Exploring *Aethina tumida* Biology and the Impacts of Environmental Factors to Generate Novel Management Strategies

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SCIENTIFIC ABSTRACT:

The small hive beetle (*Aethina tumida*) is an invasive pest from sub-Saharan Africa that has posed increasing threats to European honey bee (*Apis mellifera*) colonies in the United States over the past two decades. While control has been attempted, consistently effective management strategies still not been developed. This study sought to explore novel experimental methods to better understand and use *A. tumida* biology to target this pest. One aspect of *A. tumida* biology that has emerged as potential basis for improved control is olfactory manipulation, which could be used to disrupt beetles as they seek out *A. mellifera* colonies. Through olfactometry and electroantennography, key volatiles in *A. tumida* attraction and repulsion were tested and sensitivity of *A. tumida* to several attractants and repellents was quantified on behavioral and physiological levels. An additional source of attractive volatiles is the *A. tumida* fungal symbiont *Kodamaea ohmeri*, which ferments larval waste and is present throughout the *A. tumida* lifecycle, both externally and in the GI tract. This study explored the development of feeding and soil bioassays to test the effects of several insecticides on *A. tumida* larvae. Feeding and injection bioassays were also used to deliver a fungicide with the goal of repressing K. ohmeri, which was expected to detrimentally impact *A. tumida* health. The results of this work enhance our current knowledge or *A. tumida* biology and provide a useful basis for development of safe and selective management *A. tumida* management options for the future.

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GENERAL ABSTRACT:

The small hive beetle is an invasive European honey bee pest that poses a significant threat to apiaries in the United States. These beetles feed on hive products and brood, pollute the hive with fermenting waste, and, in severe infestations, cause colonies to abandon their hives. This project investigated previously unexplored control options that take small hive beetle biology into account. Small hive beetles have an exceptional sense of smell compared to other beetles, and this ability helps them to locate honey bee hives. Therefore, behavioral responses to attractants and repellants were tested through olfactometry, in which beetles were given a choice to travel toward or away from specific odors or odor blends. Responses to these odors on a physiological level were also quantified through electrical recordings of beetle antennae. Small hive beetles are also known to have a yeast-like symbiont, which is present throughout the small hive beetle lifecycle, both externally and internally. Feeding bioassasy for small hive beetle larvae, along with soil bioassays for the delivery of insecticides were also developed and used to test several compounds against small hive beetle larvae. Feeding bioassays were also used to deliver a fungicide to larval and adult beetles with the goal of repressing internal fungal activity The results of these studies help expand the knowledge of small hive beetle olfaction and provide a background for the development of novel control options to effectively manage this destructive pest.

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Biology and Management of Small Hive Beetles (Coleoptera: Nitidulidae): A Pest of European Honey Bee (Hymenoptera: Apidae) Colonies

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Abstract

Small hive beetle (*Aethina tumida* Murray) control has become an issue of increasing importance for North American apiculturists throughout the past two decades. *Aethina tumida* was discovered in Florida in 1989, presumably transported from its native habitat of sub-Saharan Africa through the shipment of European honey bee (*Apis mellifera* L) queens. Estimates of damage from *A. tumida* were as high as $3 million annually in the United States by the year 2004, and *A. tumida* was found in nearly every state by 2008. When adult beetles emerge from pupation in soil surrounding the hive, they are attracted to *A. mellifera* hives through a variety of pheromones and volatile organic compounds (VOCs) from bees and hive products. *Aethina tumida* larvae and adults consume hive products and bee brood, generating fermenting waste (or slime), which can eventually lead to hive abandonment in cases of severe infestation. Pest management efforts for *A. tumida* have focused on trapping adults, applying lime, diatomaceous earth, pyrethroid soil drenches, and entomopathogenic nematodes to the soil surrounding *A. mellifera* hives. Understanding the biology and life history of *A. tumida*, along with current control methods, can aid apiculturists in making informed integrated pest management decisions. Additionally, understanding critical knowledge gaps in the current research is an important step in identifying promising future management tactics in the ongoing efforts to manage this invasive pest.

Keywords: (3-5) apiary, invasive, olfactory, behavior, damage

Overview

Small hive beetles (*Aethina tumida* Murray) are an invasive species that have impacted beekeepers in the United States. *Aethina tumida* is a difficult pest to manage, due in part to the fact that the lifecycle of this beetle takes place both within the hive and in the surrounding environment. These beetles have olfactory systems attuned to hive location, and their lifecycle is also specifically adapted to exploit hive resources, all of which results in a highly invasive pest that is difficult to manage. Use of any treatment within the hive also impacts the *A. mellifera* colony by default, so ideal treatments must be both effective in beetle management and safe for bees (Neumann et al., 2016). Years of research and attempted management have led to several widely available management tactics, ranging from mechanical controls to chemical treatments, however, there are many future directions to be explored in the ongoing quest for safe and effective *A. tumida* control options. This profile will explore the biology and life history of *A. tumida*, in addition to detailing current control methods and relevant research information that may impact future *A. tumida* management practices.

Aethina tumida **Lifecycle and Biology**

Adult

Aethina tumida adults (Figure 1) are 5-7 mm long and 3-4.5 mm wide, are reddish brown or black in color, and have short, strong elytra (Neumann et al., 2013). The sex of *A. tumida* adults can easily be determined by gently squeezing their abdomens, which causes the ovipositor to protrude in females and the 8th tergite to extend in males (Neumann et al., 2013). The adult *A. tumida* lifespan is variable and is dependent on environmental conditions and sex of the beetle, with the female-biased

sex ratio suggesting shorter female *A. tumida* lifespans, as the female beetles would need to repopulate at a faster rate (Papach et al., 2019). Adult beetles in the wild are thought to live up to six months (Pettis and Shimanuki, 2000). However, it has been estimated that *A. tumida* adults in the laboratory could live up to 16 months, with female life span dependent upon egg laying frequency (Somerville, 2003; Neumann et al., 2016). It is well known that arthropods can exchange fitness factors such as longevity for increased reproductive output, so it is likely that higher egg numbers can result in shorter *A. tumida* female lifespans (Jervis et al., 2007). *Aethina tumida* adults are also capable of surviving and reproducing on various decaying fruit species; however, pupation success is significantly greater when larvae feed on hive products (Ellis et al., 2002). Unfortunately, bumblebee (*Bombus impatiens* Cresson) colonies can also be alternate hosts for *A. tumida*, with one study revealing that *A. tumida* adults did not display preference between *A. mellifera* and *B. impatiens* colonies and that *A. tumida* females will oviposit in *B. impatiens* colonies (Hoffmann et al., 2008). Infestations of *B. impatiens* colonies, along with other potential *A. tumida* infestations of native pollinators and wild *A. mellifera* colonies, pose a risk to managed colonies, since infestation levels in the environments surrounding apiaries could become high without any intervention by apiarists until *A. tumida* is well established in that geographic range.

Egg

Once *A. tumida* mating occurs, females preferentially oviposit in cracks and crevices of the hive (Neumann and Härtel, 2004) in clusters of 10-30 eggs (Stedman, 2006; Figure 2). Female beetles can also oviposit their eggs on pollen and brood comb

(Cuthbertson et al., 2003), sometimes even chewing holes in capped brood for oviposition (Ellis et al., 2004). *Aethina tumida* sex ratios are significantly female-biased, with 1.58 females for each male (Papach et al., 2019), and females can lay 1,000-2,000 eggs throughout their lifetimes (Sommerville et al., 2003). Consequently, several adult beetles can lead to large larval populations (Somerville, 2003). *Aethina tumida* eggs are a translucent white color, cylindrically shaped, and measure approximately 1.4 mm in length, and 0.26 mm in width (Stedman, 2006). Egg hatching time is dependent on temperature and humidity, with hatching generally thought to take place after three days in the hive environment (Somerville, 2003). In one study, eggs were observed to hatch after two days at temperatures of 34°C, while a three-day egg incubation period was noted at temperatures ranging from 24-28°C (Guzman and Frake, 2007).

Larva

Upon their emergence, *A. tumida* larvae (Figure 3) are approximately 1.3 mm in length, growing swiftly throughout their development to reach approximately 1 cm in length in their last instar (Neumann et al., 2013). *Aethina tumida* larvae are light-yellow in color with two rows of short dorsal spines which run along the length of the larval body, ending in two larger spines on the final dorsal segment (Neumann et al., 2013). *Aethina tumida* larvae can be easily distinguished from other larvae that may be present in the hive, as *A. mellifera* larvae are immobile and stay in the comb cells until capping, while greater wax moth (*Galleria mellonella*, Fabricius) larvae are much larger than *A. tumida* larvae and lesser wax moth (*Achroia grisella*, Fabricius) larvae lack the distinctive dorsal spines of *A. tumida* larvae. As larvae produce waste, this

leads to increased growth of the *A. tumida* yeast symbiont *Kodamaea ohmeri* within the hive, causing fermentation of honeycomb, pollen, and beetle waste, which is sometimes referred to as “slime” (Benda et al., 2008; Hayes et al., 2015). In addition to feeding upon hive products, *A. tumida* larvae will also eat *A. mellifera* eggs (Elzen et al., 1999) and brood (Neumann et al., 2001). *Aethina tumida* larval development is heavily dependent upon temperature, with one study finding that larvae could reach their peak weight in less than 8 days when maintained at 35°C, while this same process of development took 17 days when the temperature was 21°C (Meikle and Patt, 2011). Thus, warm weather and cycles of population increase and foraging in colonies best facilitate *A. tumida* development. Once *A. tumida* larvae reach their third and final instar, which usually takes 10-14 days, *A. tumida* larvae exit the hive and migrate to the surrounding soil to begin pupation, sometimes crawling distances of over 200 meters (Stedman, 2006).

Pupa

The larvae of *A. tumida* burrow into the soil, reaching depths of at least 4-8 cm (Meikle and Diaz, 2012), where they molt into free pupae (Neumann et al., 2013). Pupation time varies greatly depending upon soil temperature (Neumann et al., 2001). Although pupation time would generally be between 15 and 33 days (Meikle and Patt, 2011), it has been observed to last for 100 days in sufficiently cold weather (Stedman 2006). The pupae (Figure 4) start as an opaque white color, with some observable darkening in the eyes and wings as they near emergence (Neumann et al., 2013). After emergence, the adults will stay in the soil to sclerotize (cuticle hardens and darkens in color), and can remain in the soil for up to 35 days after pupating (Muerrle and

Neumann, 2004). It has also been noted that mating can occur in the soil (Neumann et al. 2013).

Fungal Symbiont *Kodamaea ohmeri*

The ascomycetous yeast *Kodamaea ohmeri* from family Saccharomycetaceae has been primarily studied in human medicine since the late 1990s in the context of fungal infections, generally in immunocompromised patients (Al-Sweih et al., 2011; Diallo et al., 2019). *Kodamaea ohmeri* is also closely related to the common yeast *Candida albicans* and the two can be easily confused, although *K. ohmeri* colonies have distinct wrinkled edges, while the edges of *Candida spp.* colonies are smooth (Leeman, 2012; Diallo et al., 2019). *Kodamaea ohmeri* is present throughout each *A. tumida* life stage and is thought to be important in larval nutrient provision (Leeman, 2012). It is suspected that *K. ohmeri* is a key part of *A. tumida* digestion, considering its prevalence in the guts of larvae and adults, on their food sources, and in slime (Amos et al., 2018). Recent sequencing of the *K. ohmeri* genome also revealed that *K. ohmeri* has invertase and hexose transporter genes, which means that *K. ohmeri* may be performing important functions in sucrose metabolism in *A. tumida*, as these invertase genes have not been found in the *A. tumida* genome (Tauber *et al*., 2019). It is also possible that *K. ohmeri* is involved in aiding the reproductive system, as mucilage (an adhesive and protective coating) surrounding *A. tumida* eggs has been shown to contain *K. ohmeri* (Amos *et al*., 2018).

Yeasts have long been associated with beetles, as various species of bark beetle (*Dendroctonus spp.)* are known to carry and vertically transmit yeasts of the family Saccharomycetaceae (Davis, 2015). Though the role of this yeast in the bark

beetle lifecycle is yet to be fully characterized, this relationship is thought to be symbiotic, as yeast-produced volatiles can attract or repel other bark beetles depending upon the volatile concentration, along with providing other chemical cues and enriching nutrient sources in the bark beetle environment (Davis, 2015). *Kodamaea ohmeri* also produces volatiles that are important for *A. tumida* attraction, including compounds found in isopentyl acetate (an *A. mellifera* alarm pheromone) through breakdown and fermentation of hive products, which is instrumental in attracting large numbers of adult beetles to the hive (Torto et al., 2007). Pollen is especially important in creating the volatiles produced by *K. ohmeri*, as only *K. ohmeri* grown on media containing pollen was shown to produce attractive volatiles, such as isopentyl acetate (Benda et al., 2008). Because pollen appears to be a key element in *K. ohmeri* volatile production, it has also been speculated that *K. ohmeri* could be brought into the hive via pollen gathered by workers, although *K. ohmeri* only builds up and overwhelms other hive yeasts in colonies that are infested with *A. tumida*, so it is likely that beetle feeding is mainly responsible for pollen inoculation (Benda et al., 2008).

When hives are healthy, several types of yeast can generally be isolated, but in cases of high *A. tumida* infestation, only *K. ohmeri* can be isolated, which suggests competitive displacement of natural hive yeasts (Benda et al., 2008), which is not unlike the competition that is speculated between bark beetle yeast symbionts and other fungi found in trees that bark beetles inhabit (Davis, 2015). A more recent study has shown that *K. ohmeri* is not detectable in hives when *A. tumida* is absent, suggesting that *A. tumida* feeding, movement, and defecation are necessary for *K.*

ohmeri growth (Amos et al., 2019). Two strains of *K. ohmeri* have been isolated from Kenya and Florida, respectively, and both strains produced attractive volatiles, including the components of *A. mellifera* alarm pheromone (Benda et al., 2008). While *K. ohmeri* has been used to attract *A. tumida* adults to traps, the actual role that *K. ohmeri* plays in *A. tumida* fitness and success is still largely uncharacterized (Leeman, 2012; Torto, 2010). Because *K. ohmeri* is known to cause severe fungal infections in humans (Diallo et al., 2019), it is also important to understand the impacts that potentially tainted hive products could pose to human health (Neumann et al. 2016).

Aethina tumida Olfactory System

Insect olfaction begins when odorant molecules enter pores in the olfactory sensilla of the antennae, are bound by odorant binding proteins, and are transported through lymph to an odorant receptor (Leal, 2005). Odorant receptors have corresponding neurons that pass olfactory information to the antennal lobes, which are integration centers for olfactory information, made up of spheroidal compartments called olfactory glomeruli (Kollman et al., 2016). Local interneurons process information in the antennal lobes, and then pass this information to other neurons, which take the information to mushroom bodies or the lateral horn (Schachtner et al., 2005).

The antennal lobes are known to contain excitatory acetylcholine neurotransmitters and inhibitory gamma-aminobutyric acid (GABA) neurotransmitters, as well as biogenic amines, gaseous signaling molecules, and neuropeptides (Schachtner et al., 2005). Antennal lobe size can vary between species, ranging from approximately 40 (Diptera) to 250 (Hymenoptera) olfactory glomeruli (Schachtner et al., 2005). Although sexual dimorphism has been observed in other insect species

(Koontz and Schneider, 1987)*, A. tumida* antennal lobes were shown to contain approximately 70 olfactory glomeruli in both males and females (Kollmann et al., 2016). *Aethina tumida* adults also have unusually large antennal lobes when compared with other beetles of family Nitidulidae, which provides further confirmation of their strong olfactory senses (Kollmann et al., 2016). Studies vary on the olfactory differences between the sexes, as one study noted that *A. tumida* females display stronger responses to beehive volatiles than males (Suazo et al., 2003), while another study found no significant difference in the olfactory responses of males and females to these VOCs (Bobadoye et al., 2018). As there is no significant structural difference between male and female antennal lobes, this supports the work demonstrating that both sexes have the same olfactory capabilities (Kollman et al., 2016).

Aethina tumida attraction to *A. mellifera* hives is initiated by various volatiles emitted from worker bees, brood, unripe honey, pollen, beeswax, wax byproducts, and alarm pheromone (Suazo et al., 2003). As *A. tumida* larvae feed on hive products, this facilitates the release of volatiles, which can attract more adult beetles to the hive (Torto et al., 2007). *Apis mellifera* alarm pheromone acts as an aggregation kairomone, drawing *A. tumida* adults to the hives, which is crucial, as this is where the beetles mate (Kollman et al., 2016). *Aethina tumida* adults prefer to travel to hives in the evening, mostly before and after dusk (Schmolke, 1974), indicating that olfactory cues are likely more important than visual information when attempting hive location.

Aethina tumida History and Damage

Taxonomy

Aethina tumida was originally discovered by Murray in 1867 as a member of family Nitidulidae, commonly known as sap beetles, which contains approximately 2800 species in 172 genera (Murray, 1867; Neumann and Elzen, 2004). Though generally easily distinguishable from other members of family Nitidulidae, *A. tumida* is sometimes confused with its near relative *Cychramus luteus* Fabricius, which can also be found within *A*. *mellifera* colonies in Europe (Neumann and Ritter, 2004). However, close examination easily elucidates the correct identification of the specimen, as they differ morphologically in many ways (Neumann et al., 2013), with pronounced differences in ovipositor and antenna shape, and the color of the elytra and pronotum (Neumann and Ritter, 2004). Additionally, *C. luteus* has not been shown to reproduce or cause damage within *A. mellifera* colonies, so it is more likely that only hives provide a source of shelter and perhaps insignificant amounts of food (Neumann and Ritter, 2004).

Host Range and Travel Impacts

For many years after its discovery in 1867, *A. tumida* received little attention, as it is not considered to be a significant *A. mellifera* pest in its native range of sub-Saharan Africa (Murray, 1867; Neumann and Elzen, 2004). African *A. mellifera* subspecies are not generally threatened by *A. tumida,* as these beetles only cause significant damage in weak, diseased, or abandoned hives in their native habitat (Ellis and Hepburn, 2006). Some even consider *A. tumida* to be beneficial quality control agents in sub-Saharan Africa (Ellis and Hepburn, 2006). However, *A. tumida* infestations proved a serious threat when observed in Florida in 1998 (Elzen et al., 1999); extensive damage was noted throughout the United States, primarily in southern states, by

2004, with *A. tumida* presence throughout the Midwest and in California by 2008 (Neumann and Ellis, 2008).

Feeding Damage and Behavior

The most significant damages to *A. mellifera* colonies are caused by *A. tumida* larvae as they prey upon *A. mellifera* honey, pollen, and even brood; this larval feeding cycle leads to a buildup of fermenting waste in the hive (Hayes et al., 2015; Figures 5-6). When damage becomes extensive there is a risk that *A. mellifera* colonies might abscond, leaving a mass of *A. tumida* larvae and fermenting waste in their former hives (Hayes et al., 2015).

Adult beetles are attracted to the hive through volatiles (Suazo et al., 2003), and they can detect stressed hives through these olfactory cues at distances of 13-16 km (Wenning, 2001). Digestion of hive products by *A. tumida* increases the release of these volatiles and additional adult beetles can be subsequently attracted to the hive as a result of the initial infestation (Torto et al., 2007). While *A. tumida* aggregation pheromone use is not well-characterized, it is strongly suspected that these pheromones contribute to long-range hive location and *A. tumida* aggregation within hives, which is bolstered by the fact that other beetles of family Nitidulidae are known to emit aggregation pheromones (James et al., 2000; Neumann et al., 2016) *Aethina tumida* adults tend to prefer dark and cooler areas of the hive, such as bottom boards or hive corners, where they will congregate (Cuthbertson et al., 2013; Neumann et al., 2016). *Aethina tumida* adults also tend to hide in cells in the comb, small hive crevices, or hive debris (Cuthbertson et al., 2013), and are also thought to congregate at these sites within the hive, as mating takes place in aggregations (Schmolke, 1974;

Neumann and Elzen, 2004; Neumann et al., 2016). *Aethina tumida* adults are also able to steal heat and resources from clustering bees to successfully overwinter (Hood, 2009).

Apis mellifera workers will often try to attack *A. tumida* adults, corral beetles on the tops of frames with propolis, and remove *A. tumida* eggs and larvae (Neumann et al., 2016). However, these beetles have developed hiding and evasive behaviors to avoid attack, such as dropping, running, hiding, and assuming defense postures such as pulling their legs beneath their bodies in a turtle-like stance (Neumann *et al*. 2001), which helps them avoid attacks by *A. mellifera* workers (Neumann and Elzen, 2004). *Aethina tumida* adults are also known to successfully induce mouth-to-mouth food exchange known as trophallaxis (Neumann *et al*., 2015). Inducing trophallaxis is an innate *A. tumida* behavior during which both male and female beetles engage in a "begging" behavior by touching the mandibles of *A. mellifera* workers with their antennae, with one study revealing a 50% success rate among begging *A. tumida* adults (Neumann et al., 2015). Even when *A. tumida* adults are trapped by *A. mellifera* workers and guarded in propolis cells or corrals, they have been shown to obtain nutrients needed for survival by inducing trophallactic interactions with their guards (Ellis *et al*., 2002).

Although *A. tumida* is of lesser economic importance than *Varroa destructor* (Anderson and Truman) it is estimated to account for three million dollars in damages on an annual basis in the United States (Hood, 2004). The worldwide economic impacts of this pest have also been severe, with *A. tumida* related damages in Queensland, Australia alone amounting to approximately eight million dollars from the

years 2008 to 2011 (Lamb and Leemon, 2011; Hayes et al., 2015). However, an update of the economic impact of *A. tumida* is needed, especially since the geographic range of *A. tumida* has continued to expand since that time (Neumann and Ellis, 2008).

Aethina tumida Management

Treatment options are still being explored, with little progress made since initial *A. tumida* discovery, especially in the area of chemical controls (Hood, 2004; Ellis and Ellis, 2016). Additionally, there is not a well-established economic threshold for *A. tumida*, making treatment decisions difficult. Although one source defined extensive damage as over 1000 adult beetles in a single colony (Ellis and Ellis, 2016), this is not a helpful metric in informing beekeepers regarding when and how to treat, rather it reflects the number of beetles that can be seen when management is not undertaken in time to save the colony from serious damage. Having an economic threshold is a critical aspect of determining which management option to use, especially as integrated pest management (IPM) techniques rely on an established economic threshold for management decisions. Thus, treatment for *A. tumida* tends to be somewhat arbitrary, based mainly on apiculturist observations and preferences; however, the methods detailed below include some of the more commonly used *A. tumida* management techniques.

Sampling

Aethina tumida detection is frequently accomplished by visually searching for *A. tumida* adults for *A. tumida* larvae and adults and/or trapping for adult beetles. Adult beetles can also be collected easily by hand or through an aspirator (Neumann *et al*., 2013). One study also made use of corrugated plastic strips in bottom boards, which

were attractive to *A. tumida* adults and demonstrated significant correlations between the number of beetles trapped and the overall infestation level in the hive (Schäfer *et al*., 2008). Additional detection methods include digging in surrounding soil for *A. tumida* pupae or performing polymerase chain reaction (PCR) on hive debris samples to determine whether or not beetle-specific genes are present (Ward *et al*., 2007).

Cultural Control

Cultural and biological controls have been further explored since the discovery of *A. tumida*, and these methods have received more attention than chemical controls (Ellis and Ellis, 2016). *Aethina tumida* cultural controls include the removal of excess hive products, reduction of hive humidity, and placement of traps, diatomaceous earth, and/or slaked lime around the hives (Cuthbertson *et al*., 2013; Ellis and Ellis, 2016). Diatomaceous earth and slaked lime work in similar ways, and both approaches have high pH levels and are hygroscopic (Buchholz *et al*., 2009), which can kill *A. tumida* larvae and adults and prevent pupation (Cuthbertson *et al*., 2013). Diatomaceous earth tested in traps killed 100% of beetles tested through dehydration (compared to 8.6% mortality in the control treatment), although cuticles were not abraded, which suggested that the diatomaceous earth absorbed the water directly from cuticular hydrocarbons (Cribb *et al*., 2013), essentially destroying the wax layer and binding to lipids (Buchholz *et al*., 2009). It is also speculated that diatomaceous earth that clings to *A. tumida* antennae and sensilla may affect olfactory albitites and orientation (Cribb *et al*., 2013). Slaked lime is thought to work similarly, and it was noted in one set of experiments that high level of slaked lime (15%) prevented pupation, with 0% pupation success, while 99±3.2% of control larvae successfully emerged from pupation over

the same 16 day time period (Buchholz *et al*., 2009). This treatment is thought to reduce pupation success through absorption of moisture from the soil (Buchholz *et al*., 2009). In the same study, it was also noted that low levels of slaked lime in soil that was not autoclaved also resulted in high *A. tumida* larval mortality, suggesting that the disruption of soil pH levels may lead to mortality through increased susceptibility to soil-borne pathogens (Buchholz *et al*., 2009).

Additionally, general practices of maintaining proper apiary sanitation, minimizing colony stressors, managing other pests and diseases, removal of excess honey, and ensuring that beetles do not have access to stored hive products can help minimize beetle presence, bolster colony defenses, and ensure that beetles are detected quickly (Hood, 2004; Ellis and Ellis, 2016). It is also important that any unnecessary equipment that creates extra cracks and crevices within the hive be reduced to eliminate desirable areas for *A. tumida* adults to hide and oviposit (Neumann and Härtel, 2004). Eliminating unnecessary hive equipment also helps reduce the area that must be patrolled by *A. mellifera* workers, and this practice allows the colony to better defend *A. tumida* entrance points and contain adult beetles that have already entered the hive (Hood, 2004). Pollen patties and sugar water feeders can also provide excellent food sources and harborages for *A. tumida*, so if supplementary feeding of the colony is necessary, close monitoring is essential (Hood, 2004). Finally, being mindful of the amount of time spent during hive inspections is important, as prolonging this time can stress colonies and lead to the release of *A. mellifera* alarm pheromone, which is known to attract *A. tumida* adults (Kollman et al., 2016).

Mechanical Control

The most popular mechanical control method available for *A. tumida* management is trapping, primarily through use of in-hive traps targeting adult beetles. The first *A. tumida* trap that was commercially available in the United States was a bottom board trap called the West Beetle Trap (Hood, 2011), followed by the Freeman Trap, which allowed for removal of the catching tray from the back of the hive (Hood and Tate, 2010). The design of this original bottom board trap has been modified in various ways, such as inclusion of cider vinegar as an attractant source (Hood 2011). However, the basic structure of bottom board traps comprises a removable tray filled with viscous liquid, such as vegetable oil, beneath a cover with slots or screening that is too small for bees but allows beetles to enter and become caught in the liquid (Hood, 2006). Bottom board traps can have the advantage of catching other small hive pests and parasites that might enter hive bottoms or fall from their hosts. Recent research has also demonstrated that cardboard bottom board traps are significantly more effective than cardboard traps placed on the top of frames in the hive (Muturi *et al*., 2022). Disadvantages of bottom board traps include the potential trapping of bees in the viscous liquids, and this type of trap can be difficult to maintain (Hood, 2011). There are also entrance traps, such as the pitfall traps made by Beetle Jail™, which are placed outside the entrance of hives to catch entering adult beetles in a viscous liquid as they enter the hive (Hood 2011). Various pitfall traps can also be inserted between frames in the hive, as seen in the use of popular commercially available traps such as the Better Beetle Blaster™, AJ's Beetle Eater™, and Beetle Jail Jr. Traps, which are filled with a viscous liquid to trap adult beetles (Hood, 2011; Zawislak, 2014).

Fabric entanglement traps, such as Beetle Bee-Gone are also popular (Roth *et al*., 2019), with numerous home-made versions, including kitchen wipes or other materials (Chauzat *et al*., 2016). Unfortunately, entanglement traps can have the disadvantage of snagging bees in addition to the targeted adult beetles (Roth *et al*., 2019). Finally, the Beetle Barn is another popular commercially available plastic trap that is placed on top of frames, with small entrances that only allow beetle entry (Bernier *et al*., 2015).

Numerous custom traps for *A. tumida* have also been designed and used in experiments. Bottom board traps made of corrugated cardboard or corrugated plastic sheets attached to synthetic acaricide CheckMite+ strips™ (10% w/w coumaphos) have also been used in *A. tumida* adult trapping with success, although the primary function of these traps was in estimating the severity of *A. tumida* infestations (Neumann and Hoffman, 2008). Experiments involving light traps have also yielded promising results in the capture of *A. tumida* adults and larvae post-feeding and prior to pupation, although light attraction did not outperform traps baited with pollen dough in open settings, so addition of light attraction to baited traps in enclosed areas would be a more likely future application of this method (Duehl *et al*., 2012). Although there are other custom traps that have been used in various studies, including bucket traps (Elzen *et al*., 1999), bottom bar box traps (Hood and Miller, 2003), and baited flight traps (Arbogast *et al*., 2007), the general trap types detailed above, including the pitfall, entanglement, and bottom board traps, are those frequently used by apiarists and available commercially in various forms.

Attractants

As the olfactory abilities of *A. tumida* adults are well established (Kollman *et al*., 2016), attractant compounds are often used in conjunction with mechanical controls to promote trapping success. Over the years, popular baits for *A. tumida* traps included cider vinegar (Nolan and Hood, 2008) and pollen dough, which is a commercially available mixture of pollen, carbohydrate, and protein sources, with two popular brands including Global Patties® (Arbogast *et al*., 2010) and Bee Pro® (Stuhl 2017). Additionally, this pollen dough can be impregnated with the *A. tumida* fungal symbiont *K. ohmeri* for increased attractiveness (Torto *et al*., 2007). *Apis mellifera* adults and brood have also been used in traps in the past to increase attractancy (Hood 2000). More recently, an *A. tumida* attract-and-kill bait, which included attractive compounds derived from *K. ohmeri* spiked pollen patty added into a beetle diet spiked with 2% boric acid, demonstrated high *A. tumida* mortality (Stuhl, 2020). It has also been shown that both natural and synthetic blends of fruit volatiles are attractive to *A. tumida* adults and offer a promising option for baited traps in the future (Stuhl, 2021). Finally, an attractant (patent filed in 2017) that imitates *A. tumida* aggregation pheromone containing a mixture of 6-methyl-5 hepten-2-one, nonanal, and decanal, was developed (Stuhl and Teal, 2017), which offers a promising bating option in future trapping efforts.

Biological Control

The pupation of larval *A. tumida* in the soil outside of *A. mellifera* hives has led to control tactics targeting this life stage. These methods include the placement of slaked lime and diatomaceous earth around hives to kill larvae and limit soil access, and the release of the entomopathogenic nematodes *Steinernema riobrave* and

Heterorhabditis indica, which have been found to reduce *A. tumida* populations (Cuthbertson *et al*., 2013; Ellis *et al*., 2010; Meikle and Diaz, 2012). The release of sterilized *A. tumida* adults for potential reduction of population dispersal is another potential future management method that is being developed (Downey *et al*., 2015). Finally, various strains of the bacteria *Bacillus thuriengiensis* (*Bt*) have been effective in controlling other coleopteran pests, as ingestion of *Bt* results in production of lethal crystalline proteins, which leads to insect death (Jisha *et al*., 2013). Although three strains of *Bt* have been tested against *A. tumida* larvae, they did not cause significant larval mortality when compared to the larvae exposed to negative and positive controls of water and coumaphos, respectively (Buchholz *et al*., 2006). While *Bt* has been most efficacious among lepidopterans and dipterans, there are *Bt* strains that have been successful in control of other beetle species, which may prove to be effective in *A. tumida* control (Naimov *et al*., 2001), along with development of new *Bt* strains, which could be more species specific (Buchholz *et al*., 2006). While *Bt* resistance has been observed in other species (Tabashnik, 1994; Loseva *et al*., 2002; Pauchet *et al*., 2016), the lack of prior *Bt* use against *A. tumida* indicates that *Bt* resistance would not be present (initially) in *A. tumida* populations, thus *Bt* use could be a viable management option in the future (Buchholz *et al*., 2006).

Biotechnology

The use of RNA interference (RNAi) to manage *Aethina tumida* has received some attention over the past three years. Although this method has not been applied in the field, there have been some promising laboratory results (Powell *et al*., 2017). RNAi works by silencing target genes through the injection, soaking, or feeding of

double stranded RNA (dsRNA), which can result in mortality of the pest (Joga *et al*., 2016). RNAi has also been successfully used against other coleopteran pests (Miller *et al*., 2012) and is more environmentally friendly control option, due to its species specificity (Joga *et al*., 2016), when compared to conventional synthetic insecticides. One study noted that 25 of the 47 previously discovered RNAi-related genes were present in the *A. tumida* transcriptome, indicating that RNAi would be a viable management method (Kim et al., 2018). Additionally, 100% larval mortality was noted in an RNAi injection study, although feeding only resulted in 50% larval mortality, indicating the need for a better delivery system prior to use in the field (Powell *et al*., 2017).

Chemical Control

Over the years, various insecticides have been tested for use in *A. tumida* management. The most common chemical treatment is coumaphos strips, which are also frequently used for *V. destructor* management, although bees can also be detrimentally affected by this compound (Cuthbertson *et al*., 2013; Kanga and Somorin, 2012). Soil drenching with permethrin (GardStar) is also labeled for use in the United States, but this method is not thought to be highly effective due to treatment timing complications (Hood, 2000; Kanga and Somorin, 2012).

Concluding Remarks

Although *A. tumida* has been present in the United States for the past 20 years, there is still plenty of progress to be made in *A. tumida* management. The *A. tumida* lifecycle is uniquely adapted to the hive environment and the adult beetle olfactory system, combined with the effects of the symbiotic fungi *K. ohmeri*, make *A. tumida* a

formidable pest (Hayes et al. 2015; Amos et al., 2018). Although methods for *A. tumida* management have been developed, there are significant challenges with each of these methods. Chemical treatments can have detrimental effects on *A. mellifera* colonies, and, though trapping methods for adult beetles can be useful, some adults can still avoid the traps and reproduce (Cuthbertson et al., 2013). Targeting the pupation stage of the *A. tumida* lifecycle also has its drawbacks, as damage to the hives has already occurred by the time *A. tumida* reach their pupal stage. The lack of a well-established economic threshold also makes treatment choices somewhat subjective and makes it difficult for beekeepers to know how severe an infestation is, when to treat, and what treatment to use. However, management methods continue to be studied and developed, with promising strides being made regarding biological control methods. Further research should be devoted to the use of *Bt* and RNAi to manage *A. tumida* in the future, as these methods are species-specific and could be highly efficacious if optimized (Buchholz et al., 2006; Powell et al., 2017). Another underutilized, yet important option for management, is the use of attractant and repellent compounds to attract *A. tumida* adults to baited traps and/or deter these beetles from discovering and invading apiaries (Stuhl and Teal, 2017; Stuhl 2020). While vigilance on the part of beekeepers, knowledge of past *A. tumida* infestations in their geographic area, and swift action upon detection of *A. tumida* is important for colony health and survival, there is hope that viable, sustainable, and species-specific management methods for *A. tumida* will emerge in the future.

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Figures

Figure 1. *Aethina tumida* adult (photo by Morgan Roth, from Roth et al., 2019).

Figure 2. *Aethina tumida* eggs (photo by Morgan Roth, from Roth et al., 2019).

Figure 3. *Aethina tumida* larva (photo by Morgan Roth, from Roth et al., 2019).

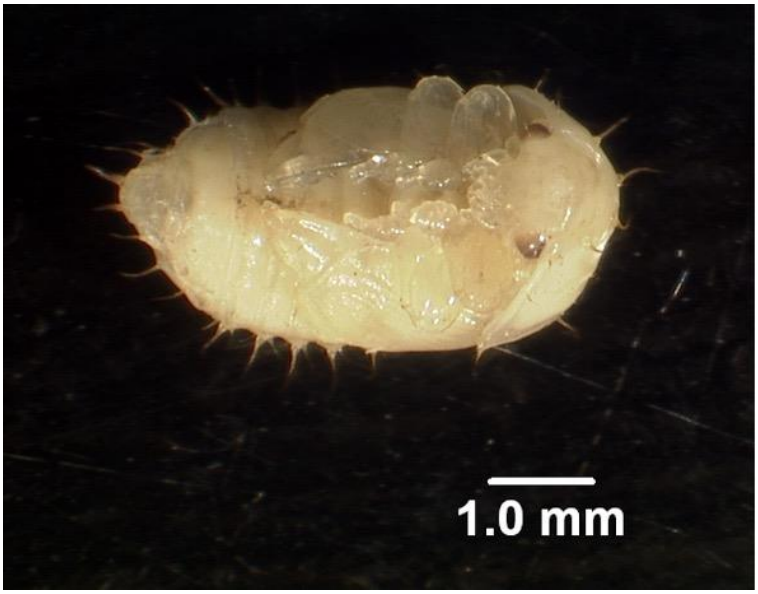

Figure 4. *Aethina tumida* pupa (photo by Morgan Roth, from Roth et al., 2019).

Figure 5. *Apis mellifera* comb infested with *A. tumida* larvae and displaying an advanced stage of larval feeding damage (left) and a comb displaying a layer of slime due to larval feeding (right).

Figure 6. *Apis mellifera* hive bottom board from heavily infested colony covered in slime, dead *A. mellifera* adults, and *A. tumida* larvae (left) and an enlarged view of dead *A. mellifera* adults covered in slime on the hive bottom board (right).

CHAPTER 2.

USING OLFACTOMETRY TO UNDERSTAND *AETHINA TUMIDA* BEHAVIORAL RESPONSES TO ATTRACTANT AND REPELLENT MOLECULES

2.1 ABSTRACT

Small hive beetle (*Aethina tumida*) management has been highly dependent upon chemical and mechanical control over the past two decades; however, many of these methods have not been consistently effective or safe for European honey bee (*Apis mellifera*) colonies. This chapter explores the behavioral effects of the attractant properties of isopentyl acetate and pollen patty upon *A. tumida* adults, and also investigates mixes of these attractants with several repellent compounds, which were previously untested against *A. tumida*. A walking-response olfactometer, designed specifically for *A. tumida*, was used for all experiments, and it was found that both pollen patty and isopentyl acetate were attractive to *A. tumida* adults, though pollen patty at 9 and 12 grams were the only weights that demonstrated significant attraction. Conversely, mixes of attractants and repellent volatiles led to less attraction or avoidance of what was previously a significantly attractive source. Of all repellents tested, pyrrolidine was found to be the most repellent, with significant avoidance of the attractive source at a 10 mg treatment of pyrrolidine. The results of this study indicate that, at the behavioral level, the repellent compounds pyrrole, pyrrolidine, 1,4-dimethylpiperazine, and piperadine are all active in deterring *A. tumida* adults from moving toward an attractive source. These results are promising, as such repellents could be used in the future in apiary settings in order to deter *A. tumida* adults from detecting *A. mellifera* colonies and entering apiaries.

2.2 INTRODUCTION

From the time of initial *A. tumida* discovery in the United States in 1998 (Elzen *et al.*, 1999), attempts at providing solutions for *A. tumida* infestations have varied widely, from cultural controls to insecticidal treatments (Cuthbertson *et al*., 2013). Despite these efforts, a consistently effective and safe management strategy is still lacking (Ellis & Ellis 2016). An underutilized aspect of *A. tumida* biology in regard to management strategy development is this beetle's highly developed olfactory system, which is key in hive location and subsequent infestation (Kollmann *et al*., 2016). To date, few studies have been focused on the *A. tumida* olfactory system, with the exception of a study that assessed the biopesticide Apicure®, which was formulated using the essential oil derived from camphor basil and was found to be repellent to *A. tumida* (Komen *et al*., 2019). The majority of *A. tumida* olfactory testing has been focused on attractive volatiles, such as hive odors (Suazo *et al*., 2003), pollen dough inoculated with the *A. tumida* symbiotic fungi *Kodamaea ohmeri* (Torto *et al*., 2007a), isopentyl acetate (Torto *et al*., 2007b; Figure 2.1a), and various fruit volatiles (Stuhl, 2021). The use of an effective repellent compound could potentially mask attractive sources, such as apiaries, and keep small hive beetles from sensing and responding to hive volatiles and thereby invading and damaging apiaries.

The story of insect repellents largely begins in the 1940s, culminating in the release of DEET (*N*, *N*-diethyltoluamide) for widespread use in the 1950s, after which DEET quickly became the "Gold standard" for insect repellency (Paluch *et al*., 2010; Leal, 2014; Figure 2.1b). Although DEET is still in use, the frequent use of this chemical for many years has resulted in widespread presence of DEET in the

environment, particularly in aquatic systems (Costanzo *et al*., 2007). There is also debate centered around the effects of DEET on human health, with some attesting to serious deleterious effects (Briassoulis *et al*., 2001) and others asserting that risks to human health are minimal (Swale and Bloomquist, 2019). In the 1970s, testing of other chemistries for repellency, including heterocyclic amines (HCAs), began, yielding promising results in the repellence of cockroaches (McGovern *et al*., 1974) and mosquitoes (McGovern *et al*., 1978). Piperidin, which was isolated from black pepper in the 1950s, was the compound that first piqued interest in the repellent abilities of HCAs (Warnhoff 1998). As a continuance of this research, the HCA Picaridin (butan-2-yl 2-(2-hydroxyethyl)piperidine-1-carboxylate), an analog of piperidine, was developed by Bayer in the 1990s (Boeckh *et al*., 1996; Figure 2.1C).

Picaridin was registered for use in the United States in 2001 (US EPA, 2005) and is now used as a mosquito repellent in at least 50 countries (Frances *et al*., 2004). Picaridin analogs are also known to effectively repel chigger mites (Klun *et al*., 2001), and lone star ticks (Solberg *et al*., 1995). The exact modes of action for DEET and Picaridin are unknown, although it is suspected that they are so offensive to the insect olfactory system that they deter host seeking and feeding (Diaz *et al*., 2016). In mosquito studies, it has been hypothesized that HCAs deter feeding through reducing or eliminating the ability of the antennae to detect odors (Klun and Schmidt, 2003); however, studies have demonstrated that mosquito antennae do respond to HCAs (Yang *et al*., 2018). While more work is needed to understand exactly how these compounds affect the insect olfactory system, the low toxicity and high efficacy of

Picaridin (Katz *et al*., 2008) suggests that the testing of other HCAs could lead to the discovery of even more effective repellent options in the future.

In this study, the HCAs chosen for testing against *A. tumida* adults were inspired by work performed by Larson *et al*., 2021, in which parent compounds piperidine, piperazine, pyrrole, and pyrrolidine, along with DEET, were tested for repellence against *A. mellifera* adults, with the goal of discovering repellent compounds that could deter *A. mellifera* adults from feeding on insecticide treated crops (Larson *et al*., 2021; Figure 2.1d-g). The long-term objective of this study was to both examine the behavioral effects of previously untested repellents against *A. tumida* adults and to discover the efficacies of these HCAs in deterring beetles from moving toward an attractive source. To that end, dual choice olfactometers were used to test the effects of these repellent compounds on *A. tumida* behavior.

Olfactometry is a well-established method of testing insect behavior that was developed almost 100 years ago by McIndoo (1926), who designed an improved Y-tube olfactometer based upon limited work published by Barrows in 1907. The early insect olfactometer design was Y-shaped, with insects released in the stem of the Y-tube and allowed to choose between a control arm and a test arm containing either an attractant or repellent odor, with a light used to encourage insects to move toward the Y-tube arms (McIndoo, 1926). Many types of olfactometers have been developed over the years, sometimes comprising as many as eight test chambers (Khoobdel *et al*., 2021). Gas Chromatography-Mass Spectrometry (GC-MS) analysis can also be paired with these systems in order to determine the exact chemistry of volatiles being pulled through the system (Turlings *et al*., 2004). Although olfactometry studies have

been performed using a diverse array of species, these tests are most frequently performed using urban pests, including mosquitoes (Posey *et al*., 1998; Omrani *et al*, 2010; Eilerts *et al*., 2018), cockroaches (Sakuma and Fukumi, 1985; Khoobdel *et al*., 2021), and bed bugs (Siljander *et al*., 2008; Gries *et al*., 2014; DeVries *et al*., 2019).

Olfactometer studies have been performed using *A. tumida* adults for almost 20 years, beginning with a four-choice olfactometer that tested *A. tumida* responses to various volatiles produced in hives and ultimately demonstrating that *A. tumida* adults were significantly attracted to volatiles from *A. mellifera* adults, including the key alarm pheromone componenet isopentyl acetate, in addition to unripe honey, fresh pollen, and the 'slumgum' generated from *A. tumida* larval waste (Suazo *et al*., 2003). A similar four-choice olfactometer was also used in a later study, confirming that *A. tumida* adults were significantly attracted to volatiles from hive products found in both *A. mellifera* and bumble bee (*Bombus impatiens*) colonies, with the exception being that *A. tumida* adults were not significantly attracted to the honey from *B. impatiens* colonies (Graham *et al*., 2011). A four-choice olfactometer was also used to confirm that *A. tumida* adults are attracted to synthetic fruit volaties (Stuhl, 2021), which is not surprising, considering that beetles of family Nitidulidae are known to be attracted to ripe or rotting fruit (Parsons, 1943; Arbogast and Thorne, 1997).

Several dual-choice olfactometers have also been designed to test *A. tumida* responses to attractant volatiles. Y-tube olfactometers were used to confirm the increased attractiveness of pollen dough when fermented through larval feeding and *K. ohmeri* inoculation (Dekebo *et al*., 2017). A series of dual choice olfactometer experiments further confirmed that *A. tumida* adults were significantly attracted to hive

volatiles and also revealed significant attraction to pollen dough inoculated with *K. ohmeri* (Torto *et al*., 2010). Anther more recent dual choice olfactometer was made specifically for *A. tumida* adults by Bobadoye *et al*. (2018), which led to the discovery that *A. tumida* adults are attracted to volatiles from several species of meloponine bee species; this is the olfactometer that inspired the design of the olfactometer used in this study (Figure 2.2). By testing both attractant compounds and mixes of HCAs and attractant compounds, this study both adds to the knowledge base of *A. tumida* behavioral responses and is a first step towards determining an optimal repellent that can be used to deter *A. tumida* adults from highly attractive *A. mellifera* colonies.

2.3 MATERIALS AND METHODS

2.3.1 Insects

All *Aethina tumida* adults used in this study came from a laboratory colony started in August of 2018. Adult beetles were collected from the Prices Fork Aiary in Blacksburg, VA, USA, and the colony currently contains F10 to F15 adult beetles and F11 to F16 larvae. Adult beetles were housed in a ventilated plastic container or glass jar and were provided with pollen patty and paper towel. Once third instar larvae are observed, they are removed from jars and subsequently transferred to either pupation conicals (multiple larvae) or pupation tubes (single larvae). Pupation conicals are made by adding approximately 30 third instar larvae to a 50 mL conical containing a moistened 1:1 sand/soil and covered in parafilm with small ventilation holes. As larvae tend to burrow down the sides of pupation conicals, it is easy to visualize at least some of the pupae to monitor adult emergence. Once visible pupae emerge as adults, the

conicals are left for several days to allow the cuticles of the newly emerged adults to sclerotize, then then adults are added to an adult rearing container. The colony is kept in a dark environmental chamber (Thermo Scientific, Waltham, MA, USA) maintained at 28°C and greater than 70% relative humidity. A specimen from the laboratory colony has been added to the Virginia Tech collection (Catalogue Number: VTEC000004965).

The *A. tumida* adults used specifically for study experiments were allowed to pupate individually in 2 mL Eppendorf tubes. These pupation tubes were prepared by adding a single larva to the tube filled with a moistened 1:1 sand/soil mix, each tube contained a a single ventilation hole in the tube's lid. As soon as adult emergence was observed, pupation tubes were emptied, and newly emerged adults were sexed and divided into separate male and female containers and were allowed to feed on pollen patty. *Aethina tumida* sex was ascertained by gently squeezing the beetle abdomen and observing the protrusion of either the ovipositor in females or the 8th tergite in males (Neumann et al., 2001). All *A. tumida* adults used in this study were not allowed to mate and were only used between 14-21 days post-emergence, which mimics the time at which they are sexually mature and ready to seek a mate in the wild (Mustafa, 2015). Similarly, all experiments were carried out in the late afternoon (4-8 PM), which is the time of day in which wild *A. tumida* adults would be most likely to engage in hive seeking behavior (Bobadoye *et al*., 2018). All olfactometry experiments were also carried out under red light to ensure that visual stimuli would not impact treatment choice, as insects are generally unable to perceive light at this wavelength (Briscoe

and Chittka, 2001). Finally, all beetles were starved for 24 hours prior to testing to increase the likelihood that they would be actively seeking out attractive sources.

2.3.2 Chemicals

Paraffin oil (analytical grade), isopentyl acetate (98%), pyrrole (98%), pyrrolidine (≥99.0%), piperidine (≥99.5%), and *N*, *N*-Diethyl-3-methylbenzamide (DEET; analytical grade) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Sec-butyl 2-(2-hydroxyethyl) piperidine-1-carboxylate (Picaridin; 98%) was purchased from AmBeed (Arlington Heights, IL, USA). The acetone (HPLC grade) used for dilution of all repellents used in this study (Figure 2.1) was from Fisher Scientific (Hampton, NH, USA). AP23® (Artificial Pollen) patties were purchased from Dadant and Sons Inc. (Hamilton, IL, USA) and was warmed prior to use in an incubator set at 28°C.

2.3.3 Olfactometer Construction

All olfactometers used in this study were made specifically for *A. tumida* adults using modified methods of Bobadoye *et al*. 2018 (Figure 2.2) and were constructed. First, a Dremel was used to make two equidistant holes on the sides of one 12-cm Petri dish and two 6-cm Petri dishes (lids were including during this step to ensure a tighter fit during later olfactometer use). A single hole was made in the bottom of the 12-cm Petri Dish. Next, two 1-cm long portals were made from the top portion of a 1,000uL pipette tip and were hot glued to both holes in the sides of the 12-cm Petrie dish and one of the holes in each 6-cm Petrie dish. Female luers (RSN LLC., 2mm PP hose barb adaptor) were then hot glued to the remaining holes in the 6-cm Petrie dishes

and to the bottom hole in the 12-cm Petrie dish. The top of the female luer affixed to the hole in the center Petrie dish was removed via dremel so that the center arena was not obstructed.

Odor delivery chambers were constructed using 20-mL glass scintillation vials with two holes drilled into the tops and fitted with female luers (RSN LLC., 2mm PP hose barb adaptor). Hot glue was added to ensure that the luers fit tightly and were sealed off from the external environment. Additionally, two charcoal filters were constructed using 15-mL conicals, which had their bases removed via Dremel to create a plastic tube. Holes were drilled into two 15-mL conical lids using a Dremel and male luers were glued to the lids to create ends for the tube. A small piece of mesh was fitted to the inside of the conical tube and one tube was screwed into the top of the conical tube and the rest of the tube was filled with API® Activated Filter Carbon pellets (Mars Fishcare North America, Inc.). The other conical lid was then fitted with mesh and hot glued to the former base of the conical, creating a charcoal filter with fittings to attach two lengths of silicon tubing with 1/8” inner diameter and 1/4” outer diameter silicon tubing (Hardness 50A).

To complete the olfactometer system, silicon tubing was connected to both ends of the 6-cm petri dishes, which converged at a Y-fitting and became one tube leading from the charcoal filter to an aquarium air pump (Danner Manufacturing Inc., Supreme OXY-FLO, Model AP-4) used on its highest setting of 275 cu. in./min. Air was carried out of the system through another piece of plastic tubing, which attached to the fitting at the bottom of the 12 cm petri dish and ran to anther charcoal filter and finally to a vacuum pump (Homespirit, 110-120V, 60 HZ, 2.8W). After the olfactometer

was constructed, steam was added to one of the scintillation vials and was observed to flow through the system and into the center Petrie dish in a matter of seconds, confirming that there was good airflow and a system that would allow for efficient odor delivery.

A platform for the olfactometer was made using a 1.3 mm thick, 8 in. by 10 in., non-glare plastic sheet (Plaskolite, Columbus OH, USA). Approximately two inches of this sheet were trimmed off one side and broken into four pieces, which were hot glued to the base to create the olfactometer platform. A single hole was made in the center of the platform to allow the tube connected to vacuum to exit the system. Tape was used to secure this platform to the base of the olfactometer enclosure. The entire olfactometer setup was placed in an enclosure constructed of a wood frame covered in black plastic sheets to keep out ambient light. The base of the enclosure contained internal lighting, which was set on red light for the duration of the experiments. A Basler acA 1300-60 gm camera (Exton, PA, USA) was mounted to the wooden enclosure approximately 18 in. above the olfactometer test area and was used to record all olfactometer experiments.

2.2.4 Olfactometer Methods

After olfactometer assembly, EthoVision XT software (Noldus, Leesburg, VA, USA) was used to record and track all olfactometry experiments. Firstly, an initial image was obtained and the three arenas, along with their diameters, were delineated and measured. The center arena was always labelled “Arena 1”, with the left and right arenas designated as arenas 2 and 3, respectively. Detection settings used included dynamic subtraction with dark images (i.e., the beetles) to be contrasted against a

lighter background (i.e., the olfactometer). The sample rate of the video recording was set at the maximum of 25.000 samples per second and the subject size was set at a minimum of five pixels. Sampling intervals were set at 10 minutes each and recordings only took place during this time. A Microsoft excel random number generator was used to randomize treatments, with odd and even numbers representing the left and right sides, respectively. Parafilm was also used to seal the olfactometer chambers to prevent beetle escape and maintain better airflow within the system. Once a beetle was added to the center Petrie dish of the olfactometer, a final piece of parafilm was used to seal the center chamber and the recording was started.

Prior to testing with any attractants or repellents, 10 mL of paraffin oil were tested on both sides of the olfactometer in order to ensure that there was no directional preference. The first attractant compound tested was isopentyl acetate (IPA), which was diluted in 10 mL of paraffin oil, with a paraffin oil control of the same volume. Next, commercial pollen patty, the food source used for the laboratory *A. tumida* colony, was tested, starting at a volume of 12 g, which filled approximately half of the scintillation vail; controls for the pollen patty treatments were empty scintillation vials (i.e., “filtered air”). All beetles were tested individually, with a single beetle comprising one replicate and a minimum of 10 replicates per treatment. All beetles were frozen for 24 hours after use in this study prior to disposal. Different test compounds were not used on the same day, new olfactometer bases were constructed, and tubes were replaced for each new test compound in order to minimize pollution of the system by multiple volatiles.

2.3.5 Olfactometer Analysis

Once olfactometer choice results were compiled, preference index was calculated using Equation 1. In some replicates beetles chose to remain in the center for the entire 10-minute test interval, so these replicates were not included in the final analysis. Preference index values can range between -1(avoidance) and +1(attraction), with a value of 0 indicating no preference or random chance.

Equation 1: $\mathbf{P} = (\boldsymbol{\alpha} - \boldsymbol{\beta})/(\boldsymbol{\alpha} + \boldsymbol{\beta})$

Where $\mathbf{P}$ = Preference Index (no units),

$\boldsymbol{\alpha}$ = number of insects in test arm, and

$\boldsymbol{\beta}$ = number of insects in control arm.

After preference index was calculated, standard error was calculated using Equation 2 below.

Equation 2: Standard Error = $\sqrt{[\mathbf{P} * \frac{(\mathbf{1}-\mathbf{P})}{\boldsymbol{n}}]}$

Where $\boldsymbol{n}$ = number of insects in used in experiment.

Finally, an exact binomial test was used to determine whether or not repellence or attractance was significantly different than expected, with 50% probability expected and a *p*-value of 0.05 or less indicating significance. Because there were numerous reps in which beetles either entered an arena and went back to the center arena or chose both arenas throughout the 10-minute test period, the percentage of arena changes for each treatment were also compared. Finally, the time it took for beetles to make their first choice (treatment or control), and the time beetles spent in each olfactometer chamber (not including time spent in center), were recorded, quantified, and analyzed using an ANOVA followed by Tukey's multiple comparisons test.

2.4 RESULTS

2.4.1 Initial olfactometer tests and attractant volatile responses

In the initial tests with paraffin oil pumped into both arenas, beetles stayed in the center arena 47% of the time, chose the left arena 29% of the time, and chose the right arena 24% of the time; a chi-square test did not result in significant preference between either side (p=0.7389). Because there was no attractant involved, it appeared that beetles preferred to simply circle the center arena, eventually coming to rest, often on the top and sides of the arena. In these initial tests, selection of the left or right arena appeared to be happening somewhat by chance. However, when attractant volatiles were added to the system, the story changed; seeking behaviors, such as concentric circling, were observed. Presumably, the beetles performed this behavior in an effort to orient themselves in the direction of the odor plume source, which is a known odor seeking technique in insects (Cardé, 1996). Throughout this study, beetles would also frequently stop near the center of the Petrie dish and were observed to move their antennae for several seconds prior to circling towards a test odor arena.

The first odorant tested was the known *A. tumida* attractant isopentyl acetate, which is a key component found in *A. mellifera* alarm pheromone (Torto *et al*., 2007). Testing of isopentyl acetate at 6ppm, a decision informed by a preliminary antennal EC_{50} value obtained through electroantennography (see Chapter 3), revealed a preference index (PI) value of 0.03 ($\pm$0.03). Because the banana-like smell of IPA was quite pungent at this concentration, even outside of the olfactometer, it was suspected that the odor was overwhelming the system and making it difficult for beetles to orient themselves to the odor source. Therefore, the concentration of IPA was reduced

tenfold to 0.6-ppm, yielding a PI value of 1.0 (±0.07), which demonstrated slight attraction. Additionally, IPA was tested at 0.3ppm (n=10), however, two beetles did not make a choice, and the remaining beetle choices yielded a PI value of -0.25 (±0.20), indicating that this concentration was also likely too high, resulting in the inability of beetles to correctly perceive the odor source (Figure 2.2A).

The second attractant compound tested was commercial pollen patty, which was first tested at a volume of 12 grams, which was the volume that half-filled the 20 mL scintillation vial used for odor delivery (empty scintillation vial for control). After preliminary pollen patty tests, it was discovered that pollen patty temperature impacted volatility, after which pollen patty was kept in an incubator at 28°C prior to use. At 12 g, pollen patty was demonstrated to be significantly attractive to *A. tumida* adults (PI=0.30±0.07; *P*=0.004), after which lower weights of pollen patty were tested to determine whether or not beetle responses were weight dependent. It was found that PI values decreased with decreasing pollen patty volume, with significant attraction still observed at 9 g (PI=0.29±0.06; *P*=0.02), followed by slight attraction at 3 grams (0.16±0.08), and 1 g (0.5±1.2; Figure 2.3B). Oddly, there was slight deterrence observed at 6 g of pollen patty (PI= -0.05±0.04), which may have been due in part to the cold season during which the majority of these replicates were performed, as there was poor temperature regulation in the laboratory space in which these experiments took place. Because volatiles emitted from 12 g of pollen patty demonstrated the highest level of beetle attraction, this treatment was chosen for combination with the potentially repellent volatiles selected for use in this study.

2.4.2 Mixed attractant and repellent volatiles

For all tests, 12 g of pollen patty tested in combination with the repellents DEET and Picaridin, along with the HCAs pyrrole, pyrrolidine, pieridine, and 1,4-dimethylpiperazine (Table 2.1; Figure 2.4). All repellents were tested in volumes of 1 mg or 10 mg, respectively, and were prepared by dilution to a final volume of 100 μL in acetone and allowed to dry onto a small disc of filter paper (~1cm diameter). This treated paper disc was placed atop of the incubator-warmed pollen patty, then experiments commenced. Surprisingly, the DEET treatments of both 1 and 10 mg did not result in avoidance of the attractive pollen patty source, with attractance demonstrated through PI values of 0.33(±0.11) and 0.30(±0.10), respectively. The HCA piperidine also did not lead to avoidance of the attractive source, with significant attraction observed at the 1 mg treatment (PI=0.54±0.12; *P*=0.03), and slightly reduced attraction at 10 mg (PI=0.23±0.12). The repellent Picaridin fared somewhat better, as significant attraction to pollen patty was reduced, however, avoidance was still not observed at 1 mg (PI=0) or 10 mg (PI=0.07±0.06) of treatment. The experimental HCA that elicited the strongest avoidant responses was pyrrolidine, with avoidance indicated at 1 mg (PI= -0.23±0.02) and significant avoidance of pollen patty at 10 mg (PI= -0.58±0.2; *P*= 0.003). Pyrrole also reduced attractant choice, with no preference seen at 1 mg (PI=0) and slight avoidance at 10 mg (PI= -0.9±0.09). Finally, 1,4-dimethylpiperazine tests resulted in moderate avoidance at 1mg (-0.45±0.28) and slight avoidance at 10 mg (-0.05±0.05). When comparing instances of beetles either leaving the first arena chosen and returning to the center arena or choosing the other test arena, it was found that over 50% of beetles from the 10-mg pyrrolidine, 10 mg pyrrole, 10 mg 1,4-dimethylpiperazine, and 1mg DEET made this choice. The only

treatments in which fewer than 30% of beetles left their first arena choice were the 12 g of pollen patty alone and both Picaridin treatments (Figure 2.5).

2.5 DISCUSSION

Overall, this study provides an overview of *A. tumida* behavioral responses to two attractants and makes a valuable first step in assessing potentially effectual repellent compounds. While there is much anectodal evidence about the attractiveness of pollen patty to *A. tumida*, one of the few studies that tested pollen patty on the behavioral level did not find pollen patty to be significantly attractive to *A. tumida* adults prior to exposer to the *A. tumida* fungal symbiont *Kodamaea ohmeri* (Dekebo *et al*., 2017). However, in this study, the commercial pollen patty used clearly demonstrated attractive properties. One reason for this attracton could be due to the essential oils found as part of the ingredients in the pollen patties used, which included lemongrass oil and spearmint oil. It is possible that these essential oils were serving as attranctants, so future studies should also examine these essential oils alone to obtain a better understanding of how they might influence *A. tumida* attraction. An additional finding of interest was that the broad-spectrum, and often highly efficacious, repellent DEET (Leal, 2014) did not induce significant avoidance of an attractive source. However, it was discovered that the HCA pyrrolidine significantly deterred *A. tumida* adults from moving toward an attractive source, with the HCAs pyrrole and 1,4-dimethylpiperazine also evoking notable avoidant behaviors. While other *A. tumida* olfactometry studies have almost exclusively tested attractant compounds to better understand *A. tumida* attractance to host colonies (Suazo *et al*., 2003; Graham *et al*.,

2011) and for use in baited traps (Torto *et al*., 2010; Stuhl, 2021); this study took a different approach in attempting to discover effective *A. tumida* repellents to keep adult beetles from entering apiaries in the first place.

Current *A. tumida* control methods mostly focus on reducing populations of beetles and larvae that have already infested apiaries (Hood and Miller, 2003; Cuthbertson *et al*., 2013). Unfortunately, years of relentless *A. tumida* infestations have made the need for a better management strategy clear and, based on the results of this study, repellents could be the answer to stopping *A. tumida* one step ahead of apiary location. Such an approach is not novel in the realm of pest management and could be applied to *A. tumida* through the implementation of a "Push-pull" strategy, where repellents push beetles from apiaries and attractants draw beetles into baited traps. Push-pull strategies originated in agricultural settings (Pyke *et al*., 1987) and have been used to combat a variety of crop pests (Cook, *et al*., 2007). Push-pull strategies have also been designed to target several urban pests, including the malaria mosquito *Anopheles gambiae* (Menger et al., 2014), *Aedes aegypti* (Salazar *et al*., 2012), and German cockroaches (*Blattella germanica*; Nalyanya *et al*., 2000). The "Push" portion of this management strategy focuses on making the crop or host unattractive or difficult to locate, while the "Pull" portion of this strategy uses an attractive stimulus, such as a baited trap or trap crop to divert and kill the pest (Cook *et al*., 2007).

In the context of *A. tumida* management, repellent compounds, such as pyrrolidine, pyrrole, or 1,4-dimethylpiperazine, could be strategically released outside of apiaries to "Push" adult beetles away and attractive baited traps could be used to

“Pull” beetles in and ultimately kill them; this method could both reduce *A. tumida* populations and keep beetles from discovering and infesting apiaries. Fortunately, a great deal of work has already been devoted to understanding and developing *A. tumida* attractants (Torto *et al*., 2007a; Dekebo *et al*., 2017; Stuhl, 2021), so focusing on potential *A. tumida* repellents is a crucial next step taken by this study. However, there is more work to be done before these repellents are ready for use in the field. In regard to future studies involving *A. tumida* repellency testing, it would be useful to, have a temperature-controlled environment in which to perform these studies, as beetles tended to be sluggish when the temperatures were colder, and the attractant pollen patty was less effective. It would also be beneficial to examine some of the numerous HCA analogs to determine whether or not there are other promising candidates that could be considered as future *A. tumida* repellents (Larson *et al*., 2021). Prior to implementation of a field study, testing a larger flying response Y-maze olfactometer (Eilerts *et al*., 2018) would be beneficial, as flying behavior more realistically mimics hive seeking behavior in the wild (Neumann and Elzen, 2004). Combinations of selected repellents with other known attractive sources (Suazo *et al*., 2003; Stuhl, 2021) would also be an important step in understanding the strength of the repellents vs. attractive sources, and would aid in optimizing repellent concentrations for deployment in eventual field studies. Such studies could emply high-tunnel setups to assess bee foraging activity in response to repellent treatments, along with assessing *A. tumida* hive discovery in a more controlled, but still field-relevant, setting (Larson *et al*., 2021).

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2.7 TABLES AND FIGURES

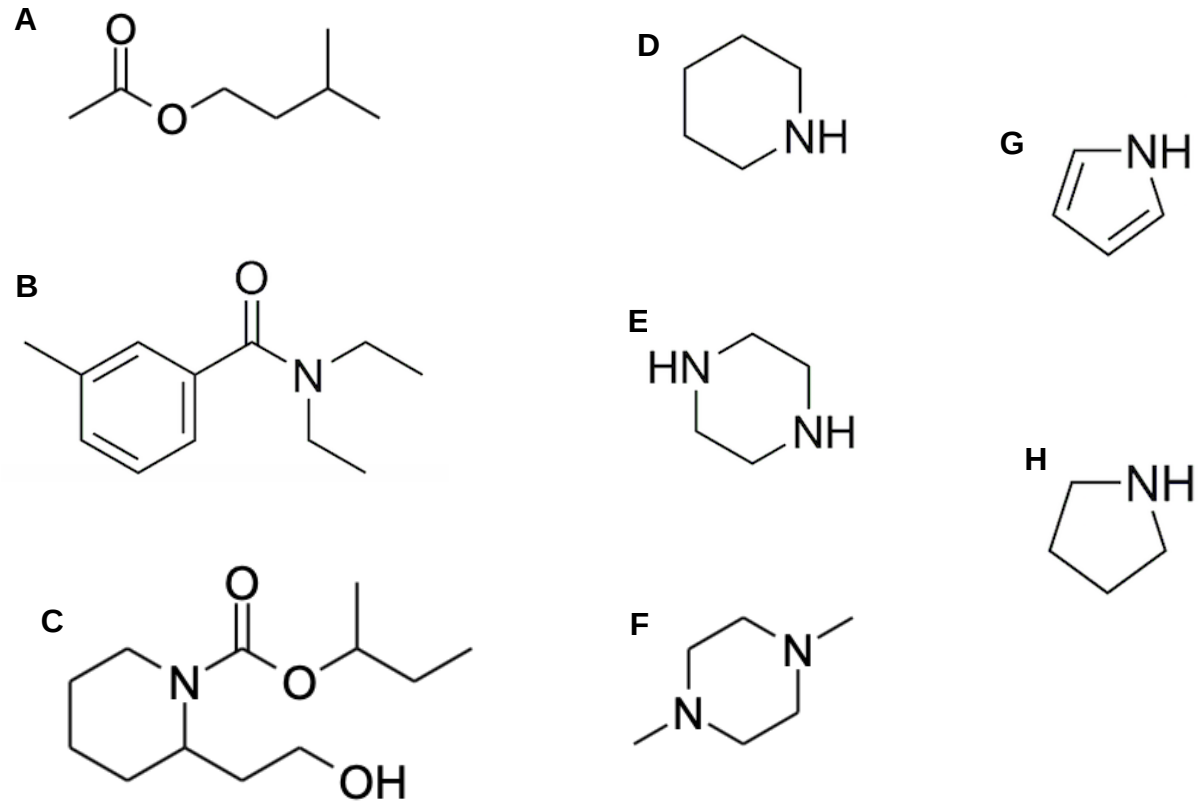

Figure 2.1 Attractant compound (A) isopentyl acetate, along with repellent compounds: (B) DEET (*N*,*N*-Diethyl-3-methylbenzamide, (C) Picaridin (butan-2-yl 2-

(2-hydroxyethyl)piperidine-1-carboxylate), (D)piperazine, (E) 1,4-dimethylpiperazine, (F) piperidine, (G) pyrrole, and (H) pyrrolidine.

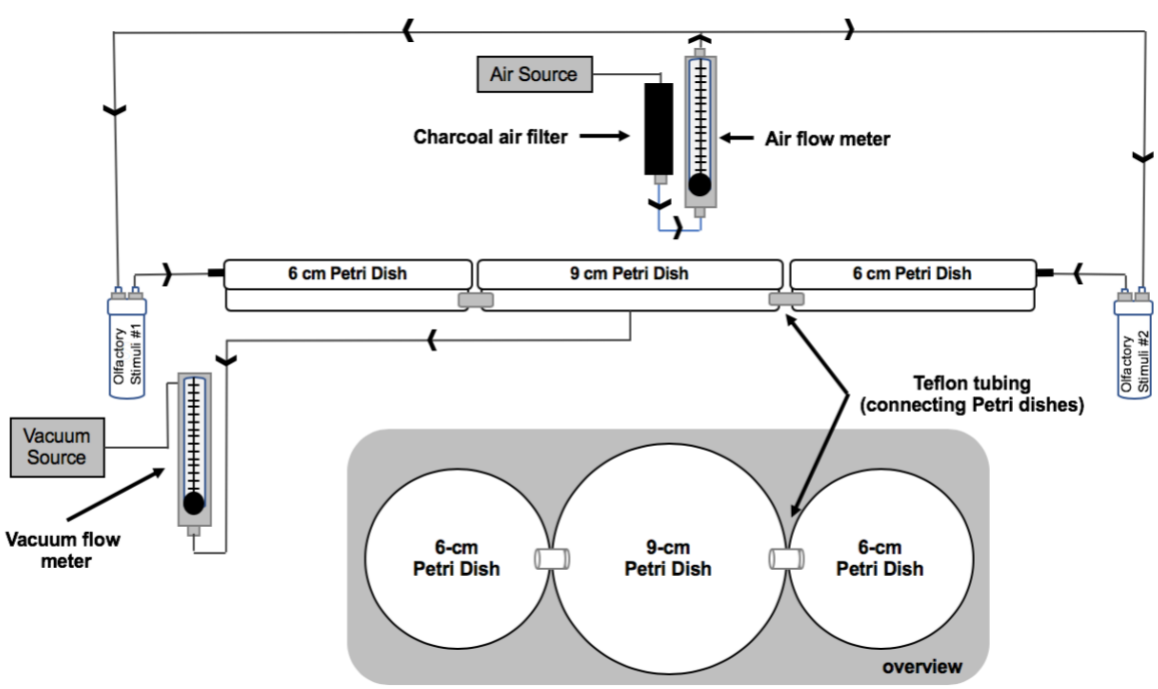

Figure 2.2: Dual choice olfactometer, based upon original design by Bobadoye *et al*., 2018; modified by Aaron D. Gross.

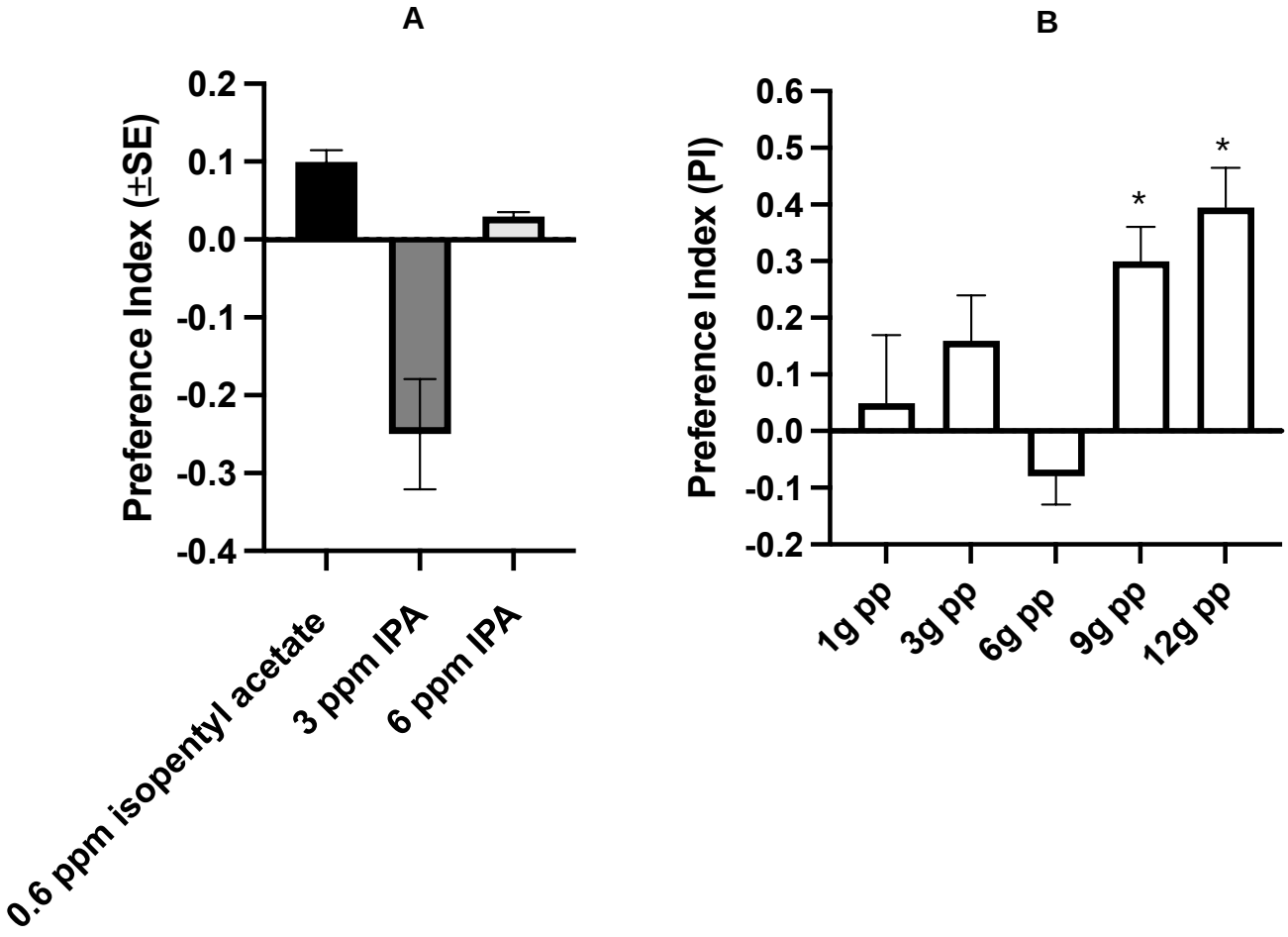

Figure 2.3 *Aethina tumida* behavioral responses to attractant odors (A) isopentyl acetate and (B) commercial pollen patty. Significance ($p \leq 0.05$) confirmed via binomial testing is denoted with an asterisk.

Table 2.1 Summary of Preference index (PI) values, standard error, number of samples, significance per exact binomial testing. Replicates in which beetles chose the center arena are not included in sample count (n) column.

Compound	PI	SE	n	significance
0.6ppm isopentyl acetate (IPA)	0.10	0.07	20	ns
3ppm IPA	-0.25	0.20	8	ns
6ppm IPA	0.03	0.03	31	ns
1g pollen patty (pp)	0.05	0.12	17	ns
3g pp	0.16	0.08	19	ns
6g pp	-0.05	0.04	38	ns
9g pp	0.30	0.06	54	*
12g pp	0.40	0.07	43	*
1mg pyrrolidine +12g pp	-0.23	0.02	13	ns
10mg pyrrolidine +12g pp	-0.58	0.20	24	*
1mg pyrrole + 12g pp	0	0	10	ns
10mg pyrrole + 12g pp	-0.09	0.09	11	ns
1mg 1,4-dimethylpiperazine +12g pp	-0.45	0.24	11	ns
10mg 1,4-dimethylpiperazine +12g pp	-0.05	0.05	19	ns
1mg picaridin + 12g pp	0	0	10	ns
10mg picaridin + 12g pp	0.07	0.06	16	ns
1mg piperidine + 12g pp	0.54	0.12	13	*
10mg piperidine + 12g pp	0.23	0.12	13	ns
1mg DEET +12g pp	0.33	0.11	18	ns
10mg DEET +12g pp	0.30	0.10	20	ns

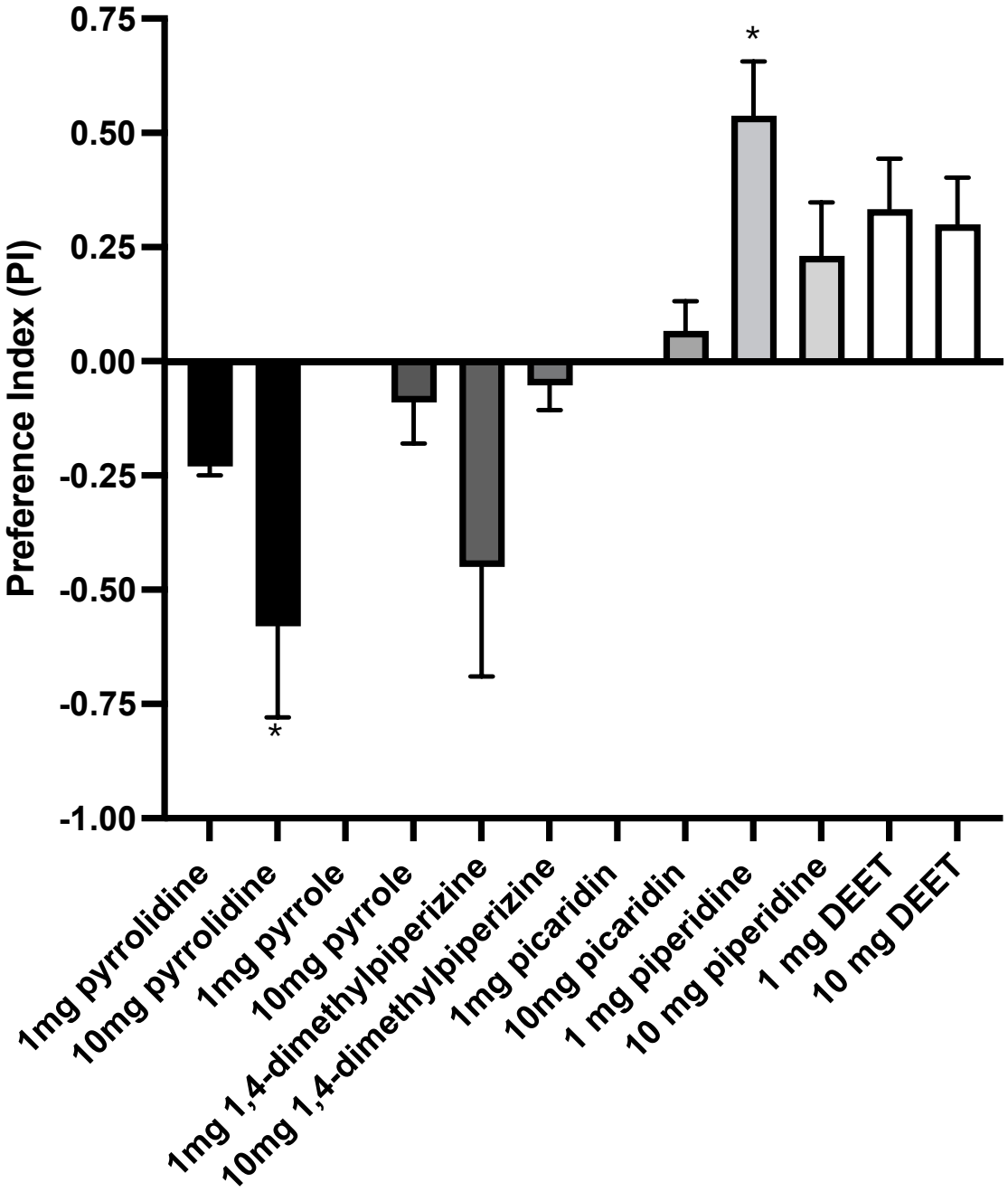

Figure 2.4 Responses to mixed attractants and repellents, with only significant repellence noted for 10 mg pyrrolidine (with 12g of pollen patty). Asterisk represents

significant ($p \leq 0.05$) attractance or repellence, calculated through binomial testing of each choice test.

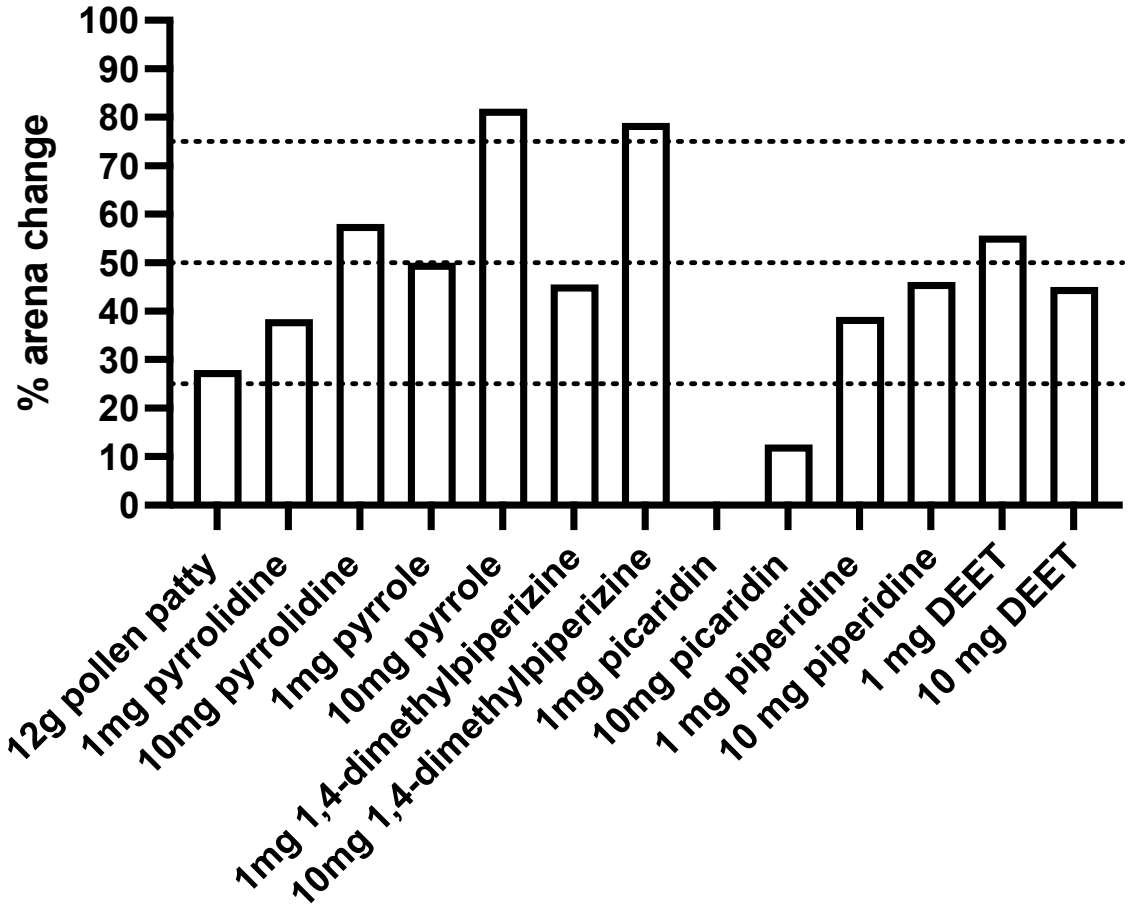

Figure 2.5 Percentage of *A. tumida* adults that left their first choice and chose to either stay in the center arena for the duration of the experiment or chose to enter the opposite test arena (minimum n=10).

CHAPTER 3.

ANALYZING *AETHINA TUMIDA* OLFACTORY RESPONSES TO ATTRACTANT AND REPELLENT MOLECULES THROUGH ELECTROANTENNOGRAPHY

3.1 ABSTRACT

Over the past 20 years, numerous efforts to manage the invasive small hive beetle (*Aethina tumida* Murray) have gone largely unrewarded, as this pest continues to damage the hives of European honey bees (*Apis mellifera* L.). These beetles use their keen olfactory systems to invade hives and lay thousands of eggs, which hatch into voracious larvae that feed on hive products and brood. To date, most of the *A. tumida* control measures in use are mechanical or insecticidal in nature, but manipulation of the *A. tumida* olfactory system through use of repellents is still a potential control method that warrants further investigation. This study sought to examine the physiological effects of two attractant compounds and several previously untested repellent compounds on the *A. tumida* olfactory system using electroantennography. Concentration-response curves were constructed to calculate the half maximal effective concentration (EC_{50}) for each compound, yielding attractant EC_{50} values of 8 ppm for isopentyl acetate and approximately 100 mg for pollen patty. Repellent EC_{50} values ranged from 0.08% for pyrrolidine to 23.1% for piperidine, with the added finding that DEET did not elicit a significant antennal response at any concentration. When EC_{50} values of attractant and repellent compounds were tested together, it was found that isopentyl acetate (IPA) combined with three most effective repellents,

pyrrolidine, pyrrole, and 1,4-dimethylpiperizine, slightly reduced antennal reasponses when compared to IPA alone. Based upon these results, pyrrolidine would be the most promising candidate for future management options. By strategically implementing this repellent source in an apiary environment, *A. tumida* adults could be deterred from entering apiaries and invading hives.

3.2 INTRODUCTION

Although honey production may be the service that *A. mellifera* colonies are best known for, the pollination services that *A. mellifera* foragers provide contribute to the approximately 20 billion dollars in agricultural benefits derived from pollinators annually in the United States and Europe (Gallai *et al*., 2009). When *Aethina tumida* (small hive beetle) adults discover hives, they are capable of hiding from, evading (Neumann *et al*. 2001), and even tricking *A. mellifera* adults into feeding them through trophallaxis (Neumann *et al*., 2015). However, the majority of damage caused by these pests occurs as a result of *A. tumida* larval feeding, which is voracious and extensive, including honey, brood, pollen, and wax (Hayes *et al*., 2015). The waste that accumulates from this larval feeding is known to ferment through the aid of the *A. tumida* fungal symbiont *Kodamaea ohmeri*, which, aside from ruining honey and leading to dirty and eventually uninhabitable hives, is known to emit isopentyl acetate, which is highly attractive to *A. tumida* adults (Benda *et al*., 2008).

Isopentyl acetate is also a key component in *A. mellifera* alarm pheromone (Wager and Breed, 2000) and exposure to isopentyl acetate is well known for its ability to instigate defensive responses in *A. mellifera* in both laboratory and field tests

(Collins *et al*., 1989), with stinging behavior increasing with isopentyl acetate concentration (Lensky *et al*., 1995). Although *A. mellifera* alarm pheromone is a releaser pheromone, and thus should only affect colonies in the short term, exposure to isopentyl acetate can lead to changes in *A. mellifera* gene expression and influences long-term behavior (Alaux and Robinson, 2007). The heightened levels of isopentyl acetate in the hive that accompany severe *A. tumida* infestations likely creates a positive feedback loop of increasing *A. mellifera* stress, which leads to even more isopentyl acetate release and *A. tumida* attraction (Torto *et al*., 2007). The highly adept olfactory systems of these beetles are corroborated by imaging studies, which examined the antennal lobes of *A. tumida* adults, finding that, compared to other sap beetles (family: Nitidulidae), *A. tumida* has unusually large antennal lobes (Kollmann *et al*., 2016). It was also discovered that there are approximately 70 olfactory glomeruli per antennal lobe in both sexes, which implies that olfactory cues are critical for both male and female beetles (Kollmann *et al*., 2016).

Although isopentyl acetate is important in *A. tumida* attraction, it is far from being the only volatile that is attractive to *A. tumida* adults. Both male and female *A. tumida* adults are also attracted to volatiles from unripe honey, fresh pollen, *A. mellifera* workers, and slumgum, which is the mix of dead bees, propolis, excrement, and other hive debris, which is often found on hive bottom boards (Suazo *et al*., 2003). It has also been demonstrated that *A. tumida* adults are attracted to *A. mellifera* brood, stored pollen, and even wax (Graham *et al*., 2011). Because *A. tumida* is ultimately a scavenger, it is not surprising that these beetles are also attracted to volatiles from Bumble bee (*Bombus impatiens*) colonies and did not preferentially chose volatiles

from *A. mellifera* colonies when choice assays were performed comparing *B. impatiens* and *A. mellifera* colony volatiles (Graham *et al*., 2011). *Aethina tumida* adults are also known to be attracted to volatiles from the colonies of the melponine bee species *Meliponula ferruginea* (black and reddish-brown morphospecies), and *Meliponula bocandei*, which are all found in the native *A. tumida* range of sub-Saharan Africa (Bobadoye *et al*., 2018). It is likely that *A. tumida* adults are attracted to volatiles from the colonies of numerous other social bees as well, since these beetles are known to infest colonies of various neotropical meliponine bee species (Bobadoye *et al*., 2018), as well as the Australian stingless bee (*Austroplebeia australis*). However, not all of these colonies are helpless against *A. tumida* attacks, as the stingless bee *Trigona carbonaria*, which is native to Australia (Norgate *et al*., 2010), protects its hive by coating *A. tumida* adults in mud, wax, and resin, essentially mummifying these pests and preventing *A. tumida* reproduction (Greco, *et al*., 2010).

Although *A. tumida* has clearly adapted to parasitize social bee colonies, these beetles can also survive on other food sources and have been recorded to feed and reproduce on a variety of fruits, including bananas, mangos, and grapes, although less reproduction took place on these alternate sources when compared to a mixture of honey and pollen (Buchholz *et al*., 2008). Peach volatiles have also been shown to be highly attractive to *A. tumida* adults, and a synthetic blend of fruit volatiles was demonstrably attractive in olfactometry tests, with responses to these volatiles confirmed on a physiological level through electroantennography coupled with gas chromatography (Stuhl, 2021). Finally, an attractant that imitates *A. tumida* aggregation pheromone and contains containing a mixture of 6-methyl-5-hepten-2-

one, nonanal, and decanal has also been developed and began undergoing the patent process in 2017 (Stuhl & Teal, 2017). Although *A. tumida* are uniquely adapted to survive in the hive environment, they do not need to be invading hives to persist in a geographic area, so eradication is an impossibility, rather, management steps should be taken to best keep *A. tumida* adults from discovering and entering hives in the first place.

When *A. tumida* infestations become severe, *A. mellifera* colonies can abscond (Ellis and Hepburn, 2006), which dramatically reduces their chances of surviving until the next year (Hepburn *et al*., 1998). In the *A. tumida* native range, Africanized *A. mellifera* colonies are known to abscond frequently (Winston, 1992) and *A. tumida* presence essentially serves to select against weak hives, however, in more temperate climates, it is crucial for colonies to build up food stores and a strong colony for the winter months, which absconding renders very difficult (Hepburn *et al*., 1999). Within the first year of *A. tumida* presence in the United States, damages incurred to hives in Florida alone was estimated to exceed three million dollars (Ellis *et al*., 2002). Since that time, the *A. tumida* geographic range quickly expanded and now covers much of the continental United States since, so the economic costs incurred by this pest are likely much higher after 24 years of *A. tumida* infestations (Neumann and Ellis, 2008). Over the past two decades, mechanical control methods, such as trapping (Hood, 2011), and chemical control methods, such as coumaphos treatments (Cuthbertson *et al*., 2013), have become the most popular ways of dealing with *A. tumida* infestations, however, trapping does not guarantee that the majority of adult beetles in the hive will be captured and does not help eliminate larvae, and the chemicals

used to treat these infestations can also be toxic to *A. mellifera* colonies (Kanga and Somorin, 2012).

The strong olfactory senses of *A. tumida* adults, and the role that olfaction plays in hive location, are well known (Hayes *et al*., 2015), but management tactics based on *A. tumida* olfaction have been slow to develop and only exist in the form of baited traps; preferred management methods still tend to be insecticidal or mechanical (Cuthbertson *et al*., 2013). Early baited trap studies tested pollen dough and pollen dough on which adult beetles were allowed to feed prior to use, thus presumably inoculating the pollen dough with *K. ohmeri*; unsurprisingly, traps with the *K. ohmeri* spiked dough were more attractive to *A. tumida* adults, due to the attractive volatiles produced by *K. ohmeri* growth (Arbogast *et al*., 2007; Benda *et al*., 2008). However, traps in these studies were shown to be most effective when close to hives (Arbogast *et al*., 2009), which means that the hive environment was still likely the most attractive source to adult beetles and traps served as a detection method rather than a means of control (Cuthbertson *et al*., 2013). Similarly, traps containing apple cider vinegar for attractancy and cooking oil for trapping the beetles have been commonly used (Hood and Miller, 2003), however, the same issue of successful beetle reproduction persists, along with the fact that decomposing beetles may decrease trap attractancy over time (Chakraborty *et al*. 2019).

Clearly the *A. tumida* olfactory system is a crucial part of hive location and further work could be done to design safe and efficient management strategies that use the olfactory system to keep *A. tumida* adults from sensing and invading hives in the first place. To that end, the following study was designed to test known repellents

DEET (*N*, *N*-Diethyl-3-methylbenzamide) and Picaridin (Sec-butyl 2-(2-hydroxyethyl) piperidine-1-carboxylate), as well as the heterocyclic amines piperazine, piperidine, pyrrole, and pyrrolidine against *A. tumida* antennae (Figure 3.1). *Aethina tumida* responses to these compounds have not previously been characterized and the goal of this study was to examine the physiological effects of these compounds on *A. tumida* antenna through a series of electroantennograms (EAGs).

Electroantennography is a well-established method of testing insect antennal responses to volatile compounds and has been in use for over 50 years (Schneider and Seibt, 1969). Specifically, EAGs are measuring the collective membrane depolarizations that are initiated when the numerous olfactory receptor neurons on the insect antenna are activated by odor molecules (Park *et al*., 2002). Therefore, the results of an EAG provide a view of the whole-antenna nervous system response (Park *et al*., 2002), which is obtained and quantified by amplification and digitization, enabling digital filtering of results and visualization of olfactory activity in voltage deflections that are larger than the baseline electrical antennal responses; the larger deflection, the stronger the response (Lahondère *et al*., 2020; Lahondère, 2021).

Early EAG studies were mostly used to study antennal responses to pheromones, as seen in EAG studies performed testing sex pheromone responses in red-banded leaf roller moths (*Argyrotaenia velutinana*; Roelofs and Comeau, 1971), queen butterflies (*Danaus gilippus*; Schneider and Seibt, 1969), and coddling moths (*Laspeyresia pomonella*; Roelofs *et al*., 1971). When the composition of volatile organic compounds is unknown, gas chromatography-mass spectrometry (GC-MS) can be coupled with the EAG apparatus to elucidate the specific chemicals that elicit

antennal responses, isolating the specific compounds that excite olfactory neurons (Arn *et al*., 1975; Lahondère *et al*., 2020). In more recent years, EAGs have been used to test insect responsiveness to odorant compounds from host plants in the context of beneficial pollinators (Blight *et al*., 1997; Honda *et al*., 1999), as well as pest insects seeking out plants to feed upon (Visser and Fu-Shun, 1995; Kendra *et al*., 2012). EAGs have also been recently used to test repellent compounds, as seen in studies examining repellence of *Aedes albopictus* mosquitos by several pyrethrins (Yan *et al*., 2021), the repellent activity of DEET and several experimental pyridyl amides against several *Anopheles* mosquito species (Cuba *et al*., 2021), and the use of heterocyclic amines in repelling *A. mellifera* foragers from insecticide-treated crops (Larson *et al*., 2021).

EAGs have been performed on many insect species (Park *et al*., 2002), and *A. tumida* is no exception; however, past *A. tumida* studies have focused on mostly on antennal responses to attractant compounds. Such studies include tests using pollen dough inoculated with *K. ohmeri* (Torto *et al*., 2007), natural and synthetic fruit volatiles (Stuhl, 2021), and the previously mentioned *A. tumida* aggregation pheromone (Stuhl, 2020), with one study focusing on testing the potential repellency of the formic acid hive treatment Apicure® (Komen *et al*., 2019). To date, the physiological effects of the repellents tested in this study have not been documented, so the series of *A. tumida* EAGs described below provides novel insights into the functioning of the *A. tumida* olfactory system and highlights promising compounds that could be incorporated into future *A. tumida* management methods.

3.3 MATERIALS AND METHODS

3.3.1 Insects

Aethina tumida adults were collected from the Prices Fork apiary in Blacksburg, VA, USA in August of 2018 to start a laboratory colony, which, at present, contains F10 to F15 adult beetles and F11 to F16 larvae has been added to the Virginia Tech collection (Catalogue Number: VTEC000004965). The *A. tumida* adults used for all experiments in this study were forced to pupate individually in pupation tubes. These pupation tubes are made by adding a single larva to a 2 mL Eppendorf tube filled with a moistened 1:1 sand/soil mix. As soon as adult emergence is noted, pupation tubes are emptied, and newly emerged adults are sexed and divided into separate male and female containers for use in experiments. *Aethina tumida* sex is easily ascertained by gently squeezing the beetle abdomen and observing the protrusion of either the ovipositor in females or the 8th tergite in males (Neumann *et al*., 2001). All *A. tumida* adults used in this study were not allowed to mate and were only used between 14-21 days post emergence, as this is the time at which they are sexually mature and ready to seek a mate in the wild (Mustafa, 2015). Although attempts were made to test beetles of both sexes against each compound used in this study, and beetle sex was noted for each EAG replicate performed, the female biased sex ratio (Papach *et al*., 2019) of *A. tumida* made it difficult to use equal numbers of both sexes, so antennal results from both sexes were combined. Additionally, all experiments were carried out between four and eight PM, which is the time of day in which wild *A. tumida* adults would be most likely to engage in hive seeking behavior (Bobadoye *et al*., 2018).

Finally, all beetles were starved for 24 hours prior to testing to increase the likelihood that they would actively seek out attractive sources.

3.3.2 Chemicals

Paraffin oil (analytical grade), isopentyl acetate (98%), pyrrole (98%), pyrrolidine (≥99.0%), piperazine (99%), piperidine (≥99.5%), 1,4-dimethylpiperazine (98%), and *N*,*N*-Diethyl-3-methylbenzamide (DEET; analytical grade) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Sec-butyl 2-(2-hydroxyethyl) piperidine-1-carboxylate (Picaridin™; 98%) was purchased from AmBeed (Arlington Heights, IL, USA). The acetone (HPLC grade) used for dilution of all repellents used in this study was from Fisher Scientific (Hampton, NH, USA). AP23® (Artificial Pollen) patties were purchased from Dadant and Sons Inc. (Hamilton, IL, USA).

3.3.3 Preparing syringes for electroantennogram (EAG) odor delivery

Modified syringes were used for odor delivery and were constructed by removing the plungers from 2 mL FORTUNA OPTIMA glass syringe tubes (Poulten & Graf GmbH, Wertheim, Germany). The tip of the syringe tube was then fitted with a small strip of Teflon tape, which was used to affix a 21-gauge needle (BD PrecisionGlide Needles, Dunedin, FL, USA) to the syringe tip. A piece of polyethylene tubing (inner diameter = 1/4 in; outer diameter = 3/8 in) of approximately 4 cm in length was then cut and a female luer was tightly fitted to this tube, with a male luer added to the top. The open side of the polyethylene tube was next fitted to the opening of the glass syringe tube, creating a closed chamber for treatment delivery. Approximately 4 cm by 0.5cm strips

of filter paper (Fisher Scientific, Waltham, MA, USA) were folded in half lengthwise and added to each tube.

3.3.4 Test compounds

To find an appropriate solvent for the test compounds in this study, ethanol, acetone, mineral oil, and paraffin oil were all tested. Unfortunately, *A. tumida* antennae responded strongly to ethanol and acetone, while mineral oil did not work well as a solvent for the compounds of interest, after which paraffin was tested and found to elicit no response in *A. tumida* antennae and worked well for dissolving the majority of the test compounds used in this study. Test compounds were serially diluted in paraffin oil, with the exception of piperazine (a crystalline solid), which was dissolved in DI water, as it was virtually insoluble in paraffin oil. Within 15 minutes prior to the start of a set of experiments, 10 μL of each test compound was added to the filter paper in the glass syringe systems and 10 μL of paraffin oil was used as a control. Because pollen patty is a solid, delivery was done based on weight rather than concentration, with pollen patty stored at room temperature (approximately 24°C) and weighed prior to the start of experiments and stored in 2mL glass vials in a freezer at 0°C. Used filter papers with treatments were never used for more than 4 hr after preparation and new papers were used for each set of experiments.

3.3.5 Electroantennograms

The procedures used for this study were based upon EAGs performed on mosquitos by Lahondère (2021), with modifications to account for *A. tumida* antennal structure. The antenna removal method began by chilling beetles on ice for 1-2 minutes and

then transferring a single beetle to a chilled aluminum block placed beneath a dissection scope. A single antenna was removed from anesthetized beetles using 2mm Vannas Spring Scissors (Fine Science Tools, Foster City, CA, USA). A V-shaped cut was snipped into the terminal antennal segment and the antenna was then and mounted on a recording electrode using Spectra 360 Electrode Gel (Simply Medical LLC., Richmond, VA, USA), while the base of the antenna was affixed to a reference electrode (also dipped in electrode gel). Both electrodes (oxidized silver wires) were enclosed in pulled glass capillary tubes containing a saline solution (pH 7; Beyenbach and Masia, 2002) and saline solution was also added to electrode holders. The electrode holders were then transported into the Faraday cage for all EAGs and were mounted on a platform beneath a compound microscope. A light source and dissection probe were then used to ensure that the antenna was properly mounted and ready for use (Figure 3.1). A glass tube with a small hole for inserting odor syringe tips was also positioned approximately 0.5 cm from the antenna and the microscope light, along with external laboratory lighting, was turned off prior EAG recordings.

Odors were pulsed over the antennae, with each replicate beginning with three sets of paraffin oil (negative control) pulses, followed by each treatment odor, with paraffin oil used between each treatment concentration to ensure that early odors used were not influencing the results obtained later in the experiment. If strong antennal responses were observed when paraffin oil was tested directly after a test compound, paraffin oil was tested until results went back to the normal near-baseline levels seen in paraffin oil tests. Concentration response curves were performed by testing compounds in order of increasing concentration, whereas treatments were

randomized when different compounds were being tested on the same antenna. The test duration for each compound was 45-seconds, with two 2-second pulses administered during this time interval; MATLAB software (MathWorks, Natick, MA, USA) was used to program odor delivery and randomize treatments when necessary. The signals from the electrodes were amplified on a microelectrode AC amplifier (Model 1800; A-M Systems, Sequim, WA, USA), the signal was filtered with a HumBug (Quest Scientific, North Vancouver, BC, Canada) 50/60 Hz noise eliminator, and the signal was recorded on a WinEDR Strathclyde Electrophysiology Data Recorder V3.8.0 (University of Strathclyde, Glasgow, UK). Each antenna was useable for approximately 20-30 minutes post-dissection, and in cases where the strongest compounds were not used last (i.e., establishing concentration-responses curves), an attractant compound previously tested to elicit strong antennal responses was tested last to ensure that the antenna was still viable, thus avoiding false negative results.

3.3.6 EAG analysis

After an EAG was completed, the signal was digitally filtered using WinEDR software, and WinEDR was used to manually establish a baseline of zero in the center of the baseline signal from each antenna. The voltage deflections (in mV) resulting from each odor pulse administered were then manually measured based off of the baseline, with the two-pulse determinate averaged in the results of each replicate and the paraffin oil control deflections from the entire replicate were averaged. GraphPad Prism (San Diego, CA, USA) was used for plotting all graphs and analyzing the EAG data. The concentration response curves for each compound were made by using the

paraffin oil control (smallest mean) to normalize the results, with 100% set by the highest mean in each data set (generally the highest test compound concentration). Odor treatment concentrations were also transformed to $\log^{10}$, and each data point was represented as percent millivolts (mV) ± standard error of the mean (SEM) and fitted with a concentration-response curve using the log agonist vs. variable response function, which generated a curve and calculated the half-maximal effective concentration (EC_{50}) for each compound. A minimum of 12 replicates was used for each attractant and repellent concentration response curve. In tests of mixed compounds, the results were not normalized in order to better compare all treatments to the paraffin oil control and results were displayed in bar graphs depicting the average deflection (mV)±SEM on the y-axis. Comparison of odor mixes was accomplished using an ANOVA followed by Tukey's multiple comparisons tests.

3.4 RESULTS

3.4.1 Observations from EAG method development

Several noteworthy results were obtained from the development of methods for this study. As noted above, *A. tumida* antennae were very sensitive to both ethanol and acetone, which was considered for use as the negative control in this study. Other compounds testing in the method development portion of this study were hexane, benzaldehyde, and nonanal, which all elicited strong responses in *A. tumida* antennae; the latter is unsurprising as nonanal is one of the active ingredients in *A. tumida* aggregation pheromone (Stuhl, 2020). As used syringes and luers were soaked in hexane for cleaning, care was taken to thoroughly rinse these tubes prior to repeated use.

3.4.2 Attractant and repellent odor tests

The first attractant compound tested in this study was isopentyl acetate, the *A. mellifera* alarm pheromone component (Wager and Breed, 2000) and known *A. tumida* attractant (Torto *et al*., 2007). As expected, *A. tumida* antennae were highly sensitive to this compound, with range finding requiring concentrations as low as 0.01 ppm to reduce deflections to a level comparable to the paraffin oil control. After analysis and over 20 replicates, an EC_{50} of 8.46 ppm was established for isopentyl acetate (Figure 3.2A; Table 3.1). Commercial pollen patty at room temperature (~24°C) was also tested for attractancy in this study. Because pollen patty is a solid, various amounts were weighed, starting with 1 g and reducing the volume down to 0.01 g, at which point the treatment could barely be seen when pressed into the odor delivery filter paper. *Aethina tumida* antennae were sensitive to pollen patty, with an EC_{50} value of 0.0981 g (Figure 3.2B; Table 3.1).

The first repellent compound tested was DEET, which was originally intended for use as the positive control for repellency in this study, due to its effectiveness as a broad-spectrum insecticide in the past (Paluch *et al*., 2010). However, antennal responses significantly greater than the paraffin oil control were not elicited at any concentration of DEET (Figure 3.3). As it was suspected that DEET could still be working as a repellent by masking antennal responses to attractant compounds by impairing the ability of odorant receptors to perceive attractant compounds, a mixture of 100 ppm isopentyl acetate was tested in combination with the relatively high concentration of 10% DEET. However, antennal responses were not reduced and responses to the attractant/repellent mixture were not significantly different than

responses to the attractant alone (Figure 3.3). Thus, the well-established and highly effective repellent picaridin (Katz *et al*., 2008) was next selected for testing and revealed far more promising results, yielding an EC_{50} of 23.1% (Figure 3.4, Table 3.1).

Understanding the responsiveness of *A. tumida* antennae to picaridin lent favorable support to testing the other heterocyclic amines (HCAs) proposed for use in this study. Further EAGs demonstrated that *A. tumida* antennae were most sensitive to pyrrolidine (EC_{50}=0.0875%), followed by pyrrole (EC_{50}=2.17%), and piperadine (EC_{50}=28.4%), which was slightly less effective than picaridin (Figure 3.4, Table, 3.1). Although testing the parent compound piperazine was a goal of this study, this compound was a crystalline solid, unlike the other HCA's tested, which were all viscous liquids. Because *A. tumida* are so sensitive to acetone and ethanol (in which piperazine is highly soluble), and piperazine was virtually insoluble in paraffin oil, DI water was the only alternative solvent. While several concentrations of piperazine were tested, the antennal responses to water tended to be somewhat stochastic and, above a 3% concentration, the responses to piperazine plateaued, which meant that obtaining a concentration-response curve for this compound would not be possible (Figure 3.5). In view of this, the piperazine analog 1,4-dimethylpiperazine was tested and was found to elicit strong antennal responses (EC50=2.57%) with a slightly higher EC_{50} than pyrrole (Figure 3.4; Table 3.1).

3.5 DISCUSSION

Based upon the results of this study, the HCA pyrrolidine is the most promising candidate for a future *A. tumida* repellenency tests, although pyrrole and 1,4-

dimethylpiperazine are also elicited stronger antennal responses than the widely used repellent picaridin (Frances et al., 2004). Converesly, DEET did not elicit an antennal responses at any concentration, nor did it appear to be blocking the effects of IPA when the two were combined, indicating that DEET would be a poor choice for an *A. tumida* repellent; this was somewhat surprising, as DEET is such an efficacious broad-spectrum repellent (Paluch *et al*., 2010). *Aethina tumida* antennae were by far most sensitive to IPA, which was consistent with the known role of IPA in beetle attraction and previous studies testing *A. tumida* antennal responses to IPA (Torto *et al*., 2007). Interstingly, one study testing three volatiles from fire ant (*Solenopsis invicta*) alarm pheromones (one of which is IPA) on a variety of insects found that *A. tumida* antennae were more sensitive to the other two pheormones than to IPA (Du *et al*., 2019), highlighting the need to continue searching for optimal *A. tumida* attractants, in addition to repellents. Another future step in this study is to perform EAGs testing the components fo the recently identified *A. tumida* aggregation pheromone (nonanal, decanal, and 6-methyl-5-hepten-2-one; Stuhl and Teal, 2020) to understand how the attractancy of this compound compares to the attractancy of IPA and pollen patty.

Combinations of the EC_{50} values of the three strongest repellents with isopentyl acetate indicate slightly diminished responses when compared to isopentyl acetate alone. By further examining attractant/repellent combinations and ratios, the levels at which repellent compounds reduce or limit responses to attract coumpounds could be established. There are numerous other HCAs that could be tested against *A. tumida* antennae, as this study focused primily on the parent compounds piperidine, piperazine, pyrrole, and pyrrolidine. Understanding the levels at which repellent

compounds could keep *A. tumida* adults from sensing and entering apiaries could be applied in deterring beetles in a field setting, while enhanced knowledge of *A. tumida* attractants could assist in the assembly of baited traps; the overall goal would be to incorporate this information into a “Push-pull” strategy to stop *A. tumida* from entering apiaries, while reducing overall *A. tumida* populations (Cook *et al*., 2007).

Because HCAs can also be repellent to *A. mellifera* workers (Larson *et al*., 2021), field tests would also need to take apiary proximity and bee responses into account. However, recent work has demonstrated that, at an antennal level, *A. mellifera* adults are most sensitive to piperidine (Larson *et al*., 2021), which is the HCA in this study that beetle antennae were least sensitive to. Differences in responses to repellent compounds between *A. mellifera* and *A. tumida* adults is promising, inferring that a selective repellent could be selected for dispersal around apiaries. Although information pertaining to HCA dispersal in the environment is somewhat limited, since insect repellents, such as those containing picaridin, are generally applied to skin or clothing (Antwi *et al*., 2008), with reapplication necessary in a matter of 3-4 hours for continued protection (Scott *et al*., 2014). An attract-and-kill bait was recently developed for *A. tumida* adults using an artificial diet containing the three attractive volatiles that are emitted when *K. ohmeri* grows on pollen patties (ethyl propionate, isobutyl propionate, and ethyl butyrate; Stuhl, 2020). When 2% boric acid was added to this attractive food mixture, lab trials noted 100% mortality of feeding beetles, however, this treatment has not been optimized for the field and the bait mixture was also detrimental to *A. mellifera* health (Stuhl, 2020). Althogh there is more work to be done, the combination of an effective repellent to push *A. tumida* adults away from

apiaries and a baited trap to pull adult beetles to their deaths offers a solution to *A. tumida* infestations that could be implemented around apiaries to stop beetles before they have the opportunity to enter hives; this approach also limits the use of insecticides to control *A. tumida* in and around *A. mellifera* colonies.

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3.7 TABLES AND FIGURES

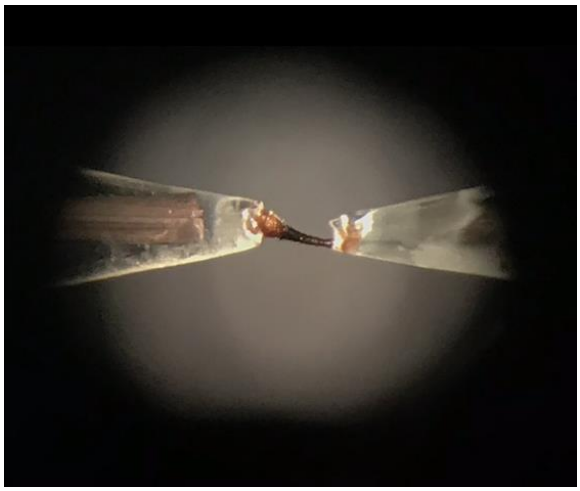

Figure 3.1 *Aethina tumida* antenna in place and prepped for EAG recording, positioned between a recording electrode (left, antenna tip) and reference electrode (left, antenna base). Antenna is held in place between saline-filled capillary tubes using electrode gel.

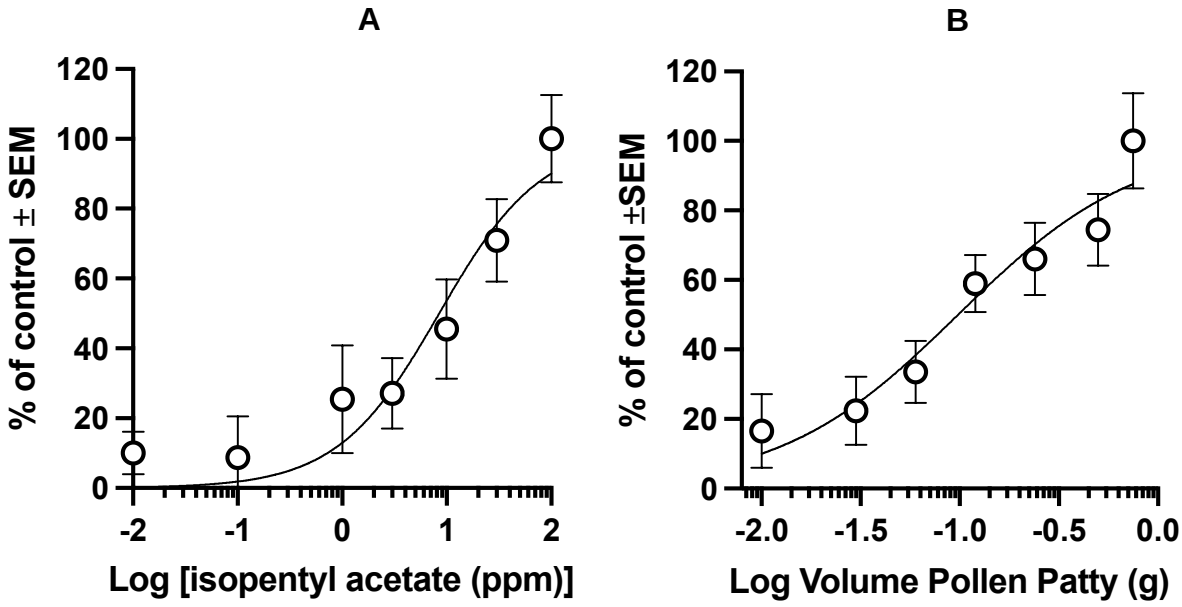

Figure 3.2 Results of EAGs testing attractant compounds on *A. tumida* antennae. Concentration response curve for isopentyl acetate (A) and volume response curve for pollen patty (B).

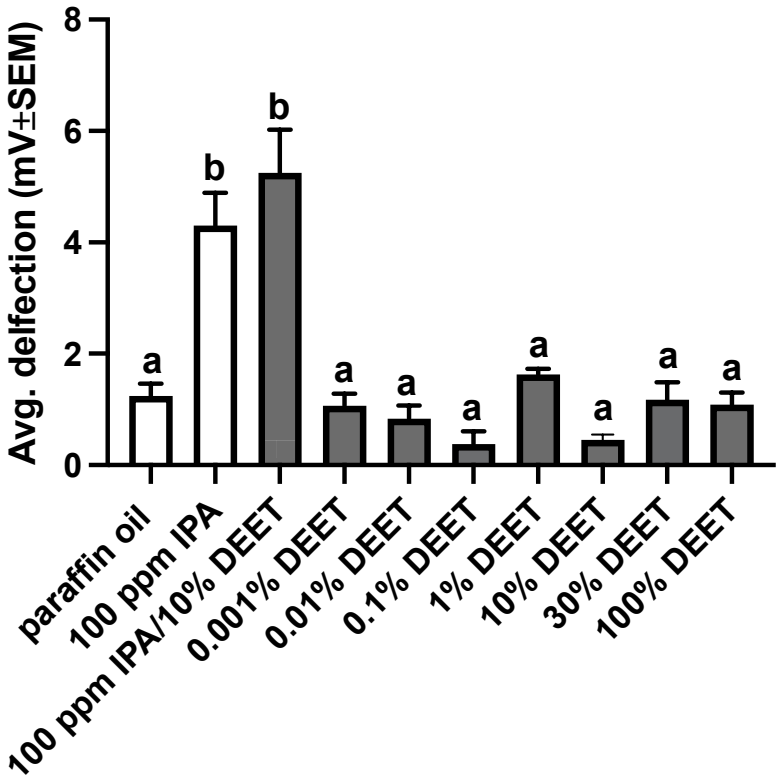

Figure 3.3 Results of EAGs testing an array of DEET concentrations, along with mixes of 100ppm IPA and 10% DEET, followed by one-way ANOVA and Tukey's multiple comparisons test. No significant difference between control and any DEET concentrations alone was seen, whereas IPA and the IPA/DEET mix were significantly different from the control and any DEET concentration alone.

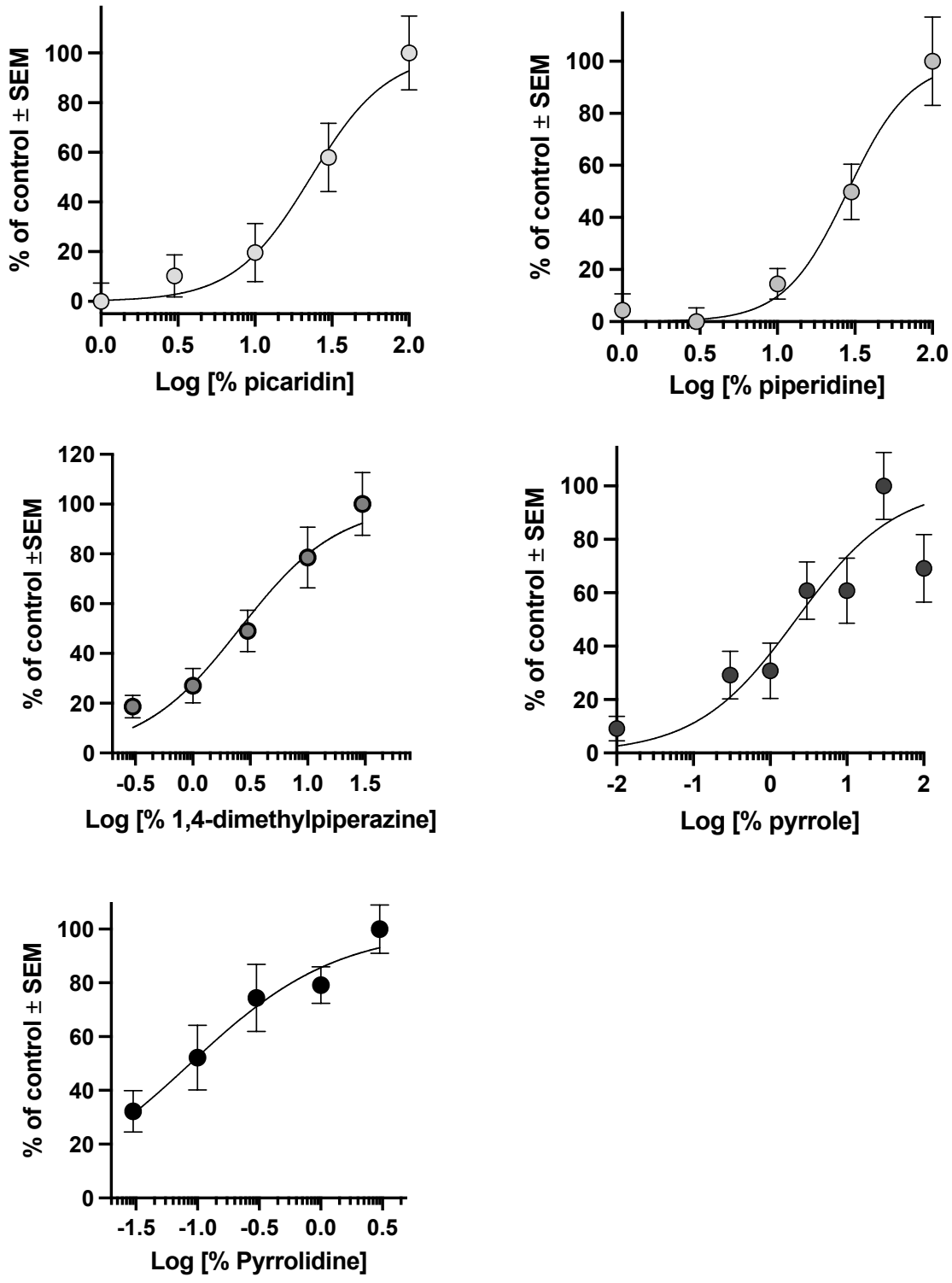

Figure 3.4 Concentration response curves for repellents used in this study: picaridin, piperidine, 1,4-dimethylpiperazine, pyrrole, and pyrrolidine.

Table 3.1 Half-maximal effective concentrations (EC_{50}), 95% confidence interval, and hillslope obtained for each test odor through *A. tumida* EAGs.

Treatment	EC_{50}	95% CI	Hillslope
Isopentyl Acetate	8.46 ppm	4.00-16.8	0.891
Pollen Patty	98.1 mg	62.4-14.8	0.962
Picaridin	23.1 %	14.2-36.1	1.74
Piperadine	28.4 %	19.4-40.1	2.13
1,4-dimethylpiperazine	2.57 %	1.39-4.48	1.01
Pyrrole	2.17 %	0.948-4.48	0.672
Pyrrolidine	0.0875 %	0.0217-0.185	0.734

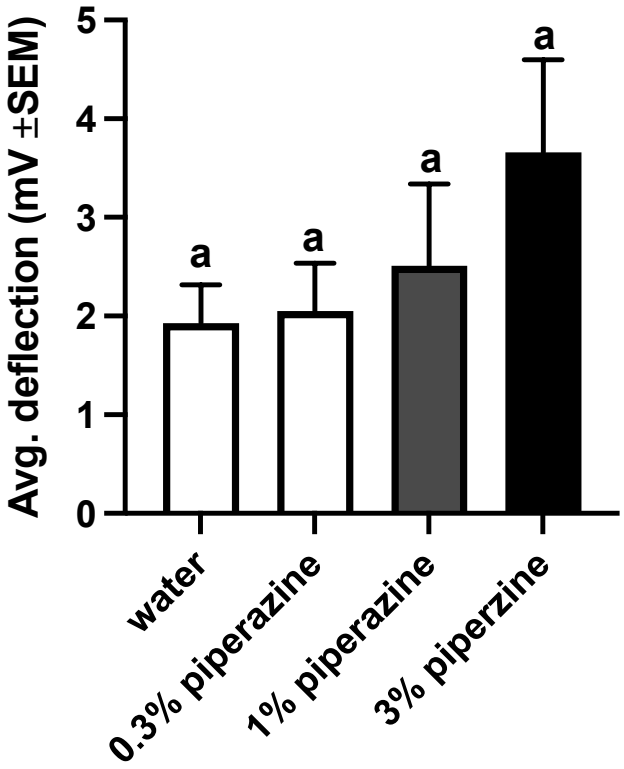

Figure 3.5 Results of piperazine EAGs (n=9), analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Other piperazine tests were run at higher concentrations, however, responses plateaued at concentrations higher than 3% and establishing an EC_{50} for this compound was abandoned due to solubility issues.

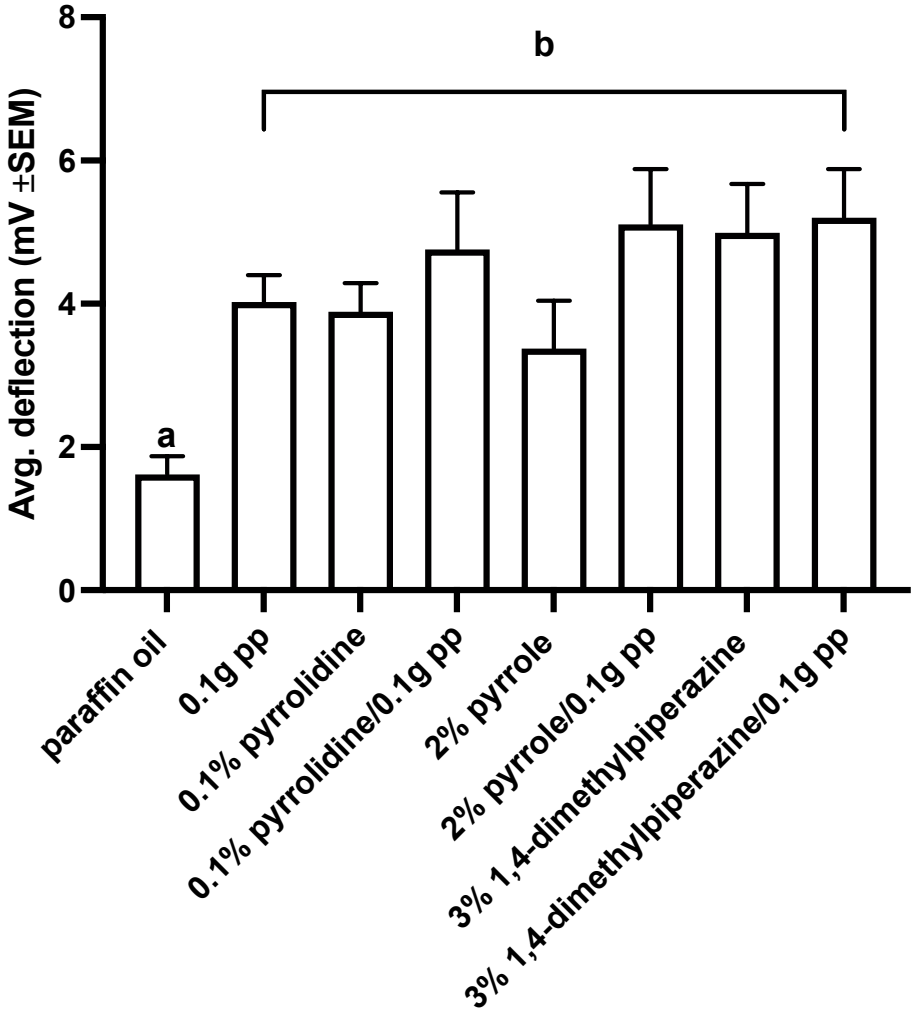

Figure 3.6 Results of EAGs combining approximate EC_{50} values of three repellents that antennae were most sensitive to, alone and in combination with pollen patty (pp). Letters indicate columns that differ significantly, calculated via ANOVA followed by Tukey's Multiple Comparisons test (n=10)

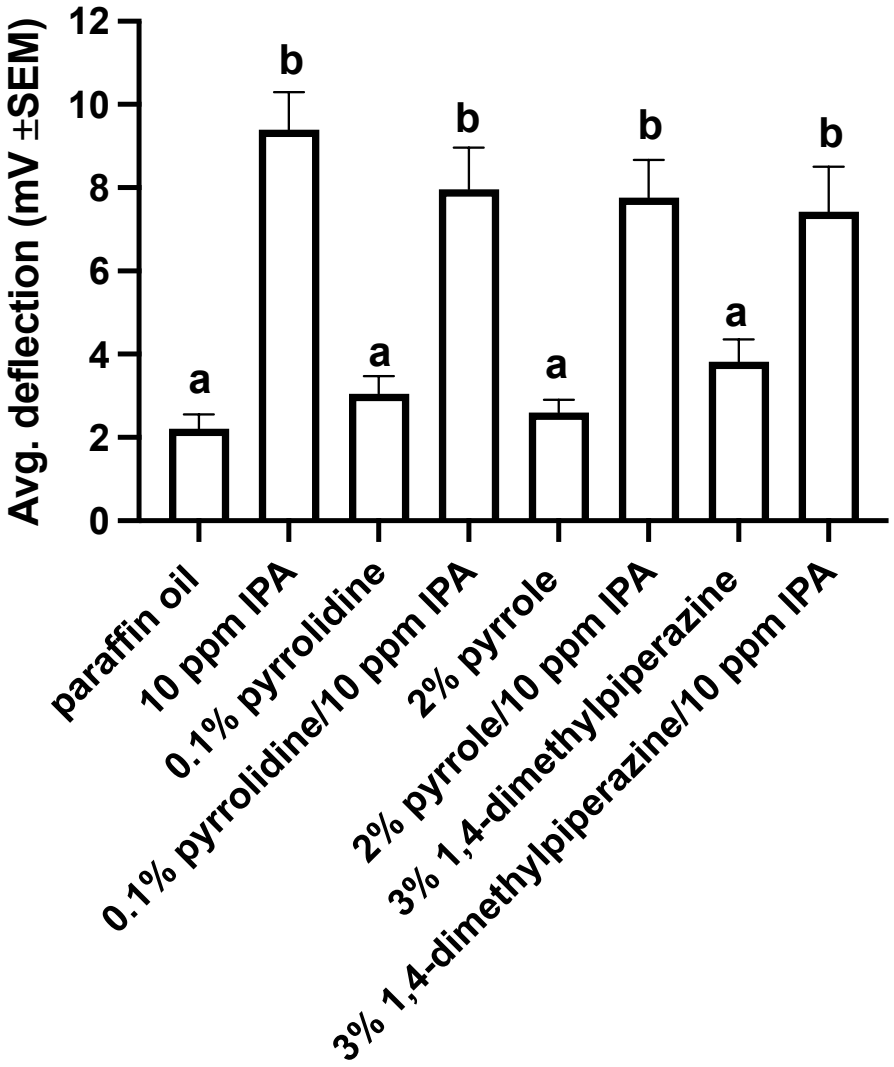

Figure 3.7 Results of EAGs combining approximate EC_{50} values of three repellents that antennae were most sensitive to, alone and in combination with pollen patty (pp). Letters indicate significant differences calculated via ANOVA followed by Tukey's Multiple Comparisons test (n=10).

CHAPTER 4.

OPTIMIZING FEEDING AND PUPATION BIOASSAYS TO ASSESS THE EFFECTS OF INSECTICIDAL AND REPELLENT TREATMENTS ON *AETHINA TUMIDA* LARVAL DEVELOPMENT AND PUPATION SUCCESS

4.1 ABSTRACT

The introduction and spread of the invasive small hive beetle (*Aethina tumida*) has been an ongoing concern to beekeepers in the United States for over 20 years. European honey bee (*Apis mellifera*) colonies are an ideal host to *A. tumida*, providing a nutrient rich environment that is protected from the elements and ideal for beetle reproduction. Although various management techniques and chemical treatments for *A. tumida* have been developed, understanding the efficacy of these treatments and techniques and how they impact *A. tumida* biology is limited. Throughout this study, a variety of methods for impairing *A. tumida* development and delivering insecticidal, repellent, or antifungal treatments were examined. A series of *A. tumida* larval feeding bioassays developed and optimized feeding pellets for delivery of insecticidal treatments and revealed that *A. tumida* larvae are sensitive to the two of the three common Varroa mite (*Varroa destructor*) treatments used in hives, which are coumaphos (EC_{50} = 25.6 ppm) and *tau*-fluvalinate (EC_{50} = 21.2 ppm), and also demonstrated that *A. tumida* were more sensitive to the pyrethroid compounds permethrin (EC_{50} = 3.37 ppm), deltamethrin (EC_{50} = 2.69 ppm), and bifenthrin (EC_{50} = 0.365 ppm), which have been used in targeting *A. tumida* in the past. Additional feeding bioassays revealed that the antifungal drug Amphotericin B, which is known to be effective in treating *Kodamaea ohmeri* infections, could be delivered to *A. tumida*

larvae and through injections and to *A. tumida* larvae and adults via feeding pellets. Finally, two types of pupation bioassays were developed to test the effects of several insecticidal and repellent treatments on pupation burrowing and pupation success, revealing that soil coumaphos and permethrin treatments over 50 ppm led to larval death, which burrowing depth was lessened in soil treated with these compounds. In pupation success bioassays, it was found that deltamethrin treatment at 50 ppm resulted in high larval mortality on the top of the soil, whereas treatment with several other insecticidal and repellent compounds did not reduce burrowing or prevent pupation. Overall, this work details specific toxicity information regarding common insecticidal treatments found in the apiary setting study and also provides groundwork and methods for testing insecticidal and repellent compounds on *A. tumida* larvae in in the future.

4.2 INTRODUCTION

Aethina tumida adults have been invading *Apis mellifera* hives in the United States since 1998 (Elzen *et al*., 1999), but consistently effective management options have remained somewhat limited throughout this time, though not for lack of effort. There are several key difficulties that make targeting *A. tumida* a complicated task, the first of which is that any treatment used within the hive environment has the potential to harm the *A. mellifera* colonies being treated. Thus, one of the most popular management methods to date is trapping, which mostly targets adults and only reduces adult numbers (Hood, 2011). Although reducing adult beetle populations in the apiary environment is unequivocally beneficial, *A. tumida* larvae are responsible

for most of the hive damage incurred by *A. tumida* infestations (Hayes *et al*., 2015). One *A. tumida* female can lay 1,000-2,000 eggs, so a few adult beetles can still generate numerous larvae (Sommerville *et al*., 2003).

Despite the potential risks of chemical treatments, there are several synthetic chemicals that are commonly used in apiary settings in the context of *Varroa destructor* control, including the octopamine receptor agonist amitraz (Blenau *et al*., 2012), the organophosphate and acetylcholinesterase inhibitor coumaphos (Elzen *et al*., 2004), and a sodium channel modulator and pyrethroid *tau*-fluvalinate (Baxter et al., 1998). As early as 1999, coumaphos was evaluated and recommended for use in *A. tumida* management (Elzen *et al*., 1999), but it is now known that coumaphos can have numerous detrimental impacts on *A. mellifera* colonies, ranging from reproductive damage to both queens and drones (Chaimanee *et al*., 2016), lifespan reduction (Wu, *et al*., 2011), and olfactory impairment (Williamson, *et al*., 2013) to buildup in wax over time (Bajuk *et al*., 2017). Other chemical treatments that have been used exclusively for *A. tumida* management are pyrethroids, the most popular of which is permethrin (GuardStar), which has been used in soil drenching (Hood, 2004). However, pyrethroids can also be toxic to honey bees, causing a host of health issues, including reproductive system impacts, reduction in foraging, learning, and olfactory functions (Decourtye *et al*., 2004), which is likely the result of the suppressed neuronal activity that results from pyrethroid exposure (Zhou *et al*., 2011).

Repeated exposure to similar chemistries over the years has led to organophosphate and pyrethroid resistance, which has been documented in *A. tumida* adults (Kanga *et al*., 2021a). In one study it was demonstrated that *A. tumida* adults

developed 5.4-fold *tau*-fluvalinate resistance and 43.7-fold coumaphos resistance over a 10-year time period (Kanga *et al*., 2021b). Within the last year, the first study examining *A. tumida* resistance mechanisms was performed using insecticides alone and with synergists, revealing that the likely mechanism of resistance to these compounds is the activity of esterase and mixed function oxidases (Kanga et al., 2021b), but more work is needed to better understand *A. tumida* biology and responses to insecticide treatments. Resistance development aside, weather conditions can lessen the effectiveness of soil treatments (Levot and Haque, 2006). It is also difficult to time when the majority of larvae will be exiting the hive for pupation; thus, soil treatments can be highly ineffective and necessitate frequent re-application (Kanga and Somorin, 2012), all of which leads to the second important difficulty in establishing effective *A. tumida* control methods: the *A. tumida* lifecycle and the uniquely adapted biology of this pest to the apiary environment.

The *A. tumida* lifecycle takes place both inside the hive (eggs and larval development) and outside of the hive (pupation and emergent adults; Neumann *et al*., 2016). A further management complication is added by the fact that *A. tumida* adults are ultimately scavengers and can survive and reproduce on a variety of fruits (Buchholz *et al*., 2008), as well as in the hives of other bee species, including *Bombus impatiens* (Graham *et al*., 2011) and several species of melponine bees (Bobadoye *et al*., 2018). Since *A. tumida* populations can thrive outside of the managed apiary environment, it is virtually impossible to eliminate *A. tumida* from a geographic area. Additionally, there is not an established *A. tumida* economic threshold to inform beekeepers regarding appropriate treatment measures to incorporate based on

infestation severity. Because *Varroa destructor* is such a disastrous *A. mellifera* pest, resources and research have been highly focused on management for this pest, which *A. tumida* has received relatively little attention. Therefore, there are many aspects of *A. tumida* biology and management options that are yet to be explored.

One of the most fascinating aspects of *A. tumida* infestations is the fungal symbiont *Kodamea ohmeri* (Amos *et al*., 2018), which is an ascomycetous yeast that is closely related to *Candida albicans* (Leemon, 2012). Although limited work has been done examining the importance of *K. ohmeri* to *A. tumida* growth and development, this fungus has been isolated from each *A. tumida* life stage, starting in the mucilage of the eggs to external surfaces and the digestive tracts of adult beetles (Amos *et al*., 2018). The results of one study indicated that *K. ohmeri* is merely a facultative symbiont and is unnecessary for the successful completion of the *A. tumida* lifecycle, however, this study was not carried out for more than one generation and was performed under laboratory conditions (Amos *et al*., 2019). Even though *K. ohmeri* may simply be a facultative fungal symbiont, its prevalence throughout the *A. tumida* lifecycle and its role in facilitating the breakdown of hive products and subsequent production of volatiles that are attractive to *A. tumida* adults (Benda *et al*., 2008), suggests that further steps could be taken to test repression of this fungus throughout the *A. tumida* lifecycle to better determine the value of *K. ohmeri* to the *A. tumida* lifecycle.

In view of the lack of knowledge pertaining to *A. tumida* chemical treatments, along with the need for clearer insights regarding the function of *K. ohmeri* throughout the *A. tumida* lifecycle, the following study was developed. The goal of this work was

to establish effective methods for *A. tumida* feeding and soil bioassays and to understand whether or not fungicide feeding could impact internal *K. ohmeri* presence and detrimentally affect *A. tumida* development and survival. The first step in this work was to develop a feeding bioassay that both sustained larval development and was an effective means of drug delivery. Building upon the successful feeding bioassays developed, a series of experiments testing the effects of the fungicide Amphotericin B were carried out. Amphotericin B is known to inhibit *K. ohmeri* and has been principally used in human medicine in cases of fungal infection (Kanno *et al*., 2017). Injection of Amphotericin B into third instar *A. tumida* larvae and subsequent *K. ohmeri* tests were also performed. Finally, two types of soil toxicity bioassays were developed, with one bioassay focused on pupation depth and the other focused on pupation success. These bioassays tested coumaphos and permethrin, along with several repellent compounds, to determine whether or not such treatments could deter larvae from burrowing and pupating.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals

Amitraz (>98%), bifenthrin (99%), deltamethrin (>99%), and permethrin (47.6% cis; 50.4% trans) were all purchased from Chem Service Inc. (West Chester, PA, USA). Coumaphos (99%), *tau*-fluvalinate (98.7%), pyrrole (98%), pyrrolidine (≥99.0%), amphotericin B (solubilized, suitable for cell culture), and potassium chloride (≥99.0%)

were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Sec-butyl 2-(2-hydroxyethyl) piperidine-1-carboxylate (Picaridin™; 98%) was purchased from AmBeed (Arlington Heights, IL, USA). Sodium chloride (≥99.0%), HEPES (≥99.0%), calcium chloride dihydrate (≥99.0%), Difco Sabouraud Dextrose Broth, and the acetone (HPLC grade) used for dilution of all compounds in this study was from Fisher Scientific (Hampton, NH, USA). AP23® (Artificial Pollen) patties were purchased from Dadant and Sons Inc. (Hamilton, IL, USA). All granulated sugar (99% sucrose) used was from the Smidge and Spoon™ brand (Novi, MI, USA). Soy protein (isolated)was purchased from MP Biomedicals LLC (Solon, OH, USA). Apex™ Bioresearch Products Agarose (General Purpose LE) and Apex™ chloramphenicol were purchased from Genesee Scientific (San Diego, CA, USA).

4.3.2 Insects

Aethina tumida adults were collected from the Prices Fork apiary in Blacksburg, VA, USA in August of 2018 to start a laboratory colony, which, at present, contains F10 to F15 adult beetles and F11 to F16 larvae. Once larvae are observed to reach the third instar, pupation conicals are made by adding approximately 30 third instar larvae to a 50 mL conical containing a moistened 1:1 sand/soil and covered in parafilm with small ventilation holes. Because larvae often burrow down the sides of pupation conicals, it is easy to visualize at least some of the pupae to monitor adult emergence. When visible pupae emerge as adults, the conicals are left for several days to allow the cuticles of the newly emerged adults to sclerotize, then then adults are added to an adult rearing container of the appropriate generation. The colony is maintained in a dark environmental chamber (Thermo Scientific, Waltham, MA, USA) at 28°C and

greater than 70% relative humidity. Additionally, a specimen from the laboratory colony has been added to the Virginia Tech collection (Catalogue Number: VTEC000004965).

4.3.3 Feeding bioassays

To carry out feeding bioassays, the first step was optimization of feeding pellets. Although ground pollen granules were first testing in formulation of food pellets, these granules were very large and, even after being frozen and ground to a fine powder with a mortar and pestle, did not readily disperse in agarose mixtures. Thus, tests with soy protein powder in agarose were carried out, along with tests of various sugar concentrations in agarose, to determine optimum nutrient ratios to keep larvae alive. Sugar concentrations at 10%, 20%, 30%, 40%, and 50% were tested, along with positive controls of pollen patty and agarose pellets with no sugar or protein. Soy protein was tested using concentrations of 1.5%, 3%, 6%, 9%, and 12%, at which point the powder became too thick to dissolve into the agarose. To make these pellets, 50 mL of a 1% agarose solution was made in a 250 mL Erlenmeyer flask and appropriate volumes of either sugar or soy protein were added while the solution was still above 65°C. Transfer pipettes were used to add the agarose solutions to wells of a flat-bottomed 96-well microplate, with each well holding approximately 300μL of the agarose mixture. Once the agarose mixture in the wells solidified, a micro spoon spatula was used to remove pellets, which readily dislodged and retained their cylindrical shape.

After testing the sugar and protein pellets on their own, it was decided that the feeding pellets would be made using a 25% sugar and 2% soy protein solution, which

was distributed in two mL volumes into 4-mL Fisherbrand disposable culture tubes (Fisher Scientific, Waltham, MA, USA). A Thermolyn Dri-bath heater (Thermo Scientific, Waltham, MA, USA) was used to melt the agarose to approximately 80°C and keep it in its liquid form while the different concentrations of the test insecticides were added. Parafilm was added to the top of the agarose tubes allowing each treatment to be vortexed prior to making feeding pellets, as described above.

Five late first instar/early second instar *A. tumida* larvae were added to 2oz clear plastic souffle/portion cups, with a small square of mesh glued to an approximately 1-cm^2 hole in the lid for ventilation. Prior to the start of experiments, the average weights of each container of larvae were obtained, as was done at the end of all feeding bioassays. The experiments ended when larvae reached the late third instar stage, which was easily discernable, visually, and was generally reached 7-10 days after the experiment commenced. All larvae were frozen for 24-hours prior to disposal after each experiment and at least three replicates were performed for each test compound (Figure 4.1A-F).

4.3.4 Antifungal feeding and injection bioassays

The first test carried out using amphotericin B treatments was a test of feeding effects upon *A. tumida* larval development (Figure 4.1J). Because *A. tumida* instar can be determined based upon head capsule width, five first instar larvae per treatment were obtained and head capsule width was measured every other day for 7 days atop a chilled aluminum block covered in a Kimwipe. *Aethina tumida* first instar head capsules range from 250-350μm, while head capsule widths range from 490-670 μm for second instar larvae, and 790-1170 μm for third instar larvae (Amos *et al*., 2018).

Treatments for this bioassay consisted of a pollen patty control, an amphotericin B pellet (1 ppm), and a pellet containing the vehicle control (0.1% DMSO). Additionally, two sets of larvae were only fed on treated pellets for 24 hours and then switched back to pollen patty for the remainder of the experiment to better understand the effects of limited fungicide feeding.

The second set of experiments fungicide feeding tests were performed by feeding second instar larvae pollen patty, agarose pellets, 1-ppm Amphotericin B and 0.1% DMSO (negative control) pellets for 24 hours, after which larvae were surface sterilized beneath a laminar flow hood following the methods of Amos *et al*. 2018. Briefly, larvae were submerged for 30 seconds at a time in 70% ethanol, sterile water, 5% bleach in aqueous polysorbate (Tween 80, 0.25%), and finally two rinses of sterile water, after which they were placed in a 1.7mL Eppendorf tube containing 250μL of sterile water. A plastic pestle and mechanical mortar were then used to homogenize the larva and the homogenate was diluted 5-fold prior to spreading 50 μL on a Petri dish of Sabouraud's Dextrose Agar (SDA). *Kodamaea ohmeri* is known to successfully grow on SDA and can easily be morphologically identified (Leemon, 2012) and 0.5 mg/mL of chloramphenicol is also added to the SDA in order to inhibit bacterial growth. After spreading homogenate, plates were incubated in the dark and images obtained after 48- and 72-hour intervals using an iPhone 11 camera beneath a single light source. Colonies were quantified using ImageJ software (imagej.nih.gov), which allowed conversion of color photos to 8-bit grayscale images. Colonies were then quantified and outlined under circularity settings of 0.30-1.00, with edges excluded.

Fungicide feeding was also carried out with adult beetles. Prior to feeding, adults were surface sterilized and then fed for two days on each of the treatments described above. Adults then were all fed upon pollen patty for the next 10-12 days and egg laying was monitored over five days. The adult beetles were then surface sterilized again, homogenized, and plated, and colonies were quantified as described above. Eggs were also counted over this time period.

The next *K. ohmeri* repression experiments were performed by modifying the delivery system to melted pollen patty, as the goal was to try to feed larvae on an amphotericin B treatment for the entirety of their development. To accomplish this, pollen patty was melted into a 0.1% agarose solution and larger agarose disks were created using 3.5-cm Petri dishes. Similar feeding disks were made using 0.1% DMSO and 2 ppm amphotericin B. Glass microscope slides containing *A. tumida* eggs were added to jars with each treatment and larvae were then fed to the third instar, after which internal *K. ohmeri* colony counts were quantified per the above methods. In a follow up experiment, amphotericin B levels were raised to a range of 10 to 1,000 ppm, which was again fed to test larvae throughout the entirety of their development. Higher homogenate dilutions (10, 25, and 50-fold) were also tested using these third instar larvae. Several untreated wild-caught *A. tumida* adults and larvae were also compared lab-reared adults and larvae, at homogenate dilutions of 25 and 50-fold.

Finally, *A. tumida* injections were performed to test the delivery of 50 nL of 1 ppm amphotericin B, 0.1% DMSO, and CNS buffer control (157 mM NaCl, 3mM KCl, 2mM $CaCl_2$, 4mM HEPES) using a nanoliter injector (World Precision Instruments, Sarasota, FL, USA). After each injection, 50 nL of treatment was expelled onto a

Kimwipe to ensure that the needle had not become blocked. Ten second instar larvae were tested per treatment and mortality was assessed after two days when all larvae had reached their third instar. Additionally, injected second instar larvae were homogenized and tested for internal *K*. *ohmeri* counts after treatment delivery and compared with third instar larvae that had survived for at least two days post injection.

4.3.5 Pupation depth bioassays

To test effects of insecticide treatments on pupation depth, 12 in. glass tubes were procured, inches were marked out on the tubes, and it was found that 1 inch of the tube held approximately 1.4 g of autoclaved 1:1 sand/soil mix. A 50 mL conical was used to form a base for each tube, with a hole made by a dremel in the top of the conical lid to allow the tube to glass fit tightly. The inside of the conical was sprayed with 2-3 pumps of water and was then filled approximately 2/3 full. The glass tube was then inserted into the soil and another pump of water was added before the conical was closed. The glass tube was next filled with the 1:1 sand/soil mixture up to the three-inch mark (no additional water added). All treatments were made by weighing out 1.4 g of sand/soil and placing this under the fume hood on small squares of aluminum foil. One milliliter of each treatment (and 1 mL acetone control) was pipetted (500μL at a time) on to the sand/soil, which was moved using the foil to help coat sand and soil particles more thoroughly. Once all treatments were administered and the sand/soil was dry, it was poured into the glass tube, and another inch of sand/soil was added over the treatment. A single third instar larva was then added to each tube (five tubes per replicate) and the tops of the tubes were covered in parafilm, with a single small hole added for ventilation. All tubes were then placed in a 50 mL conical holder

and incubated at 28°C until pupae were observed on the sides of the tubes. Because the tubes were so narrow, visualization of pupae was not difficult. As tubes were empty, the pupation depth of each larva was noted and measured.

4.3.6 Pupation success bioassays

Pupation success bioassays were developed to assess whether or not larvae would burrow past a treated range and whether or not pupation could successfully occur after passage through the treatment. Conical tubes (50 mL) were used for this experiment and centimeter increments were marked out on the sides of the tubes. It was found that a 1cm conical depth equated to approximately 5 grams of sand/soil and 2 mL of each treatment was prepared and added to the sand/soil mixture on aluminum foil beneath a fume hood. The 2 mL treatment (and acetone control) saturated the 5 grams of soil, and movement of the foil helped move the sand/soil to help ensure that it was well coated in treatment. The 50mL conicals were again dampened using a spray bottle and sand/soil was added up to 3-cm below the top of the conical. Once the treated sand/soil was dry, it was added to the conical and 1 cm of clean sand/soil was added on top of this. Twenty third instar larvae were added to each conical and parafilm was used to cover the conical tops, with several small holes added for ventilation. All conicals were kept incubated at 28°C until pupae could be observed along the sides of the conicals (approximately 2 weeks), after which they were emptied and larvae/pupae above, within, and below the treatment were counted and mortality was noted.

4.4 RESULTS

4.4.1 Feeding bioassays

The results of sugar feeding pellets showed that there was 100% survival in both replicates at the 20%, 40% and 50% concentrations, however, average weight gain in all treatments was far lower than weight gain observed with pollen patty feeding, with an approximately 8 mg difference between all sugar treatments and pollen patty (Table 4.1). With the feeding pellet made solely of agarose, 40% mortality and weight loss were observed. Overall, there was no marked difference between weight gain, with overall average weight gain of 1.88mg (10%), 1.4mg (20%), 2.9mg (30%), 1.5mg (40%), and 2.2mg (50%). Interestingly, the highest average weight gain and mortality (30%) occurred with the 30% sugar treatment. Although food was provided *ad libitum* it is possible that surviving larvae may have cannibalized their dead or dying fellow test subjects, thus obtaining some protein, and growing a bit larger. Regardless, this test indicated that as long as a sugar source was present, the concentration did not greatly influence larval survivorship or growth.

The results of the soy protein feeding bioassays told a different story. Once again, larvae thrived on the pollen patty positive control and languished with 80% mortality in the agarose negative control. Although average weight gain for each protein treatment was again much lower (1.2 to 1.9 mg) than average weight gain for pollen patty fed larvae (13.3 mg), survivorship was also quite low with average mortalities ranging from 87 to 93% for all soy protein treatments. Again, it appeared that regardless of soy protein concentration, mortality and weight gain did not greatly differ, and a carbohydrate source was imperative for beetle survival and growth.

Based upon the above information, an agarose pellet containing 25% sugar and 2% soy protein was tested and used for all insecticide and feeding bioassays. At these sugar/protein ratios, control larvae thrived and reached the third instar, and larval development was comparative to that observed with pollen patty feeding. Of the six chemicals tested (Figure 4.1A-F), the most potent oral half maximal-lethal concentration (LC_{50}) resulted from bifenthrin feeding (LC_{50}=0.465 ppm) followed by the other pyrethroids: deltamethrin (LC_{50}=2.69 ppm), permethrin (LC_{50}= 3.47 ppm), and *tau*-fluvalinate (LC_{50}= 21.2 ppm; Figure 4.3). The common in-hive treatment coumaphos was only slightly less potent than *tau*-fluvalinate, with an LC_{50} of 25.6 ppm. Finally, an LC_{50} value could not be established for amitraz, with high larval survival at concentrations of 100 ppm (Figure 4.3).

4.4.2 Antifungal feeding and injection bioassays

The larval growth antifungal feeding tests revealed that all larvae reached second instar head capsule widths by day 3. Growth trends appeared similar for all insects until day 7, at which time the pollen patty fed larvae and the larvae that had only been fed upon test compounds for 24 hours reached their third instar, while larvae fed on the treatments through the entirety of the experiment remained in their second instar until day 9 (0.1% DMSO) or died before day 9 (1ppm amphotericin B; Figure 4. 2). Comparison of homogenizing and plating of 1 vs. 5 larvae was also carried out, with a colony counts of approximately 132 for the 1 larva homogenate and 378 for the 5 larvae homogenate.

Based on the above experiment, second instar larval feeding in the second instar amphotericin B feeding experiment took place for 24-hours prior to larval

homogenization, supernatant plating, and plate incubation. After 48-hours, it was found that colony counts from SDA with 0.1% DMSO, 1ppm amphotericin B, and 0.1% DMSO were comparable, while the colony counts from the pollen patty feeding treatement varied widely, with no significant difference between treatments (Figure 4.3A). A similar variability was observed in the adult fungicide feeding bioassays, with SDA plates from larvae fed on pollen patty having fewer *K. ohmeri* colony counts than adults fed on amphotericin B and wide variability between treatments, especially the 0.1% DMSO and agarose treatments (Figure 4.3B) and a Wilcoxin signed rank test revealed no significant difference between treatments.

The results from the pollen patty/agarose disk feeding bioassays, which delivered amphotericin B for the entirety of development, were also rather perplexing. In the first set of feeding bioassay results, it was seen that 2 ppm amphotericin B feeding treatments yielded higher colony counts than both the pollen patty agarose and 0.1% DMSO controls (Figure 4.4A). It was then speculated that perhaps the concentration of amphotericin B was too low to inhibit *K. ohmeri*, so the concentration was raised to a range of 10-1,000 ppm, however, average colony counts were still comparable and the highest average colony counts resulted from the 1,000ppm amphotericin B treatment (Figure 4.4B). It was next hypothesized that perhaps the homogenate dilution (5-fold) was too high to allow for discrimination between treatments, so dilutions of 5-fold, 10-fold and 25-fold were tested using an untreated beetle (Figure 4.4C). When a numeric difference was observed between the 5-fold dilution had approximately 1,000 more colonies than the 10-fold and 25-fold dilutions, the decision was made to further dilute the homogenate to 50-fold. Subsequent tests

of 25-fold and 50-fold dilutions of several concentrations of amphotericin B yielded somewhat stochastic results, with lower average colony counts for the 25-fold dilutions of the 10 and 100 ppm amphotericin B treatments (Figure 4.4D). Comparisons of laboratory-reared and wild caught beetles were also singularly unpredictable, as 1 or 0 colonies were observed at most 25-fold and 50-fold dilutions, with the notable exception of one wild caught beetle that had a colony count of 1 at the 25-fold dilution and 883 at the 50-fold dilution.

Beginning injection bioassays proved to be a somewhat delicate undertaking, as determining the best way to inject beetles without killing them was somewhat difficult at first. Additionally, if the aluminum block was too cold and injection took too long the larvae could freeze. However, over nine replicates of 10 beetles per treatment, survivorship greatly increased, with mortality ranging from 0-30% for the first rep and from 70-90% by the sixth rep. Injections were most successful when carried out dorsally between the second and third thoracic segments, as abdominal injections tended to result in more cuticle tearing and hemolymph leakage. When *K. ohmeri* levels (50-fold dilution) were tested second instar larvae directly after injection, colony counts of 6, 14, and 5 resulted from CNS buffer, 0.1% DMSO, and 1ppm amphotericin B treatments, respectively. Conversely, injected third instar larvae allowed to feed on pollen patty for two days post-injection showed colony counts of 3 for the CNS buffer, 27 for 0.1% DMSO, and 26 for 1ppm amphotericin B.

4.4.3 Pupation bioassays

Overall, pupation depth bioassays revealed that larvae in the acetone control tended to burrow the entire 12 inches of the glass tube and into the conical below. The

same deep burrowing was observed at 10 ppm coumaphos, 10-20 ppm permethrin, and 100 ppm picaridin treatments. However, at 100 ppm coumaphos burrowing depth tended to be much shallower, with an average of 3-4 inches. Similarly, treatments of permethrin at 50 and 100 ppm essentially stopped beetles from burrowing past the treatment (Figure 4.5A-C). Unfortunately, several subsequent pupation depth bioassays resulted in larvae that would not burrow and essentially desiccated and died atop the soil. One hypothesis for this lack of burrowing is that these larvae were potentially not ready to pupate, or moisture levels were incorrect within the system. Since these later reps occurred in the winter months, a seasonal effect could also be possible. One solution to explore in the future would be to add 3rd instar larvae to a container with a food source and soil and wait until they moved away from the food to add them to the pupation tubes.

Preliminary pupation success experiments have thus far appeared to be a promising pupation test method. Despite the fact that most of the control larvae from the first pupation depth experiment escaped, the results still helped inform treatments, as 85% of larvae successfully pupated beneath the 100 ppm coumaphos, whereas only 45% larvae successfully burrowed and pupated at the 100 ppm permethrin, and all larvae died in or above the 100 ppm deltamethrin treatment. The next range of treatments revealed that at 50 ppm deltamethrin 75% of larvae remained alive but were slowly desiccating and only one larva had successfully pupated. None of the other treatments, including picaridin (100-200 ppm), pyrrole (100-200 ppm), and pyrrolidine (100-300 ppm; Figure 4.1B, E-I) reduced pupation success to below 80%

(Figure 4.5D). Future tests could implement the same methods to both increase the concentration of these chemicals and test new compounds.

4.5 DISCUSSION

Understanding how insecticides impact pests is crucial to establishing optimal treatment methods, and *A. tumida* is clearly a pest to which more study needs to be devoted. Even though chemical treatments have been used over the years, these tended to be chosen based on chance and ease of availability, as seen in the use of coumaphos, a treatment that was already widely used in hives and was already well-known to beekeepers (Elzen *et al*., 1999). However, results of this study alone indicate that coumaphos is less effective than several pyrethroids, including the common hive-treatment *tau*-fluvalinate. As *A. tumida* resistance to *tau*-fluvalinate has been documented (Kanga *et al*., 2021b) it is possible that the 6- to 20-fold difference in the LC_{50} values between *tau*-fluvalinate and the other pyrethroids used in this study could be a product of resistance development in local populations from which the lab colony was collected.

Previous resistance studies with coumaphos and *tau*-fluvalinate were performed using glass vial bioassays (Kanga *et al*., 2021b), but the feeding bioassays developed in this study could provide a useful way to measure oral toxicity and monitor resistance development in future studies. *Aethina tumida* toxicity tests in the past have primarily performed via contact through glass vials or topical applications (Kanga *et al*., 2021a&b; Bisrat and Jung, 2020; Kanga and Somorin, 2012), with one study

testing a drinking bioassay (Powell *et al*., 2020). Further, soil bioassays have been performed in the past, especially in reference to testing lime and diatomaceous earth, which essentially work to desiccate migrating larvae (Buchholz *et al*., 2009; Cuthbertson *et al*., 2013), but, in insecticide tests, beetles have been directly exposed to treated soil, rather than having the option to burrow through soil treatments (Kanga and Somorin, 2012). Knowing whether or not non-lethal soil treatments could still disrupt pupation would be useful information to uncover, and further soil tests, especially tests of less toxic repellent compounds (Larson *et al*., 2021) would be a beneficial continuation of this work.

Although oral delivery and injection of amphotericin B were not consistently effective in reducing internal *K. ohmeri* growth in *A. tumida* larvae and adults in this study, continued attention should be devoted to understanding this symbiotic relationship. *Kodamaea ohmeri* is present throughout the entire *A. tumida* life cycle, starting in egg mucilage, and it is thought that laying eggs in clutches may be to better ensure that all eggs are inoculated with this fungi (Amos *et al*., 2018). Even though one laboratory study concluded that *K. ohmeri* was merely a facultative symbiont, since *A. tumida* could still successfully survive and reproduce when external *K. ohmeri* was removed via surface sterilization (Amos *et al*., 2019), this is far from conclusive evidence that repression *of K. ohmeri* would not eventually be harmful to *A. tumida*, especially over multiple generations or in a field setting, which is yet to be studied. Sequencing the *K. ohemri* genome revealed hexos tramsporter and invertase genes, which means that *K. ohmeri* may be aiding *A. tumida* in metabolizing sucruose, since

the *A. tumida* genome was not found to contain a sucrose metabolism invertase (Tauber *et al*., 2019).

There are multiple strains of *K. ohmeri* that have been isolated from *A. tumida* (Benda et al., 2008), and this fungi has been even more widely studied in human medicine (Kanno *et al*., 2017). Thus, exploring the speicifc strains of *K. ohmeri* that may be present in *A. tumida* population, along with testing different antifungal drugs that have been used in medical settings, such as floconazole, posaconazole, 5-flucytosine, caspofungin, micafungin , and others (Al-Sweih et al., 2011; Tashiro *et al*., 2018), could help determine the optimal fungicide to deal with *K. ohmeri* in an apiariy setting. While acetic, lactic, and formic acid have all been shown to inhibit *K. ohmeri* and kill *A. tumida* adults and larvae (Schäfer et al., 2009), these treatments can be difficult to apply correctly and can be detrimental to brood and hive products (Rosenkranz *et al*., 2010). Examining the ability of specific fungicides to target and repress *K. ohmeri* is an important future step in understanding the beneifits to *A. tumida* derived by *K. ohmeri* and helping to repress the growth of this fungi in *A. mellifera* colonies.

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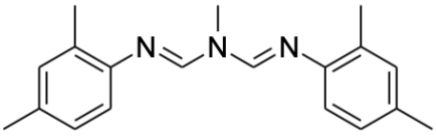
4.7 TABLES AND FIGURES

A



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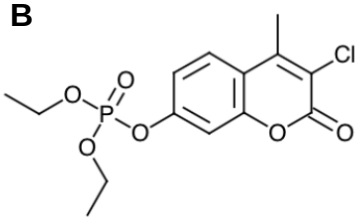

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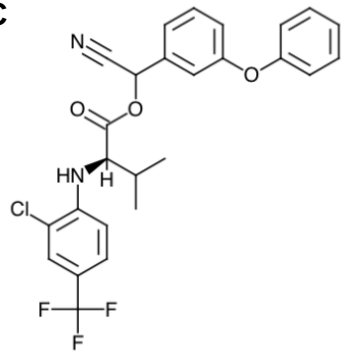

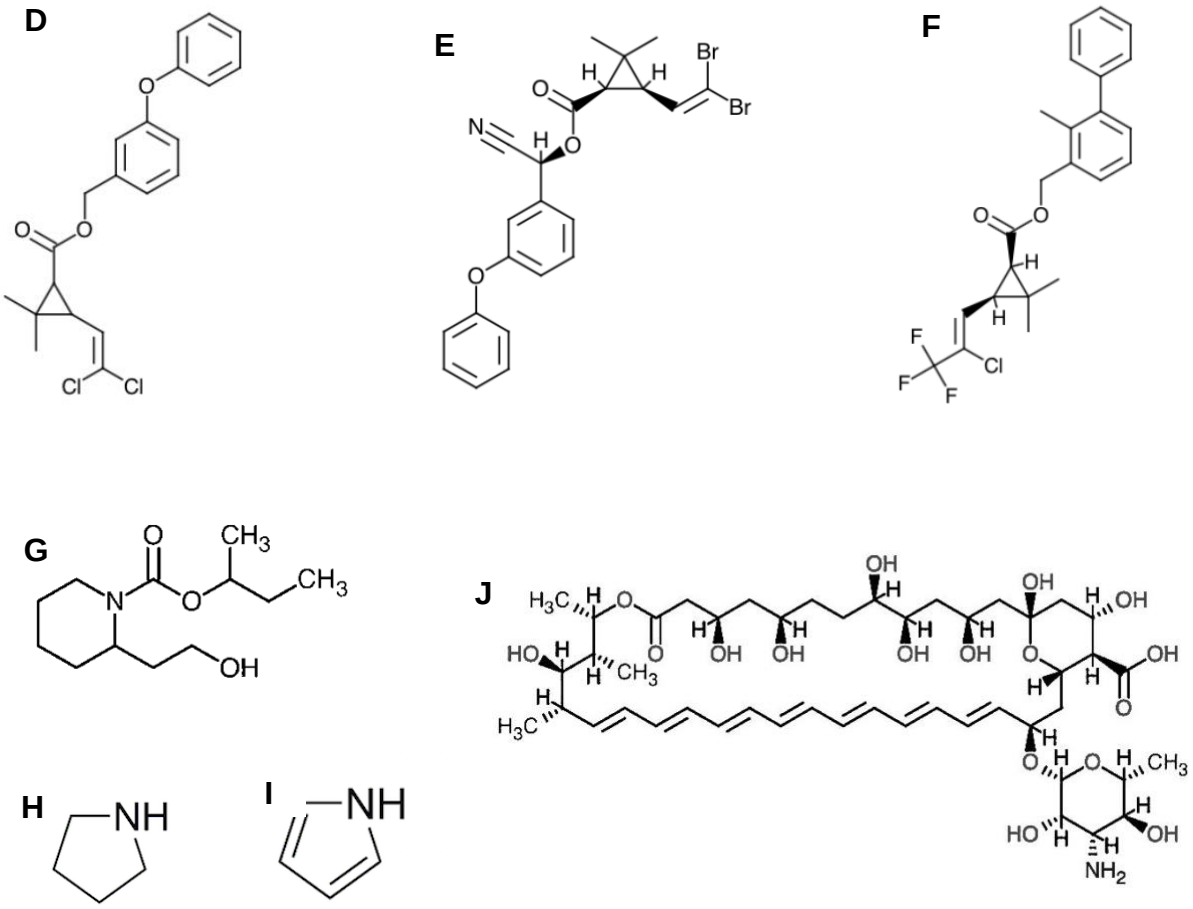

Figure 4.1 Chemicals: Amitraz (A), coumaphos (B), *tau*-fluvalinate (C), bifenthrin (D), permethrin (E), deltamethrin (F), picaridin (G), pyrrole (H), pyrrolidine (I), and Amphotericin B (J).

Table 4.1 Results of sugar feeding bioassays (2 reps combined).

Food	Avg. start weight (mg)	Avg. % Mortality	Avg. end weight (mg)	Avg. weight change (mg)
Agarose	5.3	40	4.59	-0.71
Pollen patty	3.7	10	14.3	+10.6
10% sugar	4.5	10	6.38	+1.88
20% sugar	3.8	0	5.2	+1.4
30% sugar	4	30	6.9	+2.9

40% sugar	3.9	0	5.4	+1.5
50% sugar	3.9	0	6.1	+2.2

Table 4.2 Results of soy protein feeding bioassays (3 reps combined).

Food	Avg. start weight (mg)	Avg. % Mortality	Avg. end weight (mg)	Avg. weight change (mg)
Agarose	1.4	80	4.5	+3.1
Pollen patty	1.2	17	14.9	+13.7
1.5% protein	1.3	87	2.5	+1.2
3% protein	1.1	93	3	+1.9
6% protein	1.3	93	3	+1.7
9% protein	1.4	87	3	+1.6
12% protein	1.3	87	2.5	+1.2

Table 4.3 Feeding bioassay toxicity Data (3-5 reps per treatment)

Compound	LC_{50} (ppm)	95% Confidence Interval	n	Rep #
Amitraz	>100	-----	60	3
Coumaphos	25.6	20.2-30.7	150	5
***tau*-Fluvalinate**	21.2	13.3-30.3	95	3
Bifenthrin	0.365	0.226-0.519	150	5
Deltamethrin	2.69	1.30-4.80	90	3
Permethrin	3.47	2.67-4.45	90	3

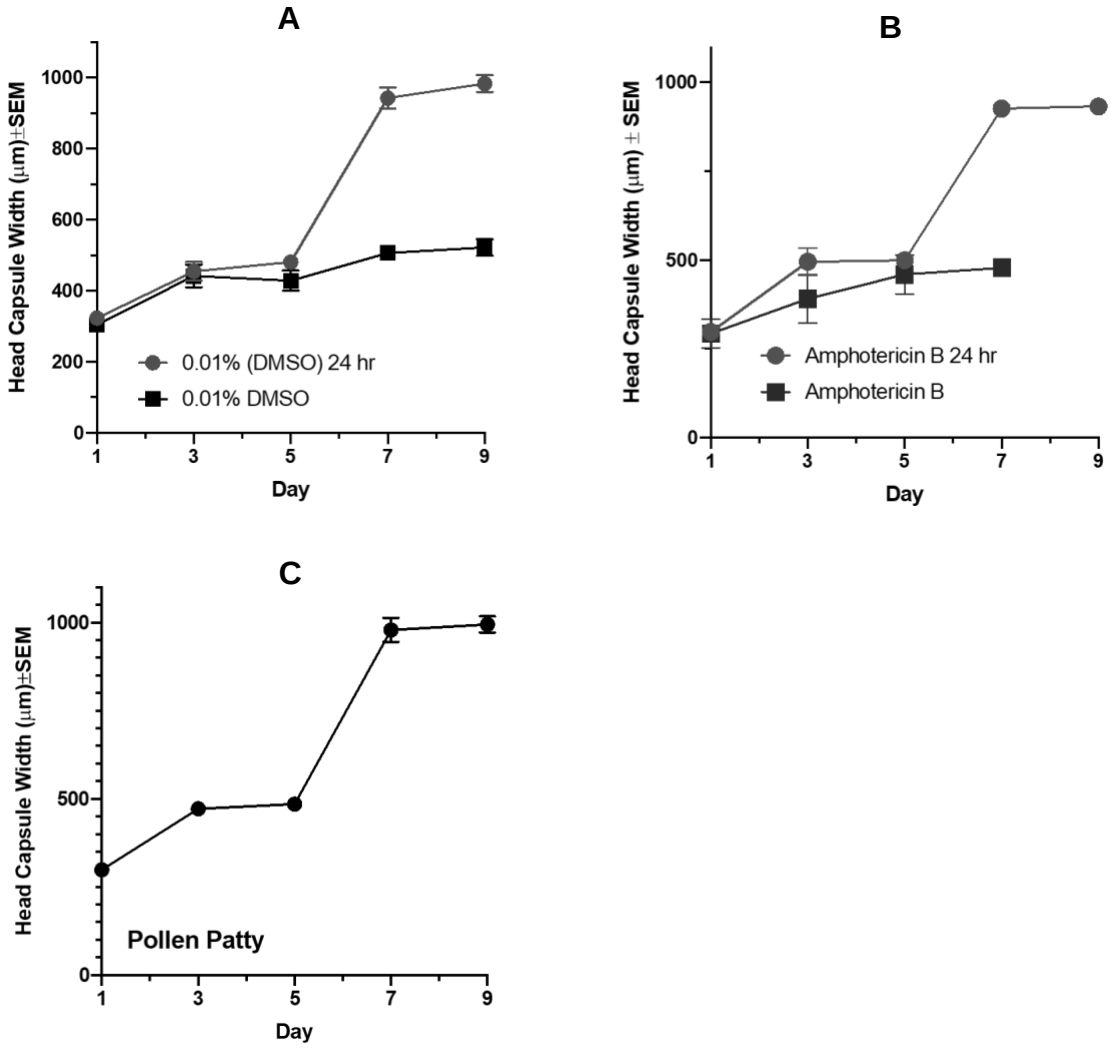

Figure 4.2 *Aethina tumida* growth based on head capsule width (first instar=230-350μm; second instar=490-670μm; third instar=790-1,170μm) measured every other day over a 9-day test period. Treatments consist of 0.1% DMSO until day 9 and 0.1% DMSO feeding for 24 hours followed by pollen patty feeding until day 9 (A), 9 day amphotericin B feeding and 24 hour amphotericin B feeding followed by pollen patty feeding until day 9 (B), and pollen patty feeding from days 1-9 (C).

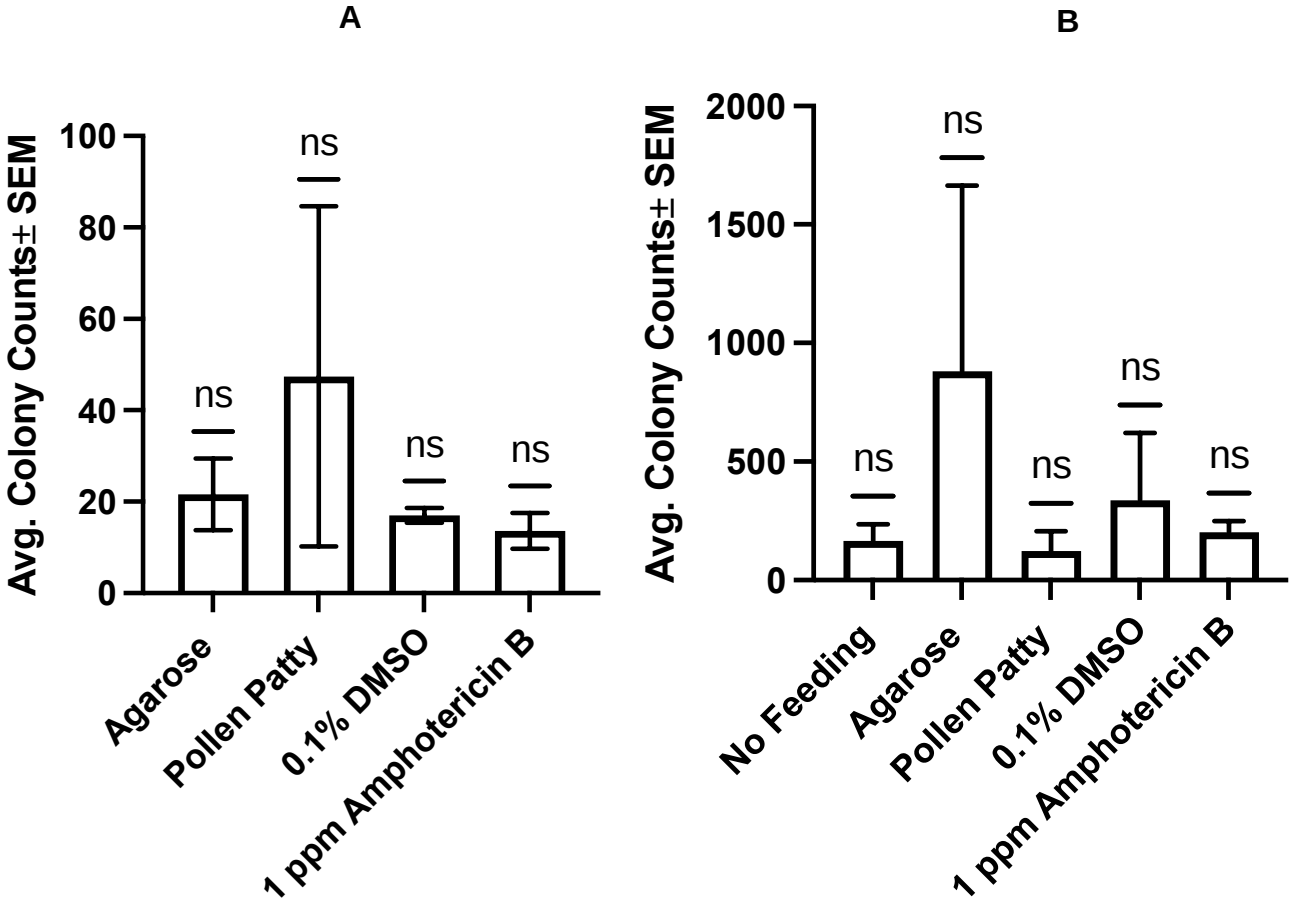

Figure 4.3 *Kodamaea ohmeri* colony counts 48 hours post-incubation resulting from *A. tumida* amphotericin B feeding tests, as observed in (A) second instar larvae (n=5) and (B) adult beetles (n=4), with results analyzed via Wilcoxon signed-rank test, no significance.

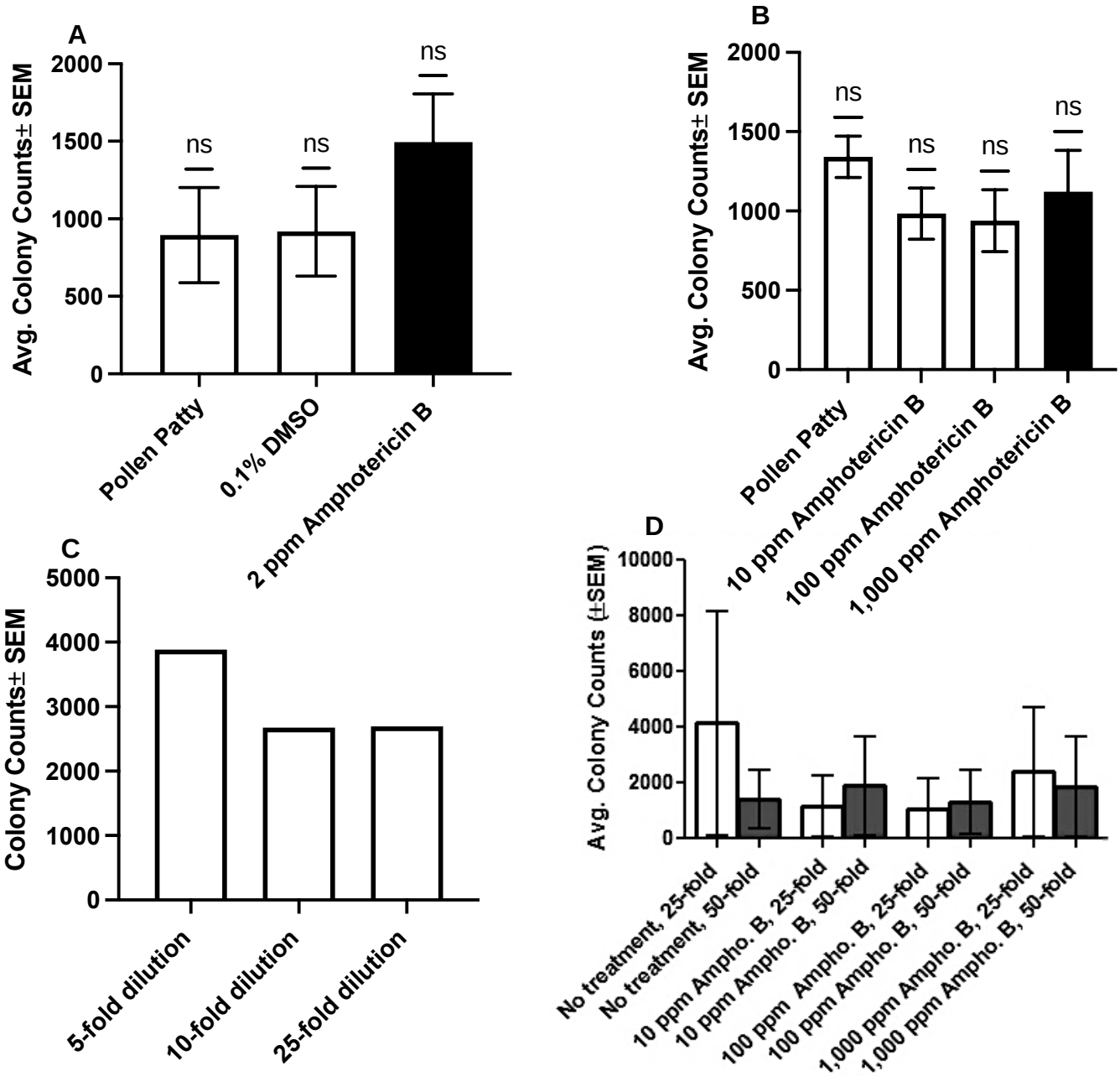

Figure 4.4 Results of fungicide feeding throughout *A. tumida* larval development through pollen patty/agarose feeding discs, starting with lower amphotericin concentrations (A; n=4) and ranging to higher concentrations without use of DMSO as the vehicle control (B; n=4). Figures C and D depict attempts to further dilute amphotericin B homogenate. Wilcoxon signed-rank

test revealed no significant difference (Figures A and B), not enough reps for testing Figures C and D.

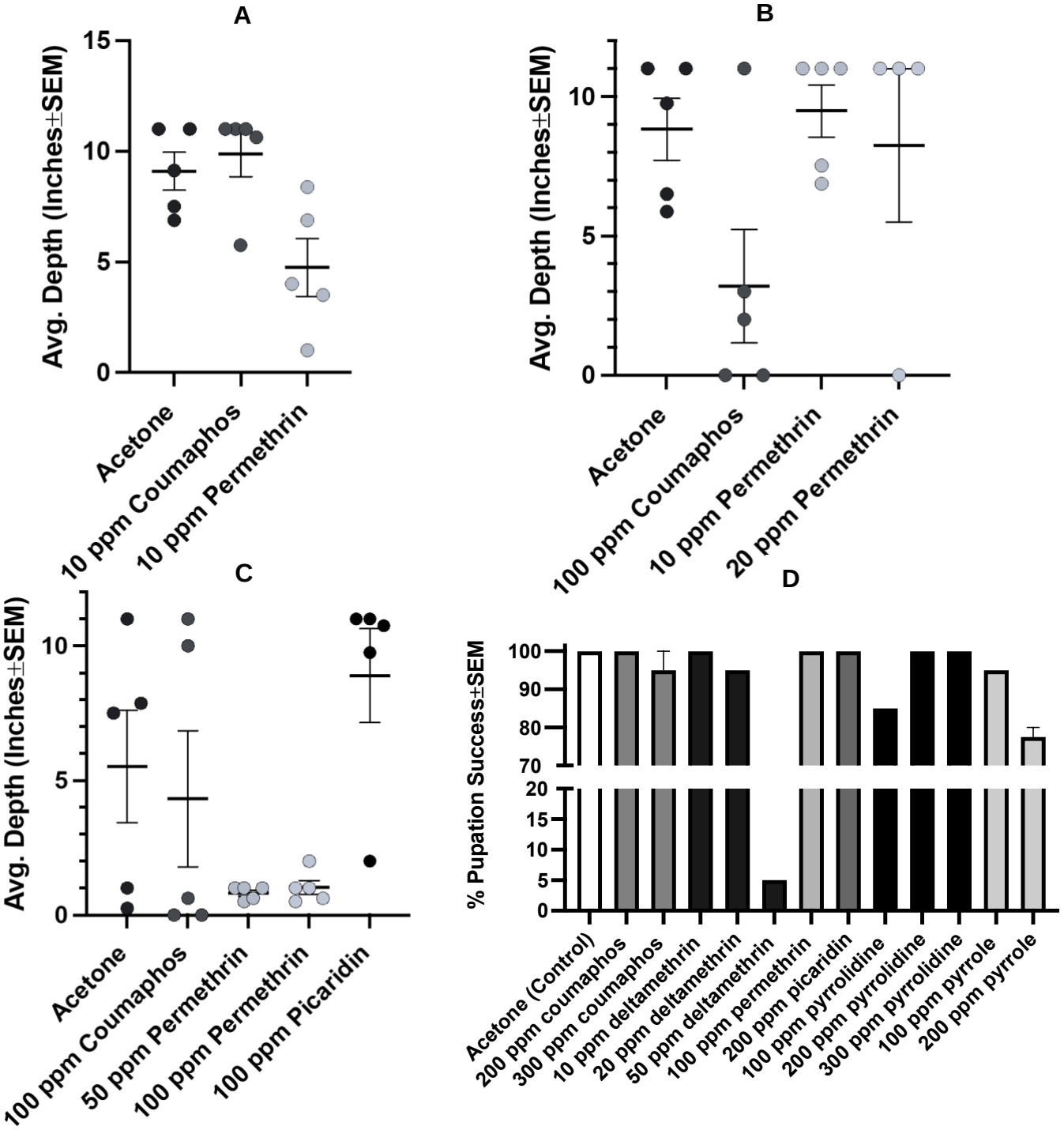

Figure 4.5 Results of pupation depth bioassays 1-3 (A-C) and pupation success bioassays (D).

CHAPTER 5: SCREENING LOCALLY COLLECTED FUNGAL ENDOPHYTES TO DISCOVER NOVEL COMPOUNDS FOR *A. TUMIDA* MANAGEMENT

5.1 ABSTRACT

Small hive beetles (*Aethina tumida*) are invasive and persistant European honey bee *Apis mellifera* pests, which are now widely distributed across the United States. Effective management options for these pests have been slow to develop, and the fact that any treatment used in the apiary setting will consequently affect *A. mellifera* colonies adds another level of difficulty to beetle management efforts. In order to discover novel, and potentilly species specific, options for *A. tumida* management, several locally collected fungal endophyte extracts were investigated in hopes of discovering an endophyte extract that was toxic to *A. tumida* larvae. Throughout this study, endophyte extracts were tested tested against *Aedes albopictus* (*C6/36*) and *Spodoptera frugipurda* (Sf21) cell lines, and the half-maximal inhibitory concentration (IC_{50}) of rotenone (positive control) was also obtained for both cell lines. While this study never moved past the *in vitro* stage, progress was made in determining several endophyte extracts that would be good candidates to advance to *in vivo* testing. Other endophyte extracts have been found to be entomopathogenic and species-specific, and this study outlines both effective methods for determining endophyte extract effectiveness *in vitro* and important future directions in the use of fungal endophyte

extracts for insect control. There is a need for selective and efficacious *A. tumida* management options, and this need could be met in the future by fungal endophyte extracts.

5.2 INTRODUCTION

Endophytes are fungi (or bacteria) that can be found in plant tissues but do not lead to symptoms of infection in the host plant (Wilson, 1995). Several plants are known to possess fungal endophytes, which are believed to provide protection from nematodes, plant pathogens, and insects (Vega *et al*., 2008). While there are an estimated one million fungal endophytes, few have been investigated for their insecticidal properties (Petrini, 1991; Yu, 2010). Integration of fungal endophyte data from 2014-2017 revealed that 97% of fungal endophytes belonged to phylum Ascomycota, and the remaining 3% belonged to phyla Basidiomycota (2%) and Mucoromycota (1%; Li *et al*., 2018). Fungal endophytes can also produce natural product metabolites with antimicrobial properties, including alkaloids, steroids, phenols, peptides, terpenoids, flavonoids, and quinones (Yu, *et al*., 2010). Despite the fact that a century of study has been devoted to entomopathogenic fungal endophytes, development of endophyte extracts for use as insect controls has been slow (Vega, *et al*., 2008).

Few experiments have tested fungal endophytes as potential insect controls, but the results thus far have been promising. One study examined the use of the fungal endophytes *Purpureocillium lilacinum* and *Beauveria bassiana* for cotton aphid control (*Aphis gossypii*), which resulted in negative impacts on aphid health and reproduction (Lopez, *et al*., 2014). Other experiments tested crops including corn, barley, tomato,

coffee, and sorghum, which resulted in detrimental impacts on pests by various fungal endophyte species (Quesada-Moraga *et al*., 2014). Endoyphyte extracts from *Cochliobolus spicifer* were shown to have larvicidal effects on two mosquito species (*Aedes caspius* and *Culex pipiens*), while the same extracts were not toxic to zebrafish, demonstrating selective activity (Abutaha *et al*., 2015). The entomopathogenic fungi *Paecilomyces lilacinus* was tested on the Chagas disease vector the kissing bug (*Triatoma infestans*) resulting in 100% morality 30 days post treatment (Marti *et al*., 2006). The few entomopathogenic fungal endophytes that have been assessed thus far have shown encouraging insecticidal activity, however there is still much work to be done in expanding this promising pest management option (Vega *et al*., 2008).

Entomopathogenic fungal endophytes could provide a novel approach to the biological control of *A. tumida*, therefore, this study was developed to test approximately 600 locally collected fungal endophyte crude extracts to isolate ideal candidates for *A. tumida* control. Although all endophyte extracts could not be tested in this project, the goal was to discover a fungal endophyte that was selective toward *A. tumida,* which could be used to design future *A. tumida* controls, whether that be through trapping or other forms of in-hive treatment. The study plan was to employ MTT assays to narrow down the number of fungal endophytes to test *in vivo*, with the goal of decreasing the number of isolates used. Rotenone, a natural environmental neurotoxin that inhibits cellular respiration (Chiaradia *et al*., 2019), served as the positive control in this study. Several concentrations of rotenone were screened against *Aedes albopictus* (C6/36) and Spodoptera frugiperda (Sf21) cell lines and the

resulting absorbances were used to calculate the half-maximal inhibitory concentration (EC_{50}) of rotenone. After establishing the IC_{50} of rotenone, different concentrations of several extracts from the locally collected fungal endophytes were screened against the *C6/36* and SF21 cell lines; the effects of the endophyte extracts were compared to the IC_{50} of rotenone using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assays.

5.3 MATERIALS & METHODS

5.3.1 Chemicals

Triple 0.1 micron filtered Fetal Bovine Serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO, USA). Trypsin (0.25%) + EDTA in Hank's Balanced Salt Solution (HBSS), and Dulbecco's Phosphate Buffered Saline (PBS; sterile filtered) were purchased from GenClone (San Diego, CA, USA). Trypan Blue Stain (0.4%), Grace's Insect Medium (1X), MEM NEAA (100X), HEPES Buffer Solution (1M), and Tryptose Phosphate Broth (1X) were all purchased from Gibco (Gaithersburg, MD, USA). MTT was purchased from Tocris Bioscience (Avonmouth, Bristol, UK). Schneider's Insect Medium, Penicillin-Streptomycin, Rotenone (≥95%), and Dimethyl sulfoxide (≥99% ACS reagent) was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

5.3.2 MTT Assay Methods

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assays were performed in microtiter plates and ultimately produced a colorimetric response to the enzymatic reduction of tetrazolium (Pascua-Maestro *et al*., 2018), which allowed

for quantitative measure of cell proliferation, viability, and cytotoxicity (Mosmann, 1983; Figure 1). Briefly, cells were removed from flasks via scraping and a 50 μL aliquot of the cell suspension was combined with 50 μL of trypan blue. The remaining cells were then centrifuged for five minutes at 5xg (120 RPM) and old media was aspirated followed by the addition of new media being added. This mixture was then pipetted onto a hemocytometer and the number of cells in the center and four corner squares were counted under a microscope. The concentration of cells (viable cells/mL) was quantified by taking the average number of cells per square, multiplied by the dilution factor and volume of each square. Cells were then diluted added to wells of a 96-well microtiter plate, starting with tests of *C6/36* seeding densities of 50,000, 75, 000, and 100,000 cells/mL and Sf21 seeding densities of 10,000, 20,000, and 30,000 cells/mLto determine the optimal cell density for discriminating between treatments and later testing rotenone and endophyte extracts. After cells were seeded, plates were incubated overnight to allow cells to adhere to the substrate.

The next day, cell culture media was replaced with 180μL of fresh media. Each toxicant was initially dissolved in DMSO and then diluted in HBSS final DMSO concentration of 0.1% desired) and 20μL of the toxicants were added to the microplate wells, with at least two determinates per treatment, along with a row of the negative control (PBS) and vehicle control (PBS and 0.1% DMSO). The plates were again incubated for 24-hours and then the media and toxicants were aspirated and 20 μL of MTT solution (5mg/mL in PBS) were added to each well. In order to determine the effects of aspirating versus pouring off cell media, MTT assays and using rotenone treatment to compare the two methods were performed using C6/36 cells. Plates were

then incubated at room temperature for 4 hours. After incubation, cells were washed twice in PBS and finally 150μL of DMSO were added to each well to solubilize precipitated Formazan crystals. Absorbance of each well at 490 nm was then read using a SpectraMax microplate reader (Molecular Devices, San Jose, CA, United States). Absorbances from toxicants were then divided by absorbances of appropriate controls, with results reported in % of control ± standard error of the mean (SEM).

5.3.3 Testing locally collected fungal endophyte extracts against Aedes albopictus (C6/36) and Spodoptera frugiperda (Sf21) cell lines

Once optimal cell density for MTT assays had been established, fungal endophyte extract testing began. Endophyte extract treatments were labeled based on their location in 96-well microtiter plates (i.e., A1-H12). Endophyte extracts were diluted to concentrations of 1 and 10% to determine whether or not a concentration-dependent response could be observed. Rotenone treatments of 1 ppm and 0.03 ppm, along with solvent and vehicle controls (PBO and 0.1% DMSO), were also tested alongside the endophyte extracts to determine whether or not the endophyte extracts inhibited cells at a level comparable to that of rotenone. All results were plotted in GraphPad Prism and absorbances were converted to percent of control. The rotenone controls, along with the PBO and 0.1% DMSO controls from the endophyte extract MTT assays were also combined and analyzed using ANOVA followed by Tukey's multiple comparisons to determine whether or not the negative and positive controls were working to providing a good comparison to assess endophyte extract effectiveness.

5.4 RESULTS

5.4.1 Establishing rotenone EC_{50} values for C6/36 and SF21 cell lines

Results of pouring vs. aspirating MTT assays were compared using an unpaired t-test, demonstrating no significant difference between methods (P=0.896), after which media was aspirated for the remainder of experiments (Figure 5.2A). When comparing *C6/36* cell densities of 75,000 vs 100,000 cells/mL, it was found that the best concentration-dependent response was observed using the 75,000 cells/mL concentration, which was then used moving forward (Figure 5.2B-C). Further MTT assays revealed that the IC_{50} value of rotenone in *C6/36* cells (75,000 cells/mL) is 0.291ppm (Figure 5.3). Testing rotenone concentration-dependent responses of Sf21 cells at densities of 10,000, 20,000, and 30,000 cells/mL was carried out and IC_{50} values of 0.03ppm, 1.36ppm, and 0.5ppm were established for each cell concentration, respectively (Figure 5.4). Moving forward to endophyte extract testing, SF21 cell densities of 20,000 cells/mL were used, as the confidence intervals as the narrowest confidence intervals were observed (0.218-8.47ppm).

5.4.2 Preliminary results of fungal endophyte extract testing

Comparison of C6/36 controls from combined MTT assays resulted in no significant difference between control absorbances and the absorbance of rotenone an 0.03 and 0.003ppm, while the 1ppm rotenone treatment was significantly different from all controls, except the 0.03ppm concentration (Figure 5.5A). Combined controls of Sf21 endophyte extracts also revealed that, at a concentration of 1ppm, absorbance of rotenone was differed significantly from the absorbance of PBS and 0.1% DMSO). Although endophyte extracts A1-H12 were tested against *C6/36* and Sf21 cells, the results displayed are highlight the most relevant endophyte extracts. When testing

C6/36 cells, the B2 (10%) endophyte extract did reduce absorption to below 50% of the control, as did H8 (1ppm), and H11 (1% and 10%); however, a combined graph demonstrates that results were not consistent, with a large margin of error between replicates (Figure 6). This highlights a problem observed throughout these tests; endophyte extracts could vary dramatically from one replicate to the next. Results from testing endophyte extracts on Sf21 cells were slightly more promising, with a combination of three replicates testing extracts A1-A12 yielding consistent results in which extract A8 (10%) reduced absorbance to less than 50% of the control value, which was consistent the 1ppm rotenone control (Figure 5.7A). Similarly, extracts A8 (10%) through A9 (1%) exhibited consistently low % of control values, although they were higher than 50% of the control (Figure 5.7B).

5.5 DISCUSSION

Due to unforeseen circumstances, including the departure of the endophyte extract supplier and the covid-19 pandemic, research into the effects of the locally collected fungal endophytes ceased after preliminary tests of extracts A1-H12. Although this study could not be completed and applied to *A. tumida* management, a useful basis for continuing this work has been established. If tests had gone on as anticipated, approximately 25 of the fungal endophyte extracts with absorbance values within three standard deviations of the IC_{50} of rotenone would have been explored in *in vivo* bioassays. *In vivo* screening of the fungal endophyte extracts would have first been carried out using first instar *Aedes albopictus* larvae, following the methods of Pridgeon *et al*. (2009). Five first-instar larvae would have been added to 24-well

plates, with food, deionized water, and fungal endophyte extracts (in varying concentrations) dissolved in ethanol. Once these bioassays were performed, the ten most effective remaining compounds would have been screened against *A. tumida* first instar larvae from the laboratory colony through feeding bioassays. In these feeding bioassays, five larvae would have been separated into containers and allowed to feed on pellets (see chapter 4) spiked with endophyte extracts, and mortality and other effects would have been noted. Although this study could not be completed, examination of local fungal endophytes for their insecticidal ability is an endeavor that could potentially lead to novel (Lopez *et al*., 2014) and species-specific (Abutaha *et al*., 2015) future *A. tumida* controls.

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5.7 FIGURES

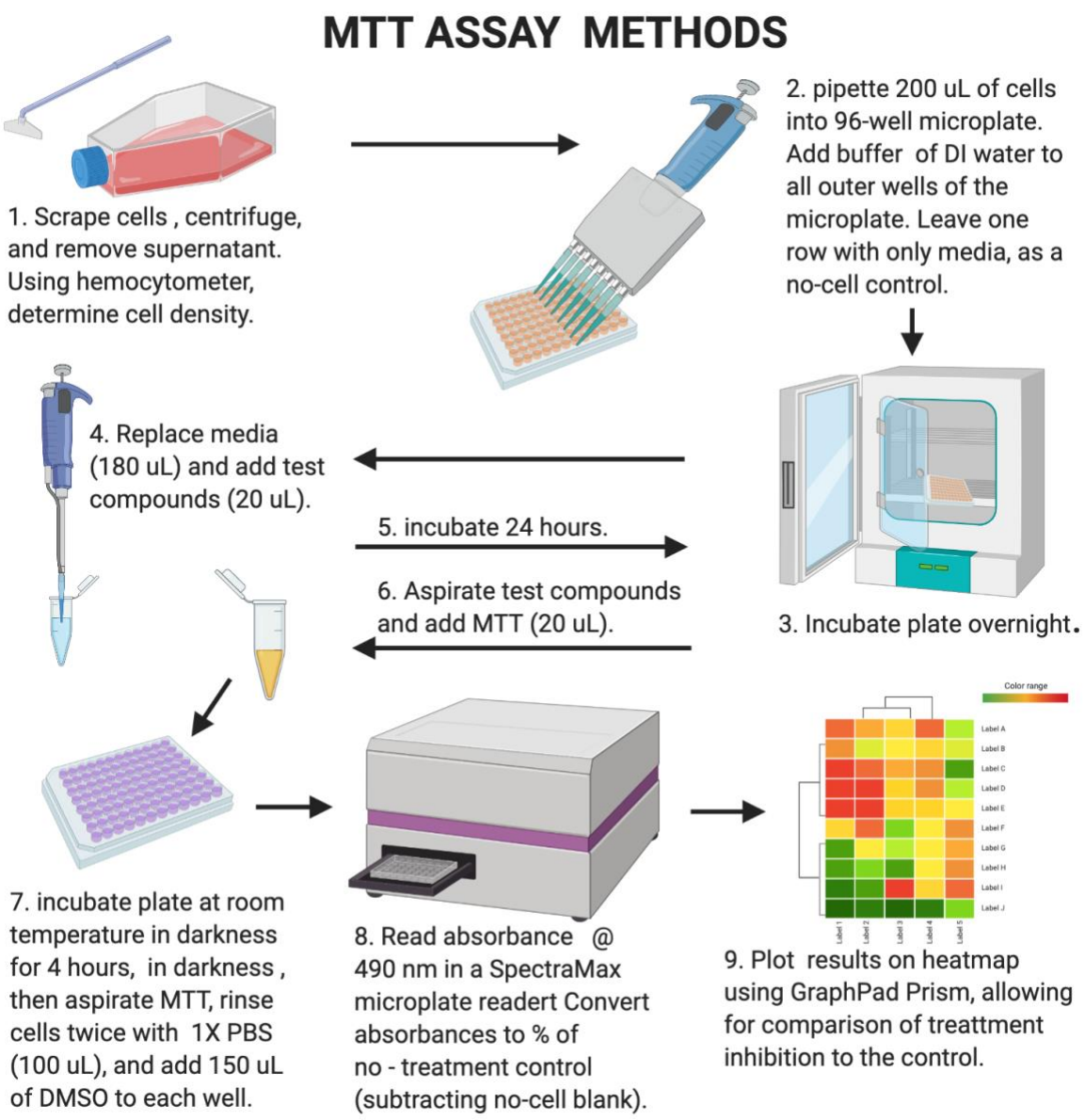


Figure 5.1 Step-by-step materials and methods for performing MTT assays, created with BioRender.com.

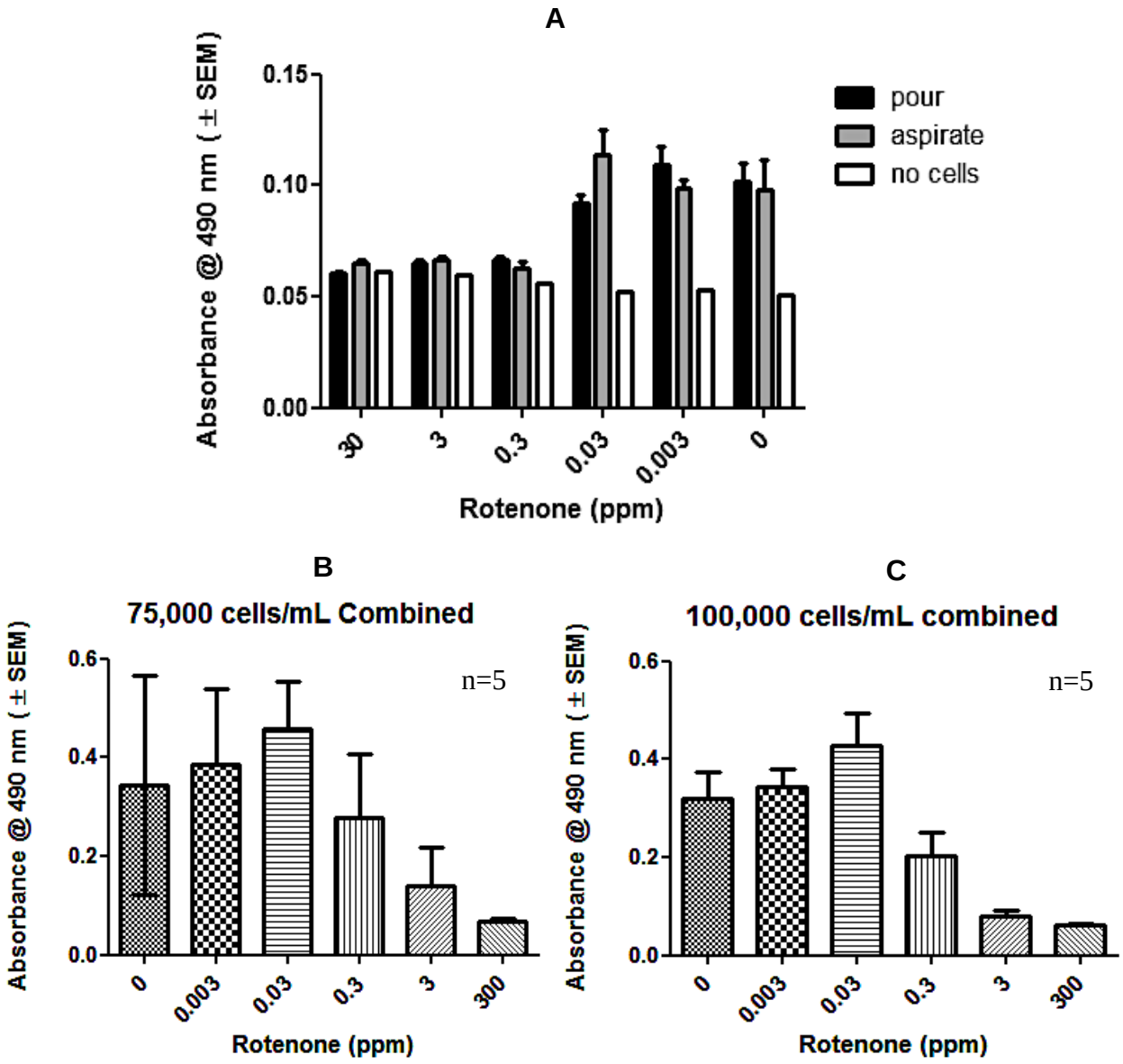
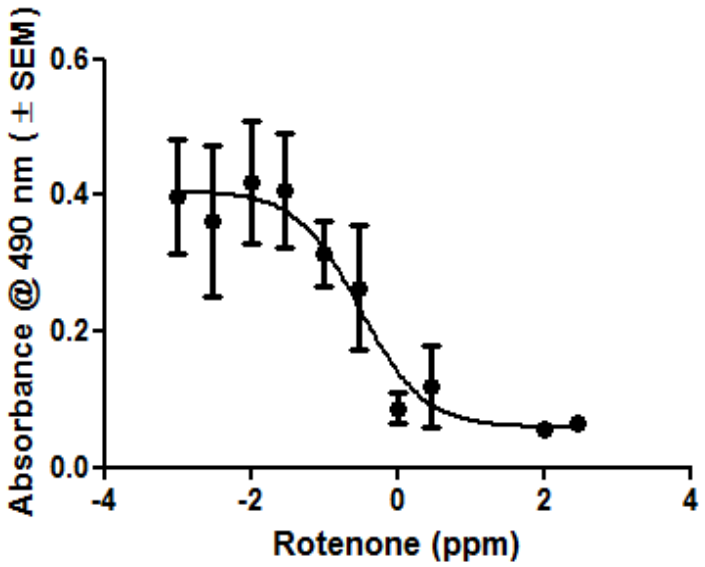


Figure 5.2 Testing effects of pouring vs. aspirating cell media (A) followed by comparison of rotenone effects of cell densities of 75,000 cells/mL (B) and 100,000 cells/mL (C)

A



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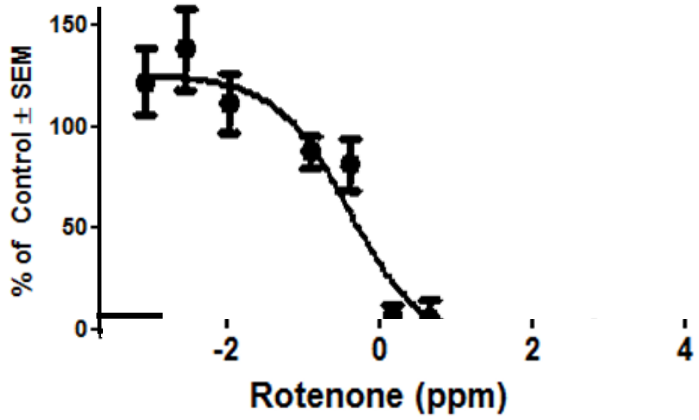


Figure 5.3 Rotenone concentration response curve using C6/36 cells (75,000 cells/mL), displayed in Absorbance (A) and as a % of the control (B). IC_{50} calculated using GraphPad Prism.

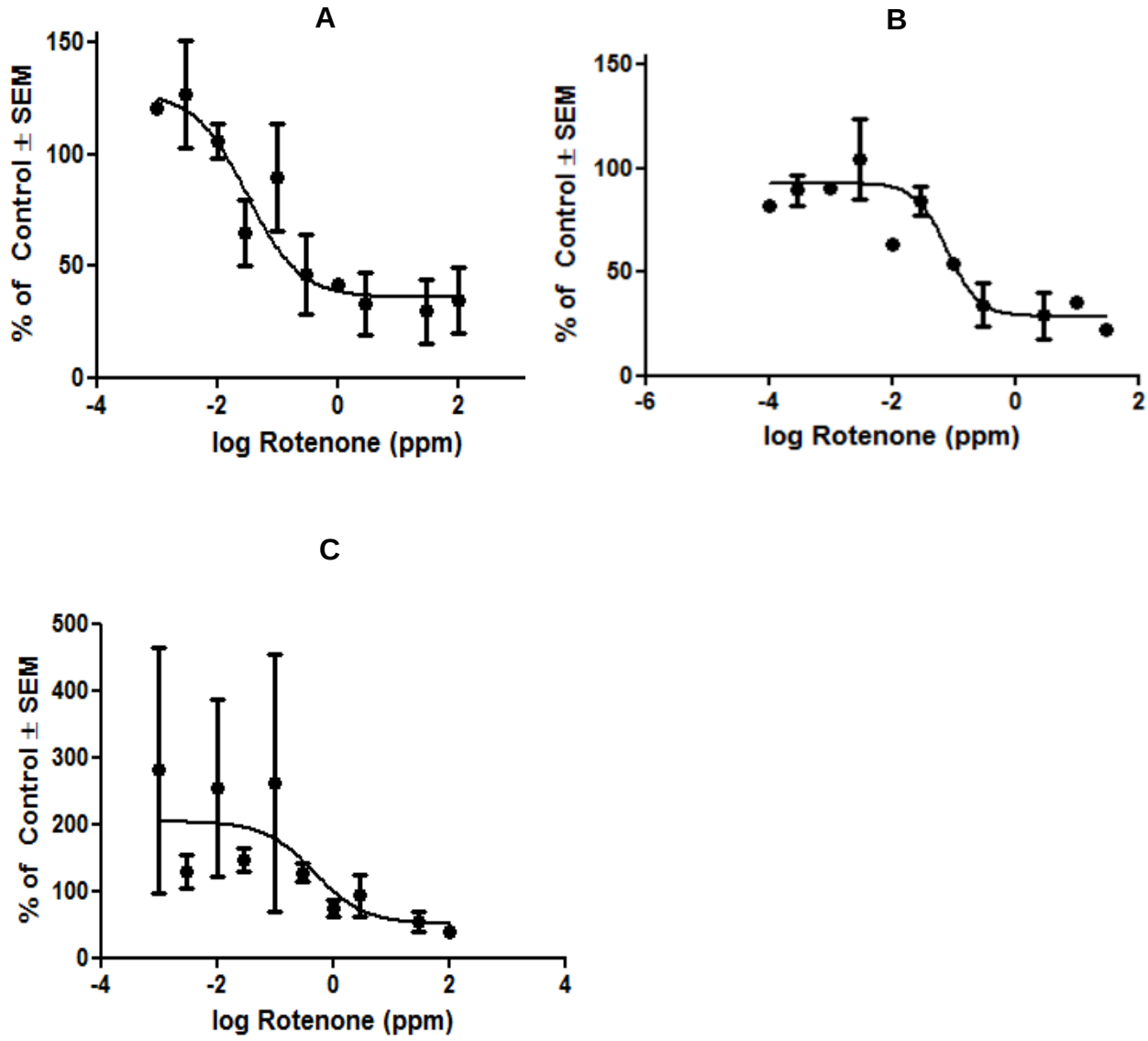
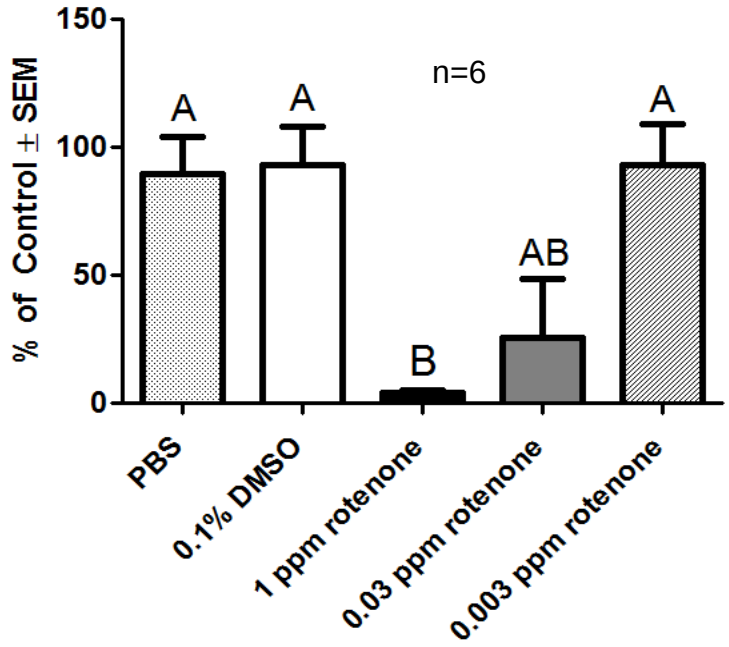


Figure 5.4 Rotenone concentration responses curves for SF21 cells at densities of 10,000 cells/mL (A), 20,000 cells/mL (B), and 30,000 cells/mL (C).

A



B

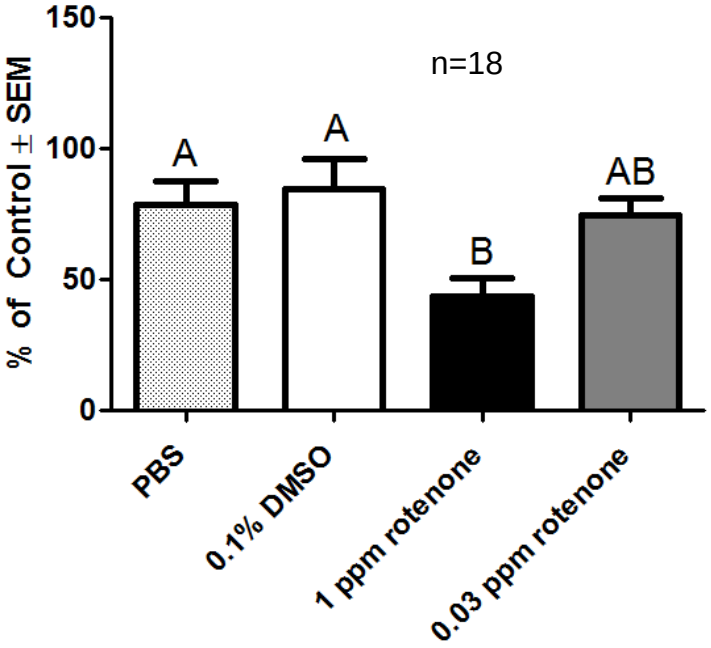


Figure 5.5

Comparison of positive and negative controls from MTT assays testing fungal endophyte extracts using C6/36 (A) and SF21 (B) cells. Analyzed via ANOVA followed by Tukey's multiple comparisons test.

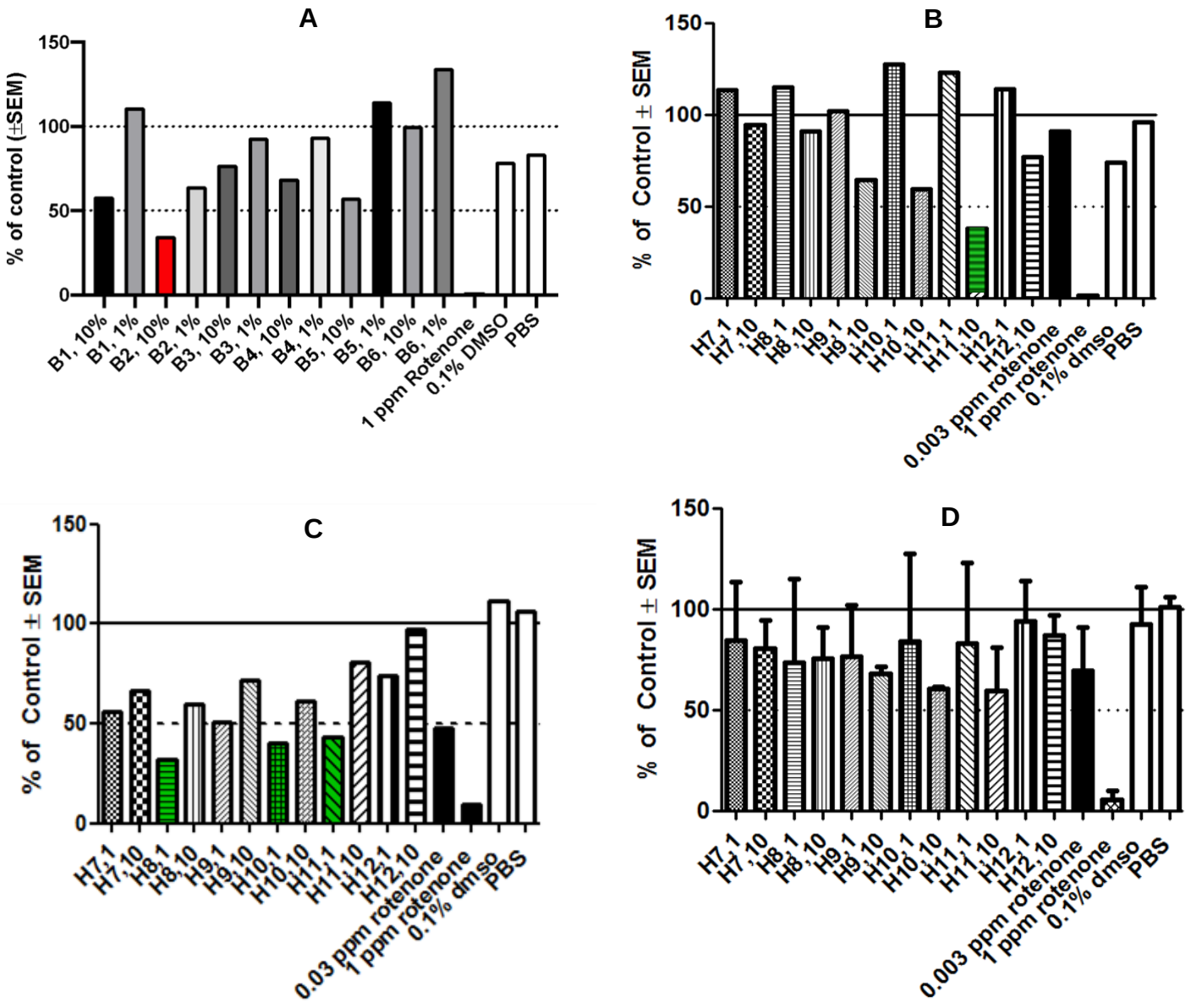


Figure 5.6 Examples of MTT assays testing fungal endophyte extracts of C6/36 cells. Extract B2 (10%) reduced absorbance to less than 50% of the control (A), as did H8(1%) and H11(1% and 10%) extracts (B-C). When combined, none of the H7-12 extracts reduced absorbance to less than 50% of the control.

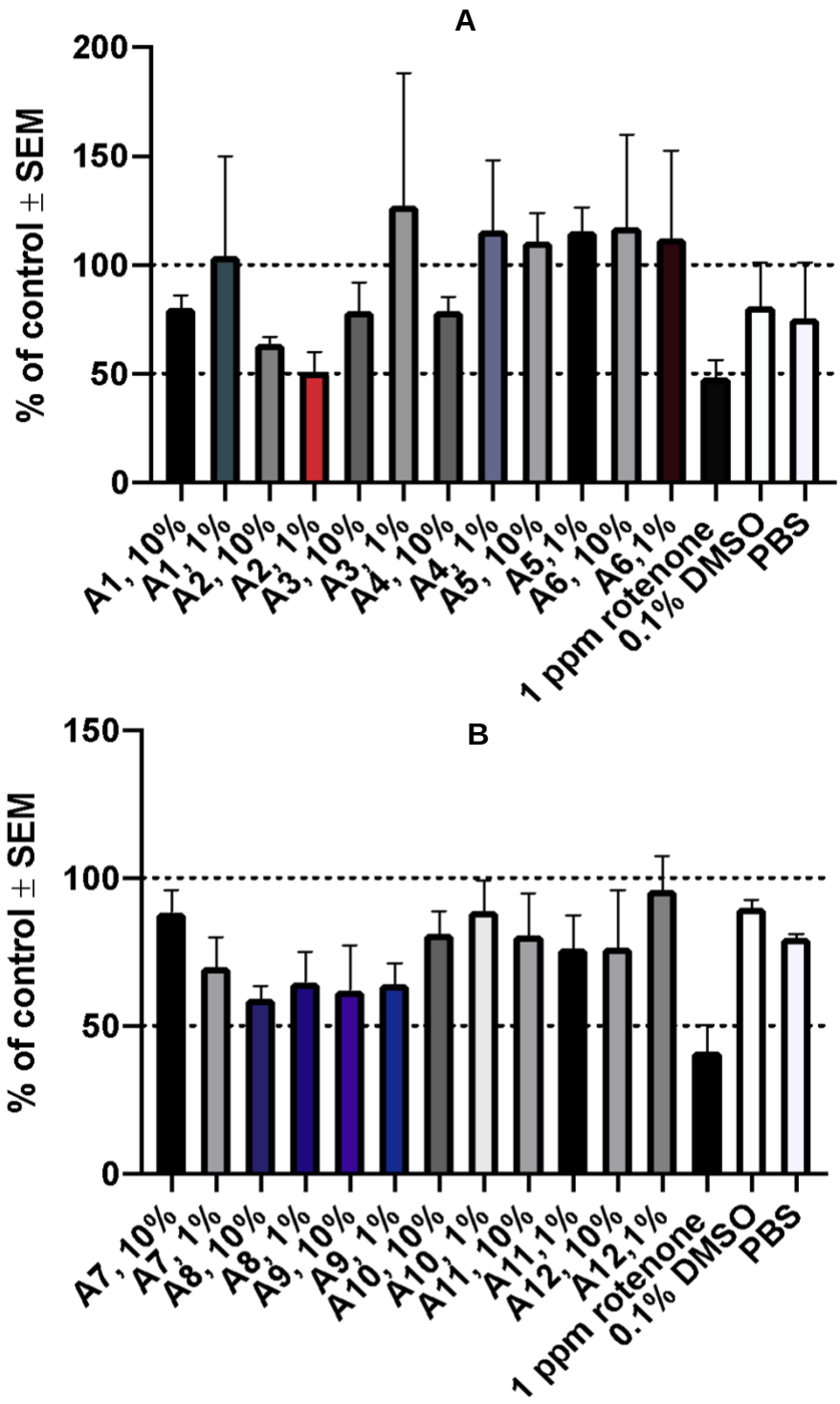


Figure 5.7 Results of three combined replicates of endophyte extracts A1-A12, tested against SF21 cells, demonstrating that (A) extract A2 (1%) and (B) extracts A8 (10%) through A9 (1%) consistently showed similar activity to 1ppm rotenone.

CHAPTER 6: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

6.1 GENERAL CONCLUSIONS

6.1.1 Examining behavioral and physiological effects of attractant and repellent compounds on Aethina tumida through electroantennography and olfactometry

The *A. tumida* olfactory system has long been known to play an important role in hive location, as these beetles are highly sensitive to numerous volatiles emitted from *Apis mellifera* colonies (Suazo et al., 2003; Torto et al., 2007). Past studies involving the *A. tumida* olfactory system have mostly focused on attractant compounds for use in baited traps (Nolan and Hood, 2008; Stuhl, 2020; Stuhl 2021), but this study took a different approach by examining the behavioral and physiological effects of several previously untested repellent compounds on the *A. tumida* through olfactometry (Chapter 2) and electroantennography (Chapter 3). Electroantennograms (EAGs) report olfactory receptor neuron excitation (Park *et al*., 2002), and thus cannot confirm attractancy or repellency. However, when EAG results can be compared to behavioral responses from olfactometer testing, a clearer picture of attractancy and repellency emerges.

Based upon the collective results from olfactometer and EAG tests, it was discovered that beetle antennae were most sensitive to the repellent pyrrolidine (EC_{50}=0.0875%), which was correlated with behavioral test results, in which 10 mg of pyrrolidine caused significant avoidance of an attractive odor source (Preference Index = -0.58). After pyrrolidine, antennae were most sensitive to pyrrole and 1,4-dimethylpiperazine, and olfactometry reveled that these compounds also led to avoidant responses to a formerly significantly attractive source. Surprisingly, the well-

known repellents DEET (*N*, *N*-diethyltoluamide) and picaridin (Sec-butyl 2-(2-hydroxyethyl) piperidine-1-carboxylate), which have been highly effective in repelling other insect species (Paluch *et al*., 2010), including *Apis mellifera* (Larson *et al*., 2021), were not strongly repellent in this study. In fact, at the antennal level, DEET did not elicit a response at any concentration, and did not lead to significant avoidance of the attractant source in olfactometer tests. Picaridin and piperidine had similar EC_{50} values (23% and 28%, respectively), although behavioral tests demonstrated that picaridin did not elicit significant avoidance, it did reduce attractant responses, whereas piperidine at 1mg still allowed significant attraction and 10 mg caused only slightly reduced attraction.

EAGs combining the three strongest repellents (pyrrolidine, pyrrole and 1,4-dimetylpiperazine) with the two attractants (isopentyl acetate and pollen patty) did not result in significant reduction in antennal responses, but this does not mean that the repellent compounds cannot overpower attractants, as was clearly demonstrated via olfactometry. There can be tens of thousands of olfactory sensilla on insect antennae (Kaissling, 1986), which differ in the substances that they detect (Gomez-Diaz et al., 2018), and further, olfactory receptors can be activated by numerous odorant ligands or by a single compound (Bohbat and Pitts, 2015). Thus, the attractants and repellents used here may be detected by different olfactory receptors, or different ratios of attractant/repellent mixes may be necessary to differentiate the level at which physiological differences between a single odor and odor mixes can be quantified. Overall, this study conclusively demonstrated that *A. tumida* antenna respond to the

known repellent picaridin and the other four heterocyclic amine test compounds, with the highest sensitivity and strongest repellency exhibited in response to pyrrolidine.

6.1.2 Fungal endophyte testing, assessing effectiveness of fungicides on A. tumida larvae, and development of bioassays for insecticide delivery

The prevalence of fungal endophytes in the environment has long been known (Vega *et al*., 2008), and the insecticidal properties of several endophyte extracts has been established (Quesada-Moraga *et al*., 2014). There is a need for further exploration of novel management methods for *A. tumida* control, and a first step in this direction was made through the testing of locally collected fungal endophyte extracts at the *in vitro* level. While this study could not be carried out *in vivo*, several endophyte extracts did demonstrate toxicity at the cellular level. By determining optimal cell densities and establishing the IC_{50} values of rotenone tested against *Aedes albopictus* (C6/36) and *Spodoptera frugipurda* (Sf21) cell lines, this study helped provide a useful metric for comparing effectiveness of endophyte extracts and screening large numbers of these extracts. Management of *A. tumida* through use of fungal endophytes could provide both an environmentally safe and potentially selective means of controlling these beetles, and *in vivo* testing of local endophyte extracts, starting in the laboratory setting, could help lead to such discoveries in future.

The presence of the *A. tumida* fungal symbiont *Kodamaea ohmeri*, and its activity in fermenting larval waste to release attractive volatiles (Hayes *et al*., 2015), is well known and *K. ohmeri* has even been used to increase attractiveness of baited traps (Torto *et al*., 2007). In view of the prevalence of *K. ohmeri* throughout all *A. tumida* life stages (Leemon, 2012), the fungicide amphotericin B was delivered to *A.*

tumida adults and larvae through a series of feeding and injection bioassays. Although amphotericin B has been shown to be an effective course of treatment against *K. ohmeri* infections in humans (Yang et al., 2009; Chakrabarti *et al*., 2014), results from the tests in this study did not reveal consistent suppression of *K. ohmeri* in *A. tumida*, indicating a need for optimization of fungicide choice, delivery, treatment timing, and treatment duration.

The feeding pellets developed in this study and used for *K. ohmeri* repression studies were also used for oral delivery of several insecticides commonly used in apiary settings (amitraz, coumaphos, and *tau*-fluvalinate; De Mattos *et al*., 2017), along with several pyrethroids (bifenthrin, permethrin, and deltamethrin; Somerville, 2003; Benuszak et al., 2019), which have been used against *A. tumida* in the past. The feeding pellets proved to be a quick and effective means of testing insecticides against *A. tumida* larvae, observing concentration-dependent responses, and quantifying half-maximal lethal concentrations (LC_{50}) for each compound. The soil bioassays developed in this study also allowed for observations of pupation depth and pupation success in insecticide treated soil. Through several experimental trials, it was found that *A. tumida* pupation depth and success was most effectively reduced through permethrin and deltamethrin treatments. These soil bioassay methods differ from other *A. tumida* soil treatment studies, in that only part of the soil was treated in attempts to determine the concentration at which larvae will not burrow to pupate, rather than adding larvae directly to treated soil and assessing mortality (Kanga and Somorin, 2012).

6.2 FUTURE DIRECTIONS

6.2.1 Further testing of attractant and repellent compounds with applications to A. tumida management

While the results of the olfactometer and EAG studies described in this study supply valuable information regarding compounds that could serve as effective *A. tumida* repellents, real-world application in an apiary setting will be the true test of repellent effectiveness. The long-term goal of this study was to discover effective management methods that could be translated into safe and practical *A. tumida* treatments in the field, with the most effective repellents identified in the laboratory setting eventually incorporated as part of a "push-pull" strategy in the field. Push-pull strategies have been commonly used in agricultural settings in the past, helping divert a pest insect from the crop of interest to an attractive trap crop (Bhattacharyya, 2017). In the context of *A. tumida* management, repellents could be strategically placed around apiaries to "push" beetles away from hives, while attractant sources baited with an insecticidal compound could be used to "pull" beetles in, subsequently killing them. In the future, further testing of repellent and attractant mixes, along with testing of a somewhat recently developed *A. tumida* aggregation pheromone (Stuhl and Teal 2017), should be carried out in the laboratory setting. Identification of effective repellents in this study was a critical first step in moving toward less toxic and more effective means of deterring *A. tumida* adults from entering hives, thereby alleviating the burden of heavy larval feeding and toxic apiary treatments.

6.2.2 Enhancing knowledge of A. tumida by optimizing K. ohmeri inhibition and developing soil bioassays for toxicity studies

Aethina tumida has been infesting apiaries in the United States for over two decades (Elzen *et al*.,1998) and there is clearly a need for improved understating of *A. tumida* biology and the ability to efficiently assess the suitability of current and future control treatments. The methods developed for feeding and soil bioassays in this study provide a scaffold upon which future treatment strategies can be built; the toxicity data obtained through this study demonstrate that the ubiquitous in-hive treatments used for *V. destructor* are less toxic to *A. tumida* larvae than pyrethroid treatments used outside the hive. It is important to understand the effects of insecticides on *A. tumida* survival and pupation success and oral toxicity and pupation success bioassays should be performed to expand this knowledge by testing more insecticides and test compounds that are potentially less toxic to *A. mellifera*. Finding ways to harm *A. tumida* without damaging *A. mellifera* colonies is a difficult objective to achieve. However, enhanced knowledge of how *A. tumida* responds to insecticides at different life stages, in addition to exploration of other aspects of *A. tumida* biology, such as the fungal symbiont *K. ohmeri*, are important steps to take toward eventually incapacitating this pest.

While the *K. ohmeri* may not have been inhibited by amphotericin B in the experiments performed in this study, testing of this fungal symbiont should not be abandoned. Although one study characterized *K. ohmeri* as a facultative symbiont after surface sterilization did not impact the *A. tumida* lifecycle (Amos *et al*., 2019), the buildup of *K. ohmeri* in infested colonies, function of *K. ohmeri* in *A. tumida* attraction (Benda *et al*., 2008), and prevalence throughout *A. tumida* life stages (Leemon, 2012) indicate that *K. ohmeri* is an important part of *A. tumida* health and effectiveness as a

pest. The goal of this study was to manipulate the *A. tumida* relationship to *K. ohmeri* to develop species-specific control methods that could undermine the foundations of *A. tumida* biology and limit beetle effectiveness.

Even though effective repression of *K. ohmeri* may not have been established, this study did take first steps in designing a system of orally delivering and injecting a fungicidal compound into *A. tumida* and subsequently testing internal *K. ohmeri* levels. In the future, identifying the strain of *K. ohmeri* present in test beetles (Benda *et al*., 2008) and examining the effectiveness of other fungicides in repressing *K. ohmeri* would be useful. Multi-generational studies of *A. tumida* success after *K. ohmeri* repression would also aid in better determining the long-term effects inhibiting this symbiont. If an effective fungicide is discovered, and if repression of *K. ohmeri* does detrimentally affect *A. tumida* vitality, fecundity, and longevity, this fungicide could be deployed in an apiary setting to effectively reduce *A. tumida* populations. Further work would also need to investigate potential *A. mellifera* fungal symbionts; an area in which current knowledge is extremely limited (Anderson, *et al*., 2011). It is known that there are several common hive yeasts that are displaced by *K. ohmeri* growth (Benda *et al*., 2008), but the role of these yeasts remains to be characteraized, thus, fungicide effects on any potentially beneficial hive yeasts should also be explored in future.

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