G10.

- Han, W., S. Zhang, F. Shen, M. Liu, C. Ren, and X. Gao. 2012. Residual toxicity and sublethal effects of chlorantraniliprole on *Plutella xylostella* (Lepidoptera: Plutellidae). *Pest Management Science* 68: 1184–1190.
- Heller, P. R., D. Kline, and A. Houseman. 2008a. Mid-April application of Acelepryn and Merit formulations to preventively suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G16.
- Heller, P. R., D. Kline, and A. Houseman. 2008b. Early May application of Acelepryn, Meridian, and Merit formulations to preventively suppress scarab white grubs, 2007. *Arthropod Management Tests* 33: G17.
- Laub, C., S. Gyawaly, and T. Kuhar. 2016. Virginia turfgrass insect management survey. Virginia Cooperative Extension Publication. ENTO-219NP.  
<http://www.pubs.ext.vt.edu/ENTO/ENTO-219/ENTO-219-PDF.pdf>
- Pontieri, L., S. Vojvodic, R. Graham, J. S. Pedersen, and T. A. Linksvayer. 2014. Ant colonies prefer infected over uninfected nest sites. *PLoS ONE* 9.
- Potter, D. A. 1998. Destructive turfgrass insects: biology, diagnosis, and control. Ann Arbor Press, Chelsea, MI.
- Rath, A. C., D. Worledge, G. C. Anderson, and C. J. Carr. 1995. Virulence of the entomogenous fungi *Metarhizium anisopliae* (Metschnikoff) Sorokin, *M. flavoviride* Gams and Rozsypal and *Beauveria bassiana* (Balsamo) Vuillemin to *Adoryphorus couloni* (Burmeister) (Coleoptera: Scarabaeidae). *Australian Journal of Entomology* 34: 181–186.
- Shetlar, D. J., and J. Andon. 2013. Preventive control of white grubs on a golf course rough, 2008. *Arthropod Management Tests* 38: G15.

- Thompson, S. R., R. L. Brandenburg, and G. T. Roberson. 2007. Entomopathogenic fungi detection and avoidance by mole crickets (Orthoptera: Gryllotalpidae). *Environmental Entomology* 36: 165–72.
- Villani, M. G., S. R. Krueger, P. C. Schroeder, F. Consolie, N. H. Consolie, L. M. Preston-Wilsey, and D. W. Roberts. 1994. Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. *Environmental Entomology* 23: 502–513.
- Yee, W. L. 2008. Effects of several newer insecticides and kaolin on oviposition and adult mortality in western cherry fruit fly (Diptera: Tephritidae). *Journal of Entomological Science*, 43: 177–190.



Table 3.1. Effect of single or combined applications of insecticides and entomopathogenic fungi treatments on number of eggs laid by *Popillia japonica* females caged on perennial ryegrass (One-way ANOVA).

Treatments	Eggs laid at < 4 cm depth per container (mean ± SE)	Eggs laid at > 4 cm depth per container (mean ± SE)	Total eggs recovered per container (mean ± SE)
chlorantraniliprole (½ rate) <sup>a</sup>	7.8 ± 3.27	0.0 ± 0.0	7.8 ± 3.26 ab
chlorantraniliprole (¼ rate) <sup>b</sup>	12.4 ± 4.03	0.0 ± 0.0	12.4 ± 4.03 b
imidacloprid (½ rate) <sup>a</sup>	1.0 ± 1.0	0.89 ± 0.40	1.4 ± 0.97 a
imidacloprid (¼ rate) <sup>b</sup>	1.2 ± 0.3	1.30 ± 0.58	2.0 ± 0.83 a
<i>M. brunneum</i> (Met 52)	6.0 ± 2.74	4.91 ± 2.20	8.2 ± 4.83 ab
<i>B. bassiana</i> (BotaniGard)	7.2 ± 1.99	0.89 ± 0.40	7.6 ± 2.11 ab
chlorantraniliprole (½ rate) + <i>M. brunneum</i>	10.0 ± 2.88	1.78 ± 0.80	11.2 ± 3.02 b
chlorantraniliprole (¼ rate) + <i>M. brunneum</i>	8.0 ± 1.09	2.60 ± 1.16	9.6 ± 1.83 ab
imidacloprid (½ rate) + <i>M. brunneum</i>	0.8 ± 0.58	0.0 ± 0.0	0.8 ± 0.59 a
imidacloprid (¼ rate) + <i>M. brunneum</i>	0.8 ± 0.80	1.78 ± 0.80	2.0 ± 1.54 a
chlorantraniliprole (½ rate) + <i>B. bassiana</i>	4.0 ± 0.89	0.0 ± 0.0	4.0 ± 0.89 a
chlorantraniliprole (¼ rate) + <i>B. bassiana</i>	17.2 ± 1.39	3.46 ± 1.55	19.2 ± 1.15 b
imidacloprid (½ rate) + <i>B. bassiana</i>	0.8 ± 0.80	0.0 ± 0.0	0.8 ± 0.80 a
imidacloprid (¼ rate) + <i>B. bassiana</i>	0.2 ± 0.20	0.0 ± 0.0	0.2 ± 0.20 a
untreated control	13.6 ± 2.47	3.0 ± 1.34	16.6 ± 3.47 b
Treatment effect	--	--	significant ( $P < 0.05$ )

Means within a column with same letter are not significantly different, Tukey's test ( $P < 0.05$ ).

<sup>a</sup> ½ rate of the highest labelled rate of respective insecticide.

<sup>b</sup> ¼ rate of the highest labelled rate of respective insecticide.



Fig. 3.1. Oviposition arena with treated soil and caged *Popillia japonica* females.



Fig. 3. 2. *Popillia japonica* eggs deposited in the soil.

## Chapter 4

### Interactions of earthworms, entomopathogenic fungi and white grubs in turfgrass.

#### Abstract

White grubs (Coleoptera: Scarabaeidae) cohabitate with many other organisms in the soil environment. The interspecific relationships between many of those organisms and white grub populations have not been well studied. A series of laboratory and greenhouse experiments were conducted to elucidate the influence of earthworms on white grubs. First, a laboratory experiment was conducted to determine whether earthworms could transport the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin in the soil. In the laboratory and greenhouse, follow-up experiments were carried out to test the effect of earthworm presence on the efficacy of *B. bassiana* on white grubs as well as oviposition by *Popillia japonica* Newman. The results of the study showed that the earthworms *Eisenia fetida* (Savigny) and *E. hortensis* consistently transferred *B. bassiana* spores in soil. The presence of *E. hortensis* earthworms in *B. bassiana* infected soil did not result in increased mortality of white grubs. In the laboratory the presence of earthworms in soil resulted in significant reduction in numbers of eggs oviposited by *P. japonica* in a no-choice experiment but not in a choice experiment.

Key words: White grubs, *Beauveria bassiana*, earthworms, oviposition

## **Introduction:**

Earthworms (Oligochaeta: Megadrilacea) are important soil organisms that are known for their beneficial ecosystem services (Bhadauria and Saxena 2010). Earthworms burrow in the soil and increase soil porosity, which improves soil aeration and water infiltration. Earthworms are excellent litter decomposers. Earthworms ingest large amounts of litter, soil and organic matter and deposit fertile casts in the soil that have high levels of nutrients. The ecosystem services offered by earthworm activities in soil include: soil formation and soil structure development, regulation of soil moisture, and recycling of plant nutrients (Blouin et al. 2013).

Because of their cohabitation in the soil, it is not surprising that earthworm populations can affect other soil-dwelling organisms. Simply by improving soil health, earthworms impact the populations of other organisms. For instance, corn rootworms, *Diabrotica* spp., are reported to benefit from earthworms by utilizing their burrows for oviposition (Kirk 1981). The interspecific relationships between earthworms and insects may be more complex. For instance, certain collembolan species are attracted to earthworms (Wickenbrock and Heisler 1997) and their mucus and urine can partially determine collembolan population distribution (Salmon 2001). Other insect, such as the ant *Formica aquilonia* Yarr, has been shown to be repelled by the mucus of the earthworm *Dendrodrilus rubidus* Savigny, which inhabits ant nests (Laakso and Setälä 1997). Such findings highlight the possibilities of interspecific interactions that may occur between earthworms and the soil insects with which they cohabitate.

Earthworms are also prey of many generalist predators including invertebrates such as ants, carabids, and mole crickets and vertebrates such as raccoons and skunks (Lee 1985, Rochefort et al. 2006, King et al. 2010, Xu et al. 2012). Many of these predators also eat white grubs (Coleoptera: Scarabaeidae) in cool season turf in North America (Rochefort et al. 2006,

King et al. 2010) suggesting that the presence of earthworms in a habitat might also influence the population dynamics of white grubs by impacting predator populations. Addition of earthworms, *Amyntas cortices* Kinberg, to corn fields reduced densities of stalk borer, *Sesamia calamistis* Hampson, probably because of an increased population of natural enemies of the pest due to improved soil habitat (Boyer et al. 1999). On the other hand, earthworms are found to negatively affect the spotted tentiform leafminer, *Phyllonorycter blancardella* (F.) (Lepidoptera: Gracillariidae) and some of its associated hymenopteran parasitoids (Laing et al. 1986). The effect of earthworms on white grubs (Coleoptera: Scarabaeidae) has not been well studied, despite the fact that these species co-inhabit the same environment.

The effect of earthworms on entomopathogenic organisms has also not been well studied. Yeats (1981) reported that earthworms reduced the populations of nematodes in pot experiments, probably by unintentionally feeding on them (Yeates 1981). Entomopathogenic fungi (EPF), in particular, could be transported and distributed by earthworms as they move through the soil and interact closely with insects such as white grubs feeding on roots. Among various soil organisms, collembolans are found to transmit EPF species *Beauveria bassiana* (Balsamo) Vuillemin, *B. brongniartii* (Saccardo) Petch, and *M. anisopliae* (Metchnikoff) Sorokin *sensu lato* (Dromph 2003). Recently, Shapiro and Brown (2013) reported that the earthworm, *Lumbricus terrestris* L. was able to mechanically transfer *B. bassiana* in the soil. However, it is still unclear if such transfer of EPF by earthworms results in increased infection of agricultural pests such as white grubs, which are susceptible to *B. bassiana*. This study was conducted to investigate the interaction of earthworms with white grubs in turfgrass as well as their interaction with EPF. The specific objectives of this study were to: i) determine whether earthworms, *Eisenia fetida* and *E. hortensis* could transport the EPF *B. bassiana* in the soil (Experiment 1), ii) examine if the

presence of earthworms in soil enhance fungal infections of white grubs (Experiment 2), iii) and examine if the presence of earthworms in soil affects oviposition of *Popillia japonica* (Experiment 3)

### **Materials and Methods:**

**Earthworms:** Two species of earthworms, *E. fetida* and *E. hortensis*, were purchased from a commercial supplier (Earthworm4sale, Raleigh, NC) and were kept at room temperature for 1-2 days to acclimatize before use. Only healthy and active earthworms were used. Any worm that did not burrow into the soil in 4 h was replaced with a new one.

***Popillia japonica* adults:** *Popillia japonica* adults were collected from the Virginia Tech Kentland Research Farm near Blacksburg, VA using *P. japonica* traps baited with floral/sex dual lure (Trécé Inc, Adair, OK). Collected beetles were kept in a screen cage to allow them to mate for 2-4 days and were provided with the smartweed, *Polygonum* spp., as a food source prior to starting the study.

**White grubs:** Third instar *Cyclocephala* spp. grubs used in the experiment were collected from the Virginia Tech Turfgrass Research Center in Blacksburg, VA in October. After collection, grubs were stored in a cold room (4°C) for 2-3 d. One day (24 h) before the experiment, grubs were removed from the cold room to acclimatize to room temperature (21 to 24°C). Grubs that were healthy and active were used in the experiment. Grubs were surface sterilized by washing them with 1 % sodium hypochlorite, 70 % ethanol and water (Lacey and Brooks 1997).

***Galleria mellonella* larvae:** *Galleria mellonella* (L.) larvae used in the study were purchased from a commercial supplier (Backwater Reptiles, Austin, TX). The larvae were stored at room temperature (21 to 24°C) for 3-4 d before using in the experiment.

**Entomopathogenic fungi:** A commercial formulation of *Beauveria bassiana* GHA strain BotaniGard ES (emulsifiable suspension) (Laverlam international Co., Butte, MT) was used in the experiment. Botanigard ES contains  $2.1 \times 10^{10}$  viable spores/ml.

**Soil:** Field collected sandy loam soil (52.8 % sand, 34.3% silt, and 12.9 % clay) with pH 6.37, and 3.2 % organic matter was used in the laboratory and greenhouse experiment. The soil was sterilized at 72°C for 3 h in a soil sterilization cabinet.

### **Experiment 1. Can earthworms transport *Beauveria bassiana* in the soil?**

In 2014, two separate laboratory experiments were carried out using each of two earthworm species (*E. fetida* and *E. hortensis*) in 1.89 liter plastic pots (Airlite Plastic Co., Omaha, NE) (Fig. 4.1). On the bottom of each pot, about 10 holes were drilled to facilitate water drainage. Each pot was seeded with perennial ryegrass, *Lolium perenne* L., 10 days prior to introducing earthworms. The top of the pot was immediately covered with a lid, which was also drilled with about 30 holes to facilitate air passage, but prevent earthworm escape. There were two treatments: i) the water treated control and ii) *B. bassiana* @  $5.3 \times 10^{10}$  spores/ m<sup>2</sup> which were applied in each of 5 pots (5 replications). The moisture content of the soil was maintained at 18-20% by volume. A total of 10 adult *E. hortensis* or *E. fetida* were introduced to each pot and left there for a week. One week later, five randomly selected earthworms from each pot were transferred (one each) to 100 ml cups (Integrity Product Inc. Grandview, MO or Medicus Health, Kentwood, MI) (total five cups per sample size) filled with 70 ml of sterilized soil (Fig. 4.2). The moisture content of the cups was 18 % by volume. The transfer of *B. bassiana* from *B. bassiana* infested pots to sterile cups by earthworms was determined by baiting for *B. bassiana* using *G. mellonella* larvae following the methods of Shapiro and Brown (2013). Briefly, one *G. mellonella* larva was put in each inside an environment chamber at  $23 \pm 4^\circ\text{C}$ , 90 % RH under



complete darkness. Cups were observed for *B. bassiana* infection and hyphal growth for 3 weeks. Transfer of fungal spores was believed to have occurred if *G. mellonella* larvae in cups died and showed growth of *B. bassiana* hyphae.

This experiment was repeated in 2016 following the same methods with the exception that an additional treatment of double the rate of *B. bassiana* ( $1.06 \times 10^{11}$  spores/ m<sup>2</sup>), was added to examine if higher concentration of *B. bassiana* in soil increases the transfer of spores by earthworms resulting in higher percentage of *G. mellonella* infection. As in 2014, there were 5 pots (5 replications) per treatment. The study was conducted using each *E. fetida* and *E. hortensis* worms in two separate experiments.

In 2016, one additional experiment was carried out with each earthworm species to examine if washing of soil and other particles attached on earthworm body surface affects their ability to transfer EPF. To do that, 5 earthworms from each of the 2016 treatments were individually washed three times in sterilized distilled water and were transferred to sterile 100 ml cups filled with 70 ml sterile soil. One *G. mellonella* larva was put in each cup and was observed for fungal infection and hyphal growth for 3 weeks. The effect of surface washing of *B. bassiana* contacted earthworms on *G. mellonella* infection was determined and compared to infection from non-washed earthworms.

## **Experiment 2. Does the presence of earthworms in soil enhance *B. bassiana* infection of white grubs?**

This study was carried out in the greenhouse in 1.89 liter plastic pots (Airlite Plastic Co., Omaha, NE) using *E. hortensis*. This earthworm species was selected because of its ability to consistently transfer *B. bassiana* in our preliminary study. Plastic pots were filled halfway with sterilized soil and seeded with perennial ryegrass seed. After 10 d, 10 third instar white grubs, *Cyclocephala*

spp., were introduced to each pot 24 h before treatment. Only healthy grubs that burrowed in the soil within 4 h of introducing to the pot were used. The average temperature in the greenhouse was  $23 \pm 5^{\circ}\text{C}$ . The moisture content of the soil was maintained 18-20% by volume. Each of five pots was assigned one of the following treatments: 1) untreated control; 2) *B. bassiana* @  $5.3 \times 10^{10}$  spores/  $\text{m}^2$ ; 3) 20 earthworms; or 4) 20 earthworms + *B. bassiana*. White grub mortality was assessed two weeks after treatment.

### **Experiment 3. Does the presence of earthworms in soil affect oviposition of *Popillia japonica*?**

Two sets of experiments, choice and no-choice, were carried out to determine effect of earthworms on numbers of eggs laid by mated *P. japonica* females. Earthworm *E. hortensis* was used in these experiments. Adult *P. japonica* were collected and maintained in cages as described previously. The experiments were carried out in 1.89 liter plastic pots (Airlite Plastic Co., Omaha, NE) pots filled halfway with sterilized soil. At 10 d prior to beginning the experiment, each pot was sown with perennial ryegrass seed to simulate a turf field. The moisture content of the soil was maintained 15-18% by volume.

In the no-choice test, a total of 5 female beetles were released inside the pot and the top was immediately covered with a cover with perforated holes to prevent beetles from escaping (Fig. 4.3). The experiment was replicated 7 times. At 10 d after beetles were introduced, the soil in each pot was hand sampled thoroughly and the number of eggs recorded.

In the choice test, female beetles were given a choice among pots with three treatments; no earthworms, 2 earthworms per pot or 5 earthworms per pot by releasing 12 female beetles inside a cage with three open top pots with three treatments (Fig. 4.4). Each container in the choice test was covered with a strip of 9 cm tall and 1.9 cm thick Velcro (Sticky Back, Velcro

USA Inc, Manchester, NH) at the top of the inner rim to prevent the earthworms from escaping. The experiment was replicated 10 times. At 10 d after beetles were introduced, the soil in each pot was hand sampled thoroughly and the number of eggs recorded.

### **Data analysis:**

The effect of earthworms on *B. bassiana* transfer and infection of *G. mellonella* larvae in 2014 was analyzed using an independent-samples t-test (Excel 14, Microsoft Corporation, Redmond, WA). The effect of earthworms on *B. bassiana* transfer and infection of *G. mellonella* larvae in 2016, the effect of *E. hortensis* and *B. bassiana* on mortality of *Cyclocephala* spp., and the effect of *E. hortensis* on *P. japonica* oviposition was analyzed using ANOVA in JMP 11.0 (SAS, Cary, NC).

### **Results:**

**Experiment 1. Can earthworms transport *Beauveria bassiana* in the soil?** In 2014, there was a significantly higher percentage of *B. bassiana* infection of *G. mellonella* larvae in pots containing *E. fetida* taken from *B. bassiana*-treated compared to untreated soil ( $t = 3.16$ ;  $df = 4$ ;  $P < 0.05$ ). Similar results occurred with the earthworm *E. hortensis* ( $t = 2.23$ ;  $df = 4$ ;  $P < 0.05$ ). The addition of *E. fetida* or *E. hortensis* from *B. bassiana*-treated soil resulted in infection of  $20.0 \pm 14.1$  and  $20.0 \pm 8.9$  % of *G. mellonella* larvae, respectively. There was no infection to *G. mellonella* from *E. fetida* or *E. hortensis* taken from untreated soil.

In 2016, with the addition of a higher rate of *B. bassiana* spores, there was a significant treatment effect on *B. bassiana* infection of *G. mellonella* for *E. hortensis* ( $F = 22.80$ ;  $df = 2, 14$ ;  $P < 0.05$ ), but not for *E. fetida* ( $F = 2.40$ ;  $df = 2, 14$ ;  $P > 0.05$ ). For *E. hortensis*, there was significantly higher infection of *G. mellonella* for earthworms taken from soil treated with higher rates of *B. bassiana* than for earthworms from soil treated with lower rate of *B. bassiana* or soil

treated with water/control ( $P < 0.05$ ) (Table 4.1). There was no significant difference in *B. bassiana* -infection of *G. mellonella* for earthworms from soil treated with lower rates of *B. bassiana* or control ( $P > 0.05$ ). It should be noted that similar trends were observed with *E. fetida*, in that a higher (but not-significantly different) level of *B. bassiana* infection of *G. mellonella* was observed for earthworms from higher rate of *B. bassiana* (Table 4.1).

In the 2016 experiment, there was a significant treatment effect on *B. bassiana* infection of *G. mellonella* from *E. hortensis* from soil treated with higher rates of *B. bassiana* ( $F = 9.6$ ;  $df = 2, 14$ ;  $P < 0.05$ ), but not from *E. hortensis* from soil treated with lower rates of *B. bassiana* ( $F = 0.5$ ;  $df = 2, 14$ ;  $P > 0.05$ ) (Table 4.1). Also, washing soil and other particles from the body surface of *E. hortensis* did not significantly reduce the *B. bassiana* infection of *G. mellonella* compared with than unwashed worms that were exposed to the high rate of *B. bassiana* ( $P > 0.05$ ) (Table 4.2). For *E. fetida*, there was no significant treatment effect on *B. bassiana* infection of *G. mellonella* for earthworms from soil treated with higher rates *B. bassiana* ( $F = 2.60$ ;  $df = 2, 14$ ;  $P > 0.05$ ); however, there was a significant treatment effect on *B. bassiana* infection of *G. mellonella* from earthworms from soil that was treated with lower rates of *B. bassiana* ( $F = 2.66$ ;  $df = 2, 14$ ;  $P < 0.05$ ; Table 4.1). Also, for *E. fetida* from soil treated with lower rate of *B. bassiana*, the washing of earthworms significantly reduced the *B. bassiana* infection of *G. mellonella* compared with unwashed worms ( $P < 0.05$ ; Table 4.2).

For all of the bioassays, there was no *B. bassiana* infection of *G. mellonella* from *E. fetida* or *E. hortensis* obtained from water-treated control soil. In all experiments, the highest percent infection occurred from unwashed earthworms (Table 4.2).

**Experiment 2. Does the presence of earthworms in soil enhance *Beauveria bassiana* infection of white grubs?** There was no significant effect of treatment on mortality of white

grubs in the first experiment ( $F= 2.56$ ;  $df= 3, 19$ ;  $P>0.05$ ) or the second experiment ( $F= 2.69$ ;  $df= 3, 19$ ;  $P>0.05$ ). Presence of earthworms with *B. bassiana* did not enhance grub mortality.

However, it should be noted that the highest grub mortality occurred in the *B. bassiana* plus earthworm treatments in both trials (Table 4.3). No fungal infection symptoms were observed in any of the dead grubs.

### **Experiment 3. Does the presence of earthworms in soil affect oviposition of *Popillia***

*japonica*? In the no-choice experiment, there was a significant treatment effect on number of *P. japonica* eggs deposited in each pot ( $F = 3.63$ ;  $df = 2, 20$ ;  $P < 0.05$ ), with significantly more eggs found in control pots than pots containing either 2 or 5 earthworms per pot ( $P < 0.05$ ; Fig. 4.5).

There was no difference between 2 earthworms or 5 earthworms per pot treatment ( $P > 0.05$ ).

In the choice-test experiment, there was no significant effect of treatment on numbers of eggs laid in each pot ( $F = 1.21$ ;  $df = 2, 29$ ;  $P > 0.05$ ). An average of 18.6, 21.4 and 29.1 eggs were found in the control, 2 earthworms per pot and 5 earthworms per pot treatments (Fig. 4.6).

### **Discussion:**

Earthworms cohabitate with many other organisms in turfgrass ecosystems and likely affect the population dynamics of those organisms. Shapiro and Brown (2013) reported that the common earthworm *L. terrestris* can distribute *B. bassiana* spores through ingestion of live spores or more commonly via attachment of spores to the earthworm body surface. Herein, we found that *G. mellonella* larvae were infected with *B. bassiana* when placed in containers that contained earthworms (either *E. fetida* or *E. hortensis*) that previously contacted *B. bassiana* infested soil; thus confirming that earthworms can transfer viable *B. bassiana* spores to uninfected soil. It was also found that *G. mellonella* were infected by *B. bassiana* from earthworms whose bodies were thoroughly washed with sterile water, suggesting that fungal spores attached on earthworm

bodies are not easily removed or may be carried internally by earthworms, which can later distribute *B. bassiana* in the soil.

In the greenhouse experiment with *Cyclocephala* spp. grubs, the overall infection rates by *B. bassiana* were low particularly compared with *G. mellonella*. Consequently, the presence of earthworms and *B. bassiana* did not significantly enhance the mortality of *Cyclocephala* spp. compared with other treatments. Though there was no significant increase in percent grub mortality in the earthworm plus *B. bassiana* treatments, a trend of higher mortality was observed for *B. bassiana* and earthworms (Table 4.3). Thus, the lack of significant differences in grub mortality among treatments with or without earthworms and *B. bassiana* indicated that the presence of earthworms in soil may not be a significant factor that can directly influence white grub populations.

Selection of suitable oviposition sites is important in insects with larvae that have reduced ability to actively search for suitable hosts (Johnson et al. 2006 and references therein). For example, Wurst and Jones (2003) found that the presence of the earthworm species, *Aporrectodea caliginosa* (Savigny) in soil increased the reproduction of aphid, *Myzus persicae* (Sulzer), in *Cardamine hirsuta* L. because of improved plant quality. Moreover, insects which spend part or all of their life cycles in soil change their oviposition behavior if the soil environment is unfavorable for the survival and fitness of their eggs and offspring. Such stressful conditions include low or high soil moisture, or presence of insect pathogens in the soil (Fry et al. 1997, Hertl et al. 2001).

*Popillia japonica* female oviposition behaviors have been previously found to be influenced by factors including soil moisture, soil type, and presence of insect pathogens (Fry et al. 1997). For example, *P. japonica* selectively laid more eggs in soil with slightly higher soil

moisture and in soil relatively low in percent sand content (Allsopp et al. 1992) and lower percent microbial biomass (Wickings 2016). The no-choice greenhouse experiment that I conducted on *P. japonica* oviposition showed that the number of *P. japonica* eggs in earthworm-infested soil was significantly lower than in soil without earthworms. The reasons for this may be either that *P. japonica* females detected the earthworms in the soil and deposited fewer eggs, or that the earthworms possibly ingested the eggs before they could be counted. With regards to the former explanation, earthworms produce kairomones that are detected by insects (Morris and Pivnic 1991). For instance, certain collembolan species are attracted to earthworms (Wickenbrock and Heisler 1997) and their mucus and urine can partially determine collembolan population distribution (Salmon 2001). In addition, earthworm mucus and urine in soil has been reported to serve as a kairomone for oviposition of *Coenosia tigrina* F., a dipteran predator of earthworms at larval stage (Morris and Pivnic 1991). Other insects such as the ant *Formica aquilonia* Yarr has been shown to be repelled by the mucus of the earthworm *Dendrodrilus rubidus* Savigny, which inhabits ant nests (Laakso and Setälä 1997). Whether scarab beetles can detect earthworm odors is not known.

Regarding the latter explanation, previous studies have found that *E. fetida* earthworms can ingest the nematodes, *Steinernema feltiae* (Filipjev) (Campos-Herrera et al. 2006). Owing to the fact that the *P. japonica* eggs when first deposited are comparable to the size of nematodes (Fleming 1972, Nguyen 1993), and that the earthworm species used in our study, *E. hortensis*, is larger than *E. fetida*, it is possible that earthworms consumed the beetle eggs in the soil. However, we are not aware of this reported before in the literature and further study is required to confirm this hypothesis.

In summary, earthworms, *E. fetida* and *E. hortensis* were observed to transfer EPF, *B. bassiana* leading to infection of *G. mellonella* larvae. However, the ability of earthworms to transfer *B. bassiana* did not enhance the *B. bassiana* infection of *Cyclocephala* spp. in plastic containers in the laboratory when *B. bassiana* spores and the earthworm *E. hortensis* were combined in containers with *Cyclocephala* spp. grubs. Number of eggs from *P. japonica* was affected by the presence of earthworms when no choice was present. However, there was no effect of presence of earthworm in soil on *P. japonica* oviposition when choice of earthworm infested or non-infested soil was present.



## References:

- Allsopp, P. G., M. G. Klein, and E. L. McCoy. 1992. Effect of soil moisture and soil texture on oviposition by Japanese beetle and rose chafer (Coleoptera: Scarabaeidae). *Journal of Economic Entomology* 85: 2194–2200.
- Bhadauria, T., and K. G. Saxena, 2009. Role of earthworms in soil fertility maintenance through the production of biogenic structures. *Applied and Environmental Soil Science* 2010: 1–7.
- Blouin, M., M. E. Hodson, E. A. Delgado, G. Baker, L. Brussaard, K. R. Butt, J. Dai, L. Dendooven, G. Pérès, J. E. Tondoh, and D. Cluzeau, 2013. A review of earthworm impact on soil function and ecosystem services. *European Journal of Soil Science* 64: 161–182.
- Boyer, J., R. Michellon, A. Chabanne, G. Reversat, and R. Tibere. 1999. Effects of trefoil cover crop and earthworm inoculation on maize crop and soil organisms in Reunion Island. *Biology and Fertility of Soils* 28: 364–370.
- Campos-Herrera, R., D. Trigo, and C. Gutiérrez. 2006. Phoresy of the entomopathogenic nematode *Steinernema feltiae* by the earthworm *Eisenia fetida*. *Journal of Invertebrate Pathology* 92: 50–54.
- Dromph, K. M. 2003. Collembolans as vectors of entomopathogenic fungi. *Pedobiologia* 47: 245–256.
- Fleming, W. E. 1972. *Biology of the Japanese beetle*. USDA Technical Bulletin 1449.  
<http://naldc.nal.usda.gov/download/CAT87201410/PDF>
- Fry, R. C., L. A. Fergusson-Kolmes, S. A. Kolmes, and M. G. Villani. 1997. Radiographic study of the response of Japanese beetle larvae (Coleoptera: Scarabaeidae) to soil-incorporated

- mycelial particles of *Metarhizium anisopliae* (Deuteromycetes). Journal of the New York Entomological Society 113–120.
- Hertl, P. T., R. L. Brandenburg, and M. E. Barbercheck. 2001. Effect of soil moisture on ovipositional behavior in the southern mole cricket (Orthoptera: Gryllotalpidae). Environmental Entomology 30: 466–473.
- Johnson, S. N., A. N. E. Birch, P. J. Gregory, and P. J. Murray. 2006. The ‘mother knows best’ principle: should soil insects be included in the preference–performance debate? Ecological Entomology 31: 395–401.
- King, R. A., I. P. Vaughan, J. R. Bell, D. A. Bohan, and W. O. Symondson. 2010. Prey choice by carabid beetles feeding on an earthworm community analysed using species- and lineage-specific PCR primers. Molecular Ecology 19: 1721–1732.
- Kirk, V. M. 1981. Earthworm burrows as oviposition sites for western and northern corn rootworms (*Diabrotica*: Coleoptera). Journal of the Kansas Entomological Society 54: 68–74.
- Laakso, J., and H. Setälä. 1997. Nest mounds of red wood ants (*Formica aquilonia*): Hot spots for litter-dwelling earthworms. Oecologia 111: 565–569.
- Laing, J. E., J. M. Heraty, and J. E. Corrigan. 1986. Leaf burial by the earthworm, *Lumbricus terrestris* (Oligochaeta: Lumbricidae), as a major factor in the population dynamics of *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) and its parasites. Environmental Entomology 15: 321–326.
- Lee, K.E., 1985. Earthworms: Their Ecology and Relationships with Soils and Land Use. Academic Press, Sydney, Australia.

- Morris, D. E., and K. A. Pivnick. 1991. Earthworm mucus stimulates oviposition in a predatory fly (Diptera: Anthomyiidae). *Journal of Chemical Ecology* 17: 2045–2052.
- Nguyen, K. B. 1993. Identification to entomopathogenic nematode species of the genus *Steinernema*. *Nematology Circular* 202: 190–193.
- Rocheffort, S., D. J. Shetlar, and J. Brodeur. 2006. Ground beetle assemblages and their seasonal abundance in cool season turf grass lawns in Quebec. *Environmental Entomology* 35: 1508–1514.
- Salmon, S., 2001. Earthworm excreta (mucus and urine) affect the distribution of springtails in forest soils. *Biology and Fertility of Soils* 34: 304–310.
- Shapiro, D. I., and I. Brown. 2013. Earthworms as phoretic hosts for *Steinernema carpocapsae* and *Beauveria bassiana*: Implications for enhanced biological control. *Biological Control* 66: 41–48.
- Wurst, S., and T. H. Jones. 2003. Indirect effects of earthworms (*Aporrectodea caliginosa*) on an above-ground tritrophic interaction. *Pedobiologia* 47: 91–97.
- Wickenbrock, L., and C. Heisler. 1997. Influence of earthworm activity on the abundance of Collembola in soil. *Soil Biology and Biochemistry* 29: 517–521.
- Wickings, K. 2016. Improving predictions of white grub distribution in turfgrass. New York State Turf Association Online Newsletter.  
<http://www.nysta.org/news/nystapr/2016/grubsenews.pdf>.
- Xu, Y., D. W. Held, and X. P. Hu. 2012. Potential negative effects of earthworm prey on damage to turfgrass by omnivorous mole crickets (Orthoptera: Gryllotalpidae). *Environmental Entomology* 41: 1139–1144.

Yeates, G. W. 1981. Soil nematode populations depressed in the presence of earthworms. *Pedobiologia* 22: 191–195.

Table 4.1. Percent *Beauveria bassiana* infection of *Galleria mellonella* larvae exposed to earthworms (*Eisenia hortensis* or *Eisenia fetida*) that were previously exposed to different concentrations of *Beauveria bassiana*-infested soil (One-way ANOVA).

Treatments	Percentage (mean $\pm$ SE) of <i>Galleria mellonella</i> larvae infected with <i>Beauveria bassiana</i>	
	<i>E. hortensis</i>	<i>E. fetida</i>
Water control	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0
<i>B. bassiana</i> lower rate <sup>a</sup>	4.0 $\pm$ 3.6 b	16.0 $\pm$ 6.7
<i>B. bassiana</i> higher rate <sup>b</sup>	32.0 $\pm$ 4.4 a	8.0 $\pm$ 4.4
Treatment effect	significant ( $P < 0.05$ )	non-significant ( $P > 0.05$ )

Means within the same column with same letter are not significantly different, Tukey's test ( $P < 0.05$ ).

<sup>a</sup> Low rate: *Beauveria bassiana* applied at  $5.3 \times 10^{10}$  spores/ m<sup>2</sup> rate.

<sup>b</sup> High rate: *Beauveria bassiana* applied at  $1.06 \times 10^{11}$  spores/ m<sup>2</sup> rate.

Table 4.2. Percent *Beauveria bassiana* infection of *Galleria mellonella* larvae exposed to earthworms (*Eisenia hortensis* or *Eisenia fetida*) that were previously exposed to different concentrations of *Beauveria bassiana*-infested soil then washed or unwashed prior to exposure (One-way ANOVA).

Treatments	Percentage (mean $\pm$ SE) of <i>Galleria mellonella</i> larvae infected with <i>Beauveria bassiana</i>			
	<i>Eisenia hortensis</i> worms exposed to high rate <sup>a</sup> of <i>Beauveria bassiana</i>	<i>Eisenia hortensis</i> worms exposed to low rate <sup>b</sup> of <i>Beauveria bassiana</i>	<i>Eisenia fetida</i> worms exposed to high rate <sup>a</sup> of <i>Beauveria bassiana</i>	<i>Eisenia fetida</i> worms exposed to low rate <sup>b</sup> of <i>Beauveria bassiana</i>
Control	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0 b
Washed earthworm	16.0 $\pm$ 6.7 a	4.0 $\pm$ 3.6	12.0 $\pm$ 4.4	0.0 $\pm$ 0.0 b
Unwashed earthworm	32.0 $\pm$ 4.4 a	4.0 $\pm$ 3.6	16.0 $\pm$ 6.7	8.0 $\pm$ 4.4 a
Treatment effect	significant ( $P < 0.05$ )	non-significant ( $P > 0.05$ )	non-significant ( $P > 0.05$ )	significant ( $P < 0.05$ )

Means within a column with the same letter are not significantly different, Tukey's test ( $P < 0.05$ ).

<sup>a</sup> High rate: *Beauveria bassiana* applied at  $1.06 \times 10^{11}$  spores/ m<sup>2</sup> rate.

<sup>b</sup> Low rate: *Beauveria bassiana* applied at  $5.3 \times 10^{10}$  spores/ m<sup>2</sup> rate.

Table 4.3. Effect of *Beauveria bassiana* with and without earthworms on mortality of third instar *Cyclocephala* spp grubs in a laboratory bioassay (One-way ANOVA).

Treatments	% mortality* (mean $\pm$ SE) of <i>Cyclocephala</i> spp. larvae	
	Trial 1	Trial 2
Control	40.0 $\pm$ 7.5	18.0 $\pm$ 3.7
<i>B. bassiana</i>	52.0 $\pm$ 8.6	40.0 $\pm$ 8.3
<i>E. hortensis</i>	34.0 $\pm$ 7.2	28.0 $\pm$ 5.8
<i>B. bassiana</i> + <i>E. hortensis</i>	64.0 $\pm$ 6.0	40.0 $\pm$ 7.0
Treatment effect	non-significant ( $P > 0.05$ )	non-significant ( $P > 0.05$ )

\* Grub mortality was assessed 2 wk after treatment.



Fig. 4.1. Experimental container used for exposing *Eisenia hortensis* and *Eisenia fetida* to *Beauveria bassiana* infected soil.



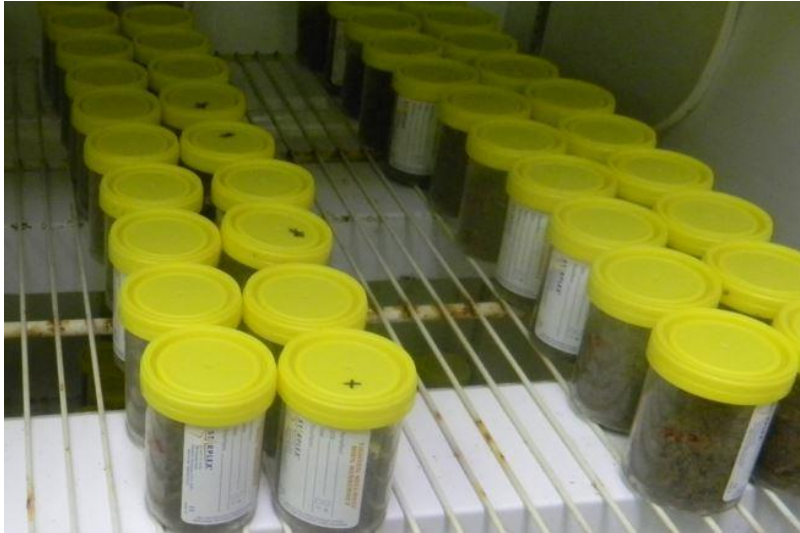


Fig.4.2. Plastic cups for baiting *Beauveria bassiana* transferred by *Eisenia fetida* and *Eisenia hortensis*.



Fig.4.3. Experimental arena to determine effect of earthworm presence on *Popillia japonica* oviposition in no choice test.

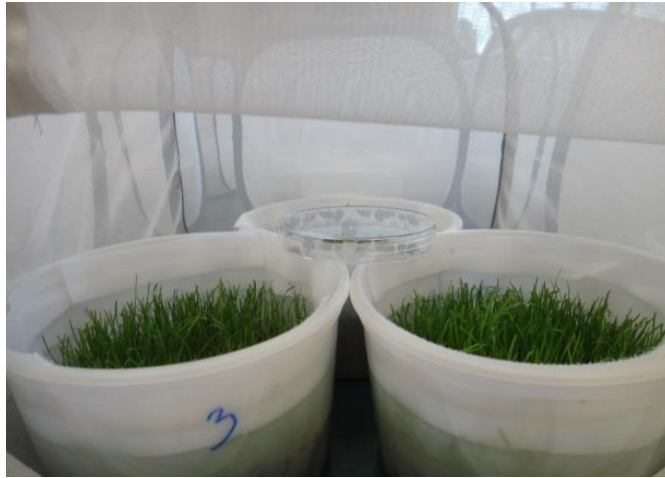


Fig.4.4. Experimental arena to determine effect of earthworm presence on *Popillia japonica* oviposition in choice test.

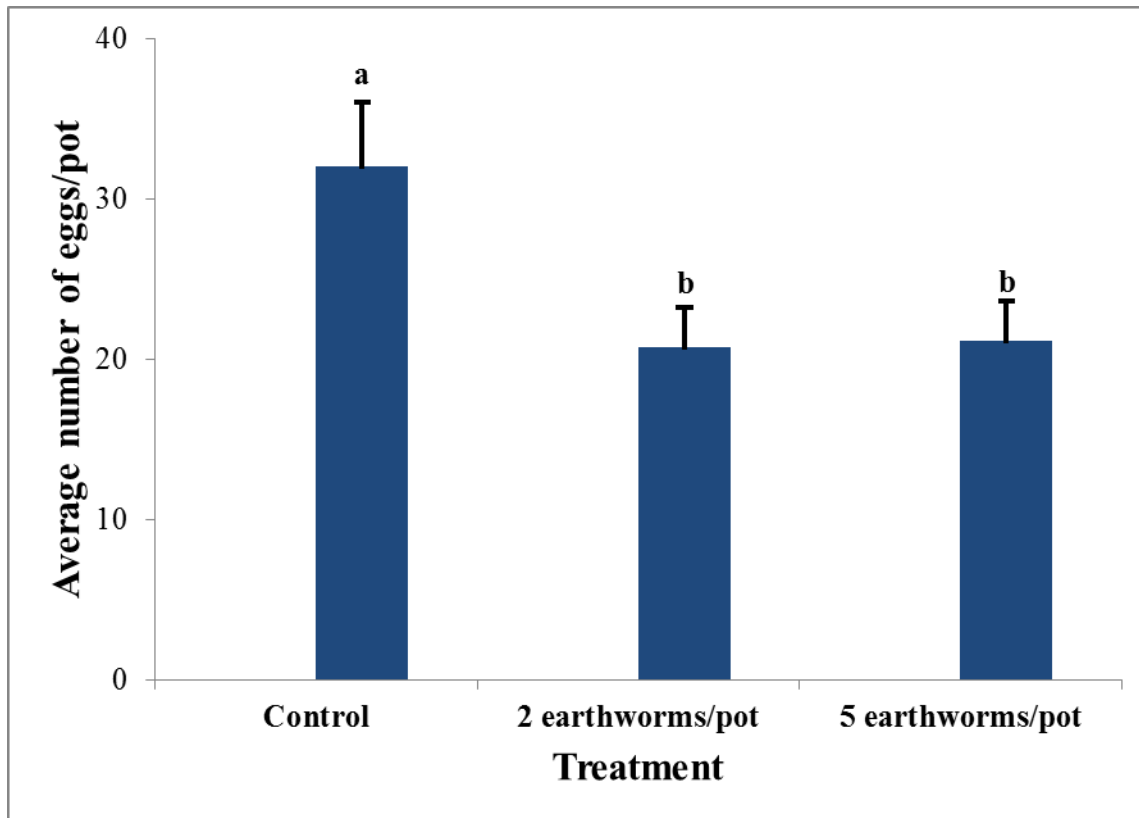


Fig. 4.5. Effect of earthworm presence on numbers of eggs deposited by *Popillia japonica* females in the laboratory in no-choice test (One-way ANOVA,  $P < 0.05$ ).

Means with same letter are not significantly different, Tukey's test ( $P < 0.05$ ).

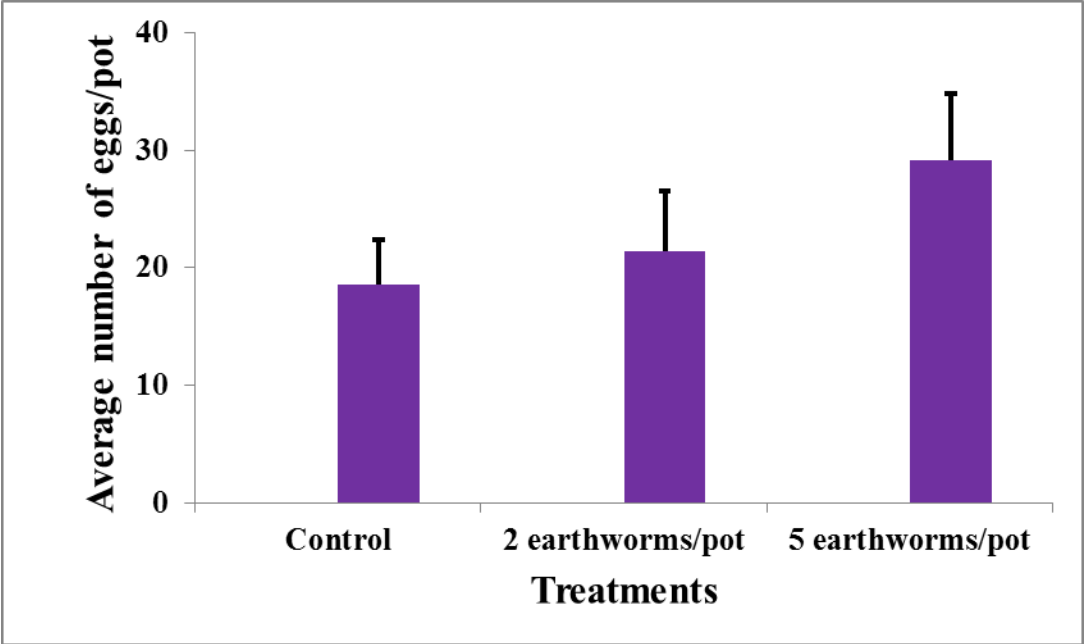


Fig. 4.6. Effect of earthworm presence on numbers of eggs deposited by *Popillia japonica* females in the laboratory in choice test (One-way ANOVA,  $P > 0.05$ )

## Chapter 5

### Effects of field rates of common turfgrass insecticides on the earthworm, *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae)

#### Abstract:

Insecticides are commonly applied to turfgrass to control insect pests, mainly white grubs (Coleoptera: Scarabaeidae). Although all registered pesticides in the U.S. must undergo ecotoxicological studies as mandated by the U.S. Environmental Protection Agency, we still do not fully know the potential harmful effects that these insecticide applications may have on non-target organisms, such as earthworms under typical field conditions. Laboratory and field studies were carried out to evaluate the effect of field rate applications of several commercial white grub control products on *Lumbricus terrestris* L., which is the dominant earthworm species found in turfgrass in the eastern U.S. In laboratory container bioassays, the neonicotinoids, dinotefuran and imidacloprid caused significantly higher mortality to adult *L. terrestris* ( $P < 0.05$ ) than two other neonicotinoids, thiamethoxam and clothianidin, or a combination of clothianidin plus bifenthrin, or the diamide chlorantraniliprole, or the organophosphate trichlorfon. However, when applied as a drench to turfgrass in spring, summer, and fall at the Virginia Tech Golf Course, none of the insecticides resulted in a significant effect on earthworm densities compared with a water control. This study indicates that a single application of any of these insecticide products in turfgrass may not negatively impact earthworm populations. The findings of this study further confirm that the results of insecticide toxicity studies in the laboratory may not necessarily be indicative of results in the field.

Key words: Earthworms, white grub, turfgrass, toxicity

**Introduction:**

White grubs, the root feeding larvae of scarab beetles (Coleoptera: Scarabaeidae), are the major edaphic pests of turfgrass in the U. S. (Potter 1998). Insecticides are commonly applied to control these pests in order to maintain a good quality turf for residential and commercial lawns and golf courses. Common turf insecticides include neonicotinoids such as imidacloprid, dinotefuran, thiamethoxam, and clothianidin; combination products such as clothianidin plus the pyrethroid bifenthrin; the diamide chlorantraniliprole; or the organophosphate trichlorfon (Gyawaly et al. 2016a). Overall, these insecticides provide very good control of white grubs when applied at the proper time (Gyawaly et al. 2016 and references therein). However, because many of these insecticides have a broad spectrum of activity and persist for a long period in soil, there is serious concern among people about their negative impact on non-target organisms. Some studies have found that repeated use of imidacloprid could have a significant negative effect on non-target organisms such as soil-inhabiting beneficial arthropods (Peck 2009).

Of the various non-target organisms in turfgrass, earthworms (Oligochaeta: Lumbricidae) are important soil macro fauna and have long been known for their beneficial effects on soil physical and chemical properties (Bhadoria and Saxena 2010). Earthworms are excellent litter decomposers, and help improve soil structure. They break down large quantities of litter, incorporate organic matter into the soil, and deposit casts in the soil that contain high levels of nutrients. By burrowing in the soil, earthworms increase soil porosity, which improves soil aeration and water infiltration. Earthworms also play an important role in turf thatch degradation (Potter et al. 1990a) and soil formation in the temperate regions (White 2006).

As part of the ecotoxicological testing requirements of the U. S. Environmental Protection Agency for pesticide registrations, all products are evaluated and ranked for their

toxicity to the “red wiggler” compost worm *Eisenia fetida* (Savigny) following laboratory protocols demonstrated by Roberts and Dorough (1984) (Table 5.1). These authors also proposed the toxicological groupings for LC<sub>50</sub> values between 1 – 10 µg a.i./ cm<sup>2</sup> of soil to be extremely toxic, between 10 – 100 µg a.i./ cm<sup>2</sup> to be very toxic, and between 100 – 1,000 µg/ cm<sup>2</sup> to be moderately toxic to *E. fetida*. Wang et al. (2012a,b) tested a large number of pesticides and showed that most organophosphates are either extremely toxic or very toxic to *E. fetida*, which is not surprising given their relatively high toxicity to most animals. These same authors reported that the neonicotinoid clothianidin had LC<sub>50</sub> values <1.0 µg/cm<sup>2</sup> and categorized it as super toxic to *E. fetida*. Thus, neonicotinoids, which are widely used in turfgrass partly due to their reduced toxicity to vertebrates, might seemingly pose an even greater threat to earthworms. However, the aforementioned bioassays were conducted on *E. fetida* in the laboratory and may not be representative of the true threat to other species of earthworms that inhabit the soil under turfgrass, such as the introduced species *Lumbricus terrestris* L. Though such studies give a general idea about relative toxicities of chemicals to earthworms in general via direct exposure, they may not exactly represent their effect under field situations (Edwards and Bohlen 1992).

In the field, edaphic conditions can vary significantly with regard to soil physical, chemical, and biological properties, vegetation cover, and weather elements compared to the laboratory. Such differences in abiotic and biotic factors in the field compared to highly controlled laboratory situations might logically lead to high variability in both exposure and response of organisms to insecticides between the two settings. Moreover, insecticide toxicity to earthworms may also be influenced by inherent earthworm biological factors (determined by species), which affect burrowing, feeding behavior, and seasonal activity (Edwards and Bohlen 1992).



Although there is a lot of information available on insecticide toxicities to earthworms, there is very limited field-based scientific literature available on their effects on earthworms in general and in turfgrass systems in particular. Knowledge of the effects of insecticides on earthworms in turfgrass systems is highly warranted because of the frequency of soil-applied drenches and the perennial nature of this ecosystem. Therefore, this study was conducted to determine the effect of white grub insecticides on *L. terrestris*, the predominant earthworm found in Virginia turfgrass.

## **Materials and Methods:**

### **Laboratory experiment:**

The laboratory experiment was conducted using adult *Lumbricus terrestris* L., purchased from a commercial earthworm supplier (The Worm Dude, San Jose, California), in 2015 and 2016.

Plastic pots with 1.89 liter capacity (Airlite Plastic Co., Omaha, NE) were half filled with top soil (Shottower Loam, pH 6.18, 2.9% organic matter) and perennial ryegrass, *Lolium perenne* L., was seeded 10 days prior to introducing 15 earthworms per pot in 2015 (trial 1) or 10 earthworms per pot in 2016 (trial 2). On the bottom of each pot, ~ 10 holes were drilled to facilitate water drainage. The top of each pot was covered with a lid, which was drilled with about 30 holes to facilitate air passage. Treatments included high label recommended rates of seven insecticides recommended for white grub control in turfgrass in Virginia and a control of water (Table 5.2). All insecticide treatments were drenched in pots at a rate that simulated field application followed by 3 cm of irrigation. Pots were checked one week after treatments for number of live worms.

In trial 2, for treatments that resulted in earthworm mortality comparable to control treatments, earthworms were also evaluated for sub-lethal effects because of the exposure to

insecticides. Earthworms from all pots for each treatment were washed with tap water and collected in a container. Then, eight earthworms for each treatment were randomly selected and earthworms were individually released on the top of the soil in each container filled half way with moist soil. Time required for each earthworm to burrow to the depth of its clitellum was recorded.

### **Field experiments:**

Three separate field experiments were carried out during the spring, summer, and fall of 2015 at the Virginia Tech Campus Golf Course in Blacksburg, VA, where the soil is a Groseclose loam with a pH of 6.17. The grass composition is a mixture of fescues, ryegrasses, annual bluegrass (*Poa annua* L.), and bermudagrass (*Cynodon* spp.). Treatments used in the field experiments were the same as the laboratory experiment. For spring and summer experiments, insecticides were applied on plots (1.5 m × 1.5 m size) arranged in a completely randomized block design with four replications of each treatment. For spring and summer experiments, all liquid insecticide treatments were applied as foliar sprays using a CO<sub>2</sub> backpack sprayer. The backpack sprayer was equipped with four, 8002VS stainless steel spray tips and calibrated to deliver 187 L per ha at  $2.76 \times 10^5$  Pa. The granular insecticide treatment of trichlorfon for each plot was mixed with 453 g sand which was then hand sprinkled over the plot.

For the spring field experiment, insecticides were applied to the turf plots on May 9, 2015. Earthworms were sampled by using a mustard powder suspension method as described by Hale (2013). Two weeks after the treatments were applied, a 20 cm diameter PVC pipe was inserted into the ground to the depth of about 3 cm in each plot and one liter of a mustard powder suspension [10 g of ground yellow mustard (McCormick and Company, Hunt Valley, Maryland) mixed in one L of water] was poured into the PVC frame to agitate any earthworms in the soil

and bring them to the surface. Within 5 minutes after application, all earthworms that moved to the soil surface were recorded.

In the summer experiment, all treatments were applied to the plots on August 17, 2015 (except trichlorfon, which was applied on September 23, 2015). Rather than the mustard extraction technique used previously, earthworms were sampled on October 9, 2015 by cutting two turf plugs per plot to a depth of 10 cm with a 10.8 cm diameter golf cup- cutter.

A third field experiment was conducted at four different locations at the Virginia Tech Campus Golf Course in fall 2015. Treatments were applied on October 9, 2015 to individual plot sizes of 0.6 m by 0.6 m at each location. A 0.3 m untreated buffer between each plot was maintained to prevent the runoff of insecticide between plots. Insecticide treatments were applied as foliar sprays using a watering can with 4.5 L of water. Granular insecticide was applied by following the method similar to spring and summer treatment. There were 4 replicates (at 4 different locations). Earthworms were sampled on October 12, 2015 (3 days after treatment) using the golf cup cutter technique described above.

### **Data analysis:**

The laboratory data on the effect of insecticide treatments on earthworm mortality and average time required to burrow into the soil were analyzed using ANOVA in JMP 11.0 (SAS, Cary, NC). Tukey's HSD test was used to compare means among different treatments. The field data on the effect of insecticides on earthworm abundance were analyzed separately for each season using the same procedure as for the laboratory experiment. All data were subjected to square root transformation but actual data are presented.

### **Results:**

#### **Laboratory experiment**

There was a significant effect of insecticide treatment on earthworm mortality in trial 1 ( $F = 17.91$ ;  $df = 7, 31$ ;  $P < 0.05$ ) and in trial 2 ( $F = 10.65$ ;  $df = 7, 31$ ;  $P < 0.05$ ).

In trial 1 the highest percent mortality (significantly greater than all the other treatments including the control) to *L. terrestris* adults was caused by dinotefuran (98.3%) and imidacloprid (88.3%). Chlorantraniliprole, thiamethoxam, clothianidin, and trichlorfon each caused very low mortality ( $< 12\%$ ). The combination of clothianidin + bifenthrin caused slightly higher but not significantly different mortality (28.3%) than the previously mentioned group or the control (Table 5.3).

In trial 2 dinotefuran (97.5%), imidacloprid (50%), and the clothianidin + bifenthrin combination (35.0%) all caused significantly higher mortality to earthworms than thiamethoxam (10.0%), clothianidin (7.5%), and the control (5%). Also, dinotefuran caused significantly higher mortality than trichlorfon (25.0%) and chlorantraniliprole (12.5%). Chlorantraniliprole, thiamethoxam, clothianidin, and trichlorfon each caused higher but not statistically different mortality than the control ( $P > 0.5$ ) (Table 5.3).

There was no significant effect of insecticides on average time required for earthworms to burrow into the soil one week after exposure to those insecticides ( $F = 0.27$ ;  $df = 4, 44$ ;  $P > 0.05$ ). On average, earthworms exposed to chlorantraniliprole, clothianidin, thiamethoxam, trichlorfon, or control treatment required (Mean  $\pm$  SE)  $222.33 \pm 23.19$ ,  $221.44 \pm 36.31$ ,  $215.00 \pm 27.93$ ,  $195.33 \pm 21.62$ , or  $191.33 \pm 31.79$  seconds respectively to burrow in the soil.

### **Field study**

In all of the field experiments, the earthworm species sampled was *L. terrestris*, the same species used in the lab bioassays. Following the spring time (preventive) insecticide treatments in May,

there was no statistically significant effect ( $F = 1.12$ ;  $df = 7, 31$ ;  $P > 0.05$ ) of insecticide treatment on the quantity of earthworms coming to the surface after mustard extract drench (Table 5.4). Similarly, in the second (Aug) and third (Oct) field experiments, following curative insecticide applications, there were, no significant effects of treatment ( $F = 1.03$ ;  $df = 7, 31$ ;  $P > 0.05$  [Aug]) and ( $F = 1.82$ ;  $df = 7, 31$ ;  $P > 0.05$  [Oct]), on quantity of earthworms sampled. However, in both the August and October experiments, the water control plots had numerically the highest density of earthworms and the clothianidin plots had the lowest density (Table 5.4).

### **Discussion:**

The turfgrass insecticides products used in this study belong to three classes of insecticides that have different mode of actions. The neonicotinoids are agonists of nicotinic acetylcholine (nACh); they bind to and affect the opening of the nACh receptors. The neonicotinoids used in this study (imidacloprid, clothianidin, thiamethoxam, and dinotefuran) are systemic and contact poisons. Chlorantraniliprole is a diamide, which is a muscle ryanodine receptor modulator and has both systemic and contact effects. Trichlorfon is an organophosphate, which is an acetylcholinesterase inhibitor, and is a contact poison. Such differences in insecticide mode of actions could result in differences in their toxicity to an organism (Sánchez-Bayo 2012).

Insecticides vary in their persistence in the environment. Factors such as moisture, temperature, soil type, pH, and sunlight affect the persistence of insecticides in the environment. Various enzymes and microbes present in plants and soil further break down insecticides. A soil half-life of 40 d has been reported for imidacloprid in sugar beet fields not receiving fertilizer applications; whereas application of organic fertilizer increased the residual activity of imidacloprid to as high as 124 d (Rouchaud et al. 1994). Sarkar et al. (2001) found that imidacloprid may degrade by half as quickly as 28.7 d in some soil types and more than 80%

will have dissipated after 120 d. A review of soil half-lives of various neonicotinoids shows that half-lives range from 28–1250 d, 7–353 d, 148–6931 d, and 75–82 d for imidacloprid, thiamethoxam, clothianidin and dinotefuran, respectively (Goulson 2013). For chlorantraniliprole, a half-life of 366 d has been reported in tomato fields (Malhat 2012). Organophosphates are among the fastest degrading compounds in the soil and can degrade by half in soil within 2.5 d after application (Li et al. 2011).

Insecticides applied to turfgrass are degraded over time due to the exposure to weather and management practices. Moreover, in turfgrass a layer of dead and live plant parts known as thatch exists between aerial foliar parts and the soil surface. The thatch contains comparably higher populations of microbes than bare soil and is one of the major factors in soil degradation of insecticides (Gardner 2001). Generally, the half-life of insecticides is shorter in turf than in bare soil (Gardner 2001). In turfgrass, a half-life of 107 d has been reported for imidacloprid in Georgia (Cox 2001); however, it can be longer in colder regions. In turfgrass, chlorantraniliprole may persist for a longer time. A half-life as long as 258 days has been reported for chlorantraniliprole in turfgrass (Australian Pesticides and Veterinary Medicines Authority 2008). EPA estimated the half-life for chlorantraniliprole in turf foliage to be 30 d and in bare soil to be 1130 d. Regardless of residual activity, this study indicated that none of the tested insecticides significantly decreased the abundance of *L. terrestris* earthworms in turfgrass within a few days after application.

Our laboratory study showed that, except for imidacloprid and dinotefuran, none of the other insecticides tested resulted in very high mortality to *L. terrestris*. Additionally, imidacloprid and dinotefuran caused no significant negative effects on earthworms in the field. This difference in effect between laboratory and field experiments for imidacloprid and

dinotefuran can be explained due to the fact that earthworms were exposed to insecticides via surface contact in the laboratory experimental arena, which was a closed plastic pot. In the field, earthworms are less likely to come in direct contact with high concentrations of insecticides than in the laboratory because of the dilution factors in the soil and the availability of more space to move. Previous studies have indicated that direct contact exposure to insecticides is more toxic to earthworms than oral exposure (Wang et al. 2012a). Furthermore, imidacloprid and dinotefuran have been reported to be highly unstable when exposed to sunlight in the presence of water (Hahn et al. 2011). It is possible that the exposure of imidacloprid and dinotefuran to sunlight in the field, in addition to several other factors, caused those insecticides to rapidly degrade to a non-toxic level to earthworms. The effect of imidacloprid to earthworms in turfgrass has been found to be affected by its formulations, application season, and length of study. Kunkel et al. (1999) found that imidacloprid significantly reduced earthworm density 10 d following a spring application, but that there was no similar negative effect 10 d after fall treatments were applied and 40 d after both spring and fall applications. The result for the effect of imidacloprid on earthworms in this study is consistent with their study.

Our laboratory and field study showed that trichlorfon has relatively low toxicity to *L. terrestris* adults. Potter et al. (1990b) also found trichlorfon to be relatively less toxic to earthworms and other arthropods in turfgrass. Clothianidin was reported to be extremely toxic to *E. fetida* in the lab by Wang et al. (2012b) and to negatively affect abundance of earthworms, mainly of *Apporectodea* spp. population, one and three weeks after field application in turfgrass (Larson et al. 2012). However, I did not find very high mortality of *L. terrestris* in lab and field experiments with clothianidin or clothianidin + bifenthrin treatments. This difference in effect of same treatments to earthworms between two studies could partially be explained by the

differences in worm species. Previous studies have found that insecticide toxicity may differ among earthworm species (Haque and Ebing 1983). Mostert et al. (2002) reported imidacloprid to be very toxic to earthworms in the genus *Pheretima*, which occur in Asia. I also demonstrated similar toxicity in the lab with *L. terrestris*. I did not observe any negative effects of the diamide chlorantraniliprole on earthworms in lab or field experiments. These results are consistent with those of Larson et al. (2012) who found no apparent negative effect of this insecticide on *Aporrectodea* spp. earthworms in turfgrass. This study also showed that chlorantraniliprole, clothianidin, thiamethoxam, and trichlorfon, which consistently caused very low mortality to earthworms in both laboratory and field studies, apparently had no negative effects on the burrowing ability of earthworms. This indicated that earthworms were not intoxicated by these insecticides and that a single exposure of these insecticides at field rates is not very toxic to *L. terrestris*.

White grub control products might take multiple applications or seasons to show their effects on non-target organisms in the field. Peck (2009), for example, found that imidacloprid, trichlorfon, halofenozide, and nematodes in general had no effects on the abundance of soil arthropods after only a single application. However, repeated applications of imidacloprid for three years significantly reduced the total abundance of hexapods, collembolans, adult coleopterans and thysanopterans. Furthermore, earthworm exposure to lower concentrations of chemicals may cause various sub-lethal effects including reduction in total biomass, reduced fertility, and decreased number of cocoons produced (Capowiez et al. 2005). This study did not consider any such effects on earthworms. Moreover, a typical turfgrass management scenario comprises the repeated use of an insecticide for longer periods, or application of several insecticides plus other agrochemicals. Therefore, coming to a conclusion of ecological



significance from this single-time mortality based study would be impetuous. However, this study confirms that insecticide toxicity observed in the laboratory may not necessarily replicate in a field situation, and that a single time application of field rates of commonly used white grub control products might not be very toxic to earthworms.

## References:

- Australian Pesticides and Veterinary Medicines Authority. 2008. Evaluation of new active chlorantraniliprole in the products DuPont Coragen Insecticide, DuPont Altacor insecticide, and DuPont Acelepryn insecticide. Canberra, Australia.  
<http://apvma.gov.au/sites/default/files/publication/13651-prs-chlorantraniliprole.pdf>
- Bhadauria, T., and K.G. Saxena. 2010. Role of earthworms in soil fertility maintenance through the production of biogenic structures. *Applied and Environmental Soil Science* 7: 150–167.
- Capowiez, Y., M. Rault, G. Costagliola, and C. Mazzia. 2005. Lethal and sublethal effects of imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). *Biology and Fertility of Soils* 41: 135–143.
- Cox, C. 2001. Insecticide factsheet imidacloprid. *Journal of Pesticide Reform* 21: 15–21.
- Edwards, C. A., and P. J. Bohlen. 1992. The effects of toxic chemicals on earthworms. *Review of Environmental Contamination and Toxicology* 125: 23–99.
- Gardner, D. 2001. Pesticide fate in turfgrass. *Turfgrass Trends*. 10: 5–9.  
<http://archive.lib.msu.edu/tic/tgtre/article/2001feb5.pdf>
- Goulson, D. 2013. An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology* 50: 977–987.
- Gyawaly, S., R. R. Youngman C. A. Laub, and T. P. Kuhar. 2015. Evaluation of insecticide application timings against white grubs in cool season turfgrass in Virginia, 2013. *Arthropod Management Tests*. *Arthropod Management Tests* 39: G10.

- Gyawaly, S., A. M. Koppenhöfer, S. Wu, and T. P. Kuhar. 2016a. Biology, ecology, and management of masked chafer (Coleoptera: Scarabaeidae) grubs in turfgrass. *Journal of Integrated Pest Management* 7: 1–11.
- Gyawaly, S., C. A. Laub, and T. P. Kuhar. 2016b. Effects of common turfgrass insecticides on earthworms. *Virginia Turfgrass Journal*. May/June 2016, pp. 14-15.  
<http://www.thepaginator.com/view.php?pubID=45>
- Hahn, J., D. A. Herms, and D. G. McCullough. 2011. Frequently asked questions regarding potential side effects of systemic insecticides used to control emerald ash borer. University of Minnesota, Michigan State University, and The Ohio State University Extension Emerald Ash Borer Fact Sheet.  
<http://pest.ca.uky.edu/ext/eab/potential%20side%20effects%20of%20eab%20insecticides%20faq.pdf>
- Hale, C. 2013. *Earthworms of the Great Lakes* 2nd ed. Kollath + Stensaas publishing, Duluth, MN.
- Haque, A., and W. Ebing. 1983. Toxicity determination of pesticides to earthworms in the soil substrate. *Journal of Plant Diseases and Protection* 90: 395–408.
- Kunkel, B. A., D. W. Held, and D. A. Potter. 1999. Impact of halofenozide, imidacloprid, and bendiocarb on beneficial invertebrates and predatory activity in turfgrass. *Journal of Economic Entomology* 92: 922–930.
- Larson, J. L., C. T. Redmond, and D. A. Potter. 2012. Comparative impact of an anthranilic diamide and other insecticidal chemistries on beneficial invertebrates and ecosystem services in turfgrass. *Pest Management Science* 68: 740–748.

- Li, W., Y. Ma, L. Li, D. M. Qin, and Y. J. Wu. 2011. The dissipation rates of trichlorfon and its degradation product dichlorvos in cabbage and soil. *Chemosphere* 82: 829–833.
- Malhat, F., A. Hend, and H. Islam. 2012. Dissipation of chlorantraniliprole in tomato fruits and soil. *Bulletin of Environmental Contamination and Toxicology* 88: 349–351.
- Mostert, M. A., A. S. Schoeman, and M. V. D. Merwe. 2002. The relative toxicities of insecticides to earthworms of the *Pheretima* group (Oligochaeta). *Pest Management Science* 58: 446–450.
- Peck, D. C. 2009. Comparative impacts of white grub (Coleoptera: Scarabaeidae) control products on the abundance of non-target soil-active arthropods in turfgrass. *Pedobiologia* 52: 287–299.
- Potter, D. A. 1998. *Destructive Turfgrass Insects. Biology, Diagnosis, and Control*. Ann Arbor Press, Chelsea, MI.
- Potter, D. A., A. J. Powell, and M. S. Smith. 1990 a. Degradation of turfgrass thatch by earthworms (Oligochaeta: Lumbricidae) and other soil invertebrates. *Journal of Economic Entomology* 83: 203–211.
- Potter, D. A., M. C. Buxton, C. T. Redmond, C. G. Patterson, and A. J. Powell. 1990 b. Toxicity of pesticides to earthworms (Oligochaeta: Lumbricidae) and effect on thatch degradation in Kentucky bluegrass turf. *Journal of Economic Entomology* 83: 2362–2369.
- Roberts, B. L., and H. W. Dorough. 1984. Relative toxicities of chemicals to the earthworm *Eisenia fetida*. *Environmental Toxicology and Chemistry* 3: 67–78.
- Rouchaud, J., F. Gustin, and A. Wauters. 1994. Soil biodegradation and leaf transfer of insecticide imidacloprid applied in seed dressing in sugar beet crops. *Bulletin of Environmental Contamination and Toxicology* 53: 344–350.

- Sánchez-Bayo, F. 2012. Insecticides mode of action in relation to their toxicity to non-target organisms. *Journal of Environmental and Analytical Toxicology* S4: 002.
- Sarkar, M. A., S. Roy, R. K. Kole, and A. Chowdhury. 2001. Persistence and metabolism of imidacloprid in different soils of West Bengal. *Pest Management Science* 57: 598–602.
- Wang, Y., T. Chang, X. Zhao, R. Yu, L. Chen, C. Wu, and Q. Wang . 2012 a. Comparative acute toxicity of twenty-four insecticides to earthworm, *Eisenia fetida*. *Ecotoxicology and Environmental Safety* 79: 122–128.
- Wang, Y., S. Wu, L. Chen, C. Wu, R. Yu, Q. Wang, and X. Zhao. 2012 b. Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*. *Chemosphere* 88: 484–491.
- White, R. E. 2006. *Principles and Practices of Soil Science: the soil as a Natural resource*. Blackwell Scientific Publication, Palo Alto, CA.

Table 5.1. List of common turf insecticide products and their United States Environmental Protection Agency designated toxicity to earthworms based on lab bioassays with the “red wriggler” compost worm *Eisenia fetida* (Table adapted from Gyawaly et al. 2016 b).

Product	Active Ingredient (AI)	Designated Toxicity to Red Wigglers
Acelepryn 1.67SC	chlorantraniliprole	Acute toxicity
Arena 50 WDG	clothianidin	Super toxicity
Zylam (Safari) 20SG	dinotefuran	Acute toxicity
Merit 75WP	imidacloprid	Acute toxicity
Aloft GC SC	clothianidin plus bifenthrin	Extreme toxicity
Meridian 25 WG	thiamethoxam	No toxicity
Dylox 6.2 G	trichlorfon	Moderate toxicity

Table 5.2. List of insecticides and rates used in the study.

Treatments	Product	Active Ingredient (AI)	Rate (kg AI per ha)	Amount of product per ha	Amount product in 2.83 L water
1	Acelepryn 1.67SC	chlorantraniliprole	0.23	1.16 L	4.46 ml
2	Arena 50 WDG	clothianidin	0.44	0.89 kg	3.40 g
3	Zylam (Safari) 20SG	dinotefuran	0.60	3.02 kg	11.58 g
4	Merit 75WP	imidacloprid	0.44	0.60 kg	2.27g
5	Aloft GC SC	clothianidin plus bifenthrin	0.36 clothianidin 0.18 bifenthrin	1.38 L	5.30 ml
6	Meridian 25 WG	thiamethoxam	0.30	1.20 kg	4.52 g
7	Dylox 6.2 G	trichlorfon	9.07	146.44 kg	34.02 g/plot
8	Water treated check	--	--	--	--

Table 5.3. Mortality (Mean  $\pm$ SE) of *Lumbricus terrestris* 1-wk after treatment with different insecticides in laboratory experiments (One-way ANOVA).

Treatments	<u>Trial 1</u> Percent earthworm mortality (Mean $\pm$ SE)	<u>Trial 2</u> Percent earthworm mortality (Mean $\pm$ SE)
chlorantraniliprole	3.3 $\pm$ 1.9 b	12.5 $\pm$ 4.1 bcd
clothianidin	11.7 $\pm$ 4.2 b	7.5 $\pm$ 6.5 cd
dinotefuran	98.3 $\pm$ 1.7 a	97.5 $\pm$ 2.2 a
imidacloprid	88.3 $\pm$ 7.9 a	50.0 $\pm$ 15.4 ab
clothianidin plus bifenthrin	28.3 $\pm$ 17.3 b	35.0 $\pm$ 4.3 b
thiamethoxam	8.3 $\pm$ 5.0 b	10.0 $\pm$ 3.5 d
trichlorfon	10.0 $\pm$ 4.3 b	25.0 $\pm$ 4.3 bc
water check	1.7 $\pm$ 1.7 b	5.0 $\pm$ 2.5 cd
treatment effect	significant ( $P < 0.05$ )	significant ( $P < 0.05$ )

Means within a column with same letter are not significantly different by Tukey's test ( $P < 0.05$ ).



Table 5.4. Effect of different insecticide treatments on number of earthworms present per plot in the field study (One-way ANOVA).

Treatments	Number of earthworms (Mean $\pm$ SE) per sample (May experiment)	Number of earthworms (Mean $\pm$ SE) per sample (Aug. experiment)	Number of earthworms (Mean $\pm$ SE) (Oct. experiment)
chlorantraniliprole	3.25 $\pm$ 1.11	2.5 $\pm$ 0.75	4.25 $\pm$ 1.24
clothianidin	2.50 $\pm$ 0.29	1.25 $\pm$ 1.08	2.25 $\pm$ 0.89
dinotefuran	1.75 $\pm$ 0.63	4.25 $\pm$ 0.96	5.25 $\pm$ 0.96
imidacloprid	2.00 $\pm$ 0.71	2.00 $\pm$ 0.70	4.50 $\pm$ 0.90
clothianidin plus bifenthrin	2.50 $\pm$ 0.29	4.00 $\pm$ 1.41	7.25 $\pm$ 1.55
thiamethoxam	0.75 $\pm$ 0.48	3.25 $\pm$ 1.55	6.25 $\pm$ 1.34
trichlorfon	1.50 $\pm$ 0.50	2.50 $\pm$ 0.75	6.25 $\pm$ 3.37
water check	2.25 $\pm$ 0.85	5.25 $\pm$ 1.43	8.50 $\pm$ 2.01
Treatment effect	non-significant ( $P>0.05$ )	non-significant ( $P>0.05$ )	non-significant ( $P>0.05$ )

## Conclusions

White grubs are the most important pest of cool season turfgrass in Virginia. The interactions of these insects and their control tactics with other organisms in the turfgrass ecosystem, such as entomopathogenic fungi and earthworms are complex. Various chemical insecticides and biological control products are available for white grubs control in turfgrass. However, imidacloprid and chlorantraniliprole, which are the most commonly used insecticides currently, are effective only against early instar grubs if applied preventatively. In this dissertation, I examined if the combined applications of entomopathogenic fungi (EPFs) with some of the insecticides results in improved control of mature white grubs, if earthworms can impact white grub populations, and the effect of white grub control products on *Lumbricus terrestris* L., the most common earthworm found in Virginia turf systems.

In general, single (standalone) or combined applications of EPFs and insecticide resulted in low mortality of third instar *Cyclocephala* spp. grubs in laboratory (chapter 2). The combined applications of EPFs, *B. bassiana* or *M. brunneum* with imidacloprid or chlorantraniliprole in general resulted in additive mortality against non-overwintered third instar *Cyclocephala* spp. grubs. These findings indicated that standalone or combined applications of EPFs with these two insecticides are not very effective against third instar *Cyclocephala* spp. grubs. Therefore, these insecticides, EPF, or their combined treatments are not recommended for control of large *Cyclocephala* spp. grubs. This study, however, also found that imidacloprid or chlorantraniliprole did not negatively affect viability and virulence of *B. bassiana* and *M. brunneum*, indicating that insecticides and EPFs used in this study are compatible.

Some of the combination of EPFs with lower rate insecticides consistently resulted in low numbers of *P. japonica* eggs in the greenhouse, and decreased numbers of white grubs in the

field when applied in June but not in April or October (Chapter 3). This indicated that application of such treatments, when grubs are still early instars, may make it possible to reduce the amount of insecticides applied in the field. However, the practicality and economics of such combinations need to also be considered because insecticide applied as standalone treatments during summer also provided satisfactory control of white grubs.

Earthworms were found to have no significant effects on white grub distribution (Chapter 4). In the laboratory, the earthworms, *Eisenia fetida* and *E. hortensis*, transferred *B. bassiana* spores in soil that resulted in infections of *Galleria mellonella* larvae. However, the presence of earthworms in soil infested with *B. bassiana* spores did not result in increased mortality of *Cyclocephala* spp. grubs. This indicated that although earthworms can distribute EPF spores, they may not necessarily increase the infection of white grubs in the soil. Earthworms were found to have no negative effect on *P. japonica* oviposition in greenhouse trials that represented field situations (choice test). Overall, this study indicated that earthworms may not be a critical factor determining the abundance of white grubs in the field.

Of the different white grub control products tested, we found that only the two neonicotinoids, dinotefuran and imidacloprid, consistently resulted in high mortality of *L. terrestris* in the laboratory. In the field, however, we found that none of the white grub control products that are commonly used currently were found to have decreased abundance of earthworms (Chapter 5). This study indicated that single applications of field rates of commonly used white grub control products may not be as toxic. The study also highlights the importance of field research since laboratory research on insecticide toxicity studies may not represent the field research result. In conclusion, the interactions of white grubs, insecticides, EPF, and earthworms are complex. My work helped to better understand some of these interrelationships in turfgrass ecosystems, but

additional work should be done to further investigate these questions to lay the foundation for a more sustainable approach to turfgrass management in the future.

## APPENDIX A

### **Relationship between earthworm density and white grub density in turfgrass**

**Objective: To determine the relationship between earthworm density and white grub density in Virginia turfgrass.**

#### **Introduction:**

Earthworms (Oligochaeta: Megadrilacea) often cohabitate with white grubs (Coleoptera: Scarabaeidae) in soil ecosystems such as turfgrass. Yet, little is known about the interspecific interactions between these two groups of organisms. Earthworms improve soil health by burrowing and increasing soil porosity, aeration, and water infiltration. They also increase the organic matter content of soils by depositing fertile casts (Blouin et al. 2013). Thus, the presence of earthworms in soils may benefit white grubs by creating a more favorable habitat for them and the plants that they feed upon.

However, the interspecific interactions between earthworms and the soil insects with which they cohabitate may be more complex than simply mutually benefitting from improved soil. Earthworms are prey of many generalist predators including invertebrates such as ants, carabids, and mole crickets and vertebrates such as raccoons and skunks (Lee 1985, Rochefort et al. 2006, King et al. 2010, Xu et al. 2012). Many of these predators also eat white grubs (Coleoptera: Scarabaeidae) in cool season turf in North America (Rochefort et al. 2006, King et al. 2010) suggesting that the presence of earthworms in a habitat might also influence the population dynamics of white grubs by impacting predator populations. Earthworms can also transmit entomopathogenic fungi such as *B. bassiana* (Shapiro and Brown 2013; Chapter 4 of this Dissertation). However, it is still unclear if such transfer of EPF by earthworms results in any effects of earthworms on white grub populations in the field. This study was conducted to

determine if there is any relationship between naturally-occurring earthworm densities and white grub densities in turfgrass.

### **Materials and Methods:**

In 2014, earthworms and white grubs were sampled at Virginia Tech Turfgrass Research Center and at Kentland Farm in Blacksburg with 35 and 30 sampling sites respectively at each location. At each site, an area of 0.092 m<sup>2</sup> was dug with a flat shovel to the depth of 10 cm and earthworms and white grubs were counted and collected in ethanol for identification. The soil was loam (41% sand, 41.6% silt, and 17.5 % clay), with 2.9% organic matter and 6.1 pH at Turfgrass Research Center. At Kentland Farm, the soil was silty loam (42.2% sand, 53.3% silt, and 4.5 % clay) with 4% organic matter and 5.1pH.

### **Results and Discussion:**

Regression analyses revealed a significant positive relationship ( $P < 0.05$ ) at Virginia Tech Turfgrass Research Center or no relationship ( $P > 0.05$ ) at Kentland Farm between earthworm density and white grub density (Table A.1, Fig. A.1, A. 2). There were no negative effects of earthworm density on white grubs. The white grub species common at the VT Turfgrass Research Center were primarily *Cyclocephala* spp. [79 % *Cyclocephala* spp., 17 % *P. japonica*, and 4 % *Maladera castanea* (Arrow)] and *P. japonica* at Kentland Farm sites [16 % *Cyclocephala* spp., 51% *P. japonica*, 33% *M. castanea*]. Earthworms were primarily Lumbricids.

In the field, earthworm and white grub populations were either not correlated (Kentland Farm) or were positively correlated (Turfgrass Research Center) suggesting that the interspecific relationships among soil macrofauna are complex. Additional research is needed to completely understand the interactions earthworms and white grubs. This study indicated that the abundance

of earthworms in an area does not play a direct role in determining abundance of white grubs as observed in other systems (Salmon 2001). For white grubs, several other environmental and soil factors have been found to play more important role in determining the suitability of an area for white grubs. These factors include soil moisture, soil texture, and microbial composition of soil. *Popillia japonica* female oviposition behaviors have been previously found to be influenced by factors including soil moisture, soil type, and presence of insect pathogens (Fry et al. 1997). For example, *P. japonica* selectively laid more eggs in soil slightly higher soil moisture and in soil relatively low in percent sand content (Allsopp et al. 1992) and lower percent microbial biomass (Wickings 2016).

## References:

- Allsopp, P. G., M. G. Klein, and E. L. McCoy. 1992. Effect of soil moisture and soil texture on oviposition by Japanese beetle and rose chafer (Coleoptera: Scarabaeidae). *Journal of Economic Entomology* 85: 2194–2200.
- Blouin, M., M. E. Hodson, E. A. Delgado, G. Baker, L. Brussaard, K. R. Butt, J. Dai, L. Dendooven, G. Pérès, J. E. Tondoh, and D. Cluzeau, 2013. A review of earthworm impact on soil function and ecosystem services. *European Journal of Soil Science* 64: 161–182.
- Fry, R. C., L. A. Fergusson-Kolmes, S. A. Kolmes, and M. G. Villani. 1997. Radiographic study of the response of Japanese beetle larvae (Coleoptera: Scarabaeidae) to soil-incorporated mycelial particles of *Metarhizium anisopliae* (Deuteromycetes). *Journal of the New York Entomological Society* 113–120.
- King, R. A., I. P. Vaughan, J. R. Bell, D. A. Bohan, and W. O. Symondson. 2010. Prey choice by carabid beetles feeding on an earthworm community analyses using species- and lineage-specific PCR primers. *Molecular Ecology* 19: 1721–1732.
- Lee, K.E., 1985. *Earthworms: Their Ecology and Relationships with Soils and Land Use*. Academic Press, Sydney, Australia.
- Rocheftort, S., D. J. Shetlar, and J. Brodeur. 2006. Ground beetle assemblages and their seasonal abundance in cool season turf grass lawns in Quebec. *Environmental Entomology* 35: 1508–1514.
- Salmon, S., 2001. Earthworm excreta (mucus and urine) affect the distribution of springtails in forest soils. *Biology and Fertility of Soils* 34: 304–310.



Shapiro, D. I., and I. Brown. 2013. Earthworms as phoretic hosts for *Steinernema carpocapsae* and *Beauveria bassiana*: Implications for enhanced biological control. *Biological Control* 66: 41–48.

Wickings, K. 2016. Improving predictions of white grub distribution in turfgrass. New York State Turf Association Online Newsletter.  
<http://www.nysta.org/news/nystapr/2016/grubsenews.pdf> Accessed 07/15/2016

Xu, Y., D. W. Held, and X. P. Hu. 2012. Potential negative effects of earthworm prey on damage to turfgrass by omnivorous mole crickets (Orthoptera: Gryllotalpidae). *Environmental Entomology* 41: 1139–1144.

Table A.1. Linear regression equations relating white grub populations (number of white grubs/0.09 m<sup>2</sup>) to different earthworm populations (number of earthworms/0.09 m<sup>2</sup>) in Turfgrass Research Center and Kentland Farm.

Regression equation	SEM		r <sup>2</sup>	p
	Slope	Intercept		
Turfgrass Research Center				
$y = 0.3078x + 2.9259$	0.06	1.39	0.4381	<0.05
Kentland Farm				
$y = 0.0098x + 7.3336$	0.12	1.21	0.0002	>0.05

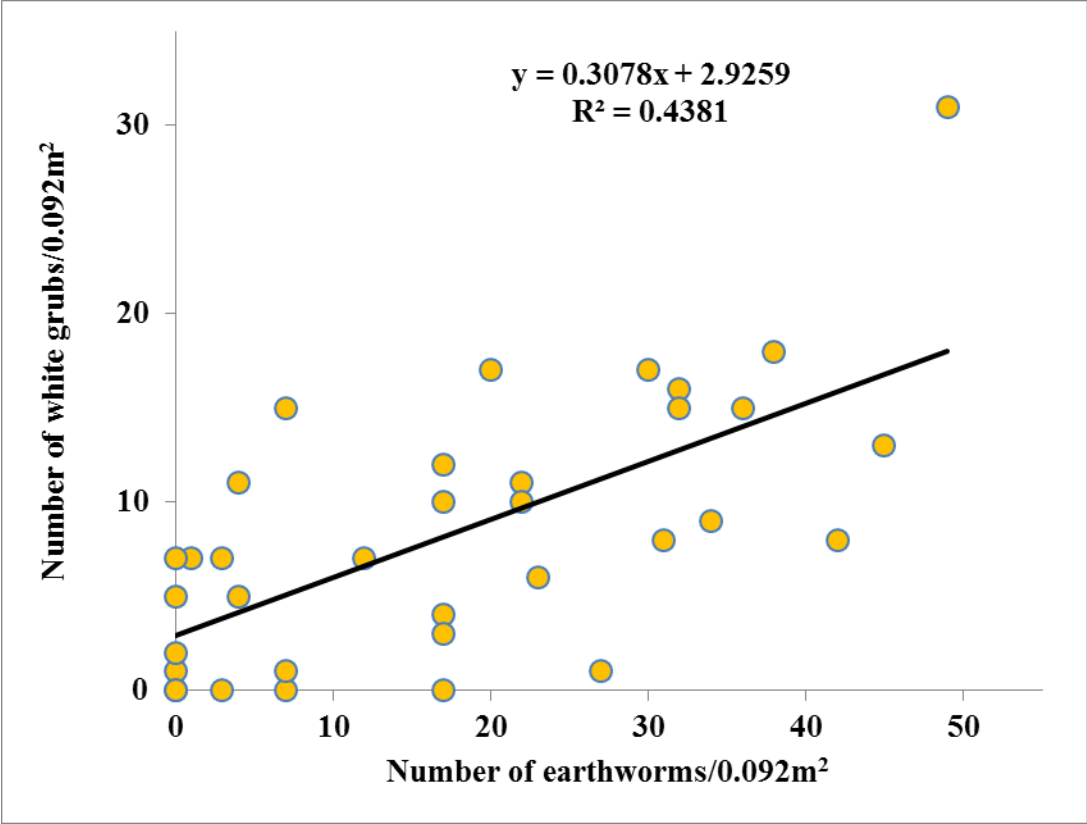


Fig. A. 1. Relationship between earthworm and white grub density at Virginia Tech, Turfgrass Research Center.

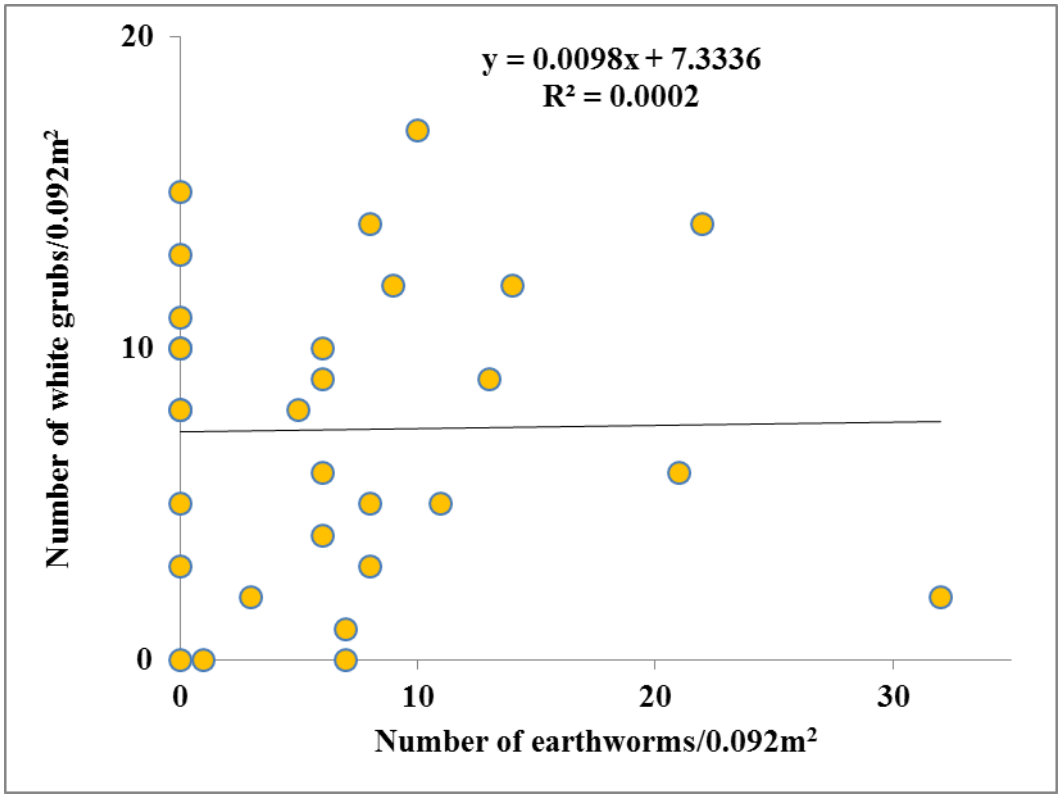


Fig. A.2. Relationship between earthworm and white grub density at Kentland Farm.