The response of *Agriotes obscurus* click beetles to pheromone and its impact on the acquisition of a fungal pathogen

by

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Abstract

Wireworms, the larval stage of click beetles, are a pest of many root crops, and are a challenge to control due to their long, subterranean life style and tolerance to chemical insecticides. An alternative approach is to target the adults. Pheromones have primarily been used as aggregants in attract-and-kill pest management tactics. However, pheromones can also alter insect movement and social interactions in other ways. I investigated whether female sex pheromone can enhance the primary transmission of the fungal pathogen *Metarhizium brunneum* Petch in *Agriotes obscurus* L. click beetles. Using video tracking, I found sex pheromone increases beetle activity regardless of season, and different light and air movement conditions. Heightened activity resulted in 58% more male-to-male contacts in a small arena. Although beetles picked up significant numbers of spores from contact with conspecifics, and an environment contaminated by conspecifics, pheromone did not enhance the level of infection obtained through these pathways.

Keywords: attract-and-kill; click beetle; horizontal transmission; *Metarhizium*; pheromone

To Ma, Pa and Stinky.

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Chapter 1.

Introduction

The study of insect pathogens has its roots in apiculture and sericulture and goes back to the time of Aristotle (B.C. 384-322), when it was recognised that domesticated insects, including honeybees and silkworms, succumb to disease (Steinhaus, 1956). However, it was not until 1938 that Sporeine, based on the gram-positive bacterium *Bacillus thuringiensis*, became the first commercially available microbial insecticide (Lord, 2005). Today, microbial insecticides, which are insecticides based on pathogenic bacteria, fungi, protozoa, viruses and nematodes, are a market worth $3.3 billion (USD) globally (Glare et al., 2012). They are used for the control of a wide range of arthropods, including those within the groups Acari; Lepidoptera; Coleoptera; Hemiptera; Diptera; Thysanoptera and Orthoptera (Arthurs and Dara, 2018; Ravensberg, 2015; Lacey et al., 2015 and references therein). Microbial insecticides based on *B. thuringiensis* have been especially successful, making up 52% of the total microbial pesticide market (CPL, 2010).

Microbial insecticides have experienced vast growth in market share over the past two decades (Bailey et al., 2009 Ravensberg 2011; Glare et al., 2012). This has largely been driven by concerns over the impact of synthetic chemicals on the environment and human health (Bruhn, 1991), which has led to wide-spread legislation regulating chemical pesticide input. The Europe Union, for example, implemented the directive on sustainable use of pesticides (2009/128/EC) in 2009 (OJEC, 2009) which aims to reduce the risk and impacts of pesticide use. Similarly, Canada has a pesticide risk reduction program in place, which is an initiative to reduce the risk of pesticides to human health and the environment (AAFC, 2015). Some of the activities of the program include providing support to smaller biopesticide companies, providing assistance for the registration of to close-to-market biopesticide products, and funding for various research programs (Kabaluk et al., 2010)

Despite the growing interest from the public and industry in adopting microbial insecticides, they still face barriers to wide-spread use. Some of the challenges they face include their ability to persist in the environment, their relatively slow speed-of-kill compared to chemical options, their limited host range, storage requirements and costs of

production. However, these barriers are largely because microbials are often viewed within a “chemical paradigm” (Jaronski, 2010); microbes are regularly applied in large quantities with existing field equipment (Feng et al., 1994), and they are expected to result in immediate control. More recently there has been an increase in novel formulation and application technologies, which overcome these shortcomings by taking advantage of the biological nature of microbials. Strain selection protocols have been optimised to take advantage of the natural diversity of entomopathogens (de Crecy et al., 2009), and advances in molecular techniques have allowed researchers to design pathogens with desirable traits (Yu et al., 2015; Wang and St. Leger, 2007). Changes in cultural practices, such as altering of chemical and tillage regimes, have vastly improved the efficacy of microbials in the field. New designs in application equipment allow microbes to be applied in a way that enhances their survival and likelihood of encountering hosts. (Beck et al., 2014).

Autodissemination is a method of microbial insecticide delivery that takes advantage of the ability of pathogens to persist and spread. The purpose of this thesis is to explore the novel delivery method of autodissemination, and to investigate whether the spreading ability of one fungal entomopathogen, *Metarhizium brunneum* Petch can be enhanced using semiochemical tools in the context of wireworm and click beetle management.

1.1. Fungal entomopathogens

There are around 700 species of fungi that have been identified as pathogenic towards insects (Roberts and Humber, 1981). Most entomopathogenic fungi infect by contact and invade through the integument and spiracles, unlike viruses and bacteria which need to be ingested. Consequently, they are especially important pathogens of sap-sucking insects such as aphids and leafhoppers (Roberts and Hajek, 1992). They are also important natural enemies of coleopterans, in which viral and bacterial diseases are rare (Hajek and St. Leger, 1994). The mode of infection of entomopathogenic fungi can be generalised as follows: fungal spores attaches on, and then penetrates into the cuticle. Once inside the coelom, the fungus may produce various structures including blastospores, protoplasts or hyphal filaments, depending on the species (Araújo and Hughes, 2016). Most species are hemibiotrophic; they will parasitize the live host and will continue to grow and develop once the host is dead. However, there are also some

groups, such as Entomophorales, that are biotrophic, which require a living host for development.

Entomopathogenic fungi have been used both in classical introductions as well as inundatively as microbial insecticides. Classical (or importation) biological control is 'the intentional introduction of an exotic biological control agent for permanent establishment and long-term pest control' (Eilenberg et al., 2001). Twenty species of entomopathogenic fungi have been used in classical biological control introductions, although recent changes in classification means that the number of species used is likely more. Introductions have had varying degrees of success. Although *Metarhizium* spp. are the most commonly introduced species (Hajek and Delalibera, 2010), the most successful introductions have been with fungi within the biotropic order Entomophthorales. Some well-known successful examples include the introduction of *Entomophaga mamaiga* in the control of gypsy moths in the United States (Hajek et al., 1996) and *Erynia radicans* in the control of spotted alfalfa aphid (*Therioaphis trifolii*) in Australia (Milner et al., 1982).

For inundative introductions, de Faria and Wraight (2007) reported that at least 12 species have been used, but again, with recent taxonomic reclassifications, this number is likely an underestimate. The most commonly used species are within four genera; *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria*, with 70% being *Beauveria* and *Metarhizium* species. These genera are ubiquitous in the environment and their saprophytic nature makes them amendable to mass-production. These fungi are predominantly used for the control of soil and greenhouse pests within the orders Coleoptera, Lepidoptera, Diptera and Hemiptera.

1.2. *Metarhizium*

Metarhizium is a genus of naturally occurring, soil-dwelling entomopathogenic fungus (Meyling and Eilenberg, 2007) in the order Hypocreales (Ascomycota). Its common name, green muscardine fungus alludes to the green conidia that are produced by infected insect cadavers (Figure 1.1). Classification of members within this genus is complex as they are anamorphic; only producing asexual spores. Consequently, classification has mainly been based on morphological similarities. Advances in molecular techniques, however, have now shown that *Metarhizium anisopliae* is composed of several morphologically similar species including *Metarhizium brunneum* (Driver, 2000; Bischoff

et al., 2009). Throughout this text, *Metarhizium anisopliae sensu latu* will be referred to as *Metarhizium* spp. unless the species is known. *Metarhizium* has been found to infect over 200 species of insects (Roberts and Hajek, 1992); however, different species and different strains have variable host ranges. For example, *M. anisopliae* var *acridum* is specific to acridids (grasshoppers and locusts) while others, like the commercial *M. brunneum* strain F52 is known to attack hosts across several orders (e.g. Erler, 2014; Liu and Bauer, 2006; Stafford et al., 2010; Weeks, 2017;). *Metarhizium*-based biopesticides have been used successfully to control locusts in Australia and Africa (reviewed in Bateman et al., 2017), spittlebugs and sugarcane borers in South America (Tiago et al., 2011) and ticks (Kanga et al., 2003). Continued research has gone into their use on other potential target pests such as malaria-vectoring mosquitos (Scholte et al., 2005) and invasive forest pests (e.g Hajek, 2006; Liu and Brauer, 2006).

The *Metarhizium* spp. infection process (Figure 1.2) begins when the asexual spores contact the host's cuticle; the hydrophobin protein layer of the spores allows them to adhere to the hydrophobic surface of the host (Holder and Keyahni, 2005). This is a highly efficient process; Brooks and Wall (2005) reported that 40% of *Psoroptes* mites exposed to *M. anisopliae* became infected "after a single touch of cadavers lasting less than one second". Conidia then germinate and produce an appresorial peg which penetrates into the coelom using a combination of mechanical pressure and cuticle degrading enzymes. Once inside, the fungus produces hyphal bodies. When the host eventually succumbs to the infection due to physical damage and toxicosis (Hajek and St. Leger, 1994), sporulation occurs under high humidity conditions.

Historically, fungal entomopathogen biology has focused on fungal interactions with the host; however, fungi form complex interactions with the external environment as well (e.g. Bidochka et al., 2001). The ability of *Beauveria bassiana* to colonize plant tissue (Bing and Lewis 1991, 1992) has motivated similar research on *Metarhizium* spp. (Jaber and Vidal., 2015). *Metarhizium* primarily disperses by infected hosts migrating to new areas before dying and releasing fungal propagules into the environment. Although female hosts carrying *Metarhizium* spores can transmit the fungus to eggs (Hajek et al., 2008), there is no evidence that this occurs in the wild, and it is thought that vertical transmission plays a minor role in the life cycle of the fungus (Fuxa, 1987). *Metarhizium* was traditionally thought of as a sit-and-wait pathogen; the fungus produces dormant conidia that remain viable in the soil for long periods of time, which become reactivated when a suitable host

comes encounters them. Spores may also be dispersed by wind or rain, or non-host vectors. However, recently *Metarhizium* spp. have been shown to form associations with plants within the rhizosphere (Bruck, 2005; Hu and St Leger, 2002). Some *Metarhizium* spp. have also been found to have the ability to live within plant tissue (Batta, 2013; Elena et al., 2011), although most studies that show this are based innoculative experiments; evidence that endophytism occurs naturally remains scant. Indeed, *Metarhizium* was not one of the four entomopathogenic genera found in coffee in an extensive three-year survey of endophytic fungi in coffee plants in South America (Vega et al., 2008). While these associations may have evolved as a pathway for environmental persistence, some researchers have also speculated that this duality in lifestyle may have been driven by the symbiotic relationship with plants, as evidenced by the ability of plants to take up insect derived nitrogen when entomopathogenic fungi are present (Behie et al., 2012). In addition, these fungi have also been found to be antagonistic to plant pathogens (Sasan and Bidochka, 2013). These new revelations have inspired new ways of thinking about how fungal bio-insecticides are applied.

1.3. *Metarhizium* as a microbial pesticide

Metarhizium aniosopliae became the first microbial pesticide to be mass produced when a production plant was established in Russia in the late 19th century by Isaac Krassilstchik (Krassilstchik, 1888), following the idea of his mentor Élie Metchnikoff, that *M. anisopliae* could be cultured on beer mash for wheat cockchafer beetle (*Anisoplia austriaca*) control. The ease of mass production has allowed it to become one of most commonly used biopesticides on the market today.

Microbial pesticide formulation is a key factor in determining the success of a microbe as a biological control agent; not only does it determine the cost of production and shelf life, but it can also dictate how the microbial pesticides are delivered to the target pest, and ultimately the efficacy. Two thirds of commercial *Metarhizium* and *Beauveria* formulations are based on the aerial conidia (Jackson et al., 2010) which have a high environmental tolerance allowing for easy storage. They can be applied dry in the form of dust or granules, or they can come in liquid formulations, which may be aqueous or oil-based. Granular formulations are generally combined with soil to target soil dwelling pests, while dust and liquid formulations can be sprayed or dusted with the same equipment that is used to apply synthetic chemical insecticides. Often additives may be added to

formulations to extend the shelf life or to improve persistence in the field (Alves et al., 1998; Liu and Lin, 2013).

Although most formulations are based on aerial conidia, some researchers have explored using alternative life-stages as biopesticide inocula. Blastospores, which are the yeast-like life stage of hypocrealan produced within the haemocoel, have been reported to have higher virulence compared to their conidial counterparts in some systems (Alkhaibari et al., 2016; Hall 1979). However, their thinner cell walls (Hegedus et al., 2992) makes them unstable during the drying process, a necessary stage in its dry formulation. This can be overcome with the use of additives in the culture medium, which in one study, can extend the survival blastospores of *M. anisopliae* to twice as long as conidia in storage (Kleespies and Zimmerman, 1998). *Metarhizium* has also been found to produce microsclerotia (Jaronski and Jackson, 2008); compact masses of melanised hyphae which are formed by pathogenic fungi as overwintering structures in soil. This iteration of the fungus can be desiccated and rehydrated to continue development. In sugarbeet root maggots (*Tetanops myopaeformis*), microsclerotia-based *M. anisopliae* granules were found to cause ~60% more larval mortality than conidiated granules at 20% soil moisture (Jackson and Jaronski, 2009). The authors of this study speculated that the difference in performance can be attributed to the faster production of conidia by microsclerotia granules and suggest that this may be a better option for soil dwelling pests.

1.4. Combining microbial pesticides with semiochemicals

Semiochemicals, which are chemicals that are released by organisms that affect the behaviour of other individuals, have been investigated in the novel application of microbial pesticides. Semiochemicals may be used for intraspecific communication (pheromones) or as interspecific cues (kairomones). The ability to change pest behaviour, coupled with low toxicity, specificity and efficacy at low volumes, has made semiochemicals a valuable tool in pest management (Witzgall, 2010). In addition, compared to other synthetic chemicals where resistance is an oft reported problem (Kranthi et al., 2002; Roberts, 1994), it is difficult for pests to develop resistance to semiochemicals as they are integral to the reproductive success of an insect (Witzgall, 2010, although see Evenden and Haynes (2001) and Tabata et al. (2007).

Semiochemicals have inspired a number of pest management strategies. Mating disruption is a strategy that uses high concentrations of pheromone to disrupt intersexual communication. This strategy has been most successfully used in lepidopteran pests including the codling moth (Cardé and Minks, 1995) and the gypsy moth (Leonhardt et al., 1996). Mass trapping, where large numbers of a population are attracted to a large-capacity trap with the goal of removing populations before feeding, mating or ovipositioning can occur has been used in the control of the bark beetle *Ips duplicatus* (Schlyter et al., 2003). Trap saturation, where traps become inundated with the pest can be problematic with mass trapping. Where trap saturation is a concern, mass trapping can be modified so that pests are attracted to a kill agent instead of a trap. These attract-and-kill strategies have been successfully used on several pest species (e.g. Butler, 2007; Smith, 1998).

Autodissemination is a method of insecticide delivery where a kill agent (can be chemical or biological) is disseminated by a target pest to members of its own species (Vega et al., 2007). Often, a semiochemical is incorporated with the kill agent in an attract-and-infect tactic. Autodissemination techniques that use pathogens as kill agents have been demonstrated in several systems including diamond back moth (Vickers et al., 2004), Japanese beetles (Klein and Lacey, 1999) and banana weevils (Tinzaara, 2007). More recently, autodissemination has gained a lot of attention due to interest in adopting this approach for malaria vector control (Chandel et al., 2016; Scholte et al., 2004). However, these techniques are still commercially immature, and there are few examples of autodissemination being used on a large scale. Variations of autodissemination techniques exist. A non-target vector can be used, such as the case for pollinator biocontrol vector technology, where pollinators are used to disseminate a pathogen to a target location (Butt et al., 2010).

For microbial control agents, autodissemination is an attractive strategy as it is possible to incorporate autoinnoculator devices into the system which protect inocula from weather elements. Autodissemination is also attractive as it does not require the entire population to have contacted the inocula for epizootics to occur. Most diseases are density dependent, which means that transmission between a susceptible and infected individual is proportional to the population density. Epizootics can occur when a threshold number of individuals are infected (Anderson and May, 1981). For example, Vickers et al., (2004) found adult diamond back moths (*Plutella xylostella*) infected with the fungus *Zoophthora*

radicans were able to transmit spores to susceptible larvae in a large cage experiment, leading to a 79% infection rate. In some cases, pathogen propagation after introduction can lead to secondary rounds of infection, which could also extend the impact of a pathogen.

1.5. Wireworms and click beetles

1.5.1. Wireworms as pests

Wireworms, which are the soil dwelling larval stage of click beetles (Coleoptera: Elateridae), feed on underground plant tissue and are a global pest of root crops (Furlan, 2005; Parker and Howard, 2001; Hokyo, 1980; Toepfer et al., 2014). In North America, they cause an estimated crop loss of 5-25% a year (Jansson and Seal, 1994). Being generalists, they are a pest of range of crops including sweet potatoes (Chalfant et al., 1993), potatoes, carrots (Parker and Howard 2001) and sugar beets (Stone, 1941). In these crops feeding in roots or tubers by larvae can create tunnels that make the crop unsellable. Crops that rest on the soil surface, such as strawberries are also susceptible to similar damage (Vernon et al., 2001). In cereals, feeding on young roots can cause death, and expose seedlings to pathogens. Of the 10,000 species of identified elaterids, 100 are regarded as pests (Vernon and van Herk, 2013).

A number of life history characteristics of wireworms make them difficult to control; in particular, they have a long life cycle, lasting anywhere from one to five years depending on the species. Many generations may be in the soil at any one time, making wireworms a problem that can persist for multiple years. As most of the elaterid life cycle takes place underground, topical applications of chemical insecticides can be ineffective. Wireworms are also resistant to a large number of the currently available chemical insecticides; those that were effective in the past, including some organochlorines, organophosphates and carbamate based chemicals (van Herk et al., 2008) have been phased out due to environmental and health concerns. In the past, a single application of the cyclodiene chlorinated hydrocarbons, Aldrin and helpatochlor gave protection to a field for at least nine years, and insecticide residues were known to be still present in quantities toxic to young wireworms after 14 years (Wilkinson et al., 1976). Changes in farming practices, such as no-till regimes, has further exacerbated the problem (Parker and Howard, 2001). Currently available options, including neonicotinoids, offer some stand protection but only

make wireworms moribund and fail to kill them (van Herk et al., 2008). Consequently, although feeding may be supressed in one year, feeding activity would likely return the following year.

Monitoring wireworm populations and establishing economic thresholds are also difficult. This is in part due to some elaterid species being similar in appearance, but having vastly different biology (Furlan, 2005), therefore what may be effective for one species may not work for another. In addition, although identifying pheromones have made it possible to trap adult beetles, the relationship between adult and larval population density is still unclear (Benefer et al., 2012).

Wireworm species that are most economically important in British Columbia include the two European species *Agriotes lineatus* and *A. obscurus*. They are believed to have been introduced from nursery stock or ship ballasts in the late 1900s (Wilkinson. 1963). Wireworms have been a persistent problem since their introduction, but was adequately managed with the availability of synthetic chemicals in the 1950's. It was not until the 1990's, when residual chemicals from previously used chlorinated hydrocarbon based insecticides started disappearing, that wireworms once again became a priority for management. Currently in Canada, the options for growers are limited; clothianidin, a neonicotinoid is registered as a seed piece treatment, while chlorpyrifos and Thimet are registered organophosphates for use on larvae. There are continual investigations into new classes of chemicals; most recently Vernon et al., (2015) was able to reduce potato tuber damage by 67-81% with companion plantings of wheat seeds treated with a combination of fipronil and thiamethoxam. For organic growers, options are limited to cultural techniques such as tilling (Andrews et al., 2008), which can mechanically damage developing pupae in the soil.

1.5.2. Dusky click beetle, *Agriotes obscurus*

Life cycle

Most of what we know about *Agriotes obscurus* is based on work done in Europe in the 1940s. The life of *A. obscurus* beetles begins when the eggs hatch in early fall. Once hatched, larvae will feed on underground plant tissue. They migrate up and down the soil column throughout the season depending on the soil moisture and will go through 13-15 moults over 3-5 years before pupating in the summer (Sufyan et al., 2014). Adults eclose

in the fall and will overwinter in the soil before emerging in early spring. Males tend to emerge earlier; in a trap study Brian (1947) found that sex ratios tend to favour males 3:1 in early spring. Later in the season, the sex ratio evens out to a roughly 1:1 ratio. Cohen (1942) reported that they mate soon after becoming active, and noted that they likely mate more than once. Adults are presumed not to survive past the fall, although a lab study had found that newly emerged adults can survive for over 150 days at simulated season temperatures (Kabaluk, personal comm.) In addition, other *Agriotes* spp. where it was though activity was limited to the summer months have been found to be active in the fall in certain regions (Kabaluk, 2016), suggesting regional differences may be present. Egg-laying takes place over a period of 4-5 weeks. Cohen (1942) reported that females can lay anywhere from 70 to 186 eggs in a lifetime (Cohen, 1942). Each laying event consisting of eggs laid singly or in clusters of up to 12 eggs. Eggs are laid in the top 2-3 inches of the soil surface and hatch 25-44 days after egg laying, after which the cycle repeats.

A. obscurus *adult activity*

During the adult active season, *A. obscurus* beetles are described as having diurnal habits. Roebuck et al., (1947) found most beetles were caught early in the morning using grass traps, while Brian (1947) and Cohen (1942) found beetle activity was greatest during the evening. Brian (1947) found that activity was limited by temperature between the hours of 0.00-12.00 h, with beetles being less activity when temperatures are lower. However, Roebuck et al., (1947) reported that attempts to correlate activity with meteorological data indicate that beetles are more likely to be influenced by humidity.

A. obscurus was believed to be only capable of short distance migrations in the past; however, recent reports describe the contrary; using carbon stable isotope techniques, Schallhart et al., (2009) found that *A. obscurus* beetles are able to disperse up to 90 m. Sufyan et al., (2011), in a mark-release-recapture study, were able to recapture beetles that were released 60 m away. Similarly, Crozier (2007) found beetles were able to travel at least 50 m over a 24 hour period. Longer distances can be covered with flight and certainly there are species of elaterids that are capable of flight. However, records of *A. obscurus* flight are rare. Crozier (2007) noted a threshold temperature of 25 ºC was required for flight.

An attract-and-kill strategy in A. obscurus

Treating seeds with synthetic chemicals has been widely used to reduce wireworm damage on crops (Vernon and van Herk, 2013). These methods work by exposing wireworms to insecticides as they move towards food sources. Recently there have been attempts to adopt this effect into attract-and-kill tactics for wireworm control. Vernon et al 2016 found interplanting potato tubers with wheat seed treated with the fipronil and thiamethoxam resulted in control comparable to in-furrow granular applications of phorate.

Attract-and-kill has also been explored with microbial control agents. Multiple strains of *Metarhizium* spp. have been found to be virulent towards wireworms (Ansari, 2009; Eckard et al., 2014; Kabaluk et al., 2007). Early studies using *Metarhizium brunneum* on wireworms have been promising; in one field study, *Metarhizium* conidia incorporated into the soil resulted in a 30% reduction in feeding damage in potatoes (Kabaluk et al., 2005). *Metarhizium* has also been used as part of an attract-and-kill strategy on wireworms: Brandl et al., (2017) combined *Metarhizium* with carbon-dioxide producing baker's yeast and managed to achieve a 37-75% reduction in tuber damage.

Traditionally wireworm management has been targeted at the larval stage; however, recently there have also been attempts to control populations by targeting the adult stage to reduce the number of mated and ovipositioning females in the field. The female sex pheromone of almost all important European pest click beetle species have been identified (Tóth and Furlan, 2005). Some elaterid sex pheromones have also been shown to be attractive to more than one species, and some are even considered to be attractive to females in addition to males (Tóth, 2013). These pheromones have been used extensively for monitoring and surveying purposes (e.g. Furlan and Tóth, 2007). However, their use in click beetle control has been limited. In Japan, Arakaki et al., (2008a) were able to reduce *Melanotus okinawensis* click beetle populations by 90% over five years using a mass trapping approach. However, there seemed to be no relationship between the numbers of beetles captured in traps and population reduction. Lower numbers of mated females were found earlier on after treatment, while those caught later had higher rates of mating. Therefore the authors postulated that the reduction in population numbers may have been due to delayed mating in females as a result of the pheromone lures used to capture males. (Arakaki et al., 2008a). Vernon et al., (2014), while exploring mass trapping in field margins as management strategy, concluded that

while mass trapping can theoretically reduce mating and oviposition, the effect was not large enough to make it an effective solution. Mating disruption has also been attempted to a lesser degree: Arakaki et al., (2008b) found that a mating disruption strategy reduced the total number of beetle catches by 74% over a period of five years.

1.6. Research objectives

An attract-and-kill strategy using female sex pheromone and *Metarhizium brunneum* has recently been investigated for the control of *Agriotes obscurus* click beetles (Kabaluk et al., 2015). A release-recapture study in open-top arenas found that a band application of pheromone-impregnated granules and *M. brunneum* conidiated rice grains was able to reduce beetle recapture by 98.2%. The aim of this thesis is to investigate whether this system can be further developed as an autodissemination strategy. To answer this question, I first investigate the robustness of the male *A. obscurus* response to female sex pheromone under different biotic and abiotic conditions in Chapter 2. The conditions considered include light quality, air movement, distance from pheromone source, and beetle age. In addition, I also examine how male beetles exhibit a positive response to female sex pheromone, and speculate as to how these changes in behaviour may be exploited in the managing of this pest. In Chapter 3, I explore whether changes in activity due to the presence of female sex pheromone affects the passive horizontal transmission and dispersal of *M. brunneum* in *A. obscurus* beetles.

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1.8. Figures

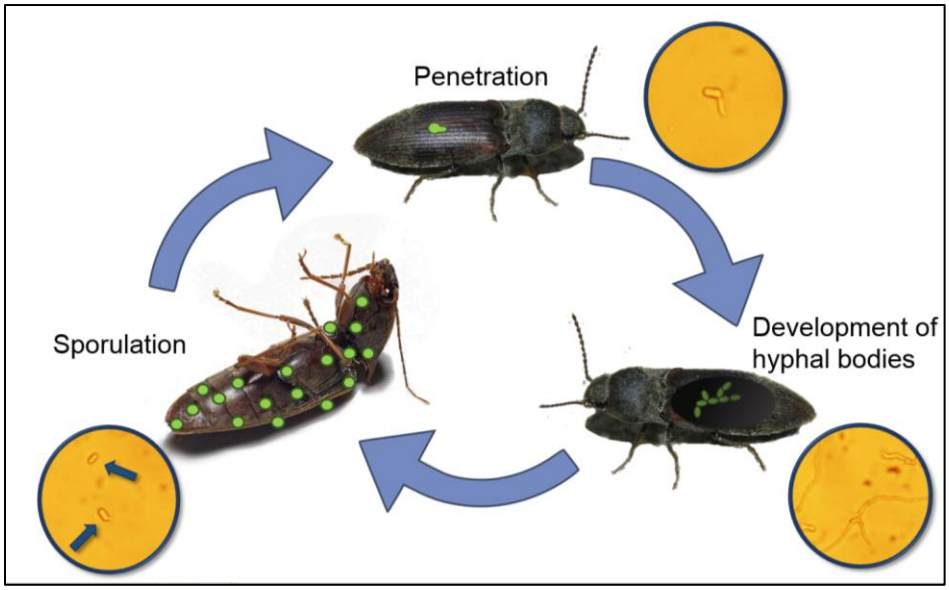

Figure 1.1 ***Metarhizium* pathogenesis.** The *Metarhizium* spp. infection process begins when the asexual spores contact the host's cuticle in which the hydrophobin protein layer of the spores allow spores to adhere to the hydrophobic surface (Holder and Keyahni, 2005). Conidia then germinate and produce an appresorial peg which penetrates into the coelom using a combination of mechanical pressure and cuticle degrading enzymes. Once inside, the fungus produces hyphal bodies. When the host eventually succumbs to the infection due to physical damage, and toxicosis (Hajek and St. Leger, 1994), sporulation occurs under high humidity conditions.

Figure 1.2 ***Agriotes obscurus*** **beetle cadaver sporulated with** ***Metarhizium brunneum***

Chapter 2.

Effect of seasonality, light, and air movement on attraction of male *Agriotes obscurus* click beetles to female sex pheromone

2.1. Introduction

Wireworms, the larval stage of click beetles (Coleoptera: Elateridae), are important pests in Europe, North America, and Asia (Eidt, 1953; Iwanaga and Kawamura, 2000). They attack underground plant tissue of a range of crops, including potatoes and sugar beets, creating holes and tunnels that make the crops unmarketable. In potatoes alone, there are an estimated 39 pest wireworm species from 21 genera (Jansson and Seal, 1994). In cereal crops, damage to seedlings can mean early death. The subterranean lifestyle of wireworms makes them particularly difficult to control. Wireworms develop over a period of 3-5 years in the soil before pupating. Topical applications of chemicals are therefore ineffective unless they penetrate the soil and are highly persistent. Effective chemical control options were established for this pest, with the rise of organochlorine based insecticides in the 1950's, which allowed this pest to be adequately managed. However wireworms have resurged in importance recently as a result of the loss of residual persistent chemical insecticides from fields. Chemical insecticides that have been used in the past, such as the organochlorine Lindane, have been phased out due to environmental and health concerns (Barsics et al., 2013). Changes in agricultural practices, including the adoption of no-till regimes, may have also contributed to the rise of wireworms (Parker and Howard, 2001). One of the current options for wireworm management are neonicotinoid seed treatments, which have been shown to provide wheat stand protection (Vernon et al., 2009). However, this class of chemicals only render wireworms moribund and do not cause mortality, making them ineffective in reducing wireworm populations (Crozier et al., 2003; Vernon et al., 2009). Consequently, there is considerable interest in developing alternative forms of control.

One possible solution is to target the adult beetles to reduce oviposition and lower population growth. In British Columbia, Canada, *Agriotes obscurus* (L.) is one of two European species that are considered economically most damaging to potatoes (Vernon

et al., 2001). Adult *A. obscurus* beetles eclose in winter and early spring and remain inactive in the soil until temperatures are favourable (Cohen, 1942), after which mating and oviposition occur. Ovipositing adults are often most numerous in pastures and cereal crops, therefore controlling adult populations is likely to be most effective in these habitats (Vernon and van Herk, 2013). Targeting the adults in conjunction with the larvae can be important, as multiple generations of wireworms may coexist at a given time. In addition, adults are the main dispersive stage of elaterids (Traugott et al., 2015), making them a key target for control. In a study by Schallhart et al., (2009), *A. obscurus* beetles were found to be capable of dispersal distances of 80 m, which was the maximum distance tested. Flight occurs in this species in some parts of Europe (Fryer, 1941; Subklew, 1935). Although flight has been reported to occur rarely in North American populations (Eidt, 1953; Wilkinson et al., 1976), a recent report suggests that mass flight may occur under certain conditions (Crozier et al., 2003)

The female sex pheromones of most major European wireworm pest species, including *A. obscurus,* have been characterized (Tóth and Furlan, 2005). They are mostly used for monitoring purposes (Vernon and Tóth, 2007); however, a number of pheromone-based management tactics that target adults have been attempted in the past, including mass trapping (Arakaki et al., 2008; Vernon et al., 2014), mating disruption (Arakaki et al., 2008; Fujiwara-Tsujii et al., 2014), and attract and kill (Kabaluk et al., 2015).

Effective lure placement in pheromone-based control strategies requires consideration for how a pest species responds to pheromone under different biotic and abiotic conditions (McNeil, 1991). Environmental cues, for example, may provide information for orientation towards chemical cues. Although chemical gradients can give directional information, other sources of directional information including celestial cues, and water and wind currents may be necessary for successful location of odour sources (Bell, 1991). Wind speed and direction have been identified as important in determining pheromone trap success in pests including lepidopterans (Riedl, 1986) and boll weevils (Sappington and Spurgeon, 2000).

In addition to environmental cues, insects may exhibit search rhythms (Daan, 1981), which can restrict pheromone response to certain periods during the day or year In most noctuid moths, for example, males respond to pheromone in the mid to late scotophase, which corresponds to periods of pheromone emission by females. This

phenomenon may be mediated by environmental cues such as photoperiod (e.g. Rosén et al., 2003), temperature, or endogenous mechanisms. There is, however, a lack of knowledge of the sensory ecology of *A. obscurus* and other European click beetles.

Recently, a granular formulation of female sex pheromone was developed and investigated for use in an attract-and-kill strategy with the fungal pathogen *Metarhizium brunneum* Petch (Ascomycota: Hypocreales: Clavicipitaceae) (Kabaluk et al., 2015). A granular formulation of pheromone may prove to be a cost-effective alternative to current liquid formulations and allow for novel application methods. Although the range of attraction for a point source of liquid pheromone has been determined previously (Hicks and Blackshaw, 2008; Sufyan et al., 2011), the attraction distance is for a granular formulation is not known. The objectives of this study were to evaluate the male *A. obscurus* click beetle response to pheromone under different abiotic conditions, and to determine the attraction range of pheromone to male beetles in the field. In our first objective, we tested whether air movement and light quality affect the response of male *A. obscurus* beetles to the pheromone. We also tested whether the response of *A. obscurus* beetles collected at different times in the season varied to determine how the efficacy of pheromone treatments would vary across seasons. Understanding how these factors affect pheromone response is vital to facilitating the development of pheromone-based techniques in the management of *A. obscurus* populations.

2.2. Materials and methods

2.2.1. Beetle collection and pheromone formulation

Due to the difficulty of rearing click beetles in the lab, our study used field collected beetles. Male *A. obscurus* beetles were collected at the Agassiz Research and Development Centre in Agassiz, British Columbia, Canada (49.22419, -121.7535) in April and May in 2014, and March, April and May in 2015, using pitfall traps baited with pheromone. Beetles collected in 2014 were used in experiments 1 and 2, whereas beetles collected in 2015 were used in experiments 3 and 4. It is not possible to know the age of the field collected beetles, although adult beetles die after one season. Fifty percent of lab-reared beetles lived for 150 days under May temperatures simulated in the lab (Kabaluk, personal communications) but their *in vivo* survival would be expected to be much shorter. For the experiments described herein, field-collected beetles were kept in

groups (≤ 300) in vented plastic containers (~20 cm X 20 cm X 10 cm). They were fed organic apple pieces *ad libitum*, and provided with dampened paper towel and fresh grass clippings (Poaceae spp.) and kept at 10°C in darkness until use. The pheromone granules were created by impregnating a cellulose based granule with a synthetic blend of *Agriotes obscurus* pheromone (1:1 blend of geranyl octanoate and geranyl hexanoate) in a proprietary process (1% wt/wt, Scotts Canada, Delta BC). Non-impregnated granules ('blank') were used as controls. A different granule was used for Experiment 4 to comply with organic certification requirements (https://www.certifiedorganic.bc.ca).

2.2.2. Experiment 1: Air movement

The effect of air movement on the response of *A. obscurus* males to pheromone was tested in an open-top arena experiment. Behavioural assays took place in July and August 2014 between 10:00 and 18:00 h at room temperature (~25-27 ^{o}C). Beetles were exposed to either pheromone or blank granules under moving (1.2 ms^{-1} air speed) and still-air conditions in a full factorial design. Beetles were removed from cold storage two to four h before testing. A single beetle was placed into a wind tunnel (Figure 2.1), 10 cm from the downwind end and covered with a 1 oz (29.6 ml) plastic soufflé cup (Solo cup company, Lake Forest IL) for five min to acclimatize. A 2 cm-wide band of granules (0.9 g) was applied by dropping granules onto a stencil placed 10 cm upwind (Figure 2.1). The stencil and cup were then removed and the beetle was video-recorded for 10 min with a GigE camera (Basler acA1300-60gc), connected to a computer (Dell, Optiplex 780). Beetles were only used once. Out of the 209 beetles tested, 42 did not cooperate (i.e. climbed on to the screen at either end or did not move after the initial 3 minutes) Failure to cooperate could potentially be influenced by treatment. For example, beetles exposed to pheromone in the field have been observed to climb to the top of grass blades (Kabaluk, 2015), therefore beetles in the pheromone treatment may be more likely to climb on to the screen at either end. Beetles in the blank treatment may also be less likely to move at all compared to the pheromone treatment. To determine whether treatment affected the likelihood of cooperation, the proportion of non-cooperators were analyzed with a binary logistic mixed model, with the date of experiment included as a random effect. I found that treatment did not influence which beetles failed to cooperate (Appendix A, Table A2), therefore they were removed from the final analysis. A total of 19-23 valid (i.e. not

discarded) replicates were conducted for each of the four air movement-pheromone combinations for each beetle collection period (April: N=87; May: N= 82).

Videos were analyzed using Ethovision XT 10 software (Noldus, Wageningen, Netherlands). Videos were sampled at 8.9 frames per second. Tracks were inspected and corrected manually where the software lost the target beetle. A 'Locally weighted scatter plot' smoothing algorithm (Noldus Information Technology, 2013) was applied afterwards to reduce noise in tracking. Beetle walking speed, distance moved, time to first contact with the pheromone band, the number of contacts, and duration of the contacts with the granules were used as measures of the beetles' response to pheromone. Duration of contact is defined as the total amount of time spent in the pheromone band in the trial. A three-way linear mixed model was conducted with pheromone treatment (pheromone, blank), air movement (moving, still) and beetle collection period (April/May) included as fixed effects (Table 2.1). The date that the experiment was conducted was included as a random effect. The frequency and duration of contacts with pheromone granules were square root transformed and natural log transformed, respectively, to normalize residuals (Table 2.1). To see if treatment influenced whether beetles were discarded or not, a logistic regression with mixed effects was performed.

2.2.3. Experiment 2: Light quality

The effect of light quality on the response of *A. obscurus* males to pheromone was tested in a circular arena. Behavioural assays took place in August 2014. Petri dish arenas (140 mm X 15 mm) were lined with paper towel, which was replaced after each test. The arena was illuminated from a single white or red compact fluorescent light bulb placed directly overhead (White: Energy Saver Twister 23 W Daylight Deluxe, Red: Energy Saver Mini Twister Red 13 W, Philips, Markham, ON, Canada). This created a light intensity of 0.8 klux and 0 lux respectively. Ambient lighting was turned off during the experiment. Our goal was to test different light intensities, and in the past, darkness has been simulated by using red light. However, some insects, such as *Anophelese gambiae* mosquitos and *Pterostichus melanarius* ground beetles, have been shown to sense red light (Gibson 1995; Allema et al., 2012). The spectral sensitivity of click beetles is unknown, therefore the treatments are referred to as light quality treatments. A single beetle was placed in a 2-cm diameter acetate ring, 1 cm away from the edge of the arena for acclimatisation. After 3 min, 0.03 g of pheromone-impregnated or blank granules were introduced into

another acetate ring placed in the centre of the arena. Both acetate rings were then removed and the beetle was video recorded for 10 min. A total of 9-12 beetles were tested for each light-pheromone treatment and beetle collection period combination across six days.

Videos were analyzed as previously described. Walking speed, distance walked, granule contact frequency and duration were analyzed as in Experiment 1, except that the third factor was light type instead of air movement. Due to zero inflation, only beetles that had reached the granule zone were analyzed for frequency and duration of contacts with granule zone. The frequency and duration of contacts with granules were square root transformed and natural log transformed, respectively, to normalize residuals (Table 2.1). The proportion of time spent moving was analyzed with a generalized linear mixed model with beta distribution and logit link function, with adaptive quadrature estimation. The probability of reaching the granule zone was analyzed as a logistic regression.

2.2.4. Experiment 3: Beetle seasonality

We found that beetles collected from different periods in the previous two experiments had different levels of activity, suggesting that seasonality of the beetles was possibly an important factor in determining beetle response to treatments. As storage time was not controlled in the previous two experiments (i.e. beetles collected earlier would have been stored for longer), we conducted a separate experiment to investigate the effect of seasonality on beetle activity and beetle response to pheromone. The effect of beetle collection period on pheromone response was tested in a 2 by 3 full factorial design, where one factor was beetle collection period (March, April and May 2015, see Appendix A, Table A2 for collection dates), and the other was pheromone treatment (pheromone vs blank). A group of beetles from each time period was tested 17 days, 18 days, 38 days, and 39 days post collection. Beetles were stored at 23 °C, 12L:D. Five 9 mm× 15 mm diameter petri dishes that were lined with filter paper, and covered with a mesh lid (Pint Size Bugdorm lids, Megaview Bug Dorm, Taiwan) were placed around a central 1 oz (29.6 ml) plastic soufflé cup (Solo cup company, Lake Forest, IL, USA) that had been trimmed down to a height of 1 cm in the middle. A single beetle was placed into each dish and allowed to acclimatize for 3 min, after which 0.75 g of pheromone granules were introduced into the plastic soufflé cup. Beetles were recorded for 11 min. Walking speed, distance walked and proportion time spent moving were measured. Each treatment-collection period

combination was tested five times each, each with five independent beetles. Beetles that did not move were discarded from the data afterwards, resulting in 1-5 valid beetles per test. Treatments had no effect on beetle validity (Appendix A, Table A3). Each beetle was used once.

Videos were analyzed as previously described (Table 2.1). Experiment 3 was analyzed with a linear mixed model, where beetle collection period and pheromone were included as fixed effects, and days post collection were included as a discrete random effect. The group mean of all the variables measured for each test was used as the response variable. Number of beetles in the test was used as a weighting variable.

2.2.5. Experiment 4: Beetle response range

We tested the attraction distance of the pheromone granules in a mark-release-recapture experiment in a pasture field located in Agassiz, British Columbia, Canada (49.260488, -121.742726). Eight 30 m X 3 m plots, spaced 20 m apart, were marked out, and the grass mowed to 10 cm in height (grass height outside of plots was left at ~50 cm). Plots were orientated length-wise in a north-south direction, with the south side being close to the perimeter of the field. Four plots had pheromone granules applied while the other four had blank granules applied as a control. Pheromone treatment was assigned to plot in randomized blocks where two adjacent plots were considered a block. A band of 1.6 g of pheromone granules was introduced across the width of the middle of the plot (Figure 2.2) by first spreading granules evenly in a 1.95-m long and 2.5-cm wide V-shaped angle iron, and then tipping the bar to lay granules in a uniform band. Organic granules were used to comply with organic regulations. The initial pheromone release rate of an analogous sample of granules indoors was 278 µg per day, which then decreased to a steady rate of 20-40 µg per day at four days post deployment (see Appendix A, Figure A1 for pheromone release rate).

Beetles were marked with enamel model paint (Testor's Enamel Paint, Rockford IL, USA) with different colours on the thorax and the abdomen to indicate plot, release distance, and release side. Two sets of 16-18 beetles each were released 1, 4, 8 and 14 m on both the north side and the south side of the band. Any beetles that did not move after 24 h were discarded from the data. Beetles were recaptured with un-baited pitfall traps made from plastic cups (⌀=7 cm) inserted to the ground so that the lip was level with

the ground. The pitfall traps were placed in the middle, and on both ends of the granule band, making a total of three pitfall traps per plot (Figure 2.2). Pitfall traps were checked after 1 h, and then every 3.5 h for the subsequent 13 h. Traps were checked every 24 h thereafter, for a total of six days. Southerly winds were blowing at a speed of 1.3 ms^{-1} on the day of the release (http://climate.weather.gc.ca, Agassiz station).

We analyzed the effects of pheromone granule treatment, release distance, and release side on the proportion of beetles recovered with a generalized linear mixed model with a binomial distribution and logit link function (Table 2.1). Release distance, release side and treatment were included as fixed effects with release distance treated as a continuous variable. Plot was included as a random effect.

In all models, non-significant interactions were removed first followed by non-significant main effects that were not part of a significant interaction. All experiments were analysed using SAS University Edition (SAS Institute, Cary, North Carolina, USA). Where measurement precision is indicated, standard error is quoted.

2.3. Results

2.3.1. Experiment 1: Air movement

Beetle walking speed, distance walked and number of contacts with the granule band were affected by a significant three-way interaction between beetle collection period, air movement and pheromone presence (walking speed: $F_{1,145}$=10.0, P=0.002; distance walked: $F_{1,145}$=10.38, P=0.002, and number of contacts with granule band: $F_{1,145}$ =5.76, P=0.018 respectively). Out of the 167 valid tests, 13 beetles did not reach the pheromone band (April: 7 out of 87; May: 6 out of 82). The three-way interaction was found to be marginally significant for the duration of contact with the granule band ($F_{1,145}$=3.37, P=0.069). For those that reached the pheromone band, a two way interaction between air movement and beetle collection was significant for the time to first contact with the band ($F_{1,136}$=36.49, P=0.041). Due to the interaction effects with collection period, and how collection period was confounded with storage time, we split the analyses for beetles collected in April and May.

Beetles collected in April walked significantly faster in the moving air-pheromone combination compared to those exposed to the still air-pheromone combination or moving

air without pheromone (pheromone*air movement: $F_{1,68}$=17.92, P<0.001; pheromone: $F_{1,68}$ = 1.58, P = 0.212; air movement: $F_{1,68}$= 3.98, P=0.05, Figure 2.3 A). The distance walked by the April beetles followed the same pattern (pheromone*air movement: $F_{1,68}$=17.8 P<0.001: pheromone: $F_{1,68}$=17.8, P<0.001; air movement: $F_{1,68}$=17.8, P<0.001). The speed and distance walked by beetles collected in May were not affected by pheromone presence or air movement (walking speed: pheromone: $F_{1,63}$=0.00, P=0.961; air movement: $F_{1,63}$=0.11, P=0.744; pheromone*air movement: $F_{1,62}$=0.30, P=0.587; distance walked: pheromone: $F_{1,63}$=0.03, P=0.852; air movement: $F_{1,63}$=0.00, P=0.974, pheromone*air movement: $F_{1,62}$=0.47, P=0.498)

The time it took for beetles collected in April to first reach the pheromone band was 1.5 times faster in the moving air treatment than in the still air treatment, but pheromone had no effect (pheromone*air movement: $F_{1,62}$=0.32, P=0.575; pheromone: $F_{1,63}$=4.97, P=0.424; air movement: $F_{1,64}$=5.11, P=0.027). Neither air movement nor pheromone treatment had an effect on the time it took beetles collected in May to reach the pheromone band (pheromone*air movement: $F_{1,55}$=0.02, P=0.896; pheromone: $F_{1,57}$=2.06, P=0.156; air movement: $F_{1,56}$=0.28, P=0.598). For the beetles collected in April, the effect of pheromone on the number of contacts with the granule band varied with air movement (pheromone*air movement: $F_{1,68}$ =4.84, P=0.031; pheromone: $F_{1,68}$=6.49, P=0.013; air movement: $F_{1,68}$=2.21, P=0.1416, Figure 2.3 C). The total time spent in the band, however, was not affected by air movement or pheromone (pheromone*air movement: $F_{1,68}$=1.18, P=0.281; air movement: $F_{1,68}$=2.83, P=0.097; pheromone: $F_{1,68}$=3.25, P=0.076). Beetles collected in May made more contacts with the band and spent longer in the band when they were in still air compared to beetles in moving air (contacts with band: $F_{1,64}$=5.03, P=0.028; duration in band: $F_{1,64}$=5.55 and P=0.022, Figure 2.3 C). Pheromone did not influence either variable (number of contacts with band: pheromone: $F_{1,63}$=1.05, P=0.309; pheromone*air movement: $F_{1,62}$=1.54, P=0.220 and duration in band: pheromone: $F_{1,63}$=1.43, P=0.237; pheromone*air movement: $F_{1,62}$=2.24, P=0.140).

2.3.2. Experiment 2: Light quality

Response data of beetles collected in April and May were analyzed together as there was no three-way interaction among the treatments (Appendix A, Table A4, Table A4). Light quality did not affect walking speed, distance moved, or movement duration (Appendix A, Table A4). Light quality also did not affect whether the beetles reached the

granule zone, or the frequency and duration of contact with the zone (Appendix A, Table A5). As expected, beetles exposed to pheromone walked faster (72% faster) and farther (31% more) than beetles in the blank control (speed: $F_{1,67}$ = 27.0, P = <0.001; distance: $F_{1,67}$=28.52, P<0.001; Table 2.2). Beetles exposed to pheromone also spent a greater proportion of time moving ($F_{1,68}$=4.92, P=0.030; Table 2.2). In the pheromone treatment, 24% more beetles reached the granule zone compared with the blank control (Wald χ^2= 8.98, P =0.003). Of those beetles that reached the granule zone, individuals exposed to pheromone had 75.9% more contacts compared to those in the blank treatment ($F_{1,46}$=6.49, P=0.014). However, pheromone did not increase the duration of contact with the granule zone ($F_{1,45}$=0.56, P=0.457).

Beetles collected in April walked faster and farther than beetles collected in May (walking speed: $F_{1,67}$=6.24, P=0.015; distance: $F_{1,67}$=5.62, P=0.025, Table 2.2). Beetles collected in May spent 85.1% more time in the granule zone compared to those collected in April ($F_{1,46}$=7.47, P=0.009). There was no difference in how long April and May beetles spent walking ($F_{1,67}$=0.58, P=0.449).

2.3.3. Experiment 3: Beetle seasonality

Beetles collected in March and April did not differ in walking speed, walking distance, or the time that they spent moving; however, beetles collected in May walked more slowly and not as far compared to those collected earlier (Table 2.3, Figure 2.4). Collection time did not alter the beetles' response to pheromone (pheromone*beetle collection in Table 2.3). As predicted, beetles exposed to pheromone walked faster, farther, and longer than beetles without exposure (Table 2.3, Figure 2.4).

2.3.4. Experiment 4: Beetle response range

In the field trial, a considerably higher proportion of beetles (72.2% $\pm$ SE 1.7) were recaptured overall in the pheromone treatment compared to the blank control (4.3% $\pm$ SE 1.4). The number of beetles recaptured decreased with the release distance, but the rate of decrease was greater for the blank treatment (pheromone*release distance: $F_{1,53}$=9.7, P=0.003, Figure 2.5).

The proportion of beetles recovered from all release distances for the blank treatment never exceeded 50%. For the pheromone treatment, over 50% of beetles were recovered within 4.4 h (± SE 0.88) for distances up to 7 m. At 14 m, approximately one third of the beetles were still recovered. Mean time to maximum recaptures at each release distance in the pheromone plots are shown in Table 2.4. More beetles were recaptured from the South than the North ($F_{1,53}$=9.32, P=0.004).

2.4. Discussion

We found that air movement affected the response of *A. obscurus* males to pheromone, but light quality did not. We also found that seasonality affected the activity of the beetles with lower activity in beetles collected later in the season. However, seasonality did not affect their response to pheromone.

2.4.1. Male click beetles respond to pheromone under both moving and still air conditions

The importance of air currents as directional cues for odour reception has been most emphasised in lepidopterans, although walking insects can also use wind as a directional cue (American cockroach *Periplaneta americana* Bell and Kramer, 1980; red flour beetle *Tribolium castaneum*: Romero et al., 2010). We found some evidence that air movement enhances the ability of beetles to locate and respond to pheromone, but this could vary with season. In our air movement experiment, beetles collected in April responded with increased activity and interaction with pheromone under moving air conditions. The observation that click beetles use air movement to orient to a pheromone source is consistent with the behaviour of click beetles in nature. In the field, it has been observed that males in the presence of female sex pheromone often climb to the top of grass blades (Kabaluk, 2015). This behaviour is likely performed so that beetles can position themselves to sense air movement cues. Beneath the canopy, air movement may not provide a reliable directional cue, as micro habitat features can “stir and dilute” pheromone plumes (Cardé and Willis, 2008). We were unable to detect a positive response to pheromone in beetles collected in April in still air conditions, and in beetles collected in May. This could relate to arena design as in our subsequent experiments, we found beetles collected from both months showed a positive response, despite the lack of introduced air movement. Click beetles exhibit strong thigmotactic behaviour, therefore in

rectangular arenas click beetles will spend a large proportion of time in the corners compared to circular arenas. This could greatly decrease our ability to detect changes in activity.

In our field experiment, a higher proportion of beetles were recaptured when released upwind of the pheromone source. The wind speed recorded on the day was comparable to the speeds that inhibited response from May-collected beetles in the air movement experiment, therefore this may reflect an avoidance of strong winds. However, it should be noted that the south side in our field experiment which afforded higher beetle captures was confounded with closer proximity to the field perimeter, which was bordered by a dirt road for vehicle access.

Our results partially corroborate data obtained in a recent study (Blackshaw et al., 2017), that tested the effect of wind on click beetle response to pheromone on a linear track. At 6 m away from a wind and pheromone source, where wind speed was similar to that of our study (2 ms^{-1}), air movement enhanced the response range of beetles to pheromone, whereas at higher wind speeds (i.e., 6 ms^{-1}) beetles were deterred. They found this effect in beetles collected in May, whereas we failed to detect a response in beetles collected in May. However, the authors also used field collected beetles; therefore our subjects are not directly comparable due to differences in emergence periods from year to year. The authors also used a different criterion to classify a positive response; Blackshaw et al. (2017) classified a positive response to pheromone as beetles orienting towards the pheromone, whereas we classified a positive response as beetles making contact with the pheromone source and/or beetles changing activity.

2.4.2. Male click beetles respond to pheromone under both white and red light

Agriotes obscurus has been reported to exhibit diel patterns of activity and quiescence (Brian, 1947; Kabaluk, unpublished data). The beetles have also been reported to be negatively phototropic (Cohen, 1942). We therefore expected that light quality could have a negative or positive effect on their response to pheromone. However, we found this not to be the case. In contrast, Blackshaw et al. 2017 observed that the proximity of released *A. obscurus* to a window, which was an untested factor in their experiment, had an effect on beetle response to pheromone. However, the authors do not

report on how the response is affected. A limitation to this study is that artificial light can differ from natural light in spectrum and the amount of polarization flicker, therefore these should also be considered when interpreting results (Shields, 1989).

Although light did not have an effect, this does not rule out other factors that signal time of day may influence male beetle response to pheromone. Although evidence remains anecdotal, previous researchers have speculated that humidity and temperature may be highly effective in determining *A. obscurus* diurnal activity (Cohen, 1942). Roebuck et al., (1947) noted that humidity appears to encourage activity of *A. obscurus* in tall herbage, whereas Brian (1947) remarked that high temperature was a limiting factor of *A. obscurus* activity. Humidity and temperature have been found to influence responses to sex pheromones in other arthropod taxa; the tick *Argas persicus* for example, responded less to an assembly pheromone under high humidity (Hassanali et al., 1989). In the field, these factors can be difficult to tease apart as they are highly correlated; in the early morning, increased light levels are associated with warming temperatures, which also causes humidity changes in the atmosphere. We therefore suggest further exploration into the effects of temperature and humidity on click beetle response.

2.4.3. Male click beetles are less active later in the season

In all three lab experiments, we found beetles collected later in the season had a lower level of activity. This was evident even when storage time in the lab was controlled for. The effect of seasonality can be due to environmental effects or beetle senescence. Although we did not control for temperature or humidity in our experiments, we contend that changes in activity level were not due to environmental factors as room temperatures did not vary greatly (25-27^{o}C). In addition, differences in activity were also found in our air movement and light quality experiment, despite beetles collected from different periods being tested on the same day. This suggests that temperature was not an influencing factor.

It is more likely that effects of seasonality are due to beetle senescence. Although it is not possible to determine the age of beetles collected in the field, beetles emerge in the field over a few months in the spring and early summer. The mean age of beetles captured at one point in time will be different than those collected at another time, although the degree to which cohorts overlap is unknown. Still, aging in insects can lead to the

degeneration of the nervous system as well as the musculoskeletal system, which can lead to a decline in spontaneous locomotion. Decline of locomotor activity due to age has been reported in *Blaberus discoidalis* cockroaches (Ridgel et al., 2003), *Drosophila melanogaster* vinegar flies (Fernández et al., 1999) and *Apis mellifera* honey bees (Tofilski, 2000). This is further corroborated by observations that beetles collected in May made more contact with the granule band in the still air versus moving air treatment in our air movement experiment, while the same effect was not seen in beetles collected in April. A deterioration of the musculoskeletal system is consistent with the inability to withstand high wind speeds. Flying insects, have been found to be inhibited by upwind movements when high wind speeds are encountered, regardless of whether pheromone is present or not (e.g. potato aphid: Goldansaz and McNeil, 2006). This is observable in walking insects too; Hardee et al., (1969), for example, found boll weevils were deterred by winds greater than 7km h^{-1}.

Although we found changes in overall activity across seasons, we did not find that beetle collection period affected the response to pheromone. Insects can exhibit circannual rhythms in resource searching, and consequently may show differential response to pheromone across seasons. For example, fewer boll weevils (*Anthonomus grandis*) are caught in pheromone-baited traps during spring (Rummel and Bottrell, 1976), and weevil age is a determinant in pheromone response (e.g., Duehl et al., 2011, Boughton and Fadamiro 1996). In our seasonality experiment, although beetles had reduced activity later in the season, they consistently walked twice as fast in the pheromone treatment, compared to the blank treatment. This shows that although there is a change in basal activity level, their response to pheromone did not change.

2.4.4. Beetle response range and recapture rates

The cellulose-based formulation of pheromone attracted male beetles from the maximal distance tested (14 m), and offers a new option for pheromone-based control strategies. Our recapture rates are comparable to those using liquid pheromone in the field (Hicks and Blackshaw, 2008, Sufyan et al., 2011). Sufyan et al., (2011) had a recapture rate of 70% for click beetles released 2 m away from a point source which gradually decreased to 40% at 15 m. Hicks and Blackshaw (2008) had recapture rates of 75% at 4 m away from a point source which decreased to 30% at 16 m. The quantity of pheromone used was not reported in either case. Similar to Sufyan et al., (2011), we found

the beetles' responses to be immediate, with most recaptures being made within the first 3 days. However, in contrast with Hicks and Blackshaw (2008) and Sufyan et al., (2011), the number of recaptures differed between the two release directions, as discussed previously. The grass was mowed to a short height of 10 cm in our experiment, whereas grass height was not reported in the previous reports. Wind effects may be stronger with a lower canopy.

2.4.5. Implications for pheromone strategies

Whether these rates of recapture are sufficient for economic management of click beetles is largely dependent on the pheromone application tactic employed; in an attract-and-kill tactic with autodissemination, only a proportion of the pest population is required to make contact with the pheromone source in order to afford control. For example, adult German cockroaches (*Blattella germanica*), through their faeces horizontally transfer insecticidal bait to coprophagic nymphs, which eliminates the need for nymphs to seek and contact the bait (Kopanic and Schal, 1999). In particular, in a microbial-based autodissemination tactic, infection of a small subset of the population can have a multiplicative impact on population dynamics. Pathogens have the ability to proliferate, and epizootics can occur when a threshold of the population becomes infected. On the other hand, attract and kill strategies that rely on a non-disseminative kill agent may require higher rates of contact.

In our study, female sex pheromone not only induced aggregation of male beetles, it also prompted greater activity of males, corroborating previous data (C. Benefer, unpublished). Possible exploitation of increased motility prompted by sex pheromone has not been investigated, but increased motility in green peach aphids (*Myzus persicae*) due to aphid alarm pheromone exposure facilitates increased infection with the fungal pathogen *Verticillium lecanii* (Roditakis et al., 2008). These findings suggest that increased motility may be exploited to increase the effectiveness of disseminative insecticides. In addition, there is evidence that increased exercise can affect the physiology of insects, which may have important implications for the use of pheromone with chemical or microbial insecticides. For example, increased activity has been associated with decreased disease resistance in *Gryllus texensis* crickets (Adamo and Parsons, 2006), and higher immune response in non-foraging bumble bees (*Bombus terrestris*) (Doums and Schmid-Hempel, 2000) which may affect the efficacy of pathogen

based insecticides. Lastly, increased activity may also expose pest species to other predators which may contribute to the control effort.

2.4.6. Conclusions

Male beetles respond to female sex pheromone under a range of conditions. Lure placement is not expected to be affected by light levels but it may be most effective to place lures upwind from beetles. The pheromone appears effective throughout the season. Pheromone deployment earlier in the season, however, will expedite beetle captures, as beetles are more active in early season. Much remains to be explored with regards to other internal and external factors that could affect responses of *A. obscurus* to sex pheromone in the field including humidity, temperature, as well as barometric pressure, mating and nutritional status. Knowing the limits and conditions of pheromone response is necessary for the selection of an effective pheromone control tactic that will achieve the ultimate goal of reducing populations of reproducing click beetle adults and/or curtailing oviposition events.

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2.6. Tables

Table 2.1 Model specifications. A linear mixed model was used unless otherwise specified.

Experiment	Response	Transformation	Fixed Effects	Random Effects
Experiment 1: Air movement	Walking speed		Air movement Pheromone	Date of experiment
	Distance walked			
	Time to first contact with granule band	Natural log		
	Number of contacts with granule band	Square-root		
	Duration of contact with granule band	Natural log		
Experiment 2: Light quality	Walking Speed		Light type Pheromone presence Beetle collection date	Date of experiment
	Distance Walked			
	Number of contacts with granules zone	Square-root		
	Duration of contact with granule zone	Natural log		
	Proportion time spent moving*			
	Probability of reaching granule zone	Squared		
Experiment 3: Seasonality	Walking speed		Pheromone Beetle collection date	Date of experiment
	Distance walked			
	Time spent moving			
Experiment 4: Response range	Proportion of beetles recovered^		Pheromone Release direction Release distance	Plot

* Generalized linear mixed model with beta distribution and logit link function

^ Generalized linear mixed model with binomial distribution and logit link function

Table 2.2 Walking speed, distance walked, and proportion of time moving (mean ±SE) recorded for *A. obscurus* males (collected in April and May 2014) in response to pheromone and light quality.

			N	Walking Speed (cms^{-1})	Distance walked (cm)	Proportion time moving
April	Red	Blank	9	0.8(0.1)	390.2(76.3)	0.53(0.10)
		Pheromone	12	1.4(0.2)	807.5(98.1)	0.84(0.04)
	White	Blank	9	0.9(0.1)	549.5(75.4)	0.71(0.03)
		Pheromone	9	1.4(0.2)	798.1(127.0)	0.85(0.03)
May	Red	Blank	6	0.6(0.1)	365.1(82.8)	0.58(0.12)
		Pheromone	9	1.1(0.2)	649.7(107.9)	0.81(0.05)
	White	Blank	10	0.5(0.1)	262.5(54.3)	0.50(0.09)
		Pheromone	11	1.2(0.2)	685.4(96.3)	0.77(0.07)

Table 2.3 **Model output for the effect of pheromone on walking speed, distance walked and proportion of time moving for *A. obscurus* males collected in March, April and May. Experiment day is included as a random effect.**

	Num DF	Den DF	F Value	Pr>F
Walking speed				
Pheromone	1	126	32.6	<.001
Beetle collection period	2	126	9.64	<.001
Pheromone*Beetle collection period	2	124	0.55	0.581
Distance walked				
Pheromone	1	126	32.75	<.001
Beetle collection period	2	126	8.72	<.001
Pheromone*Beetle collection period	2	124	0.47	0.624
Movement duration				
Pheromone	1	126	27.87	<.001
Beetle collection period	2	126	6.35	0.002
Pheromone*Beetle collection period	2	124	0.83	0.437

Table 2.4 **Mean time (h ±SE) to maximum recaptures for *A. obscurus* beetles released at different distances away from a pheromone granule band (N=4).**

Release distance (m)	Mean h to maximum recapture (±SE)
1	19.3 (4.2)
3	13.1 (0.9)
7	34.6 (2.6)
14	38.1 (8.5)

2.7. Figures

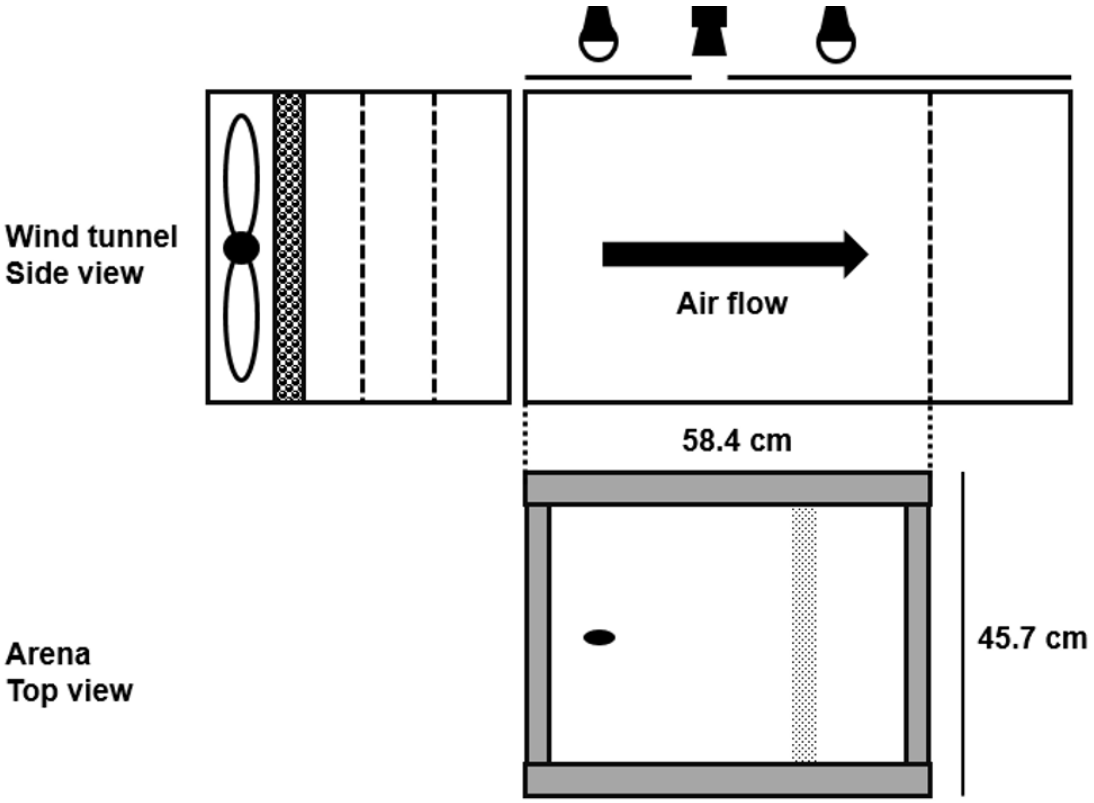

Figure 2.1 **Wind tunnel arena.** The tunnel (120 cm × 50 cm × 50 cm) was constructed using 4-mm thick plexiglass. Corrugated plastic framed screens were used to section off the middle to form a 45.7 cm × 58.4 cm arena. The base of the arena was lined with brown dry sheathing paper, which was held down by steel bars (2 cm × 50 cm × 3 mm) on all four sides. The tunnel was illuminated with two 23W CFL blubs (Daylight Mini Twister, Philips Lighting, ON, Canada) located on both sides of the arena, which were diffused with a layer of foam. At one end of the tunnel, a box fan (52 cm × 52 cm) was used to drive wind through activated charcoal

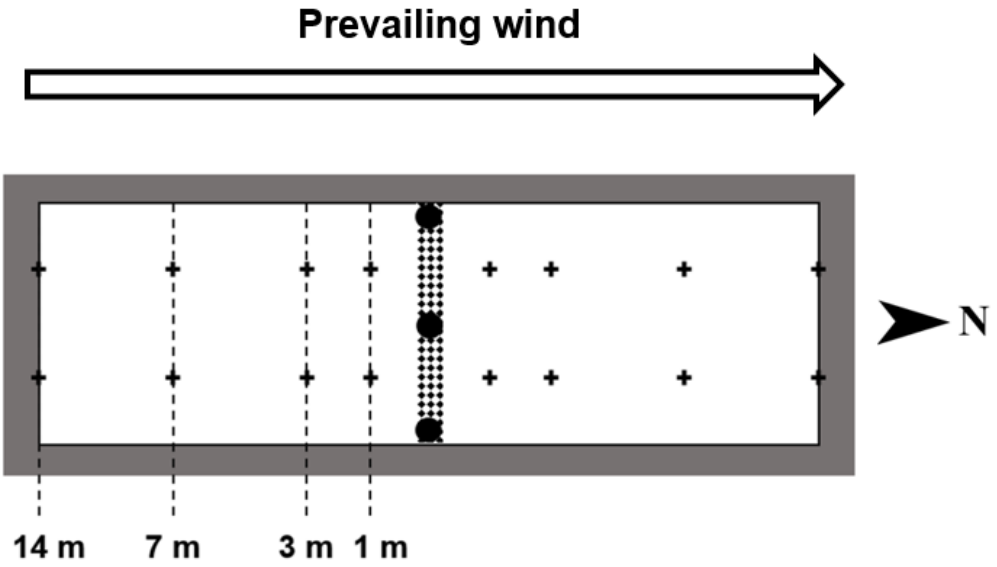

Figure 2.2 **Plot (30 m × 3 m) layout for the mark-release-recapture experiment.** The granule band (stippled area) was placed across the middle of the plot (see text for details). Three pitfall traps were placed within the granule band. Beetles were released 1, 3, 7 and 14 m away from the midline of the plot on both sides of the band. Crosses (+) show beetle release points.

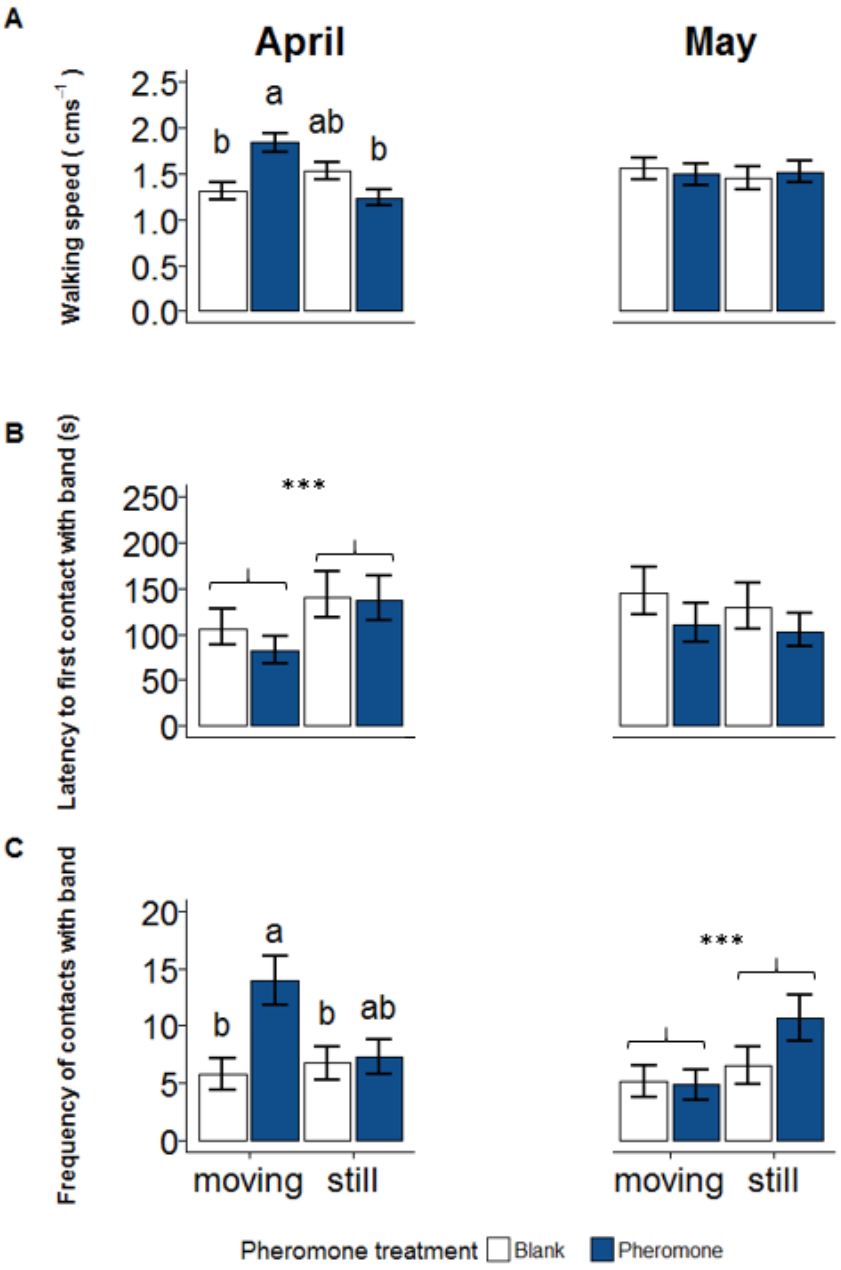

Figure 2.3 **Impact of pheromone and air movement on *Agriotes obscurus* males (A) Least squares mean (±SE) walking speed, (B) time to first contact with pheromone band and (C) frequency of contacts with band.** Beetles were collected in April (N=87) and May (N= 82) in 2014. Walking speed is a relative measure and not a true measure of speed. There was no effect of pheromone or moving air on the walking speed, time to first contact with the pheromone band, or the frequency of contacts with pheromone band in the beetles collected in May, therefore no pairwise comparisons are shown. *** represent significance ($p<0.05$) as assessed by contrasts in a generalized linear model. Letters represent significance at $p < 0.05$ as assessed by Tukey's HSD.

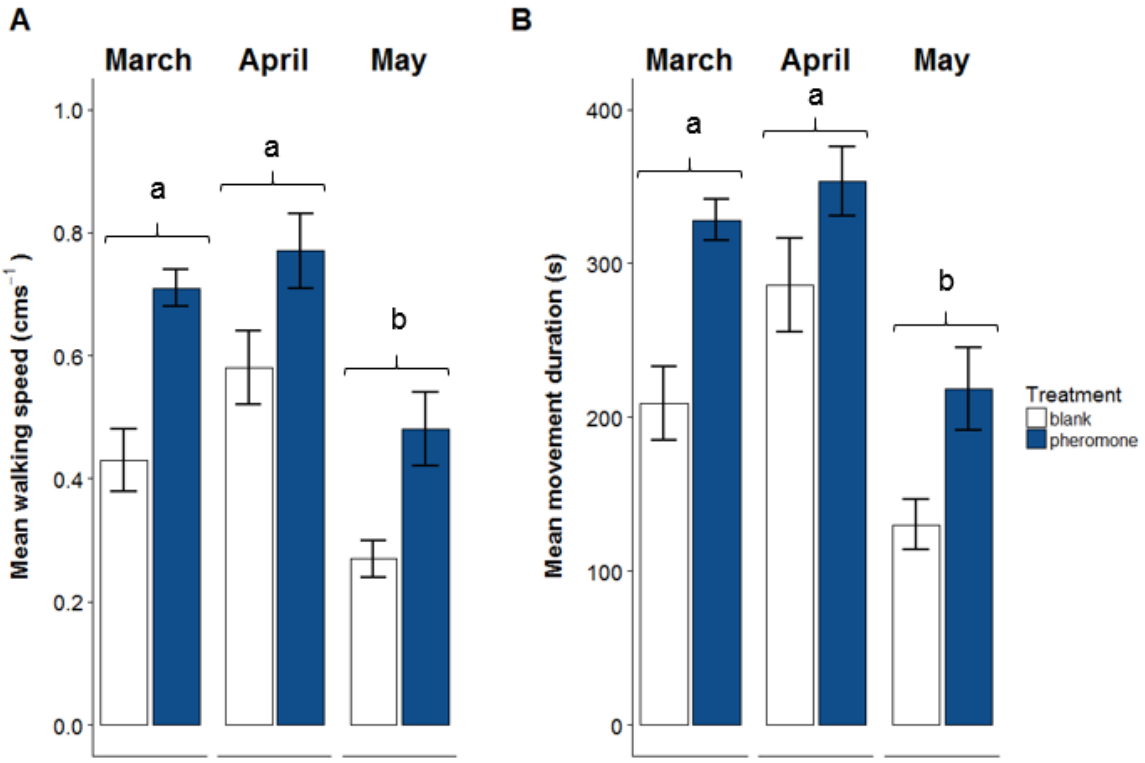

Figure 2.4 **Impact of beetle collection period (March, April, May 2015) on the response of *Agriotes obscurus* males to pheromone treatment.** Beetles were tested in groups of 1-5. Number of groups (number of beetles): March: 44 (220); April: 47 (188); May: 48 (195)). Activity is depicted by mean (±SE) (A) walking speed and (B) movement duration. Walking speed and movement duration were both influenced by beetle collection period (letters represent significance at $p<0.05$ as assessed by Tukey's HSD) and pheromone.

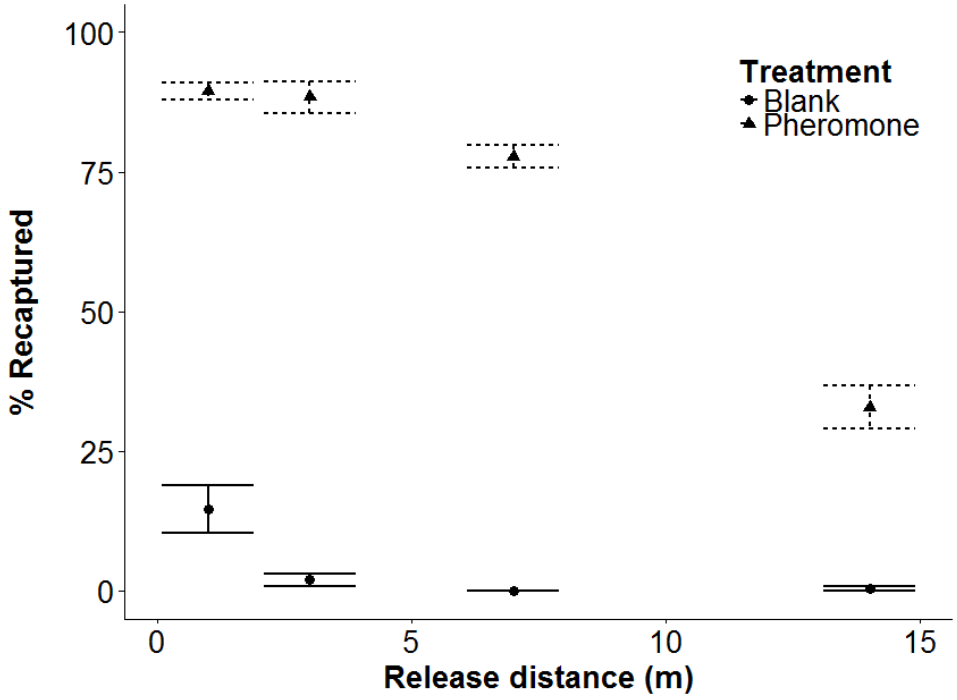

Figure 2.5 **Mean proportion (±SE) of *Agriotes obscurus* males recovered from plot (see Fig. 2.2) after release from 1, 3, 7 and 14 m away from a band of blank or pheromone-impregnated granules.** N=4 per treatment. The final model is % Recovered= 4.0045(Pheromone) -0.693(Distance) + 0.4396(Release Side)-+0.4513(Distance*Pheromone)-1.480. As there was no interaction between release side and other factors, data are pooled.

Chapter 3.

Effect of synthetic sex pheromone of female *Agriotes obscurus* click beetles on dissemination of *Metarhizium brunneum* by males

3.1. Introduction

The past decade has seen an increasing interest in using autodissemination techniques in insect pest management. Autodissemination is the technique of contaminating a pest species, or a non-target vector, with an insecticide (chemical or biological), which is then transmitted to members of its own population (Vega et al., 2007). Attractants such as synthetically produced semiochemicals are often an integral component of autodissemination systems (Baverstock et al., 2010). They are primarily used for their aggregant properties, however, they can also mediate other behaviours in insects. The effect of these behavioural changes on autodissemination efficiency has not been well explored.

Autodissemination is touted as an efficient method for delivering insecticides in instances where pest species have cryptic life styles (e.g., *Aedes albopictus* larvae; Unlu et al., 2017). For entomopathogen-based autodissemination, this strategy has the added benefit that autoinoculation devices can be incorporated into the system, to protect pathogens from environmental stressors such as ultraviolet light irradiation (Alves 1998; Fargues et al., 1997). Autodissemination has been explored in the application of baculoviruses (e.g., Jackson et al., 1992; Yu and Brown, 1997), bacteria (Skadeland, 1981) and protozoa (Shapas et al., 1977), but among insect pathogens, the application of entomopathogenic fungi has been of particular interest. This is due to their mode of infection, which is through contact with the insect integument. This infection mode contrasts with other pathogens, which need to be consumed for infection to occur. Although entomopathogen autodissemination is not yet widely available, proof-of-concept studies across a wide range of taxa have shown promising results (e.g., diamondback moth: Vickers et al., 2004; Japanese beetles: Klein and Lacey, 1999; larch beetles: Srei et al., 2017; *Aedes* mosquitos: Reyes-Villanueva et al., 2011).

Entomopathogenic fungi that have been explored most thoroughly in relation to autodissemination techniques are within the family Clavicipitaceae (Hypocreales). This family includes *Metarhizium* spp. and *Beauveria bassiana*, which together make up 80% of commercially available fungal insecticides (de Faria and Wraight, 2007). The infection cycles in these two genera are similar; it begins when conidia contact the host's cuticle. The conidia germinate and penetrate into the coelom where hyphal bodies are produced. After the death of the host, sporulation occurs under high humidity conditions, producing infective asexual conidia on the host's exterior (Roy et al., 2006)

In autodissemination, after a host has received the initial dose of inoculum, the fungus can be dispersed via two routes. The first is passive primary transmission, which is when contaminated live individuals transfer spores to clean conspecifics, either directly by host-to-host contact, or indirectly via the environment. The second route is active secondary transmission, where susceptible individuals come into contact with the next generation of fungus that has been produced on mycosed cadavers.

Sensing factors that pose mortality risks is essential to insect survival, and as such, many are able to alter their behaviour to avoid mortality risk factors. When the risk factor in question is a pathogen, insects may exhibit hygiene behaviour (Rothenbuhler, 1964; Schwarz et al., 2012; Yanagawa, 2007), emit alarm signals (Rosengaus et al., 1999) or avoid the pathogen. Avoidance may manifest as avoidance of infected conspecifics (Parker et al., 2010; Swanson et al., 2009) or avoidance of contaminated substrates (e.g., Meyling and Pell, 2006). When combining potentially repellent insecticides with attractive lures, the compatibility of the two substances must be considered. Semiochemicals, which are used in autodissemination systems may initiate behavioural sequences (Shorey et al., 1970; Vinson, 1972; Yanagawa, 2011), trigger responses towards certain stimuli (Barrows, 1975) or change host activity levels (Marsh, 1975). Although there is some work on the interaction between repellent chemical insecticides and attractive baits (Vargas et al., 2002), few studies have addressed interactions between pathogens and attractive lures.

Understanding the resulting behaviour of insects is important for predicting the efficacy of a microbial control agent. Insect behaviour can impact pathogen transmission (reviewed in Baverstock et al., 2010; Parker et al., 2010). Indeed, there are instances where pathogens use this to their advantage by manipulating host behaviour post infection

to promote the transmission of propagules (George et al., 2013; Goulson, 1997; Krasnoff et al., 1995; Trandem et al., 2015). Mobile insects are more likely than less mobile insects to encounter infected individuals or pathogens in the environment. Baverstock et al., (2005) showed that pea aphids, *Acyrthosiphon pisum,* pick up more of the fungus *Pandora neoaphidis* during colonization of new plants than when they were feeding, possibly due to the greater movement associated with colonization (however, this was not formally tested). The presence of co-occurring arthropods can also increase fungus transmission. Baverstock et al., (2008) found that introducing parasitoids or other herbivores into a cage of *Microlophium carnosum* nettle aphids, resulted in increased transmission of *P. neoaphidis* in the aphids. Parasitoids triggered escape responses in aphids, whereas other feeding herbivores forced aphids to move to alternate feeding sites. Both actions led to increased movement and thus, potentially greater pathogen acquisition. Other studies have found a similar effect of other natural enemies (Roy et al., 1998; Baverstock et al., 2009; Meyling et al., 2006).

Sex-specific behaviour has also been suggested to affect the transmission of fungal entomopathogens. The rate of *M. anisopliae* transmission between mates of the Mediterranean fruit fly (*Ceratitis capitate*) was 27.2% higher when the inoculated sex was male (Quesada-Moraga et al., 2008). The authors attributed this to not only higher motility in males, but also to differences in body positions of mates during copulation. Males carried high quantities of conidia on their genitalia, which could have been transferred more efficiently to females. These hypotheses, however, were not formally tested.

To my knowledge, only one study thus far has tested the effects of a synthetic semiochemical on pathogen transmission. Green peach aphids, *Myzus persicae*, exposed to aphid alarm pheromone, transmitted the fungus *Verticillium lecanii* at a greater rate than unexposed aphids (Roditakis et al., 2000). However, the same study demonstrated that a similar effect could be achieved with sublethal doses of the insecticide imidacloprid as the agitant, leading the authors to conclude that this latter method of enhancing fungal transmission was more practical.

An attract-and-kill tactic is currently being investigated for control of *Agriotes obscurus* (L.) click beetles (Coleoptera: Elateridae) (Kabaluk et al., 2015). *Agriotes obscurus* is one of 39 elaterid species that are considered pests of potatoes world-wide (Jansson and Seal, 1994). The larvae, known as wireworms, feed on underground plant

tissue, forming tunnels in tubers in root crops (Parker and Howard, 2001) and exposing seedlings of cereal crops to pathogens (van Herk and Vernon, 2013). Click beetles are an ideal candidate for an attract-and-kill or autodissemination tactic; they have a patchy distribution within the field (Blackshaw and Vernon, 2008), which means they may be inefficient to target with broadcast application. Click beetles also lay their eggs within the soil, making topical applications that target the larval stage ineffective. In a field-enclosure experiments, where *A. obscurus* beetles were released into an arena containing a band of granular *Metarhizium brunneum* and female sex pheromone, beetle recapture was reduced by 98% (Kabaluk et al., 2015). Kabaluk (2005) also found that passive horizontal transmission can occur between click beetles; housing two inoculated beetles with eight clean beetles, resulted in 100% mortality in the latter.

In my previous work, I demonstrated that female *A. obscurus* sex pheromone prompts increased walking speed and activity of conspecific males. In this study, I investigated whether activity changes of pheromone-exposed *A. obscurus* affect the passive transmission of *Metarhizium brunneum* Petch. First, I investigated whether the presence of *M. brunneum* affects attraction of *A. obscurus* males to synthetic sex pheromone. Second, I studied direct and indirect passive horizontal transmission of *M. brunneum* to *A. obscurus*, and whether pheromone affects this transmission.

3.2. Materials and Methods

3.2.1. Beetle collection and pheromone

Males of *A. obscurus* were collected from a grass field at the Agriculture and Agri-Food Canada's Agassiz Research and Development Centre in Agassiz British Columbia, Canada (49.22419 and -121.7535) between March and April in 2015, using pitfall traps baited with synthetic female sex pheromone. Using the same method in 2016, beetles were collected in a field in Chilliwack, BC (49.20789, -121.92917). Collected beetles were kept in vented plastic containers (~20 cm × 20 cm × 10 cm, < 300 beetles per container). They were fed organic apple pieces *ad libitum*, provided with dampened paper towel and grass (Poaceae spp.), and kept at 10°C in darkness until use. Synthetic pheromone was formulated by impregnating cellulose based granules with a 1:1 blend of geranyl octanoate and geranyl hexanoate (1.0% wt/wt, Scotts Canada, Delta BC, Canada). Non-impregnated granules ('blank') were used as controls.

3.2.2. *Metarhizium brunneum* conidiated rice granules

Metarhizium brunneum strain LRC112 conidiated rice grains ('MET') were produced using solid state fermentation on rice and were stored in vacuum bags at 4°C until use (Kabaluk, 2014). This strain of *M. brunneum* was isolated from an infected wireworm in a field in Agassiz, BC. The strain is known to be highly virulent towards *A. obscurus*, *A. lineatus* and *Selatosomus pruininus* wireworms (Kabaluk et al., 2005). Non-conidiated rice grains ('CON') were used for controls where stated. To determine spore viability, 50 µl of a 1×10^{-6} ml^{-1} suspension were plated on a 6-cm diameter plate of potato dextrose agar medium (BD Life Sciences, MD, USA), and incubated at 23 °C in darkness for 24 h. Two cover slips (18 × 18 mm) were placed onto each plate and the number of spores out of 100 that had germinated were recorded per cover slip. Two replicate plates were prepared on each experimental day.

3.2.3. Monitoring for mortality and sporulation

To monitor for mortality and sporulation, beetles were kept individually in 1 oz (29.6 ml) soufflé cups (Solo cup company, Lake Forest, IL, USA) with moistened dental cotton (Richmond Dental, Charlotte, NC, USA) and a piece of apple. Mortality was checked every day for 14 days. Apple pieces were replaced every 3-4 days. In addition to determining the cause of death, rate of sporulation was also measured to determine whether *M. brunneum* infection would lead to the production of fungal propagules for dispersal to other *A. obscurus* beetles. To determine whether death was due to *M. brunneum*, cadavers were surface sterilized by dipping in sequence into 70% ethanol (5 s), water (5 s), 1% sodium hypochlorite (30 s) and again water (3-times) (Lacey et al., 1997). Afterwards, beetles were placed into a Petri dish (30 mm × 15 mm) which was fitted with a piece of damp filter paper, sealed with Parafilm, and incubated at 25 °C in darkness. The cause of death was determined to be *M. brunneum* infection when cadavers produced green spores.

3.2.4. Experiment 1: Beetle response to female sex pheromone when *M. brunneum* is present

To determine whether *A. obscurus* males avoid *M. brunneum* spores, beetles were exposed to grains with and without the fungus (MET/CON) in the presence and absence

of pheromone (two by two full factorial design), and their activity was recorded (29-33 beetles tested per treatment). The test arena was prepared by placing a transparent plastic lid (3.8 cm diameter) into the middle of a Petri dish (140 mm × 15 mm), which was then filled with 25 g of autoclaved potting soil (Garden Works, Burnaby, BC, Canada). The edge of the plastic lid was flush with the soil surface. The plastic lid served as the treatment zone. Beetles were introduced into a tube (0.9 × 2.0 cm) at the edge of the Petri dish arena, and allowed to acclimatize for 3 min. Afterwards, 0.03 g of pheromone-impregnated granules and 0.09 g of conidiated rice grains (4.4 $\times 10^6$ viable spores per gram) were introduced into the treatment zone in the middle. Although this is a high concentration of fungus (equating to 3.9$\times$ 10^{13} viable spores/ha), it mimicks the spatial density at which it was applied at in a band application in the field (Kabaluk et al., 2015). This concentration was originally chosen in Kabaluk et al. (2015) to ensure beetles would obtain a high enough dosage for likely mortality. This density of *M. brunneum* granules was also used in Experiments 2 to 4. To improve the contrast of beetles on soil for motion detection, a dot of enamel paint (Testor's Enamel Paint, Rockford, IL, USA) was applied to the thorax of each beetle (See Chapter 2 for details). Beetle walking was recorded for 10 min.

Beetle walking speed, distance, and duration were measured using Ethovision (Noldus, Wageningen, Netherlands). Beetles were detected using the dynamic subtraction method, taking samples at a rate of 8.9 frames per second. A 'Locally weighted scatter plot' smoothing algorithm (Noldus Information Technology, 2013) smoothing algorithm was applied afterwards to reduce noise in tracking. The activity of 160 beetles was recorded (37-42 per treatment), testing each beetle only once. From these 160 beetles, 106 were monitored for mortality and cause of death, and 33 beetles (7-9 per treatment) were washed to determine the number of spores that they had picked up from the treatment zone. The remaining beetles were lost due to handling. To estimate the number of spores on the body surface of beetles, beetles were placed immediately into Eppendorf tubes containing 250 µl of 0.5% Tween 80, and were then vortexed for 5 min. The number of spores in the suspension was determined by counting two 100-µl aliquots with a haemocytometer (improved Neubauer, Hausser Scientific, PA, USA) at 400× magnification and by taking the average.

Beetle walking speed and walking distance were analyzed with a general linear model. The frequency of contacts with the treatment zone was analyzed with a generalized

linear model with a negative binomial distribution and logit link function. Mortality and sporulation were analyzed with a generalized linear model with a quasi-binomial distribution and binomial distribution, respectively, with a logit link function. The number of spores picked up was analyzed with a generalized linear model with quasi-poisson distribution with a log link function. Quasi-likelihood models were used in instances where data were overdispersed. In all analyses, *M. brunneum* treatment and pheromone treatment were included as main effects. The date of the experiment was included as a fixed effect blocking factor. In all models, non-significant interactions were removed first followed by non-significant main effects that were not part of a significant interaction. All experiments were analysed using R (R version 3.3.2, Core team 2016).

3.2.5. Experiment 2: Horizontal transfer of conidia spores between live beetles (small scale)

I tested the ability of contaminated beetles to directly and indirectly transfer *M. brunneum* conidia to clean beetles. A 'donor' beetle was inoculated by being allowed to walk for 30 min in a 2 oz (59.1 ml) soufflé cup containing 15 g of soil, which had 31 MET or CON rice grains applied evenly on top using a stencil (12586 grains m^{-2}). MET grains carried 4.4×10^6 viable spores per gram. This procedure introduced ~1.1×10^6 viable spores to the arena. After inoculation, the donor beetle was moved into a Petri dish (140 $\times$ 15 mm) arena and left to roam for 30 min (Figure 3.1). To test the effects of pheromone on the horizontal transmission, 0.03 g of either pheromone-impregnated or blank granules were placed into the middle of the arena prior to the start of the experiment. After half an hour, the donor beetle was either retained in ('Keep' treatment), or removed from ('Remove' treatment), the arena, after which a 'recipient' clean beetle was introduced (beetle density for 'Keep' treatment: 130 beetles m^{-2}, Figure 3.1). This enabled us to tease apart the effects of beetle-to-beetle conidia transfer (direct transfer), and beetle-to-environment-to-beetle conidia transfer (indirect transfer). Direct transfer and indirect transfer are both possible in the Keep-treatment, whereas only indirect transfer is possible in the Remove-treatment. The recipient beetle was allowed to walk around for 1 h before it was removed and monitored for mortality and cause of death. Five recipient beetles were tested per treatment. The experiment was repeated six times for a total of 240 beetles tested. Out of these 240 beetles, 21 were discarded from the data due to handling-related causes. The number of contacts between the donor and the recipient beetle in the

Keep-treatment was counted for a subset of pairs (N=9 each for fungus and non-fungus treatments) to determine the effects of *M. brunneum* and pheromone on the contact frequency between beetles.

A mixed effect, binary logistic regression was conducted to determine whether beetle removal, donor beetle inoculation and pheromone affected mortality. The analysis was conducted as a split plot design, with pheromone treatment nested within experiment date included as a random factor. For beetles that had died within the 14-day period, the probability of *M. brunneum* sporulation in cadavers was analyzed in the same manner.

The number of contacts between beetles in the subset of MET-Keep and CON-Keep beetles was natural log transformed to normalize residuals, and was analyzed with a linear mixed model. Model specification is the same as previous analyses, except that donor beetle treatment was not included.

3.2.6. Experiment 3A: Horizontal transfer of conidia between live beetles (large scale)

I further investigated the ability of contaminated beetles to passively transmit *M. brunneum* conidia in an experiment conducted in a greenhouse. Twenty gallon (75.7 L) nursery containers (Haviland Plastic Products Co, Haviland OH, USA) were filled with 10 gallons (37.9 L) of soil (Sun Gro Horticulture Distributions Inc, Agawam, MA, USA) and sown with 80 g of *Triticum* spp. wheat grass seed. Wheat grass was trimmed to 9 cm prior to the onset of the experiment. To apply the treatment, dishes were made up by placing a Petri dish (30×15 mm) within a Petri dish (90×15 mm). Both dishes were filled with soil and flattened until the soil surface was flush with the rim of the smaller Petri dish. The inner Petri dish contained either 0.2 g of MET or CON grains, and the outer Petri dish had either 0.1 g of pheromone-impregnated or blank granules. The spore yield of the granules was 3.03×10^9 per gram and the spore viability was 81.5 % (± SE4.0). I tested three combinations of *M. brunneum* and pheromone treatments; MET-blank, MET-pheromone and CON-pheromone. Only CON grains with pheromone were tested as a control to reduce the number of treatments for logistical reasons. Twenty marked donor beetles were introduced into the arena for 24-30 h prior to the experiment for acclimatisation (beetle density: 71 beetles m^{-2}). Treatment dishes were then placed into the centre of the arena for a 24-h infection period. After 24 h, the inner dish was replaced with a similarly prepared

dish that did not contain MET or CON grains. Immediately after, beetles were either kept or removed from the pots, making this a 2 × 3 design. Beetle removal was carried out over a 10 min interval. Any beetles that were not retrieved during this time interval were left in the pot. Forceps were sterilized with 0.5 % bleach in between each beetle collection. A group of 20 recipient beetles was then introduced into all arenas (beetle density: 143 beetles m^{-2}). An additional 20 unmarked clean beetles were introduced into arenas in the Remove-treatment in order to retain the same beetle density. After 24 h, all beetles were removed from arenas. There were six replicates for each *M. brunneum*-pheromone-donor beetle removal combination, for a total of 36 pots.

Generalized linear models with quasi-binomial distributions and log link function were used to examine beetle mortality and *M. brunneum*-sporulated cadavers per pot, where *M. brunneum*-pheromone treatment, donor beetle removal and associated interactions were included as explanatory variables. In most replicates only 10-16 out of 20 beetles were successfully removed, therefore the number of donor beetles removed was modeled as a continuous factor.

3.2.7. Experiment 3B: *M. brunneum* persistence in a greenhouse

Metarhizium spores are easily degraded by UV light exposure. In order to determine whether a change in the number of infected beetles was due to the degradation of *M. brunneum* in the greenhouse, a separate spore persistence experiment was conducted concurrently. One gallon (3.8 L) pots were filled with soil and sown with 4.3 g of wheat grass seed. Area of the soil surface was ~ 0.07 m^2. Grass was trimmed to a height of 9.0 cm prior to the onset of the experiment. *Metarhizium brunneum* spores (viability: 79.8 % ± SE 0.6) were harvested from *M. brunneum* grains using a commercial mycoharvester (Model MH-5b, Mycoharvester, Buckinghamshire, UK), and were applied to the pots as a dust, using an electric powered duster (Exacticide Technicide, San Clement, CA, USA). To prevent drift of spores, a tent was erected over each pot by propping a plastic sheet on top of a 45-cm long bamboo cane placed into the pot. Spores of *M. brunneum* were applied by inserting the nozzle of the duster into a hole on the top of a tent. Pots without *M. brunneum* were used as controls. Tents were removed after application. Fifteen ‘proxy’ beetles, which acted as proxies for the quantity of viable spores in a pot, were then introduced into 4 pots of each treatment 1, 2, 3, and 5 days post application. Proxy beetles were recollected after 24 h and monitored for mortality and

cause of death for 14 days. Because recollection rates were high in this experiment (78.1% ± SE 0.03), no covariates were used in this analysis. For each day, there were four replicates of the *M. brunneum* treatment, and two replicates of the control treatment, for a total of 24 pots.

The effect of day after application on the mortality and sporulation was tested using a generalized linear model with binomial distribution and logit link function. To describe the rate at which mortality and sporulation decays over time, mortality and sporulation rates were adjusted for the control as described in Table 3.1. Day was included as a continuous explanatory variable. The median survival time was calculated for each pot, and a linear model was conducted to compare the median survival time between the MET and CON treatments. The survival time was determined by taking the median survival time for each pot.

3.2.8. Experiment 4: Vectoring ability of *A. obscurus* beetles

I investigated the vectoring ability of *A. obscurus* by releasing a contaminated donor beetle onto soil at different times post *M. brunneum* exposure, and by determining the mortality and sporulation of uninfected beetles as a proxy for the amount of spores deposited. A spore treatment cup was prepared by filling 2 oz (59.1 ml) cups with 6 g of soil, and placing 31 rice grains with and without fungus (MET and CON grains, respectively) evenly to the surface using a stencil. MET grains carried 5.1×10^7 viable spores per g. This procedure introduced ~1.3×10^7 viable spores to the arena. A single male beetle ('donor') was introduced into the spore treatment cup and allowed to walk for 5 min. The donor beetle was then immediately placed into a test arena (140×15 mm) containing 15 g of soil. After 5 min, the donor beetle was removed and placed into a new holding arena (140×15 mm). The test arena was kept for later assessment. The donor beetle was transferred to a new test arena 20 min post exposure. The process was repeated at 0.5 h, 1 h, 3 h and 6 h post exposure, with the donor beetle being held in the test arena for 5 min at each time point. This gave a total of six test arenas per donor beetle. Twelve proxy beetles were then placed into each dish and allowed to walk for 10 min, after which time they were reared individually to determine mortality and cause of death. The experiment was repeated four times. To describe the rate at which mortality and sporulation decreased with time after donor beetle exposure, mortality and sporulation

were adjusted for background mortality using a parallel control experiment, which used a donor beetle that had been exposed to non-conidiated rice granules (Table 3.1). Twenty-six out of the 475 proxy beetles tested were removed from the dataset as they remained immobile during the experiment. I performed a generalized linear model with quasi-binomial distribution on the mortality and sporulation at 14 days, with time as the main effect. Experiment day was included as a fixed effect blocking factor. Beetles that had not died by day 14 were excluded from the sporulation analysis.

3.3. Results

3.3.1. Experiment 1: Beetle response to female sex pheromone when *M. brunneum* is present

As expected, pheromone increased the walking speed, walking distance and frequency of contacts with the treatment zone (Figure 3.2, Table 3.2). *Metarhizium brunneum* treatment did not affect beetle movement, indicating that *A. obscurus* does not avoid *M. brunneum* spores (Table 3.2).

In the presence of pheromone, beetles picked up 2.3 times more spores across fungus treated and untreated arenas (pheromone: $F_{2, 30}$=5.66, P=0.024; *M. brunneum*: $F_{2,30}$=32.25, P<0.001; pheromone**M. brunneum*: $F_{3, 29}$=0.019, P=0.892; Figure 3.3). However, this effect did not translate into greater mortality or *M. brunneum* sporulation in the pheromone treatment (Table 3.2). Fungus led to 86.2% mortality by 14 days (N=58) compared to 60.3% (N=58) in the untreated controls (*M. brunneum*: $F_{1, 103}$=4.82, P=0.028). Percentage sporulation was 42.1% and 1.8%, respectively (*M. brunneum*: $F_{1, 103}$=23.09, P=<0.001). The median survival time of beetles was 7.5 days in the fungus treatment and 12 days in the control.

3.3.2. Experiment 2: Passive transfer of conidia between live beetles (small)

In the horizontal transmission experiment, keeping the donor beetle in the arena led to 21.0% higher recipient beetle mortality in the *M. brunneum* treatment (96.4%) compared to when the donor beetle was removed (79.6%) (*M. brunneum:* χ^2 =27.049, DF=1, P=<.001*;* Keep/Remove: χ^2 =3.845, DF=1, P=0.050*; M. brunneum**Keep/Remove: χ^2 =5.812, DF=1, P=0.016, Figure 3.4A). There was no difference in mortality between

control treatments. Mortality was high in the control treatments (47.2%), likely due to senescence in the late season. Beetle removal had no effect on the likelihood of sporulation in beetles that died within the 14 days (Appendix B, Table B1).

In the subset of beetles (N=18 pairs), where interactions between beetles were studied, pheromone increased the number of contacts between the donor and the recipient beetle by 58.1% (Pheromone: χ^2 =6.169, DF=1, P=0.0130), whereas *M. brunneum* had no effect on the number of contacts between beetles (Pheromone**M. brunneum*: χ^2 =0.234, DF=1, P=0.629; *M. brunneum*: χ^2 =1.53, DF=1, P=0.216). Pheromone did not lead to more mortality of recipient beetles (Appendix B, Table B1), but did lead to more mycoses in *M. brunneum* treated recipient beetles (Pheromone**M. brunneum*: χ^2 =6.162, DF=1, P=0.013, Appendix A Table A1, Figure 3.4B).

3.3.3. Experiment 3: Horizontal transfer of conidia in live beetles (large)

As expected, in the large-scale horizontal transmission experiment, *M. brunneum*-treated donor beetles had greater mortality and mycoses than the untreated controls (CON-Pheromone: mortality=49.5%, sporulation=16.4%; MET-Pheromone and MET-Blank: mortality=96.4%, sporulation=71.6%). Pheromone did not increase sporulation in the *M. brunneum*-treated donor beetles (MET-Pheromone vs MET-Blank: mortality: Z=1.55, P=0.253; sporulation: Z=1.10, P=0.50. For full statistics, see Appendix B, Table B2).

Pheromone did not increase mortality or sporulation in the recipient beetles in the *M. brunneum* treatments (MET-Pheromone vs MET-Blank mortality: Z=1.059, P=0.580; sporulation: Z=0.796, P=0.852) but, as expected, mortality was 76.6% higher in the pots treated with *Metarhizium* compared to the control (*M. brunneum*-Pheromone: $F_{2,34}$= 30.719, P<0.001; MET-Blank and MET-Pheromone vs CON-Pheromone: Z=7.101, P<0.001, Bonferroni adjusted). Mycoses were also higher in the *M. brunneum* treated pots (74.6%) compared to the control (20.0%) (*M. brunneum*-Pheromone: $F_{2,34}$= 22.503, P<0.001, MET-Pheromone and MET-Blank vs CON-Pheromone: Z=6.458, P<0.001).

Removing donor beetles did not affect mortality in recipient beetles (*M. brunneum*-Pheromone*Donor beetles removed: $F_{2, 31}$=0.899, P=0.417; Donor beetles removed: $F_{1, 33}$ =0.015, P=0.902). However, it did result in less mycoses across *M. brunneum*-Pheromone

treatments (*M. brunneum**Number of donor beetles removed: $F_{2, 33}$=2.461, P=0.102; Number of donor beetles removed: $F_{1, 33}$=4.374, P=0.044, Fig 3.5).

Background mortality was high (53.1% deaths in the CON-Pher treatment), likely due to the high temperatures in the greenhouse. Of the control deaths, 55.1% were due to causes other than *Metarhizium* spp. However, a logistic regression showed that the level of other background fungal contamination, excluding *Metarhizium* spp.-related deaths, was similar between treatment groups (*M. brunneum*-pheromone*Donor beetles removed: $F_{2, 29}$=0.205, P=0.16; *M. brunneum*-pheromone: $F_{2, 32}$=0.324, P=0.726 and Donor beetles removed: $F_{1, 30}$=1.408, P=0.245). These deaths were therefore included in the mortality and sporulation analysis as non-*Metarhizium* deaths.

3.3.4. Experiment 3B: *M. brunneum* persistence in greenhouse

In the persistence experiment, neither the mortality (93.4% ± SE2.1) nor the sporulation (81.0% ± SE6.5) of proxy beetles decreased with time post *M. brunneum* application, after correcting for control mortality and *M. brunneum* infection, respectively (mortality: $F_{1,16}$=0.009, P=0.924; sporulation: $F_{1,16}$=0.930, P=0.349, Table 3.1). The mean median survival time in the MET treatment was 7.9 days ± SE0.44, whereas less than half of the beetles in the control died within the 14-day observation period. This suggests that minimal spore degradation occurred during our mesocosm experiment.

3.3.5. Experiment 4: Vectoring ability of *A. obscurus* beetles

The mortality of proxy beetles was not affected by time post inoculation of the donor beetle ($F_{1,14}$=1.735, P=0.209). However, sporulation of proxy beetles decreased with time ($F_{2,13}$=7.31, P=0.007), with mycoses dropping from 91.7% to 35.6% after 1 h post inoculation of the donor beetle (Figure 3.6). This result suggests that beetles exposed to *M. brunneum* initially deposited spores in significant numbers, which declined rapidly. The change in the adjusted proportion sporulated is described with the following equation:

$$P(Sporulation) = \frac{e^{0.2024 - 3.2164t + 0.4248t^2 + d}}{1 + e^{0.2024 - 3.2164t + 0.4248t^2 + d}}$$

where *t* is the time post exposure of the donor beetle and *d* is the beetle pair block.

3.4. Discussion

In this study, I demonstrate that the presence of *Metarhizium brunneum* conidial spores does not affect the response of male *Agriotes obscurus* to female sex pheromone. Passive horizontal transmission, that is transmission of spores from sources other than sporulated cadavers, can occur between male beetles. However, an increased level of activity, which results from exposure to sex pheromone, does not enhance passive horizontal transmission in male beetles under the fungus concentration applied. Horizontal transmission can be achieved indirectly, when inoculated beetles drop spores onto the surrounding substrate, and non-inoculated beetles pick up these spores. This type of transmission occurs within the first hour of beetles contacting the inoculum.

3.4.1. Beetles do not avoid *Metarhizium*

In the open arena study, male *A. obscurus* did not avoid *M. brunneum* inoculated grains; beetles interacted with pheromone-impregnated granules and exhibited pheromone-induced movement behaviours, whether *M. brunneum* was present or not. As a result, pheromone exposure led to more spores being picked up by the beetles. However, this did not lead to increased mortality or sporulation, likely due to the fact that beetles in both cases picked up sufficient numbers of spores to cause mortality. There have been reports of *A. obscurus* both avoiding and not avoiding *M. brunneum*. Non-avoidance of *M. brunneum* is consistent with a previous report that *A. obscurus* does not avoid sporulating *M. brunneum* cadavers (V. Fung, unpublished data). However, Janmaat et al., (unpublished data) found that *A. obscurus* reduced searching time and increased resting time in wheat-planted arenas sprayed with *M. brunneum*. Avoidance of entomopathogens can be context-dependent; common flower bugs (*Anthologies nemorum),* avoided spores of *Beauveria bassiana* only when foraging on host plants but not on soil surfaces (Meyling and Pell, 2006). Moreover, black vine weevil larvae (*Otiorhynchus sulcatus*) became attracted to *M. anisopliae*-treated media when plants were present but not when they were absent (Kepler and Bruck, 2006). Pathogens can trigger host avoidance behaviour through their organic compound profiles (e.g., Mburu et al., 2013; Swanson et al., 2009; Yanawaga, 2014), and the surrounding environment may play an integral role in these profiles. While I did provide soil substrate in my arenas, I did

not introduce vegetation, which could potentially alter the ability of beetles to detect pathogens.

3.4.2. Passive horizontal transmission pathways

I demonstrate that spores can readily be transferred from beetle to beetle after they are exposed to inocula. In both the small-scale and large-scale horizontal transmission experiments, beetles that were secondarily introduced into an area became infected with *M. brunneum*, despite the initial source of inoculum being removed. While I showed that beetle-to-environment-to-beetle transmission can occur, I could not determine the relative importance of this pathway compared to beetle-to-beetle transmission.

In the small-scale horizontal transmission experiment, keeping the inoculated donor beetle led to greater mortality in the recipient beetle. This would seem to suggest that beetle-to-beetle contact is an important pathway of pathogen transmission. The pathway of horizontal transmission, however, was not clear in the large-scale horizontal transmission experiment; although removing donor beetles led to less cadavers sporulating in recipient beetles, this was true for both the control and the fungus treatment, suggesting the observation was an effect of the experimental procedure.

Although most studies on passive horizontal transmission of *Metarhizium spp.* have focused on intersexual transmission, most research agrees that horizontal transmission of *Metarhizium* spp. occurs readily (e.g., *Blattella germanica*: Quesada-Moraga et al., 2004; *Glossina morsitans morsitans*: Kaaya and Okech, 1990). It has been reported that mechanical transmission of spores between individuals is a highly efficient process; in *Psoroptes* mites, 40% of mites exposed to *M. anisopliae* became infected "after a single touch of cadavers lasting less than one second" (Brooks and Wall, 2005).

In addition to direct spore transfer, an alternative explanation for the results observed in the small-scale horizontal transmission experiment is that the retained donor beetles may have had more time to shed spores within the arena. Although beetles can vector conidial spores to conspecifics, this vectoring ability is limited. The number of spores retained on the body of *A. obscurus* is known to drop dramatically one day post-exposure to *M. brunneum* conidiated rice grains (Kabaluk, 2014). The results from the

vectoring experiment (Experiment 4) show that loss of spores from *A. obscurus* occurs within the first hour. Soil that had been contaminated immediately after a donor beetle was inoculated, led to more sporulation in proxy beetles than soil contaminated later after exposure. This reduction in passive transfer over time is consistent with other studies (Sookar et al., 2014; Ugine et al., 2014; Cárcamo et al., 2015). For example, *Ceratitis* spp. fruit fly females that had picked up ~1.0×10^6 *M. anisopliae* spores, and were then introduced to a set of three "clean" males on five consecutive days, passed on fatal doses of inoculum to these males (Dimbi et al., 2013). Similarly, dip-infected males of house flies, *Musca domestica*, that were exposed to 10 clean females at time-points up to 96 h were able to horizontally infect 7.4 females when newly infected, and 1.6 females after 96 h of infection (Cárcamo et al., 2015). Although I found no effect of time post inoculation on infection after the first hour, this may be an effect of the dose of *M. brunneum* used. My study differed in that I tested for indirect transfer of spores to clean individuals as opposed to direct transfer. In addition, I tested only one concentration of *M. brunneum*, and the number of spores deposited in the soil are likely to differ depending on the spore concentration applied, as well as the time beetles spent at the inoculum source. Similarly, the number of spores that proxy beetles pick up is dependent on their exposure time to the substrate. All of these factors may have led to low enough doses of *M. brunneum* obtained, that differences in mortality was not observed. A point to consider then is the relative importance of different transmission pathways at different doses of *M. brunneum*. The effect of distance away from the initial inoculum should also be considered; presumably there is a point, at which spores remaining on the body are less easily dislodged and lost to the environment, in which case beetle-to-beetle transmission may become comparatively less important.

The lack of direct transfer of spores (i.e. from beetle to beetle) by click beetles is perhaps unsurprising, considering the life history of *Metarhizium spp.*; spores are able to remain dormant, or live saprophytically in the soil within the rhizosphere (Hu and St. Leger, 2002; Bruck, 2005) until a new host comes along, where upon the spores that receive the chemical and topical cues from an insect cuticle begin to germinate (Hajek and St. Leger, 1994). *Metarhizium* spp. can persist in soil for periods lasting from several months to several years depending on the strain and environmental conditions (Zimmerman, 2007). The conidial spores are not the main dispersive stage, and therefore are unlikely to be adapted for being mechanically vectored over long distances. Long-distance dispersal is

primarily achieved when infected hosts migrate to new locations before dying. Indeed, Entomophthorales (Zygomycete) fungi, which also kill the host to complete their cycle, have been shown to produce spores that lack the propensity to be vectored. Resting spores of the entomophoralean fungus *Entomophaga grylli* have limited propensity to be mechanically vectored (Kistner et al., 2015). Non-host ants that had been inoculated with a liquid suspension of *E. grylli* spores lost 50% of conidia within 6 h post inoculation (Kistner et al., 2015), with only 2% of spores remaining after 24 h. In contrast, transmission of the biotrophic ectoparasitic fungus *Laboulbenia slackensis* is more dependent on susceptible hosts coming into contact with live infected hosts. This fungus has an adaptation that makes it more amendable to host-to-host transmission; it produces spores that adhere to each other forming string-like structures that remain on the body of the infected host until a new host comes along (De Kesel, 1995). De Kesel (1995) tested both direct and indirect transmission of the fungus by exposing clean *Pogonus chalceus* beetles to contaminated substrate (indirect) and infected beetles with contaminated substrate (direct). De Kesel found that the density of thalli (mature ascospore of *L. slackensis*) on the host increased with dose in the direct transmission treatment, whereas in the indirect transmission treatment it did not. The author suggested that this is because *L. slackensis* does not tend to lose spores to the environment. Propensity for mechanical vectoring for a pathogen that kills its host requires delayed germination upon contact with the insect cuticle, and a slow speed of kill.

The propensity for spores to be vectored is essential when the motivation for an autodissemination strategy is to target cryptic species, such as soil-dwelling click beetles. This puts into question whether males can carry spores to egg-laying females, which may be far away from a pheromone-inoculum source. Although my experiments indicate that a large number of spores is lost from a beetle body shortly after acquisition, it is possible that the spore load that remains may be sufficient for male-to-female transmission. Furthermore, copulatory behaviour is likely to make transmission of *Metarhizium* spp. more efficient. This remains to be explored with *A. obscurus*. Transmission of *Metarhizium* spp. during copulation, however, has been found to occur in many systems tested to date. Virgin females of *Anopheles gambiae* mosquitos that had been exposed to *M. anisopliae conidia* passively transferred conidia to uncontaminated males, resulting in ~10-30% of males becoming infected (Scholte et al., 2004).

3.4.3. Horizontal transmission is not affected by pheromone exposure

A number of studies have shown that changes in host movement can affect the transmission of fungal pathogens in a population (Roditakis et al., 2000 and speculated in Furlong and Pell 1996; Roy et al., 1998). I therefore expected that sex pheromone, which increases host motility, would further pathogen acquisition. This was not the case for the small-scaled horizontal transmission experiment, where pheromone did not increase mortality in beetles on contaminated substrates. Among beetles that died, sporulation was higher in the absence of pheromone. These surprising results may be due to the large dose of inoculum used in both experiments. *Metarhizium spp.* infection is dose-dependent in click beetles (Kabaluk et al., 2015). Pheromone may increase the number of spores that recipient beetles pick up, but if recipient beetles in the blank treatment also pick up a sufficient lethal dose, the effect of pheromone will not be detectable. This was the case in the avoidance experiment (Experiment 1), where treatment and control beetles did not differ in mortality and mycoses, although pheromone-exposed beetles picked up more spores. The small size of the test arenas magnifies the effect of a high dose. This inference is further supported in that pheromone had no effect on infection of donor beetles, although pheromone increased the rate of infections in a clean beetle population in a small-enclosure experiment (Kabaluk et al., 2015). Enumeration of the spores picked up by beetles would help tease out this effect. If it was indeed the case that an excessive inoculum was used, it would be of interest to see whether pheromone enhances passive horizontal transmission, when a lower dose of innoculum is applied.

3.4.4. Conclusions

Although sex pheromone increases the motility of *A. obscurus* males, it does not enhance the passive horizontal transmission of *M. brunneum* to beetles, under the conditions tested. Based on my findings two approaches for autodissemination of *M. brunneum* in *A. obscurus* can be explored; the first approach is to devise methods that enhance mechanical transmission of conidial spores. Novel techniques, such as combining microbial control agents with electrostatic powders (Nboyine et al., 2015 and Athanassiou et al., 2017) to enhance the vectoring capability of insects have shown promise in this regard. The second approach is to enhance the likelihood that female beetles come into contact with contaminated substrates. To this end, spore persistence in the environment should be considered, which can be enhanced through conservation

biological control principals, such as limited use of pesticides that have adverse effects on the fungus (Pell et al., 2010), or through formulations that are tolerant to environmental variability.

There is considerable room for innovation in the combined use of semiochemicals and microbial insecticides. In this study, I explored how one aspect of pest response to semiochemicals affects transmission of an insect pathogen. However, semiochemicals mediate a suite of behaviours and physiological changes in insects, and it remains unclear how these may affect pathogen transmission. A holistic view of how semiochemicals impact pest species, and a better understanding of pathogen ecology can allow us to explore other avenues of achieving synergistic results with semiochemical-insecticide combinations.

3.5. References

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3.6. Tables

Table 3.1 **Corrected background mortality and infection in *A. obscurus* using Schneider-Orelli formula (Schneider-Orelli, 1947), where T is the total number of beetles tested. M is the number of beetles that died within the 14 day observation period, S is the number that sporulated with *Metarhizium*, O is the other causes of mortality, i.e. the number that either sporulated with other pathogenic or saprophytic fungi or asymptomatic causes. Subscripts refer to the treatment.**

	Formula
Correcting for background mortality	
Number of beetles tested for mortality	$T_{MET} \times \left(1 - \frac{M_{control}}{T_{control}}\right)$
Number of dead beetles	$M_{MET} - \left(T_{MET} \times \frac{M_{control}}{T_{control}}\right)$
Correcting for background *Metarhizium* infection	
Number of beetles tested for *Metarhizium* infection	$T_{MET} \times \left(1 - \frac{S_{control} + O_{control}}{T_{control}}\right)$
Number of beetles sporulated	$S_{MET} - \left(T_{MET} \times \frac{S_{control}}{T_{control}}\right)$

Table 3.2 **Effect of pheromone and *Metarhizium brunneum* on *Agriotes obscurus* activity (N=160), mortality, *M. brunneum* sporulation.** Non-significant interactions were removed first followed by non-significant main effects that were not part of a significant interaction. (N=106)

	Num DF	Den DF	F Value	P
Walking speed				
Pheromone	1	158	10.84	0.001
M. brunneum	1	157	0.01	0.930
Pheromone: *M. brunneum*	1	156	0.00	0.975
Distance walked				
Pheromone	1	158	8.23	0.005
M. brunneum	1	157	0.13	0.722
Pheromone: *M. brunneum*	1	156	0.02	0.882
	Num DF	**Den DF**	χ^2	**P**
Frequency of contacts				
Pheromone	1	158	29.9	<.001
M. brunneum	1	157	0.43	0.510
Pheromone: *M. brunneum*	1	156	0.91	0.505
Mortality				
Pheromone	1	102	0.06	0.803
M. brunneum	1	103	4.82	0.028
Pheromone: *M. brunneum*	1	101	2.68	0.102
Sporulation				
Pheromone	1	102	0.48	0.491
M. brunneum	1	103	23.09	<0.001
Pheromone: *M. brunneum*	1	101	0.55	0.814

3.7. Figures

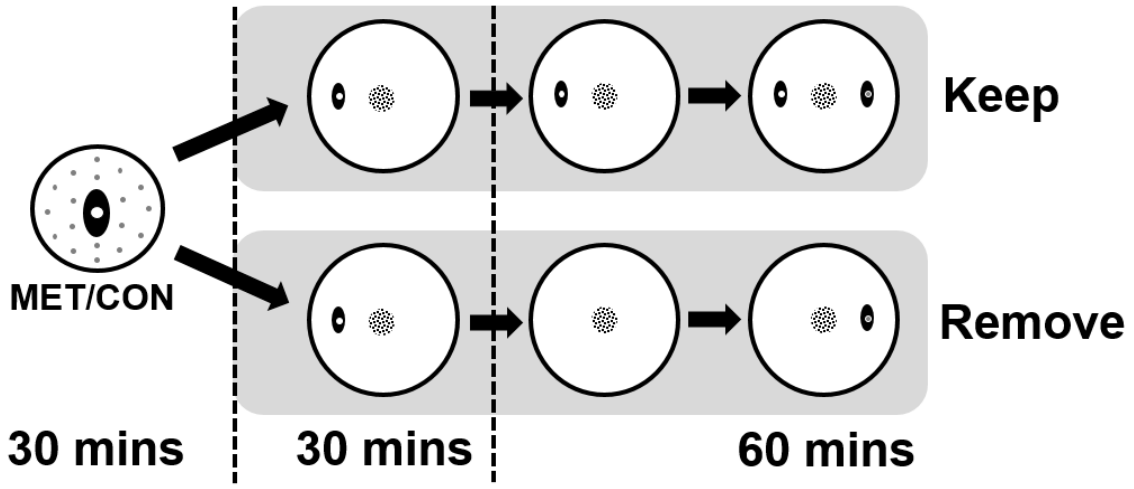

Figure 3.1 **Horizontal transmission experiment, small scale.** A marked *Agriotes obscurus* donor beetle is placed into 1 oz (29.6 ml) Solo cup containing 31 conidiated rice granules. The donor beetle is then transferred to a 14 cm Petri dish containing pheromone or blank granules in the treatment zone and allowed to walk for half an hour, after which the beetle is either kept or removed. A recipient clean beetle is then introduced and allowed to roam for 60 min

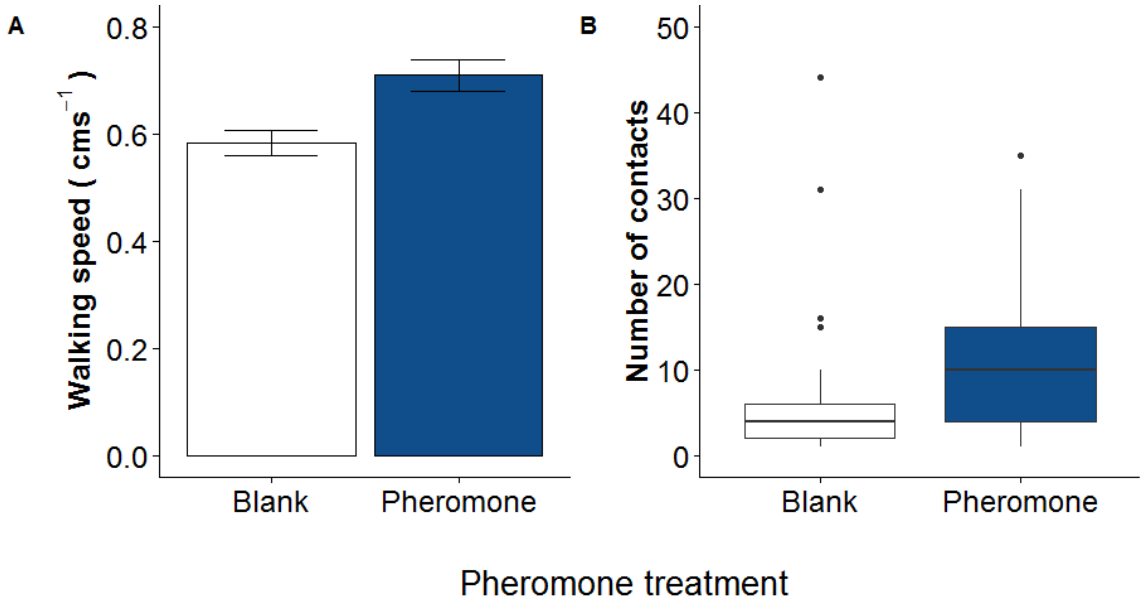

Figure 3.2 **Mean (A) walking speed (±SE) and (B) number of contacts with treatment zone for male *Agriotes obscurus* beetles exposed to blank (white bars) and pheromone (dark bars) granules, measured over 10 min.(N=160)**

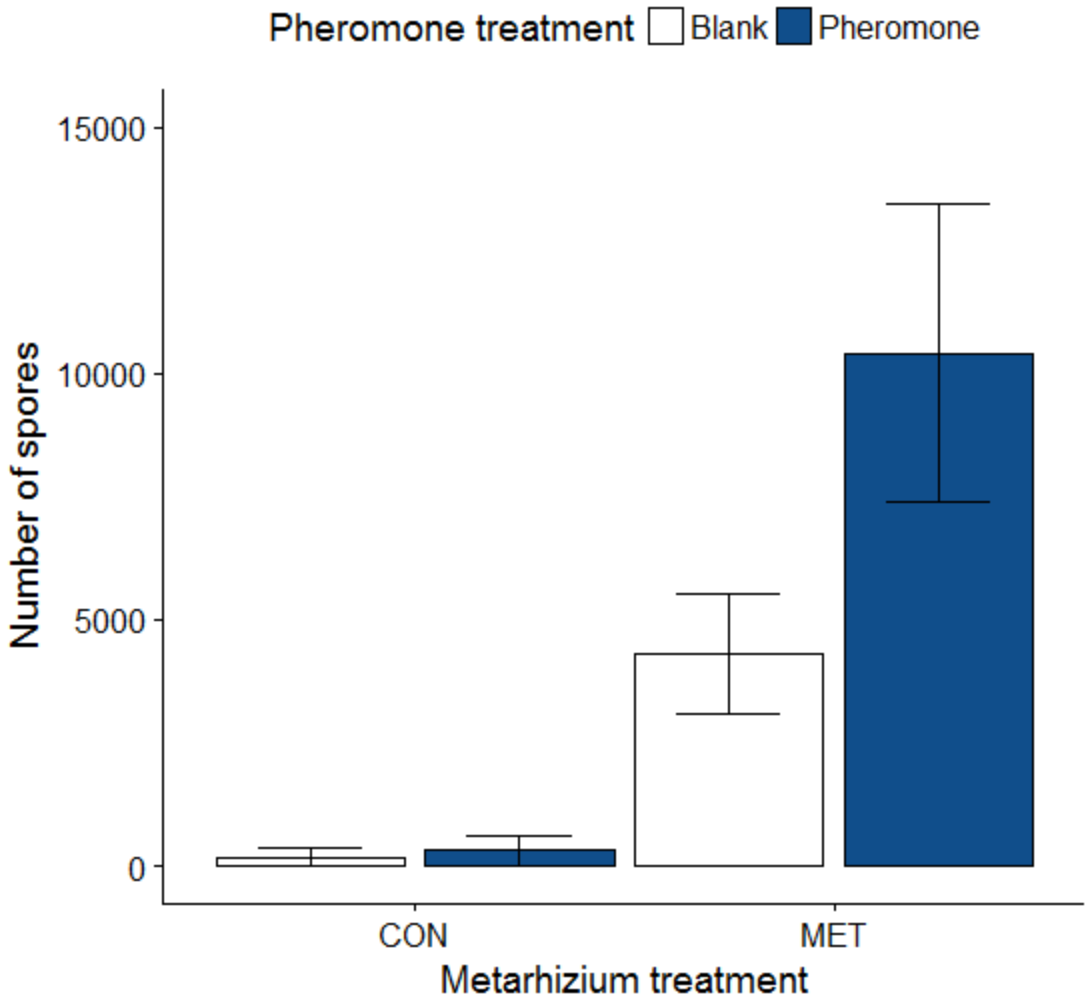

Figure 3.3 **Number of spores washed off of *Agriotes obscurus* beetles that had been exposed *Metarhizium brunneum* rice grains for 30 min in the presence and absence of female sex pheromone.** Beetles were washed with 250 µl of 0.05% TWEEN and the number of spores in two samples of 10 µl were counted using a haemocytometer. (N=33)

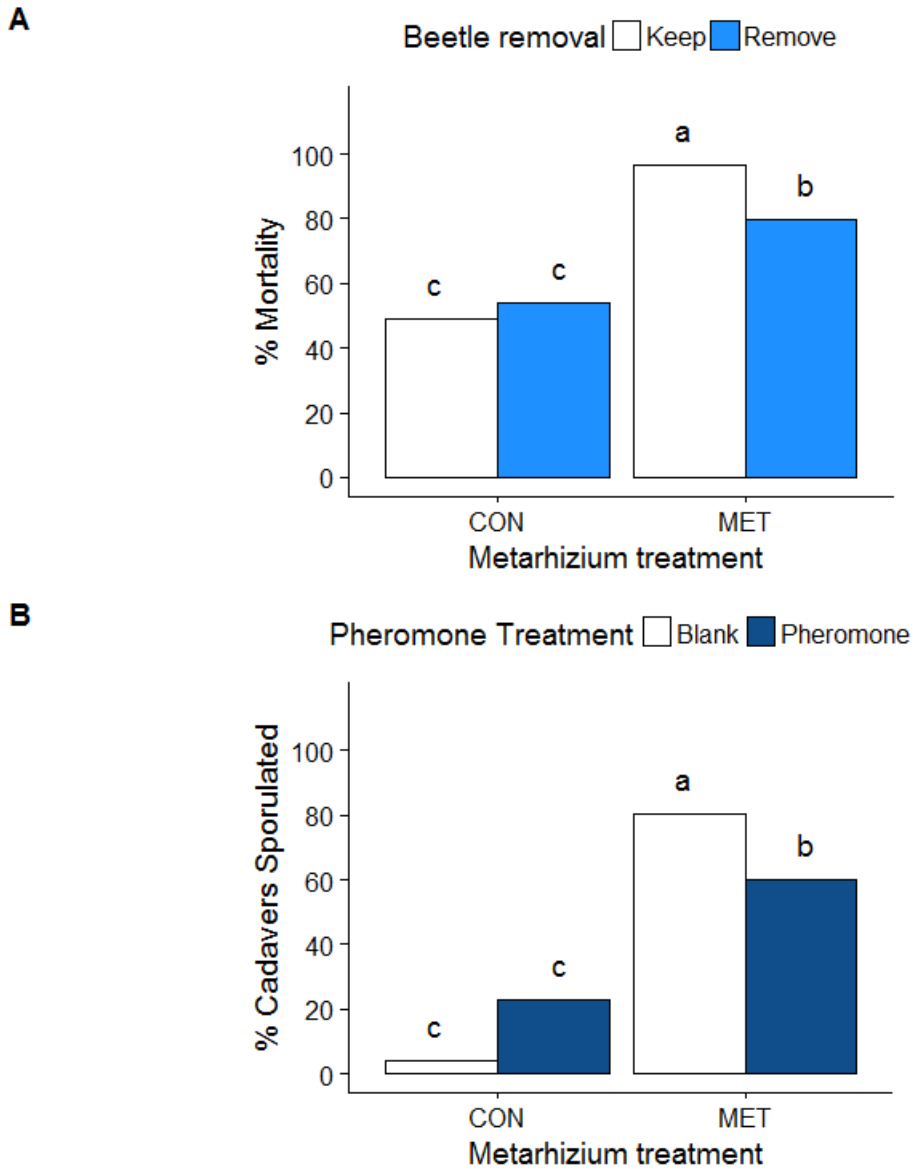

Figure 3.4 **A) % Mortality (N=219) of *Agriotes obscurus* beetles that had been exposed to substrate that had been contaminated with *Metarhizium brunneum* by another *A. obscurus* beetle, and B) % cadavers sporulated (Small-scale horizontal transmission experiment).** Only those that had died by day 14 were included in the sporulation analysis (N=148)

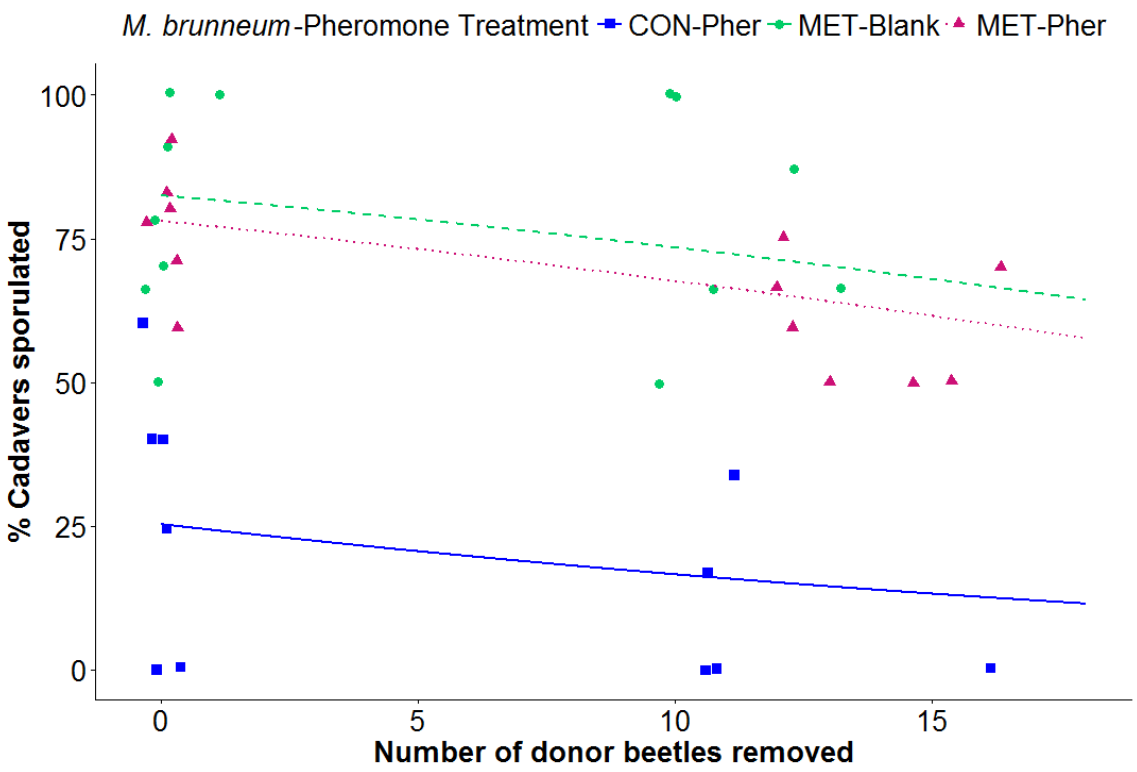

Figure 3.5 **% Sporulation of *Agriotes obscurus* beetles that had been placed into arenas with different numbers of donor beetles removed (Large-scale horizontal transmission experiment).** Only those that had died by day 14 were included in the sporulation analysis (N=36 pots)

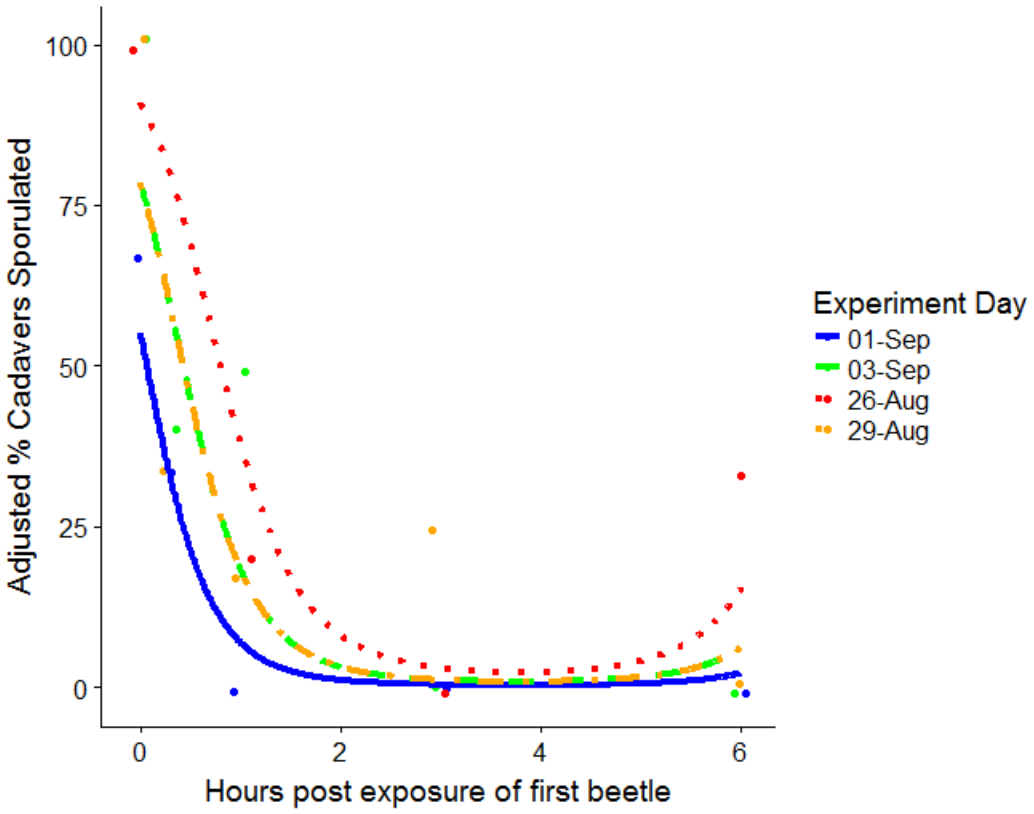

Figure 3.6 **Adjusted % cadavers sporulated.** Clean *Agriotes obscurus* beetles were exposed to substrate that had been exposed to beetles immediately after, 0.3, 1, 3 and 6 hours post exposure to *Metarhizium*. Lines show model output (see text). Lines from 03-Sep and 29-Aug are overlapping.

Chapter 4.

Concluding summary

Autodissemination of entomopathogens occurs in three steps; 1) target pest contacts the pathogen 2) target pest picks up sufficiently high numbers of the pathogen and 3) target pest transfers the pathogen to conspecifics. In this thesis, I have shown that autodissemination of the fungus *Metarhizium brunneum* should be further explored as a tactic for the control of *Agriotes obscurus* click beetles. However, the application rate required for comprehensive wireworm control, and economic viability of this option are still to be determined.

The first condition for autodissemination is readily met; in Chapter 2, I demonstrated that *A. obscurus* is attracted to a granular formulation of female sex pheromone, corroborating work done by Kabaluk et al., (2015), who found *A. obscurus* increased visitations to a band of granules that were impregnated with pheromone. In the field experiment in Chapter 2, over 25% of beetles that were released 14 m away were recollected using a combination of pitfall traps and pheromone. In Chapter 3, I also showed that *A. obscurus* pheromone response was not affected by the presence of *M. brunneum*. As a result, beetles exposed to a mixture of *M. brunneum* conidiated rice grains and pheromone granules picked up more than twice the number of spores, compared to when granules had no pheromone. These findings support the field study conducted by Kabaluk et al., (2015), in that *A. obscurus* will likely come into contact with the fungus, if combined with pheromone.

Additionally, click beetles were responsive to pheromone, under different environmental conditions tested, suggesting that the granular pheromone formulation will be effective under various scenarios. Beetles responded equally well to pheromone in white light and red light, in both cases walking faster and for longer in the presence of pheromone. In addition, click beetles responded in both moving and still air, although response was enhanced by wind in beetles collected early in the season. Consequently, placement of pheromone sources should consider how landscape features may affect air dynamics. For example, high wind speeds can inhibit pheromone-lured trap captures of boll weevils (*Anthonomus grandis*), therefore placing lures downwind of brush lines can

lead to more captures on windy days (Sappington and Spurgeon, 2000). For later season *A. obscurus* control, it may therefore be advisable to place the pheromone-fungus combination in areas where the wind can be dampened by vegetation. In my studies, only one wind speed was tested, therefore further work is needed to establish the wind speed threshold for activity.

Larvae are the damaging life stage of *A. obscurus*, while adults typically cause little impact. An adult-targeted strategy therefore requires that either adults are eliminated before oviposition occurs, or that fecundity is reduced. In Chapter 1, I demonstrated that click beetles collected in different months responded equally to pheromone. Consistent response to pheromone suggests a long window of opportunity in which autodissemination could be effective. However, there was a general reduction in activity in beetles that were collected later in the season, which could impact the success of such a strategy; beetles collected in May walked 61% slower and were 60% less active than beetles collected in March and April. *A. obscurus* oviposition occurs as early as April (Sufyan et al., 2014), lasting till late June, although this can vary depending on climate. In the lab, beetles collected in April in the UK have a preoviposition period of 39 days while those collected later have a preoviposition period of 14-15 days (Cohen, 1942). Therefore there is a greater likelihood of hitting beetles before oviposition with autodissemination early in the season.

Another aspect to consider however, is that immune function and insect susceptibility to entomopathogens can change over time (Adamo et al., 2001; Mnyone et al., 2011; Rolff, 2001). For example, although older *Monochamus alternatus* beetles were found to be generally less susceptible to the fungus *Beauveria bassiana* with age (Maehara and Kanzaki, 2014), this was no longer true near the end of the beetles lifespan. Beetles inoculated at 28 days of age died faster than those inoculated at 14 days of age. Later applications should therefore not be discounted.

The second and third condition for autodissemination, where a target pest must pick up sufficiently high doses of pathogen, and be able to transfer them to conspecifics is also met. In Chapter 2, I showed that click beetles can transmit spores to other males through two pathways; indirectly through contamination of the environment, and directly through contact with other males. Indirect transmission can be an invaluable pathway, as it may target not only other adults, but also eggs that may be laid in soil. Egg mortality due

to *Metarhizium* application in soil has been previously reported for other pests (e.g. Leles et al., 2012). However, my studies show that vectoring of *M. brunneum* spores to the environment by *A. obscurus* beetles is limited to the first hour post inoculation.

Another approach is to enhance autodissemination through beetle-to-beetle (direct) transmission. In autodissemination, transmission of spores to females is preferable as it will have a more direct effect on egg-laying. While spore transfer between males and females was not explored, I demonstrated that male-to-male transmission was possible. It would be expected that male-to-female transmission would be equally, if not more effective, due to the close contact that occurs during mating. During mating, *A. obscurus* pairs remain in copula for 60 seconds to 15 minutes (Cohen, 1942), compared to male-male contact where social interactions have not been previously reported.

One aspect that should be further explored, is whether after losing large quantities to the environment, would the quantity of spores that remain on the body of a beetle be sufficient for direct transmission. This remains an important piece of information needed for the optimisation of autodissemination. Furthermore, even if the dose transferred to the beetles was insufficient for mortality, a sublethal dose may still reduce the fecundity of females (e.g. Quesada-Moraga et al., 2004), and this should be explored more in depth.

While I explored the use of behavioural manipulation in influencing the transmission of *M. brunneum* in *A. obscurus*, my findings showed that heightened movement due to pheromone, did not lead to increased direct or indirect transmission of the fungus. These results, however, highlight the need for a thorough understanding of host-pathogen ecology, in order to use behavioural manipulation effectively. Nonetheless, autodissemination remains a promising strategy for the control of *Agriotes obscurus* beetles, and is an application method that can help overcome many of the challenges that come with using a microbial control agents such as *Metarhizium*.

4.1. References

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Appendix A

Supplementary tables and figures for Chapter 2

Table A1 **Beetle capture periods (2015) and seasonal pheromone response experiment dates.**

Beetle collection period		Test dates	Mean (SE) room temperature at time of testing	Mean (SE) room relative humidity at time of testing
From	To			
March 11th	April 9th	April 25th, 26th; May 16th, 17th	23.5 (0.14)	36.2(0.82)
April 21st	April 24th	May 26th, 27th; June 16th, 17th	24.1 (0.25)	41.8(0.67)
May 7th	May 27th	June 24th, 25th; July 15th, 16th	24.4 (0.22)	42.7(0.39)

Table A2 **The effect of air movement-pheromone treatment on the number of *Agriotes obscurus* males that failed to complete the trial in the air movement experiment (Experiment 1).** Trial was failed either by not moving, or by climbing on the screen. A binary logistic mixed model was conducted and date of experiment was included as a random effect.

	Num DF	Den DF	F Value	Pr>F
Pheromone	1	191	0.88	0.348
Beetle collection period	1	190	0.32	0.575
Air movement	1	189	0.31	0.578
Pheromone*Air movement	1	188	2.37	0.125
Pheromone*Beetle collection period	1	187	0.27	0.601
Air movement*Beetle collection period	1	186	2.39	0.124
Pheromone*Air movement*Beetle collection period	1	185	0.07	0.794

Table A3 **The effect of beetle collection period and pheromone treatment on the proportion of *A. obscurus* beetles that successfully completed the trial (i.e. moved).** A generalized linear mixed effect model with binomial distribution was conducted and date of experiment was included as a random effect.

	Num DF	Den DF	F Value	Pr>F
Pheromone	1	126	0.43	0.512
Beetle collection period	1	126	0.03	0.966
Beetle collection period*Pheromone	1	124	0.45	0.503

Table A4 **The effect of light (red/white), beetle collection period and pheromone on male *Agrotis obscurus* walking and activity.** A linear mixed model was conducted and date of experiment was included as a random effect.

	Num DF	Den DF	F Value	Pr>F
Walking speed				
Pheromone	1	67	27.04	<0.001
Beetle collection period	1	67	6.24	0.015
Light	1	66	0.02	0.884
Beetle collection period*Pheromone	1	64	0.27	0.602
Light*Pheromone	1	63	0.27	0.605
Light*Beetle collection period	1	65	0.31	0.580
Pheromone*Beetle collection period*Light	1	62	0.96	0.331
Distance walked				
Pheromone	1	67	28.52	<0.001
Beetle collection period	1	67	5.62	0.025
Light	1	66	0.14	0.707
Beetle collection period*Pheromone	1	64	0.04	0.836
Light*Pheromone	1	63	0.03	0.859
Light*Beetle collection period	1	65	0.51	0.476
Pheromone*Beetle collection period*Light	1	62	1.28	0.263
Proportion time moving				
Pheromone	1	68	22.7	<.001
Beetle collection period	1	67	2.75	0.102
Light	1	66	0.20	0.656
Beetle collection period*Pheromone	1	63	0.01	0.933
Light*Pheromone	1	65	0.54	0.731
Light*Beetle collection period	1	64	2.07	0.155
Pheromone*Beetle collection period*Light	1	62	0.54	0.466

Table A5 **The effect of light (red/white), beetle collection period and pheromone on male *Agrotis obscurus* contacts with pheromone granules.** A linear mixed model was conducted unless otherwise stated. Date of experiment was included as a random effect.

	Num DF	Den DF	F Value	Pr>F
Reach zone*				
Pheromone	1		8.98	0.003
Beetle collection period	1		2.49	0.115
Light	1		0.02	0.900
Beetle collection period*Pheromone	1		0.15	0.700
Light*Pheromone	1		1.28	0.259
Light*Beetle collection period	1		0.27	0.273
Pheromone*Beetle collection period*Light	1		0.052	0.820
Frequency of contacts+				
Pheromone	1	46	6.49	0.014
Beetle collection period	1	45	0.40	0.532
Light	1	44	0.01	0.943
Beetle collection period*Pheromone	1	41	0.06	0.807
Light*Pheromone	1	42	0.09	0.765
Light*Beetle collection period	1	43	0.40	0.530
Pheromone*Beetle collection period*Light	1	40	3.07	0.088
Cumulative duration of contact+				
Pheromone	1	45	0.56	0.457
Beetle collection period	1	46	7.47	0.009
Light	1	44	0.46	0.502
Beetle collection period*Pheromone	1	42	0.29	0.596
Light*Pheromone	1	41	0.12	0.735
Light*Beetle collection period	1	43	0.69	0.410
Pheromone*Beetle collection period*Light	1	40	1.02	0.319

* Wald Chi-Square

+ Analysis includes only beetles that reach granule zone

Pheromone release rate from granules used in field experiment at 23.1 °C (± SE 0.05)

Methods

Pheromone granules (1.6 g) were placed into an aeration chamber. Charcoal-filtered air was drawn for 24 h through the chamber and a glass column containing Porapak-Q adsorbent. Volatiles were desorbed using a mixture of pentane ether (1:1). Aliquots (N=2) were analyzed by gas chromatography.

Results

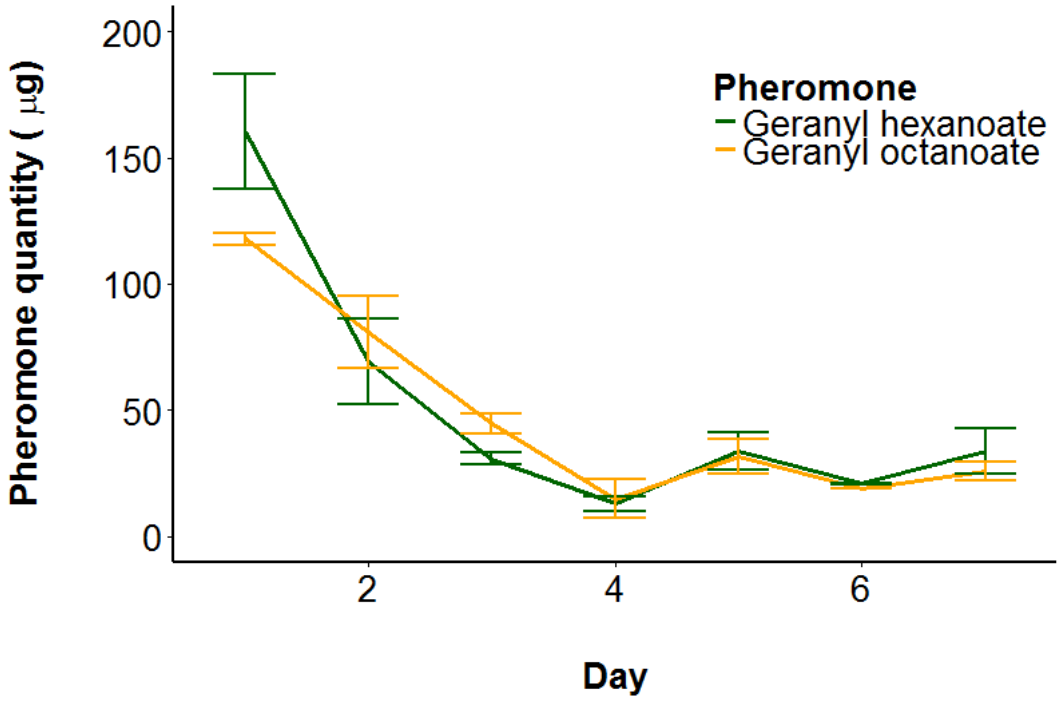

Figure A1 **Pheromone released from 1.6 g of pheromone-impregnated cellulose based granules.**

Appendix B

Supplementary tables and figures for Chapter 3

Table B1. **The effect of *Metarhizium brunneum*, donor beetle removal and pheromone on male recipient *A. obscurus* mortality and *M. brunneum* sporulation (N=221) in small-scaled horizontal transmission experiment**. A mixed effect, binary logistic regression was conducted with pheromone treatment nested within date of experiment included as a random factor. For beetles that had died within the 14 day period, the probability of *M. brunneum* sporulation was also analyzed in the same manner (N=148).

	DF	χ^2	P
Mortality			
M. brunneum	1	27.049	<.001
Keep/Remove	1	3.845	0.050
*M. brunneum**Keep/Remove	1	5.812	0.016
Pheromone	1	0.641	0.423
Keep/Remove*Pheromone	1	0.604	0.437
*M. brunneum**Pheromone	1	0.258	0.611
Pheromone*Keep/Remove**M. brunneum*	1	0.426	0.514
Sporulation			
M. brunneum	1	24.316	<0.001
Pheromone	1	0.679	0.410
*M. brunneum**Pheromone	1	6.162	0.013
Keep/Remove	1	2.961	0.085
Keep/Remove*Pheromone	1	1.374	0.241
*M. brunneum**Keep/Remove	1	0.036	0.851
Pheromone*Keep/Remove**M. brunneum*	1	0.594	0.441

Table B2 The effect of *Metarhizium brunneum*, donor beetle removal and pheromone on male donor *A. obscurus* mortality and *M. brunneum* sporulation in large-scale horizontal transmission experiment (N=36 pots, 718 beetles). Only beetles that had died by day 14 were included in the sporulation analysis (N=326 beetles).

	Num DF	Den DF	F	P
Mortality				
M. brunneum-Pheromone	2	35	30.719	<.001
Donor beetles removed	1	34	0.015	0.903
M. brunneum-Pheromone* Donor beetles removed	2	31	0.899	0.417
Sporulation				
M. brunneum-Pheromone	2	35	22.504	<.001
Donor beetles removed	1	34	4.374	0.044
M. brunneum-Pheromone* Donor beetles removed	2	31	2.461	0.102