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Effects of Pesticide Residue Accumulation on Honey Bee (*Apis mellifera* L.) Development & Implications for Hive Management

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Effects of Pesticide Residue Accumulation on Honey Bee (*Apis mellifera L.*) Development & Implications for Hive Management.

By

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Honey bees (*Apis mellifera* L.) face high annual declines in the United States and pesticide exposure is a factor. Bees may return with residues from the environment or become exposed through beekeeper-applied compounds, however the effects of pesticide accumulation in combs on bees have not been well-studied. To further examine this, chlorothalonil fungicide and beekeeper-applied acaricide amitraz, common pesticides within the hive, were applied to comb. Queen bees laid eggs onto treated and control combs (acetone solvent or untreated) then larval development and adult worker bee measures (hypopharyngeal gland size and abdominal lipids) were compared to determine potential effects of pesticide residues on bee health. Results indicates that larvae reared in comb treated with amitraz developed significantly smaller hypopharyngeal glands.

Exposure to newer chemistries, may not result in rapid losses but rather colonies may exhibit slow chronic losses over time, indicating impacts may be due to persistent residual effects. Here, we assessed the use of dead bee traps for monitoring pesticide incidents. Trap efficacy was assessed by exposing workers imidacloprid (or freeze-killed (control)) and monitoring traps to determine when dead/dying bees are removed from the hive (recapture rates). Dead bee traps recaptured 27.7% of freeze-killed control bees and significantly less of the imidacloprid-treated bees. Trap collection data from

three apiaries indicate distinct differences in timing of observed mortality by location. Results elucidate how pesticide exposures may be monitored and this thesis concludes with an instructional guide to build and use traps to better monitor for hive health issues.

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Table 4.1: List of state agencies and their contact information for reporting incidents and bee kills from suspected pesticiide exposure.

Chapter 1: Literature Review

1.1 Importance of Honey Bees and the Beekeeping Industry

Approximately one third of the plants we eat require insect pollination to have successful seed or crop production, commercially managed honey bees (*Apis mellifera* L.) contribute to 80% of those services (Thapa 2006). In fact, honey bees provide pollination to over 95 crops across the nation, including our most nutritious foods (fruits, vegetables, and nuts). The contributions to fruit and vegetable production is estimated at over $3 billion US dollars while the overall added-crop value to the economy, in 2009, was roughly $15 billion USD (Losey and Vaughan 2006; Calderone 2012). Active pollination by bees occurs as a result of foraging. As bees travel between flowers, small hairs on their body collect pollen, which is produced from male reproductive structures of a plant, called anthers. Honey bees utilize stiff hairs on their legs as a "comb" to groom pollen grains into specialized concave areas on their hind legs known as "corbicula" or pollen baskets, which are used to transport pollen loads back to the hive. And as bees forage, pollen grains from their body transfer onto the stigma, or female reproductive structure, of conspecific flowers. This in turn fertilizes the plant and allows development of seeds. Plants with higher pollen deposition occurring, typically have higher reproduction of fruit or seeds (Garratt et al. 2014; Klatt et al. 2014). Some crops receive modest gains in yield or quality of the crop, while others may be completely dependent on the pollination provided by bees. For example, in 2019, there were over 1.17 million acres of almonds that required more than a million colonies for pollination (Goodrich 2020). To meet this demand, the majority of managed honey bees

colonies across the US are transported to California just to pollinate almonds. Though almonds are a major cash crop they are only one of many crops that require honey bees to pollinate. In the last 15 years, there has been an increase of more than 300% in the need for pollination services (Aizen and Lawrence 2009), however, beekeepers struggle to meet growing demands due to high annual losses of colonies and continued challenges with bee health decline.

The beekeeping industry does not solely rely on pollination services as a source of income. In addition to contributions from pollination services, roughly 450 million pounds (lbs.) of honey is produced annually by honey bee colonies in the US (Shahbandeh 2018) and honey production, in 2018, was valued at approximately $333 million USD (Root 2019). Beekeepers will only harvest the excess honey that bees collect and will leave enough honey for bees to survive the winter. Honey is produced when Forageing bees collect excessive amounts of nectar in their honey stomachs to bring back to the hive and store. Floral nectar is a required carbohydrate or energy source for honey bees. Honey bees also forage for floral pollen, a source of protein necessary for growth and brood rearing. Beekeepers can trap bee-collected pollen when pollen sources are ample and either sell pollen grains as health supplements for human consumption and or beekeepers will feed pollen back to colonies to supplement nutrition during pollen dearths. Younger bees, or workers that remain in the hive, process the incoming nectar and pollen by incorporating digestive enzymes and removing moisture so that nectar is converted into honey and pollen into beebread for long-term storage. Honey and beebread are critical overwintering resources to sustain

energetic demands for thermoregulating winter clusters. Honey bees do not hibernate over winter but rather cluster together to maintain shared heat generated by shivering thoracic muscles. Honey bees exhibit this adaptive "hoarding" or foraging for nectar and pollen to allow honey bees to begin producing brood and building the population during late winter before there are floral resources available in the landscape. The large population size and high foraging activity makes honey bees an ideal and easily managed pollinator for large cropping systems but in any livestock system there are many challenges associated with proper management of the bees and their pests and pathogens (Shipman et al. 2013).

In addition to honey, other substances produced by honey bees such as pollen, beebread, wax, and jelly) are economically valuable products and may be used to produce other value-added products. For example, royal jelly which is a protein-rich glandular secretion fed to developing bees is often used as a key ingredient in many specialty products for health and cosmetic benefits in humans. Additionally, to keep the beekeeping industry going there are many large operations that have expanded into queen rearing and have become bee breeders or suppliers to smaller operations and hobbyist beekeepers. In fact, the current market price (in 2018) for purchasing a small nucleus colony, containing roughly 10,000 adult and developing brood is roughly $110 US and about $86 for "packages" of bees containing roughly 7,000 adult bees only (Root 2019). This, however, is the average US commercial rate for large bulk orders therefore Nebraska beekeepers, which consists mainly of small-scale operations and hobbyist

beekeepers often must pay 50-75% higher prices (~$175/nucleus and $150/package) to cover costs for transport and delivery into the state.

Hive products and services from honey bees have been highly regarded and valued for centuries around the world. However, more recently bees, both honey bees and wild bees, have played a major role in shifting perceptions regarding outdated or insufficient environmental protection policies. Media attention surrounding bee decline have spurred renewed conservation efforts and has led scientists to scrutinize the role environmental stressors (poor habitats and pesticide exposure) play in global bee health decline. Honey bees are biological indicators of the surrounding environment and colonies as well as hive products may be tested to determine the overall presence of environmental pollutants within a 2-mile radius of the hives as this is the typical foraging range for honey bees (Devillers and Minh-Hà 2002; Celli and Maccagnani 2003). The presence of these pollutants or toxicants may impact many different organisms and systems. The alarming losses in honey bees are also reflected in reductions in abundance and diversity of wild bees and other beneficial pollinators (Goulson et al. 2015), further supporting the role honey bees play as bio-indicator species. The ease of managing honey bees compared to other bee species also makes them a useful tool to help researchers continually reevaluate environmental policies and develop more effective pesticide protection guidelines.

1.2 Honey Bee Biology

The European honey bee (*Apis mellifera* L.) is one of ~20,000 species of bees worldwide. They are classified in the taxonomic order of Hymenoptera (Family: Apidae)

and are related to ants, wasps, and sawflies. As social insects, honey bees have a unique life history that includes a dynamic structure of jobs where individual bees function as a superorganism and their survival is tied to the success of the colony. In the insect world, there are only a few examples of this reliance. Eusocial or "truly social" insects exhibit traits such as cooperative brood care, overlapping generations, and division of labor. In honey bees, there is division of reproductive castes and labor or polyethism. Polyethism, in honey bees, is age-based and each individual carries out a role in the hive suited for their physiological state which changes as do their roles throughout the bee's life. This includes the feeding of brood or immature larvae, storage of food, building of wax, and other tasks that support the continued development of the colony. These worker bees make up the non-reproductive or sterile caste of the colony while queens (reproductive females) and drone bees (reproductive males) are tasked with brood production and mating responsibilities. Honey bees express haplodiploidy and the queen may lay fertilized or unfertilized eggs which results in female (diploid) or male (haploid) offspring, respectively. Unfertilized eggs result in haploid males or drones which have no role other than to mate with a virgin queen from another colony to pass on the genetic information from their mother. Eggs that are fertilized by sperm are diploid, contain genetic information from both maternal and paternal lines, and develop into a female sterile worker bee or a reproductive queen depending on the dietary care given during early larval development.

Colony tasks, for newly-emerged adult worker bees, begin with brood care and queen care by "nurse" bees (3-12 days old), then as they age their roles progress to

hygienic tasks such as cell cleaning, nestmate grooming, food processing, and comb building by “house” bees (13-20 days old), and finally the roles transition to the riskiest tasks, guarding and resource collection by “forager” bees (>21 days). Nurse bees care for brood by feeding them protein-rich glandular secretions produced from their hypopharyngeal and mandibular glands. Nurse bees ingest large amounts of beebread, or processed pollen, which stimulates the production of glandular secretions or “jelly”. All larvae are fed royal jelly, named for the family of “major royal jelly proteins (MRJP)” that make up roughly 18% of the glandular secretions. The other components of royal jelly include water (50%–60%), carbohydrates (15%), lipids (3%–6%), amino acids, and other trace minerals and vitamins. Hypopharyngeal glands are an important organ in the endocrine system that secrete this specialized jelly. They are the largest gland in the body, located within the head of adult bees, and are highly developed in young nurse bees but rapidly degrades after approximately 2 weeks of age, which triggers the transition from brood care to house tasks (Klose et al. 2017). House bees build new comb, process food, and perform hygienic behaviors important for maintaining colony health, such as removing mite-infested or disease infected brood from sealed comb cells and physically removing dead bees (brood and adults) as well as removing debris from the hive. This behavior ensures the overall health of the colony because removal occurs before the pathogens and pests become infectious or transmissible (Thompson 1963; Trumbo et al. 1997; Kim et al. 2018). The oldest bees in the colony take on the riskiest tasks and spend most of the time outside the hive guarding against robbers and collecting floral resources (pollen, nectar, and sap) and water. Foraging is energetically

taxing and involves many potential external risks such as predation, weather extremes/events, and pesticide exposure further emphasizing the importance of allocating tasks among nestmates and securing the most vulnerable individuals (queen, brood, and young adults) in the safety of the hive.

The complex roles and functions within the hive are highly regulated and controlled through multiple modes of communication that can relay a wide array of information, such as recruiting foragers to a floral source, releasing an alarm signal or warning to defend the hive from intruders and predators, and even encouraging the queen rearing process to replace a failing queen. Honey bees communicate to nestmates mainly through chemical signaling (pheromones) but also through contact (ex. antennation), vibrations, and sound. The social nature of honey bees makes them heavily reliant on effective communication among nestmates to ensure tasks within the hive are highly regulated which maximizes the productivity potential of colonies. However, normal colony functions can be disrupted by several “stressors” that may impact hive communication and alter behaviors or performance of individual bees. It is important to evaluate these “stressors” and the interaction they may have with honey bee health and behavior to fully understand the potential impacts occurring at the colony level.

1.3 Honey Bee Health Issues

Though beekeeping literature is vast and grows every day, there is still a lot we do not understand including factors behind consistently high colony losses. In fact, annual losses of honey bee hives in the United States over the past decade have averaged 40%, (vanEngelsdorp et al. 2012; Lee et al. 2015; Seitz et al. 2016; Kulhanek et al. 2017) which is 25% higher than the acceptable annual loss. According to Steinhauer et al (2014), Colony Collapse Disorder (CCD) accounted for 61.6 % of reported annual colony loss for 2012-2013. However, CCD is a general term that describes a unique set of symptoms in which apparently robust colonies rapidly depopulate leaving only a few workers, the queen, and brood and occasionally delayed infestation by pest insects. It was originally described and named in 2007-2008 (vanEngelsdorp et al. 2009; United States Congress 2010) and researchers have since identified over 60 factors contributing to CCD indicating there is no single causal agent and it is only one way in which a colony may appear as it declines. Anecdotally, beekeepers who struggle to identify clear causes for losses will often report CCD as the cause of hive losses and national surveys suggest CCD has been reported in beekeeping operations of all sizes (vanEngelsdorp et al. 2009). The precise causes for these symptoms are not fully known or understood but colony health declines are attributed to multiple stressors that may potentially interact with one another.

Major stressors in honey bee colonies include parasites, pathogens, poor nutrition, pesticides, and poor management (United States Congress 2010; USDA 2018). Each stressor has its own complex set of effects and interactions and they all present challenges in beekeeping, but the primary problems involve the parasitic mite, *Varroa*

destructor, and the chronic presence of and exposure to pesticides both in the environment as well as within the hive. How these stressors interact and how we manage them as they occur can play a large role in sustaining the health and survivability of hives.

1.3.1 Pests & Pathogens

The major pest of honey bees are ectoparasitic mites, *Varroa destructor*, that originated from a closely related species, the Asian honey bees *(Apis ceranae*), but switched host and rapidly became widespread found everywhere European honey bees are managed, with the exception of Australia (Cantwell and Smith 1970). The presence of varroa mites spread quickly in the US through the movement of colonies across states for pollination services (Cantwell Smith 1970). Varroa mites feed on the abdominal lipids or fat body and hemolymph of bees which when infected during pupal development causes significant changes in physiology, such as reductions in body weight, hemolymph volume, abdominal carbohydrates, and vitellogenin proteins that are critical for over-wintering (Amdam et al. 2004; Ramsey et al. 2019). Other impacts of varroa feeding, include physical deformities (typically caused by mite-vectored viruses) and immunocompetence that may make bees more susceptible to pathogens, including the viruses vectored by varroa such as deformed wing virus (DWV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV) (Le Conte et al. 2010). Beekeepers often seek one product or compound that will control all mite issues, however, a more integrated pest management approach that includes multiple strategies (preventive, cultural, mechanical, and chemical options) is necessary to control mites on adults bees as well as reproductive mites sealed inside comb cells. Without management, varroa mites can

cause a colony to crash within 1-2 years, therefore proper pest management is a critical component to maintain healthy productive hives.

There are many other pests that can impact the health of honey bee colonies or the equipment used by beekeepers. For example, adult moths and larvae of the lesser wax moths (*Achroia grisella*) and greater wax moths (*Galleria mellonella*) which do not typically affect the health of honey bees directly, will tunnel through comb cells and are highly destructive to bee larvae, pupae, pollen, and honey stores (Kwadha et al. 2017). Unattended stored equipment, such as empty hive boxes with comb containing leftover pollen and honey stores, may easily become invaded by wax moths and overridden until combs become covered in frass and damaged beyond recovery (Kwadha et al. 2017). Wax moth control options consists of the use of chemical deterrents, such as products containing the active ingredient paradichlorobenzene (Para-moth) to deter female moths from depositing eggs in combs and on equipment (Kwadha et al. 2017) as well as the use of biocides, such as *Bacillus thuringiensis* (Mckillup and Brown 1991). Frames already infested with wax moths can be exposed to extreme heat or cold to destroy larvae and eggs that are already present (Cantwell and Smith 1970). Beekeepers that have used “moth balls” or products containing naphthalene risk harm to hives as the residues of this compound may leech into the wooden frames and comb and later may release toxic volatiles. Other pests that are less significant to hive loss but may contribute to or indicate stress include tracheal mites, small hive beetle, and *Nosema* pathogens. Tracheal mites *(Acarapis woodi)* are ectoparasitic mites that live in the bee trachea, or airway, and feed on hemolymph or circulatory fluids, reduces oxygen

availability, and negatively affects foraging activity. Small hive beetle (*Aethina tumida),* which are a more common hive pest and are prevalent in the southern parts of the United states, feed on honey, pollen, wax, and defecate in honey causing fermentation of food stores and potential losses in beekeeping combs (Cantwell and Smith 1970). *Nosema apis* and *N. ceranae* which has more recently displaced *N. apis* from US colonies*,* are microsporidian endoparasites that infest the midgut cells of bees and disrupt nutrient absorption (Higes et al 2008a). Despite their less severe impacts on hive health, beekeepers will attempt to manage these but are unaware that these stress-related diseases may indicate more severe underlining problems that weakened the bees and made them more susceptible to other stressors. Stronger colonies with ample pollen stores can withstand high *Nosema* spore loads, however, when other stressors, such as malnutrition (Rinderer and Kathleen 1977; Huang 2012) or pesticide exposure (Pettis et al. 2012; Wu et al. 2012), co-occur, lower worker longevity is observed. This makes management of each stressor an important factor, mitigating the impact of pests can reduce the potential for interactions between stressors that cause bee health decline.

Due to the social nature and large populations of honey bees, there are a number of very communicable, common diseases that are caused by viruses, fungi, and bacterium, that afflict hives. There are over 30 known viruses commonly detected in honey bees, some cause adverse health effects while others remain asymptomatic or exhibit no known impact. Often, hives may have multiple viruses present at any time (Traynor et al. 2016; Berenyi et al. 2006). In a healthy colony the bees may not exhibit

symptoms and the virus may lay in remission within the colony (Berenyi et al. 2006). Viruses can be transmitted vertically and horizontally to the queen, brood, and other nestmates. Transmission may also occur through direct contact with infested nestmates and mite vectors or indirectly through contaminated floral resources and surfaces. The most prevalent viruses are typically transmitted through the ectoparasite *Varroa destructor* mite. The viruses that are transmitted from these parasites include deformed wing virus (DWV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV) and have been shown to cause dramatic losses of colonies (vanEngelsdorp et al. 2009b; Cox-Foster 2007; Genersch et al. 2007).

Viruses may be prevalent in honey bees but there are other pathogens impacting the hive such as fungal and bacterial infections. There are multiple types of fungal infections most of which are considered stress-related meaning infections occur when colonies are immunosuppressed, weak, or combating other stressors. For example, *Ascosphaera apis* is a common fungus that causes chalkbrood disease by infesting the gut in developing larvae. The fungi out-competes host larvae for food causing larvae to die from starvation but as the fungus continues to consume the remaining body from inside, the dead larvae become “chalky” and hardened in appearance (Aronstein and Murray 2010). The third pathogen that can cause stress to colonies are bacterial infections. The bacteria *Melissococcus plutonius* which causes European foulbrood and affects mortality in brood is transmitted when the bacteria becomes incorporated into the bee bread or honey and is consumed by the larvae (Forsgren 2010). Another, more lethal and persistent bacteria is the spore-forming *Paenibacillus larvae* that causes

American foulbrood. It is another brood pathogen that infests the gut but differs from the others in that it is very transmissible and spores may remain viable and can survive within the comb for as long as 40 years (Chan et al. 2009). American foulbrood infection can be treated using antibiotics, however, this is not recommended as antibiotics do not kill the bacteria but rather masks symptoms and prevents its growth. The recommendations for managing outbreaks of this bacteria is to destroy all infected frames and sanitize remaining equipment with heat (Roetschi et al. 2008) (Wilkins et al. 2007).

Many of these pathogens have been examined closely but the interactions that occur between pathogens and other stressors are quite complex and still relatively understudied. There is still much to examine on the impacts of pesticides on the immune system of bees, specifically how exposure to pesticides that act on the central nervous system plays a role in immune incompetence causing bees to become more susceptible to other pathogens under certain conditions (O'Neal et al. 2018).

1.3.2 Poor Nutrition

Proteins, lipids, carbohydrates, minerals and vitamins play vital roles in colony growth, development, reproduction, immunity, and behavioral transitions in honey bees, therefore, proper nutrition is key to mitigating bee health decline. Colonies rely on forager bees to collect abundant and diverse sources of floral nectar and pollen to obtain nutritional requirements, including 10 essential amino acids that honey bees cannot produce and must obtain from their diet. Malnutrition in honey bees causes decline in overall colony health (Standifer 1980) by reducing stress resistance (Huang

2012), lowering immunocompetence (Alaux et al. 2010), and impairing communication and foraging capabilities (Scofield and Heather 2015). Colonies suffering from malnutrition may not be able to forage as effectively as healthier bees (Scofield and Mattila 2015). This weakening of the hive exacerbates other hive issues and allows opportunistic stressors (pathogens and hive pests) to take over. For example, more diverse pollen diets can upregulate enzymes vital for immune defense (Grimble 2001; Mao et al. 2013) and bees with ample protein, micronutrients, and amino acids exhibited reduced mortality associated with Nosema and IAPV infections (França et al. 2009; Cotter et al. 2011; Di Pasquale et al. 2013). Other research suggests that varroa mite feeding may limit protein metabolism as well as inhibit some immunity genes which in turn increases susceptibility to pathogens, including viruses vectored by varroa mites (Aronstein et al. 2012).

The overall composition of the landscape can greatly affect the number of flowers and impact nutrient availability and overall health of colonies (Donkersley et al. 2014). Degraded landscapes that lack bee forage can be caused by many factors including the over-use of herbicides and rapid conversion of natural habitats into agricultural cropping systems and urban developments. To optimize time and reduce energy costs bees will typically forage within approximately 3.2 miles from the hive but they will go further if they must (Eckert 1933). Colonies within 4 miles of forage dearths will not gain weight because of the extensive time and energy costs associated with foraging and therefore may not survive the winter due to the inability of the colony to build sufficient food stores (Eckert 1933). Areas with high floral diversity provide ample

options for bees to obtain appropriate levels of protein and carbohydrates. Bees that are provided high floral diversity exhibit increased longevity, increased production of jelly for brood, and increased resistance to other stressors (Haydak 1970; Crailsheim 1992; Di Pasquale et al. 2013; Vaudo et al. 2015). Due to the potential for nutrition to positively and negatively (depending on abundance or lack of, respectively) impact other stressors it is invaluable to continue examining the interactions that the factors may have when they occur in tandem.

1.3.3 Pesticides

Pesticides are designed to kill pests that are harmful or undesirable to humans. They are effective at the job they are designed for (i.e. insecticides target pest insects, herbicides target weeds, etc.) however, may have unintended effects on non-target organisms, such as honey bees. Pesticides are a major concern for beekeepers given the prevalence of pesticide use in agricultural and urban landscapes, as well as beekeeper-applied compounds. In fact, over 121 different compounds have been found in bees, pollen, and wax (Johnson et al. 2009; Mullin et al. 2010; Sanchez-Bayo and Koichi 2014; Ravoet et al. 2015). Adverse effects from pesticide exposure may cause direct mortality of individual bees (Le Conte et al. 2010; Mullin et al. 2010) or may cause sub-lethal effects that weaken the colony through the inhibition of critical social behaviors such as foraging, brood development, and hygienic behavior (Johnson et al. 2009; Mullin et al. 2010). As exposed foragers return to the hive with contaminated resources, the pesticide residues begin to accumulate (vanEngelsdorp et al. 2009ab). Mortality was found to be higher in brood raised in pesticide-laden “dirty’ comb when compared to

"clean" comb containing few or no pesticide residues. Further, the bees reared in "dirty" comb exhibited shorter longevity and increased susceptibility to *Nosema spp.* infection as adults when compared to those reared from "clean" comb (Wu et al. 2011, 2012). Three compounds (chlorothalonil fungicide, imidacloprid insecticide, and amitraz acaricide) were commonly detected and found in varying levels within comb, honey, bees, pollen, and brood food. Due to the prevalence of these chemicals in hive products, there is need to further investigate potential impacts of these residues on hive health and colony functions.

Fungicides are a class of pesticides designed to control fungal growth and mitigate damage caused by infection typically during the flowering or fruit development stage and if left untreated infections may become detrimental to crops (Oldroyd 1999). Although, fungicides do not target insects and have relatively low toxicity to insects, some active ingredients have shown harmful effects on bee brood, however, current regulatory policies surrounding fungicide use lack relevant pollinator protection guidelines and continues to be a growing concern for beekeepers (Kubik et al. 1999); (Yoder et al. 2013; Johnson et al. 2013; Thompson et al. 2014; Sgolastra et al. 2016); (Mao et al. 2017). Fungicides are commonly used in crops and orchards as both foliar spray applications and seed treatments (US EPA 1999; Wallner 2009). In many circumstances, these fungicides may remain prevalent in the surrounding environment for an extended period and residues of systemic fungicides may be expressed in pollen and nectar of the treated plants, contaminating forage for bees (Kubik et al. 1999). In citrus plants treated with the fungicide (metalaxyl, fosetyl-Al, H_3PO_3 or oxadixyl), residue

persistence and inhibition of the soil borne Black Shank disease (*P.* [*nicotianae* var.] *parasitica* and *P. citropthora*.) was seen for as long as 117 days past initial treatment (Matheron 1988) and these fungicides persisted at concentrations of 238 µg per g of soil for as long as six months (Blunt et al. 2015). When the fungicides are present in nectar and pollen the residues may be ingested and or incorporated into food stores such as honey or beebread (stored pollen). The fungicides may negatively impact beneficial fungi within the beebread and disrupt nutrient absorption (Yoder et al. 2013). Further, ingestion of contaminated food by adult bees can inhibit the production of ATP energy and reduce their ability to fly (Mao et al. 2017). Exposure to fungicides to larvae through brood food have shown apoptic cell death within the midgut (Ales and Ellis 2011). These nutritional deficits mimic poor nutrition and in environments where other stressors exist can lead to a synergistic effect (Degrandi-Hoffman et al. 2017). Studies show the presence of fungicides may synergistically interact or increase the toxicity of many other pesticides, particularly insecticides, making the combination more toxic than either alone. One study found a three-fold increase in the toxicity of ergosterol biosynthesis inhibitor fungicides and several neonicotinoids through oral or topical exposure while another found that when bees were treated with the fungicide fenpyroximate a ten-fold increase in toxicity occurred with a post treatment of tau-fluvalinate (Johnson et al. 2013; Thompson et al. 2014). Additionally, bees fed chlorothalonil in combination with coumaphos, a common beekeeper-applied acaricide exhibited mortality rates 3 times greater than chlorothalonil on its own (Zhu et al. 2014).

Combinations of these pesticides showed increased mortality in not only honey bees but bumble bees as well (Sgolastra et al. 2016).

Though studies have commonly addressed the presence of fungicides in the environment and the impacts they have on adult honey bee health, few have examined the impact once present inside the hive. Chlorothalonil fungicide was one of the most prevalent compounds, detected in 49.2-52.9% of wax (max: 53700 ppb, ave: 91.4 ppb), pollen (max: 98900 ppb, ave: 35 ppb) and bees (max: 878 ppb, ave: 7.2 ppb)(Mullin et al. 2010; Sanchez-Bayo and Goka 2014). Chlorothalonil was originally released in the US in 1966 to control fungal infections, such as rusts, mildew, blight, mold and algae, that affect fruit, vegetables, flowers, and crops (EPA, 1999). The mode of action for chlorothalonil is reduced deactivation of glutathione (Pompella et al. 2003) an important antioxidant in many organisms, such as fungi, that can mitigate damage to cellular functions (Tillman et al. 1973). An estimated 15 million lbs. of this compound has been applied since it was first released (EPA, 1999) and as a result of the pervasive use of chlorothalonil, residues may be detected (range of 1-57000 ppb) within comb, honey, and pollen (Mullin et al. 2010; Sanchez-Bayo and Goka 2014). Even at levels as low as 23.2 ppb, research has shown chlorothalonil in bee bread can cause sublethal effects on bee health by reducing the beneficial microbial fungi inside of the gut of bees, decreasing beneficial microbes in stored bee bread, and loss of these microbes has been linked to the regulation of pathogen infection in brood, such as the fungal disease chalkbrood (Yoder et al. 2013). The prevalence of this compound has led researchers into the examination of the impacts it may have on beneficial insects.

The second pesticide class of interest for this research are beekeeper-applied acaricides, specifically the compound amitraz and its metabolite 2,4-dimethylphenyl-N'-methylformamidine or DMPF. Both insecticides and acaricides are considered pesticides but acaricides specifically target organisms in the class *Arachnida* not *Insecta*. Originally created in 1969 by the company Boot co. (Harrison et al. 1973), it is used as an insect repellant, possible pesticide synergist, and tick and mite control for dogs (NCBi 2019). Amitraz works by inhibiting synthesis of prostaglandin and monoamine oxidases through interactions with the octopamine receptor and is targeted at organisms in the phylum *Arthropoda* (Bonsall and Turnbull 1983). This mode of action causes over stimulation of the central nervous system by stimulating alpha adrenergic receptors and eventual paralysis (Bonsall and Turnbull 1983) of the target organism.

In beekeeping, amitraz is utilized as an acaricide for the control of *Varroa* mites and is applied directly inside of the hive. The compound amitraz has been shown to cause significant mortality to bees exposed in a caged setting at doses above 0.01 g (Vandenberg and Shimanuki 1990). Queen bees also experience negative effects when they are exposed to amitraz including a reduction in egg laying and the size of her worker retinue or the number of nurse age attendants that care for her (Walsh et al. 2020). Though the active ingredient, amitraz, breaks down within a day, the metabolite DMPF is readily absorbed by wax due to its lipophilic nature (Korta et al. 2001). Of the many compounds found within bee's wax, DMPF is one of the most prevalent and the residues persist in 60.5% of wax, pollen, and bees samples in concentrations ranging from 9.2 – 43000 ppb with a median of ~200 ppb (Mullin et al. 2010; Sanchez-Bayo and

Koichi 2014; Ravoet et al. 2015; Johnson et al. 2013). Although residues may be prevalent and at levels that may cause detrimental effects, potential impacts of DMPF exposure are highly understudied. In fact, there are only a few studies (O'Neal et al. 2005, 2017; Papaefthimiou et al. 2013; Dai et al. 2018) that examine the metabolite DMPF and how it interacts with other pesticides. The effects of DMPF on bee health has received some attention in the last years with research suggesting that amitraz and its metabolite increase bee heart rate and decreases survival of bees that are infected with viruses (O'Neal et al. 2017). Examining how the residues present in brood comb interacts with development and health is the next step.

The third compound of interest in this review are the neonicotinoid insecticides. Neonicotinoids are a class of systemic insecticides derived from the nicotine compound which exhibits insecticidal properties by binding with nicotinic acetylcholine receptors (nAChRs) and causing a stimulation of nerve cells which may lead to eventual paralysis and death (Yamamoto 1999; Pompella et al. 2003; Tomizawa and Casida 2005). The first active ingredient, imidacloprid, was developed by Bayer Crop Science and released to the market in 1985 (Yamamoto 1999). Since the release of imidacloprid six other neonicotinoid insecticides have been added to the market thiamethoxam, acetamiprid, clothianidin, thiacloprid, dinotefuran, and nitenpyrum (Gervais et al. 2010). Each of these compounds has a slightly different chemical structure, toxicities, application methods, and uses to control a board spectrum of organisms. Neonicotinoids are listed as a category II or III level of toxicity to humans and are considered highly to moderately toxic to bees with toxicity varying in each active ingredient (Fishel 2005). Neonicotinoids

may be used in agricultural and urban landscapes as seed coat treatments, sprayed on foliage, injected into trees, applied to the soil, or directly added into the irrigation system (Yamamoto 1999). As systemic pesticides, neonicotinoid residues may translocate throughout the plant which makes for an effective insecticide for controlling stem boring and root feeding pests. This, however, means that residues may also accumulate in floral structures of treated plants, contaminating pollen and nectar which then exposes visiting forager bees (Stoner and Eitzer 2012; Sánchez-Hernández et al. 2016; David et al. 2016). In many countries there are strict regulations on the use of neonicotinoids due to concerns over the level of toxicity they may have for bees (Gross 2013). Neonicotinoids are still being researched to determine the full extent of their impact on bees, other organisms, and ecosystem functions. The regulation and ban of neonicotinoids have brought up questions regarding how they move throughout the environment and their effects on beneficial organisms (Gross 2013). Research has shown that the combination of neonicotinoids (Thiamethoxam= 1 ng/bee, Clothianidin = 0.8 ng/bee) and food sources that are nutritionally poor (containing 15% sucrose) can synergistically interact and cause a 50% decrease in survival, reduced consumption of food, and reduced glucose levels in hemolymph (Tosi et al. 2017). This nutritional stress may have already existed due to the presence of monoculture limiting foraging options or the presence of fungicides in pollen, nectar, and bee bread (Mullin et al. 2010; Sanchez-Bayo and Koichi 2014; Ravoet et al. 2015) which have been shown to reduce the beneficial fungi in bee bread that affects gut microbiomes and nutrient absorption (Yoder et al. 2013). In bees that have ingested neonicotinoids there is evidence of

suppressed immunity and increased presence of viral pathogens (Prisco et al. 2013). In concentrations as low as 10 ng per bee acute mortality can occur in laboratory settings (Iwasa et al. 2004). Field level studies show decreases in foraging, communication, and colony development when colony level at 10 µg/kg oral exposure of imidacloprid (Kirchner et al. 1999).The foraging bees do not always experience acute death and may return to the hive with contaminated food stores which causes an accumulation of neonicotinoids in honey, pollen, bee bread, wax, and bees are found in concentrations from 5 to 400 ppb (Mullin et al. 2010; Stoner and Eitzer 2012; Woodcock et al. 2017; Kartal 2019). The numerous effects of neonicotinoids on honey bee health have become a concern for beekeepers and makes them a valuable insecticide class to investigate.

1.3.4 Poor Management

Among beekeepers the phrase “ask ten beekeepers and get eleven answers” is commonplace. The attitude of approaching the same problem with many solutions can be helpful in some situations but in others it can lead to more issues. Despite 400 years of domestication in the US, roughly 8% of honey bee colony mortality is attributed to improper management (vanEngelsdorp et al. 2008). Over those 400 years, beekeeping has evolved from managing colonies in woven baskets, or skeps, to wooden Langstroth boxes (named after Rev. Lorenzo Lorraine Langstroth) that hold vertical wooden frames. Frames are removable and house the bees and comb cells containing brood and food stores. This system allowed beekeepers to remove frames to inspect inside the colonies for signs of disease, assess food stores, and examine brood making honey bees much easier to manage. However, it also allowed beekeepers to more easily reuse comb

frames over multiple seasons. Equipment from colonies that died out is quickly put back into operation with a new colony of bees but over time comb frames may become contaminated by pathogens and pesticides and may continually reinfect or expose new colonies. Many of the issues beekeepers face change over time and more extensive research is needed to address outdated practices and develop new management strategies. Poor management techniques that may harm the overall health of the colony include improper or complete lack feeding colonies (Standifer 1980), insufficient inspections for queen health and brood diseases, as well as the mismanagement of pests, and prevention of swarming behavior.

Overwintering hives often require supplemental food stores and many new beekeepers may not know that it is an important part of colony management (Standifer 1980). Colonies may not be able to survive or grow appropriately because they lack the proper nutrition. Many of these management problems occur because there is a lack of extended education and a misunderstanding of biology.

Beekeepers face stressors such as the ectoparasitic Varroa mites that require proper management either through a number of nonchemical tools or through the use of chemical interventions, like acaricides. Improper use of these chemicals is common, though directions for use are on the package they are not regulated once the product is in hand. The chemicals are often applied in the wrong amount or frequency, at the wrong time, or even in a manner that causes increased toxicity in bees, such as increasing the concentration or mixing with other ingredients. Additionally, several miticides are synthetic lipophilic compounds which leave potentially harmful residues

that accumulate in wax, pollen and even bees (Mullin et al. 2010; Sanchez-Bayo and Koichi 2014; Ravoet et al. 2015). To contrast, other beekeepers, misunderstand how pests should be managed and will choose not use any control method at all. This leads to spikes in Varroa populations and causes infested colonies to weaken which then become targets for opportunistic robber bees to steal hive resources and transfer mites back to their hive. Thus, neighboring apiaries are all impacted when beekeepers mismanage mites in their hives.

In addition to beekeeper-applied pesticides, bees may become exposed to other agrochemicals through contaminated floral nectar, pollen, water, and even soil which is then brought back to the hive and is either consumed by nestmates are stored in comb cells (Kubik et al. 1999; David et al. 2015). This leads to an accumulation of pesticide residues within multiple matrices (pollen, wax, bees) in the hive over time (Mullin et al. 2010; Sanchez-Bayo and Koichi 2014; Ravoet et al. 2015) and bees reared from pesticide-laden or “dirty” comb have exhibited impacts on brood, including higher mortality, delay development, and higher susceptibility to pathogens as adults (Wu et al. 2011, 2012). These studies highlight that there are unknown interactions occurring among stressors, including exposure to pesticide residues, that may indirectly impact bee health in consequential ways. Given that Varroa mites continue to be the greatest concern for beekeepers the interaction between chronic pesticide exposure and mites is a critical knowledge gap. For example, delayed development and emergence of adult workers expressed in bees reared from pesticide-laden comb may provide a reproductive advantage for Varroa mites as mother mites produce offspring that

develop alongside developing host bees, however, further research would be necessary to assess this. Lastly, great efforts, are being made to breed Varroa resistant traits in bees, however, if mites are obtaining reproductive advantages due to delayed development of host worker bees when reared in pesticide-laden comb then these Varroa-resistant traits may be rendered ineffective or lost. Though many beekeepers and researchers recommend comb replacement there are no regulatory standards for how often it should be done.

Honey bee exposure to agrochemicals outside the hive (Kubik et al. 1999; David et al. 2015) can not only lead to accumulation within the hive but it can cause sublethal effects that include disorientation, indirect mortality through contaminated stored food, reduced foraging, among other things (Mullin et al. 2010; Johnson et al. 2009). This can be a major management problem as there are currently no standards for how to monitor or manage sub lethal pesticide exposure. There are measures that can be taken for acute pesticide mortality that can financially aid beekeepers that lose colonies from a single, lethal exposure. These measures are available after the colony has died and do not provide preemptive actions to reduce a sublethal exposure to pesticides. The ability to monitor for lethal and sublethal pesticide exposure is in part due to the lack of knowledge surrounding events. Many beekeepers do not trust apiary inspectors and do not report pesticide-related bee kills, making tracking of pesticide impacts very difficult. They also do not want to report pesticide kills in fear of losing contracts with farmers and landowners where the bees are kept. Which makes understanding when a pesticide exposure occurs and the early symptoms, quite difficult.

1.4 Conclusion

Honey bees are an important part of our agricultural system and economy. They provide pollination services that result in billions of dollars added value, and the need for these pollination services grows every year. This makes the decline of honey bees an important conversation and has prompted researchers to examine why populations are dwindling. Most of the decline is attributed to 5 major stressors; pests, pathogens, poor nutrition, pesticides, and poor management. The prevalence of biotic and abiotic factors throughout the season has generated interest in further examining their potential to interact with one another. Little is known about the impact pesticides have once within the hive.

In this chapter, I reviewed the literature on honey bee health and management challenges and in chapter 2, I present research that examined the potential impacts of pesticide residues, specifically chlorothalonil fungicide and the metabolite DPMF of the commonly used acaricide amitraz, in brood comb on honey bee health and development. Findings indicate that amitraz residues may cause developmental effects on hypopharyngeal glands but there was no evidence to suggest adverse effects on larval developmental from exposure to chlorothalonil residues. In chapter 3, I further present research evaluating the use of dead bee traps as an effective monitoring tool for pesticide incidents. Here, I introduced pesticide-treated bees into hives equipped with traps that collect dead and dying bees removed from within the hive. Bees were treated with varying sub-lethal doses of imidacloprid and paint-marked so they could be easily identified from trap collections and distinguished from dead untreated bees captured in

traps. Results suggest that the monitoring tool was more effective at capturing bees in spring when colonies were smaller and that larger traps were more effective at capturing dead bees removed from the hive than the less optimal smaller traps. Lastly, the final chapter of this thesis is an extension guide for beekeepers that outlines the construction and use of the dead bee traps as monitoring tools for pesticide exposure as well as other hive health issues. Our research seeks to better understand if our beekeeping management practices, which include application and residue accumulation of pesticides in brood comb, impacts worker bee development. Additionally, this research seeks to find better ways to monitor for pesticide incidences so that beekeepers can more readily recognize and manage hives that may have pesticide exposure. This project will help develop integrated pesticide management recommendations that will mitigate and reduce the impacts of pesticide residues in comb and improve the health and productivity of hives.

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Chapter 2: An Examination of Potential Impacts of Pesticide Residues in Brood Comb on Honey Bee Health.

2.1 Introduction

Over one third of the crops grown in the United States require active pollination from insects (Klein 2007). Commercially managed honey bees perform most of these pollination services contributing over $15 billion US dollars in added value to many crops such as almonds, blueberries, broccoli and numerous other fruits, vegetables, and nuts (Losey and Vaughan 2006; Calderone 2012). In addition to generating income from pollination service fees, beekeepers may use other hive products (honey, pollen, propolis, wax) to produce value-added commodities (lotions, soaps, health supplements, and lip balms) which has expanded the industry and economic return for beekeepers.

Within the agricultural sector, crop production output has increased by 170% and the demand for contracted pollination services provided by managed honey bees has increased by 300% , but there has only been a 45% increase in the beekeeping industry over the last 15 years (Aizen and Lawrence 2009; USDA 2018). The number of colonies available for pollination continues to lag as demand increases with higher crop production which is necessary to sustain the world's growing population. This strain on beekeepers and the agricultural industry is further exacerbated by high losses of honey bee colonies and the decline of wild bee health globally (Aizen and Lawrence 2009; NRDC 2015). Despite higher demand for honey bee services, the number of colonies present in the US has declined by more than 4 million, from 6 million colonies to the

current estimate of ~2 million (Ellis et al. 2010). This strain on beekeepers and the agricultural industry is further exacerbated by high losses of honey bee colonies and the decline of wild bee health globally (Aizen and Lawrence 2009; NRDC 2015).

Annual losses of honey bee colonies in the US during the last five years has ranged between 11% - 72% with many states experiencing consistent losses of roughly 40% (vanEngelsdorp et al. 2012; Lee et al. 2015; Seitz et al. 2016; Kulhanek et al. 2017). In most other agricultural systems, this level of loss would devastate businesses and for some beekeepers it has (Steinhauer et al. 2013). However, many can recover some losses through management by splitting the inventory of remaining hives though often at high economic expense. With overburdening losses to beekeepers and the increasing demands for pollination services, there has been considerable research into causes and factors contributing to colony health decline (Ellis et al. 2010; vanEngelsdorp et al. 2012; Steinhauer et al. 2013; Lee et al. 2015; Seitz et al. 2016; Kulhanek et al. 2017).

Multiple factors have been identified as contributing to bee decline, including what some refer to as the 5 P's: pests, pathogens, poor nutrition, pesticides, and poor management (United States Congress 2010; Goulsen et al. 2015). These factors have been studied to varying degrees but the primary focus here is on the impacts of pesticides. Bees may encounter pesticides through direct contact (dermal or inhalation exposure) during foraging or from contaminated hive surfaces, such as comb. Bees may also become exposed to pesticides through oral ingestion of contaminated forage (nectar/pollen) and water sources. For example, studies show that residues of systemic insecticides, such as neonicotinoids applied as seed treatments, foliar sprays, or

introduced directly into the soil or irrigation, can migrate throughout the plant and may be expressed in floral nectar and pollen (Bonmatin et al. 2003; Sánchez-Hernández et al. 2016; David et al. 2016). The neonicotinoid contaminated resources can be unintentionally picked up by foraging bees and potentially brought back to the hive causing further impact to the colony (Kubik et al. 1999; David et al. 2015). Bees require water for thermoregulation and food processing, Therefore, contaminated runoff water from crop fields may also be picked up by water-collecting bees, brought back to the hive, and shared among nestmates. Beyond environmental exposures, bees are exposed to pesticides through beekeeper-applied compounds, such as acaricides used within the hive to control the major ectoparasitic pest, *Varroa destructor* mites (Johnson et al. 2009; Mullin et al. 2010; Krupke et al. 2012).

The presence of pesticides in nectar and pollen becomes a confounding issue when foraging bees return to the hive and expose other nestmates, including the queen and brood, with contaminated food or through contact with contaminated bees and comb (Stoner and Eitzer 2012; Sánchez-Hernández et al. 2016; David et al. 2016). More than 121 different pesticides residues have been documented in stored pollen (beebread), honey, comb, and bees (vanEngelsdorp et al. 2009; Mullin et al. 2010; Sanchez- Bayo and Goka 2014; Ravoet et al. 2015). Pesticides vary in toxicity to bees and unintended exposure may cause acute mortality or sublethal impacts on health. (Le Conte et al. 2010; Mullin et al. 2010; Degrandi-Hoffman et al. 2015; USDA 2017). The prevalence of these pesticides outside and within the hive has resulted in further examination of how exposure may impact bees in subtle, sublethal, and or indirect ways

that disrupt colony functions rather than focusing on direct acute or chronic lethality on individual bees. Sublethal effects of pesticides on bees are highly varied, compound dependent, and may disrupt various behaviors, cognitive functions, and physiological processes including impaired foraging (difficulty navigating, loss of memory, and reduced learning capacity), impaired olfactory functions, and suppressed social immunity or immunocompetence in bees making them more susceptible to other stressors (Decourtye et al. 2003; Iwasa et al. 2004; Le Conte et al. 2010; Dively et al. 2015; Fisher et al. 2017; O'Neal et al. 2018).

Two pesticides commonly detected inside the hive and often at high levels include fungicides picked up from the environment and beekeeper-applied acaricides used to control Varroa mites. In this study, we focused on the most prevalent fungicide, chlorothalonil, and the most used beekeeper-applied acaricide, amitraz. Chlorothalonil is a fungicide frequently used in orchards on fruit and nut trees (Kubik et al. 1999; David et al. 2015) and applied as a foliar spray to combat infections from mold, mildew, algae, bacteria, and rot that would be detrimental to crop production if left unmanaged. It is considered a category IV, low toxicity compound and is listed as not acutely toxic to bees (US EPA, 1999). As a result, chlorothalonil is approved for used on numerous pollinator-dependent crops and is approved to be applied during bloom which may explain its prevalence in the hive and why residues are often at high levels in stored pollen and comb (Kubik et al. 1999; David et al. 2015; Fisher, et al. 2017). In fact, multiple studies have found chlorothalonil to be one of the most commonly detected

pesticide found within the hive in 53% of samples and at levels as high as 57 ppm in comb (Mullin et al. 2010; Sanchez-Bayo and Goka 2014; Ravoet et al. 2015).

Honey bee colonies are contracted for pollination in orchards, therefore the use of some fungicides, like chlorothalonil, during bloom, are of particular concern to beekeepers as foragers will collect contaminated nectar and pollen and bring it back to the hive (Kubik et al. 1999; David et al. 2015). Impacts from chlorothalonil exposure are wide ranging in the literature and some studies suggests chlorothalonil can exhibit interaction effects with other compounds and or hive stressors. For example, honey bee larvae fed a diet spiked with chlorothalonil (100 mg/L) exhibited reduced survival (Dai et al. 2018a), and another study showed that similar levels of chlorothalonil (100 mg/L) also lowered digestion of protein, and increased susceptibility to viral infection when fed 2,300 ppb in pollen (Degrandi-Hoffman et al. 2015). Further, chlorothalonil at low concentrations (23.2 ppb) in bee bread has shown to indirectly affect bee health by reducing beneficial gut microbes, altering microbial communities in stored bee bread, and even through regulation of pathogen infections, particularly fungal diseases such as chalkbrood (Yoder et al. 2013). These microbes play a critical role in bee health as they aid in the digestion of pollen grains so that bees may readily absorb nutrients (Mao et al. 2007). Altering or reducing microbial functions may lead to malnutrition in bees which in turn can impact that ability to fly further disrupting foraging capacity for exposed colonies. Chlorothalonil alone does not cause acute toxicity to adult bees but studies have also shown there are synergistic interactions between chlorothalonil and beekeeper-applied acaricides (Johnson et al. 2013; Zhu et al. 2014). Johnson et al.

(2013) found that when topically exposure to chlorothalonil (10 µg/ bee) was combined with acaricides, such as thymol (10 µg/bee) and tau-fluvalinate (1 µg/bee), acaricide toxicity to bees increased by 2-fold. Further, when chlorothalonil (34 mg/L) was fed to bees with the acaricide coumaphos (8 mg/L), treated larvae exhibited a 4-fold increase in mortality (Zhu et al. 2014). Another study showed that less than 50% of experimental bees survived to adult emergence when bees were fed pollen treated with chlorothalonil (0.25 µg/bee) and combined with all of the following pesticides; glyphosate (0.0086 µg/bee), imidacloprid (0.06 µg/bee), chlorothalonil (0.25 µg/bee), chlorpyrifos (0.005 µg/bee), amitraz (0.75 µg/bee), coumaphos (1.85 µg/bee), fluvalinate (4.59 µg/bee) (Tomé et al. 2020).

The other compound prevalent in brood comb, and of focus in this study, is the break-down product of the acaricide amitraz, or N-(2,4-dimethylphenyl)-N-methylformamidine (DMPF) metabolite (US EPA 1996; Johnson et al. 2009, 2013). Amitraz is a beekeeper-applied chemical that rapidly metabolizes or degrades into 2,4-dimethylformamidine (DMF) and N-(2,4-dimethylphenyl)-N-methylformamidine (DMPF). Amitraz is classified as a category II toxicant for dermal exposure, meaning that it is moderately toxic when contact is made to skin but is “practically non-toxic to bees” (US EPA 1996). Though amitraz is used within the hive it still can cause sublethal effects on the health of honey bees. Studies have shown it is persistent in honey for up to 10 days before it degrades into DMF and DMPF metabolites (Korta et al. 2001). Amitraz, is not detected in wax because it rapidly degrades into DMPF within approximately 24 hours of exposure (from 0.07 to 2.35 $mg.kg^{-1}$) (Korta et al. 2001; Martel et al. 2007). The

metabolite DMPF is detected in over 60% of combs tested at levels ranging 5-43000 ppb (Mullin et al, 2010; Sanchez-Bayo and Goka 2014; Ravoet et al. 2015), however, another study detected residue levels averaging ~16,858 ppb for DMF and DMPF metabolites and suggested some transfer of residues may have occurred to brood (Morales et al. 2019). High DMPF residue levels is attributed to the over use and dependency of amitraz to manage ectoparasitic *Varroa destructor* mites, a major pest of honey bees, which feeds on fat stores and circulatory fluids of bees and acts as a vector to several viruses.

Research on the potential impacts of amitraz on bees has shown some negative effects on survival but have been quite limited. Further understudied, are the potential impacts of amitraz metabolites in food stores and comb. Dai et al. (2018b) showed a delay in development of bee larvae when fed a diet contaminated with amitraz (46 mg/l) and decrease of approximately 25% in survival from egg to adult when fed a diet with amitraz (46 mg/l)) at levels comparable to what has been found in brood comb (Dai et al. 2018b). Additionally, exposure through abdominal injection and topical exposure to amitraz at levels of 10^{-6} M and 10^{-9} M caused a biphasic effect on the heart, or a decrease in heart rate at low levels and an increase at high levels which can impact circulatory system and therefore the ability to properly thermoregulate (Heinrich 1987; Papaefthimiou et al. 2013). While another study shows that oral exposure to amitraz and DMPF at 100 µM caused increased heart rate and decreased survival of bees when stressed by a virus formulated in a laboratory setting as a model system for non-enveloped RNA viruses called flock house virus (FHV) (O'Neal et al. 2017). Although amitraz is a treatment for varroa mites, a study completed by de Mattos et al. (2017)

showed a decrease hygienic behavior in bees to the presence of varroa when topically exposed to amitraz (2.8 μg/bee) indicating that though amitraz may control varroa it may also be inhibiting valuable varroa resistant behaviors.

While the impacts of amitraz and chlorothalonil exposure through oral ingestion and topical application have been examined, few studies have assessed the effects of DMPF metabolite or chlorothalonil residues in comb on bee health. Additionally, there are major gaps in science on the effects of accumulating pesticide residues in brood comb on developing workers, queens, and drones. Earlier studies showed worker bees reared in pesticide contaminated comb exhibited higher mortality, delayed larval development, and increased susceptibility to *Nosema* spp. infection as adults (Wu et al. 2011, 2012), However, the residues reported in this study were complex mixtures containing 4-17 compounds and, thus, the observed effects cannot be correlated to a specific compound. Given the high levels and prevalence of both chlorothalonil and amitraz metabolite (DMPF) in hive products (Mullin et al. 2010; Sanchez-Bayo and Goka 2014; Ravoet et al. 2015), further research is needed to assess potential impacts on the development of honey bees.

The aim of this study was to examine the effects of chlorothalonil and DPMF to bee larval development and adult health. It was found that DMPF caused a significant reduction in the size of acini within the hypopharyngeal glands of bees raised in treated comb sections. To determine this, we treated individual comb frames with either chlorothalonil or amitraz at concentrations that were commonly found in wax and then

assessed several health measures to determine potential effects on egg-laying and larval development in honey bee workers.

2.2 Methods

2.2.1 Pesticide Treatment & Application

To assess potential effects of pesticide residues on the development of worker bees, twelve frames of newly drawn comb were randomly assigned a compound (chlorothalonil or amitraz) and a concentration (low, medium, high). Each comb frame was then divided into three sections or blocks of 144 comb cells (12 cells X 12 cells). Blocks were adjacent to each other and located in the brood area (contained roughly 7 mm from the top and side edges and 4 mm from the bottom) of the frame. Within each frame, one block of comb was assigned a compound treatment (chlorothalonil or amitraz) which was applied at either low, medium, or high concentrations. The remaining two blocks were assigned one of two control groups (acetone solvent and untreated). There was a total of six frames treated with each compound and two frames per treatment level. The arrangement and order of the three block treatments were randomly assigned low, medium, and high treatment levels for chlorothalonil (0.1, 1, and 10 mg/L or 100, 1000, and 10,000 ppb) or amitraz (0.01, 0.1, and 1 mg/L or 10, 100, and 1,000 ppb). Treatment levels for each compound were selected to cover the range of exposure levels commonly observed in comb. (Mullin et al. 2010; Wu et al. 2011; Ravoet et al. 2015; Sanchez-Bayo and Goka 2014).

To treat the blocks of comb in experimental frames, a stock solution was made for each compound by dissolving 50 mg of the solute compound into 50 ml acetone

solvent followed by serial dilutions to obtain the appropriate high, medium, and low treatment concentrations. Solutions were sprayed onto comb blocks and during application adjacent sections were protected by sealing off comb cells using wax paper. To ensure equal treatment coverage, each 144 cell block was divided into 36 cell sections. A 32 oz. chemically resistant ZEP Professional Sprayer spray bottle was then used to mist treatment solutions onto each section 5 times. This application method yielded 3.5 ml of treatment solution per block or ~100 µl into each cell. The acetone solvent was allowed to evaporate off over 24-hours before frames were used in hives.

2.2.2 Apiary Set-up & Queen exclusion

The experimental trials took place at the University of Nebraska – Lincoln research apiary located on East Campus (40°49'44.4"N 96°39'26.7"W)) from April through October in 2019. Three European honey bees (*Apis mellifera* L.) colonies, each containing roughly 40,000 to 60,000 bees bred from Carniolan and Italian stock, were used as mother colonies to house experimental frames during all replicated trials. Queens from mother colonies were caged on randomly assigned experimental frames to allow queens to lay eggs in all three blocks of treated combs. Queens were caged onto the frame using push-in cages made from 1/8' metal mesh with a queen excluder screen that allows slim-bodied workers to pass through and care for the queen but prevents larger egg-laying queens from escaping. After 24 hours, the queens were released and secluded away from the experimental frames for the reminder of the replicate. Experimental frames with newly laid eggs were then placed next to other frames containing young brood and ample nurse bees to care for brood. Mother colonies were

maintained using standard beekeeping management practices and assessed for health issues, such as brood diseases throughout the season. Further, no pesticide treatments were applied during the experiment. Instead, varroa mite levels in mother colonies were regularly monitored and managed through cultural and mechanical control tactics (breaking brood cycles and drone brood trapping). Additionally, food stores were monitored throughout the season and supplemented when needed to ensure mother colonies had adequate pollen and nectar to rear brood in experimental hives.

2.2.3 Larval Development Measures

To assess potential impacts of residues in brood comb on worker bee development, the number of eggs, larvae, and pupae within each comb section (144 cells per block) was quantified and compared across treatment groups. Brood assessments occurred at each developmental stage: egg stage (day 1 of development), 1st instar larvae (4 d old), 5th instar larvae (8 d old), prepupae (12 d old), and pupation/pre-emergence (19 d old). On the 19th day of development, frames were removed from the hive and placed in an incubator (Darwin Chamber Company model H024) set to 33°C with humidity at between 50%-60%. Smaller push-in emergence cages were placed on each individual comb section to isolate treatment groups and prevent intermingling of newly emerged bees from different treatments. Assessment of adult emergence was quantified starting at time marker “0 hour” which indicated the time that queens were released from egg-laying cages exactly 20 d prior and assessments continued at 4, 8, 12, 24, and 28 h after (which was the latest recorded emergence time). At each time point, the number of new-emerged bees in each comb section was

quantified, collected, and set up in quart deli cups with screen lids and raised screens in the base for ventilation fed fresh pollen patty (combined with sugar water (1:1 w/v)), syrup, and water for 24 h. This continued until all bees had emerged from each section which typically took about 48 hours (after time marker “0 hour”) to complete. Newly emerged bees were then placed in falcon tubes in a Frigidaire commercial chest freezer model no. FFC07K1CW0 for later dissection and analysis. Analysis of the proportion of adult bees emerging at 0 h, 4 h, 8 h, 24 h and 28 h from treated comb (acetone, untreated control, and compound) was represented graphically but was not analyzed due to lack of replication.

2.2.4 Adult bee dissection and measures

Ten newly emerged bees (1 d old) from each treatment comb section were randomly selected and dissected for abdominal lipids or fat body and hypopharyngeal gland analysis. Fat bodies were assessed to determine potential impacts on bee nutrition or lipid stores vital for overwinter. Fat bodies are located inside the bee on the ventral side of the abdomen and serve as energy reserves vital for sustaining bees through pupae development as well as the overwintering process. The hypopharyngeal glands, located in the head between the two compound eyes, were also measured to assess impacts on their ability to produce glandular secretions necessary for brood growth and development. Bees were dissected by first removing the stinger and pulling out the entire intestinal tract, including the honey stomach, from the abdomen. The abdomen and head were then detached from the remaining body and stored individually in a Frigidaire commercial grade freezer model no. FFC07K1CW0 at -10° F or

-23.33° C in microcentrifuge tubes for fat body and hypopharyngeal gland analysis, respectively.

For fat body analysis, tubes containing abdomens were incubated and dehydrated at 70° C for 24 hours in a drying oven (Thelco model 70D). Dried abdomens were weighed prior to adding 300 µl of methanol:chloroform (1:1) solution into each tube to dissolve fat body stores (Smart et al. 2016). After 24 hours, the solution was decanted, and the abdomens were placed back into the oven to dry for another 24 hours. After, abdomens were reweighed and the change in weight was determined to be the amount of fat bodies dissolved by the methanol:chloroform solution.

Hypopharyngeal glands, are the largest gland in the honey bee, consists of long paired lobes made up of clusters of ~550 acini, and located in the head. Studies show that there is a positive correlation between the size of the gland and its glandular activity (Deseyn and Billien 2005) and that the acini are largest for young bees and peak in size by day 6 due to the use of the glands as secretory vesicles for jelly (Hrassnigg and Crailsheim 1998). To determine the average gland size for each bee, hypopharyngeal glands were removed from heads and deep focus images were taken to measure the perimeter and diameter of 10 individual acini per bee. Images were taken using a Unitron Z850 Stereomicroscope (8-50x zoom) equipped with Canon T5i camera and Quick Focus Micro 3.1 software.

2.2.5 Statistical Analyses

Larval Development

Egg-laying was not consistent across experimental frames and comb blocks, therefore the number of replicates that queen bees laid eggs in experimental frames were analyzed by treatment type (control, acetone, or compound) and level (low, medium, or high) for chlorothalonil and amitraz to determine whether the residues had any deterrent effect on queen egg-laying behavior. Additionally, the average number of eggs laid in each comb block and the proportion of individuals that reached the subsequent developmental stages (1^{st} and 5^{th} instar larvae, pre-pupae, pupae, and adult emergence) were quantified however not statistically analyzed due to insufficient sample size. The proportion of eggs that successfully reached adult emergence were statistically analyzed across treatment types (acetone, control, and compound). All data were assessed for normal distribution and equal variance and transformed using a generalized linear mixed model (GLMM)-(*link*-natural log function.) to account for the underlying distribution of the data. A Binomial distribution was used to fit the count response with repeated measures, Beta Distribution was used to fit the proportion response with repeated measures and statistical analyses were completed with Analysis of Variance (ANOVA) models followed by Tukey's HSD means separation tests using SAS 9.4 software program.

Hypopharyngeal glands and fat body

Measurements for hypopharyngeal gland size (acini perimeter and diameter) and fat body (weight) had insufficient sample size, therefore data were pooled across dose and analyzed only by compound type (control, acetone, compound) for chlorothalonil and amitraz. To assess if chlorothalonil or amitraz residues negatively

impacted hypopharyngeal glands, vital for performing proper brood care, or reduced likelihood of survival due to lower fat stores. Analysis of variance (ANOVA) models and Tukey's HSD tests were performed to compare treatment measures to control groups for each compound separately. All data were normally distributed and exhibited equal variance. Statistical differences were determined at α=0.05 and analyses were completed using SAS 9.4 software program.

2.3 Results

2.3.1 Egg Laying performance

A total of 25 replicated trials were performed with amitraz (*n* = 9) and chlorothalonil (*n* = 16) treated frames. Data showed a lower proportion of eggs (13.9%) were laid in combs treated with low doses of amitraz. To contrast, in medium and high amitraz treated frames, egg-laying was more successful and occurred in 84% and 33% of replicated trials, respectively. Additionally, eggs were successfully deposited in 60% of trials when queens were caged on frames treated with chlorothalonil at low concentrations and slightly lower egg-laying success was observed in medium (32%) and high (45%) chlorothalonil treated frames. Despite the differences observed among dose levels, the control groups (acetone and untreated comb sections) paired with each compound treatment also yielded similar trends in egg-laying success, suggesting other factors driving poor egg-laying performance in this experiment. No statistical differences were observed in egg-laying rates for either treatment (amitraz ($F_{2,12}$=1.64 p=0.23); chlorothalonil ($F_{2,12}$=0.25 p=0.78)) or dose levels (Figure 2.6.1).

Frames that had eggs laid in comb cells were then quantified at day 1 of development immediately after the queen was released. Data showed a dose response of the number of eggs laid in combs treated with amitraz with the average(±SE) number of eggs decreasing (144 ± 150, 61.9 ± 34, 8.4 ± 5.7) as dose increased from low, medium, high treatments, respectively. The average number of eggs laid in acetone solvent and untreated comb sections (averaging(±SE) 78.9 ± 10.8 and 78.4 ± 19.9 eggs across all dose levels, respectively) was more consistent in combs paired with chlorothalonil compared to those paired with amitraz. The average number of eggs laid in untreated comb (average 101.06 ± 17.3 eggs across all doses) was higher than in acetone solvent treated combs (average 58.4 ± 10.3 eggs). No statistical differences in the average number of eggs laid were observed for either treatment (amitraz ($F_{2,10}$=3.7 p=0.06); chlorothalonil ($F_{2,10}$=1.25 p=0.33)) or dose levels (Figure 2.6.2).

2.3.2 Larval Development

Of the 25 total replicated trials performed, 15 had successful egg deposition in at least one of the three comb sections for amitraz (*n* = 6) and chlorothalonil (*n* = 9) treated frames and continued for assessments on larval development. Replicated trials were examined for the proportion of eggs that survived through the larval stages and successfully emerged as adults. Comb treated with high levels of amitraz did not have any bees successfully emerge as adults, however, 24% and 33% of eggs emerged from low and medium amitraz treatments, respectively. To contrast, chlorothalonil treated frames showed similar emergence rates in low, medium, and high treatments and averaged 16%, 28% and 23%, respectively. Bee emergence from comb treated with

acetone solvent ranged from 28 to 37% for amitraz frames and 5 - 30% for chlorothalonil frames while emergence from untreated comb ranged from 15 - 45%. No statistical differences were observed in the proportion of eggs that survived to adults between controls and compound treatments (chlorothalonil ($F_{2,9}$=0.61 p=0.56)) amitraz ($F_{2,9}$=0.03 p=0.9692)) or dose levels (Figure 2.6.3).

The proportional number of brood that survived to the next developmental stage (eggs (day 1), 1st instar larvae (day 4), 5^{th} instar larvae (day 8), early pupae (day 12), late or pre-emergence pupae (day 19) in brood developing from treated comb (acetone, untreated, or compound) were quantified but not analyzed because sample size was insufficient due to the lack of replicates in which eggs were laid consistently in all treatment sections. Many times the queens would only lay in one or two sections of the frame but not all treated comb making comparisons across treatment groups difficult. The data suggest mortality was highest among young brood particularly during egg eclosion and into early larval instar stage for both amitraz and chlorothalonil. And the proportional survival rate increased as larvae approached pupal development (Figure 2.6.4). Lower survival rates in early instars follow previous research indicating that later larval stages of development are less vulnerable and more likely to survive (Sakagami and Fukuda 1968), however, more data would be needed to validate this observation.

The adult emergence data suggests that there were no evident delays in larval development time and adult emergence in bees reared from either chlorothalonil or amitraz treated combs. There were indications that the queens may have laid in control

comb (control and acetone) before choosing to lay in comb treated with chlorothalonil due to the average(±SE) proportion of bees in treated comb that emerged at 24 hours 37.8 ± 4% and at 28 hours 23.9 ± 13%. This indicates that more than 61.7% of the bees reared in comb treated with chlorothalonil emerged at the later hours whereas comparatively, acetone and control had a combined proportional emergence of 29.3 ± 11% and 44.4%, respectively, before the 24 hour time mark (Figure 2.6.5). This could imply the possibility that queens choose to lay in the control comb first before laying in the contaminated comb and are preferentially choosing less contaminated comb over comb with higher levels of pesticide residue present but more research would be needed to assess this.

2.3.3 Hypopharyngeal gland & Fat body

Bees reared in chlorothalonil-treated combs, showed no observed differences in the average size of hypopharyngeal gland acini (diameter ($F_{2,5}$=0.68 p=0.55); perimeter ($F_{2,5}$=2.88 p=0.15)) compared to control groups (Figure 2.6.6). Bees reared in amitraz-treated comb exhibited statistically smaller acini diameter ($F_{2,5}$=9.14 p=0.02) and perimeter ($F_{2,5}$=6.55 p=0.04); a 20.3% reduction in acini width and 17.3% reduction in acini perimeter compared to control groups (Figure 2.6.7). This data indicates that larval exposure to amitraz may lead to less developed hypopharyngeal glands which could then potentially further impact the quality of brood food, however, more research is necessary to assess this. Data showed that the amount fat body in each bee was similar for all treatment types for both chlorothalonil and amitraz. The average fat body weight (μg) of bees in chlorothalonil trials was 626.7 μg (acetone), 730 μg (control), and 713.3

µg (compound) and 645 µg (acetone), 740 µg (control), and 750 µg (compound) for amitraz trials. No statistical differences in fat body weights were observed for either treatment (amitraz ($F_{2,5}$=0.76 p=0.51); chlorothalonil ($F_{2,5}$=1.23 p=0.37)(Figure 2.6.7).

2.4 Discussion

Exposure to pesticides in the environment and from beekeeper-applied compounds has resulted in the accumulation of chemical residues from many compounds into hive matrices (bees, food stores, wax) (Mullin et al. 2010; Sanchez-Bayo and Goka 2014; Ravoet et al. 2015). Two of the more prevalent pesticides found at relatively high concentrations in comb, chlorothalonil and a metabolite of amitraz (DMPF) have shown significant negative effects on both adults as well as larvae (Yoder et al. 2013; Papaefthimiou et al. 2013; Johnson et al. 2013; Zhu et al. 2014; Degrandi-Hoffman et al. 2015; O'Neal et al. 2017; Dai et al. 2018ab), however, most of this previous research examined oral or topical exposures and did not assess the potential effects of residues in comb. Our goal with this research was to expand on previous research and bridge knowledge gaps about the presence of specific compounds in brood comb that may impact development. Due to the presence of both chlorothalonil and amitraz at high levels in comb (Mullin et al. 2010; Sanchez-Bayo and Goka 2014; Ravoet et al. 2015) and previous research indicating developmental delays tied into pesticide residues in comb (Wu et al. 2013) and larval death (Dai et al. 2018ab), we hypothesized that high levels of compound residue would cause negative effects on larval development or survival because developing larvae are immobile and lay directly in contact with the contaminated comb surfaces. Brood production and health is essential

to colony survival and delays in development or increases in brood mortality may affect the productive output at the colony-level.

The success of honey bee colonies is dependent on a robust population of healthy individuals performing age-dependent tasks throughout the hive, and any strain on brood production represents an unsustainable burden on the colony. The continuous use and detection of agrochemicals including the fungicide chlorothalonil and active metabolite of the acaricide amitraz (DMPF) in honey bee hives necessitates investigation into any deleterious effects that these compounds may have on brood production, adult emergence, and individual morphometric characteristics of honey bees.

In this study, individual frames were treated with either chlorothalonil or amitraz (DMPF) at concentrations that were commonly found in wax and then assessed for egg-laying, larval development, adult emergence, and overall health as determined through fat body and hypopharyngeal gland analysis. Here, we saw that chlorothalonil did not have significant effects on any of the larval development or health measures assessed. Previous literature indicates that when fed a diet containing chlorothalonil at similar levels found within the hive, larvae experienced acute toxicity as well as decreased survival (Dai et al. 2018a). Our research did not result in the same larval mortality when they were exposed dermally through comb. Our results were also not consistent with previous research indicating developmental delays associated with multiple pesticide residues in comb (Wu et al. 2013). This research examined a large array of pesticides that may have acted synergistically while here chlorothalonil (and amitraz) were

examined in isolation, indicating chlorothalonil residues in comb alone does not harm honey bee larvae. Although data suggests that the presence of chlorothalonil in comb may not have adverse effects on worker bee development, the sample size was insufficient due to low egg-laying success in experiment trials and because data were collected from only one season. This study also did not examine any potential effects on reproductive individuals (queens or drone bees) whom often express higher sensitivity to toxins than worker bees. Therefore, greater sampling efforts and another field season would be necessary to fully assess potential impacts.

The examination of amitraz (DMPF) had slightly different results. Data showed no significant differences in the average number of eggs laid or the proportion that survival from eggs to adult emergence. This is contrary to previous research that showed bees fed a diet contaminated with amitraz (46 mg/l) had increased mortality and developmental delay (Dai et al. 2018b). This could be because the highest concentration of amitraz examined was 1000 ppb (or 1 mg/L) a level much lower than the concentration used by Dai et al. (2018b) but more consistent with levels found in comb. Bees reared in amitraz-treated comb exhibited significantly smaller hypopharyngeal gland acini in both diameter ($F_{2,5}$=9.14 p=0.02) and perimeter ($F_{2,5}$=6.55 p=0.04) compared to controls, indicating a correlative impact on the productivity of the gland to produce brood food (Deseyn and Billien 2005). Previous research that examined the impact of amitraz fed to adult bees in pollen showed no significant impacts to hypopharyngeal gland size (Esmael et al. 2016), however other insecticides, such as neonicotinoids, have shown negative effects on hypopharyngeal gland acini size

(Heylen et al. 2011; Hatjina et al. 2013). The potential reduction in productivity is concerning as it could present further disruption to the hives future population. The need for bees to produce appropriate levels of nutrition to rear worker bees or queens is imperative, if the size of the glands also decreases the production, there may be potential developmental delays or health factors for brood that are reared by bees with underdeveloped hypopharyngeal glands. To our knowledge, our research is the first to examine how larval exposure to amitraz (DMPF) in brood comb may impact the development of hypopharyngeal glands as adults, however we did not examine whether reduced acini size impacted the volume of glandular secretions produced by nurse bees and or the quality of the brood food.

Due to monetary constraints we were unable to test comb sections for each frame to confirm the application method yielded residue levels at the expected treatment levels and to assess whether pesticide residues migrated into other adjacent comb sections after application. The degradation and translocation of residues in comb is not fully understood and has been identified as a source of inherent difficulty in studying pesticide effects at the colony level (Sponsler and Johnson 2016). This makes determination of the actual exposure concentration or uptake by bees difficult as well, meaning we cannot accurately describe the exact amount each bee may have been exposed to. Pesticides introduced within the colony through contaminated food sources are diluted through “shared feeding” (trophallaxis) in honey bees and are broken down naturally in the environment (Sponsler and Johnson 2016), further complicating how to determine exposure risk in bees. Finally, colony level field research faces inherently

difficult challenges due to a large number of confounding factors (Sponsler and Johnson 2016, 2017). This research was conducted with a limited number of colonies and queens in mother colonies exhibited inconsistent egg-laying performance. Bees reared in comb treated with high concentrations of amitraz (1 mg/L) did not reach the pupal stage likely due to poor egg-laying performance in queens that resulted in multiple replications with little or few eggs laid and which were later removed by worker bees before pupation, thus data lacked the sufficient sample size for statistical analysis for several measures and should be repeated another season.

Overall, our results indicate that development of crucial hypopharyngeal glands may be affected by exposure to amitraz residues in comb during larval development, however, the potential implications of that on brood food production was not assessed here. Additional research could elucidate the impact of smaller gland size on normal colony functions, like brood and queen care, as well as other subtle behavioral impacts such as precocious shifts in hive tasks. Though our research did not observe effects from chlorothalonil residues, there is the potential for synergistic interactions between chlorothalonil and other acaricides that suggests both compounds should be further studied separately and in combination with others. The data presented here is a preliminary look into the effects of pesticide residues in brood comb on bee health and colony development. However, pesticide residues are accumulating in brood comb in complex mixtures and at alarming levels, therefore, more research is critically needed to examine the role this plays in bee health decline so that we may develop management strategies to mitigate pesticide exposure and risk to bees.

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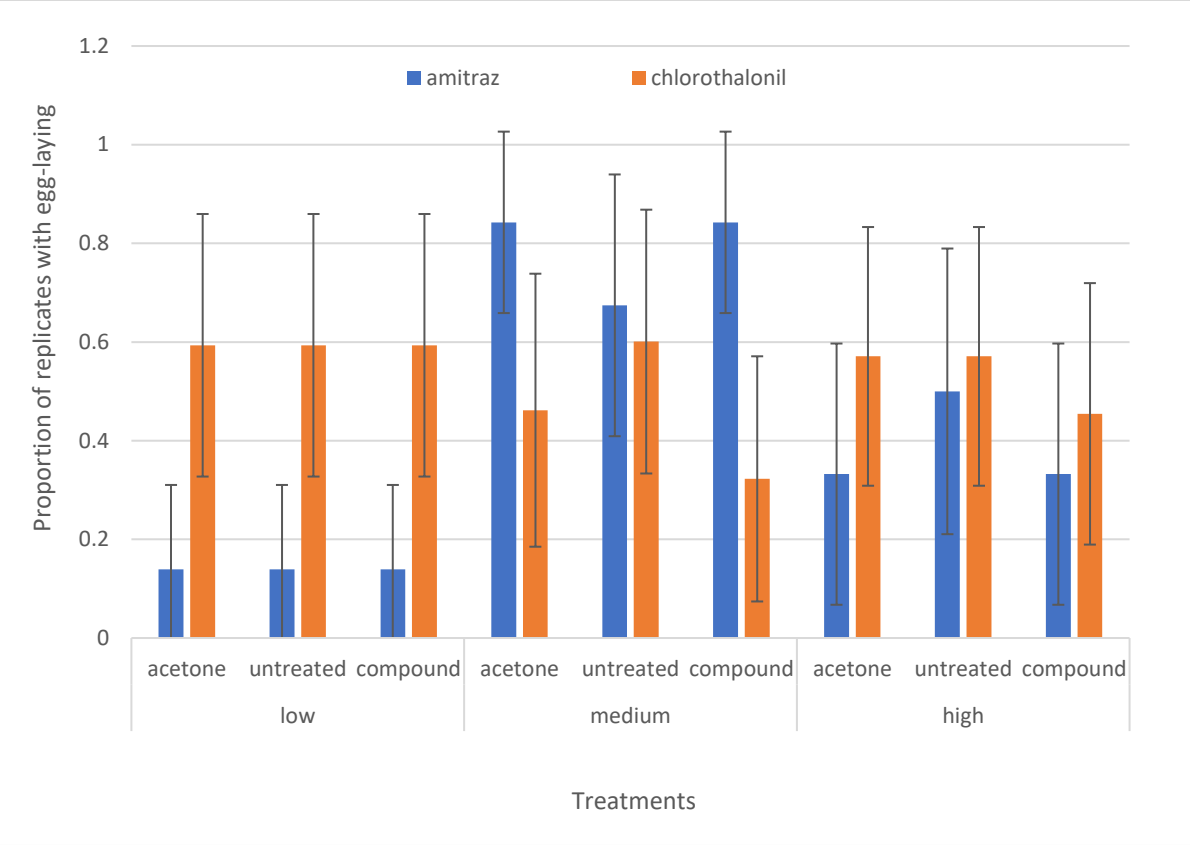
2.6 Figures

Figure 2.6.1 Proportional Egg-Laying Success in Experimental Frames. **Experimental frames consisted of**



three comb sections; one section treated with a compound (amitraz or chlorothalonil), one section treated with acetone solvent and the other left untreated. The proportion of experimental replicates (amitraz (n=6) or chlorothalonil (n=9)) in which the queen bee successfully laid in the combs was analyzed by treatment (control, acetone, and compound) and dose level (low, medium, high). Low, medium, and high treatment doses for amitraz (0.01, 0.1, and 1 mg/l) and chlorothalonil (0.1, 1, and 10 mg/l) reflect environmental relevant exposures and residues levels found in comb. Data shows a lower proportion of eggs laid in combs with low doses of amitraz, however, the control comb sections (acetone and untreated) paired with low amitraz also yielded low egg-laying success. No statistical differences in egg-laying rates were observed for either treatment (amitraz ($F_{2,12}$=1.64 p=0.23); chlorothalonil ($F_{2,12}$=0.25 p=0.78)) or dose levels.

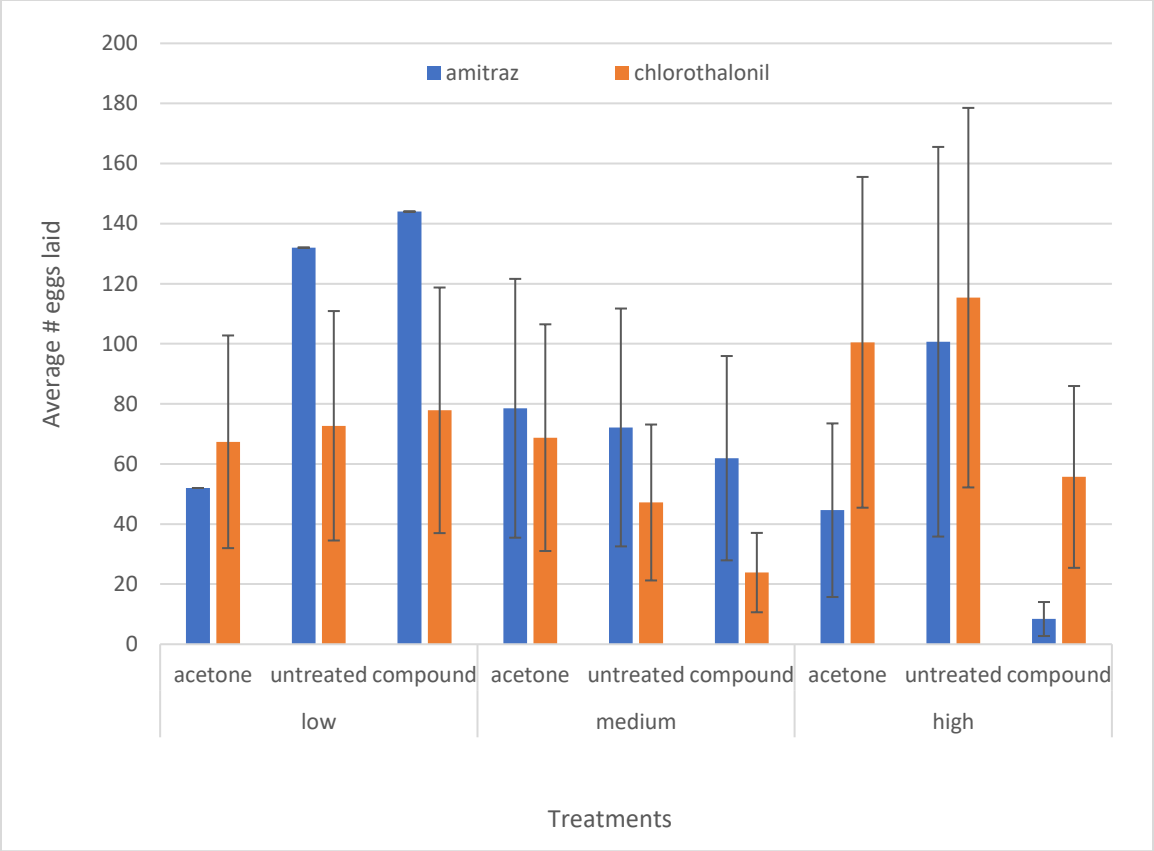


Figure 2.6.2. Average Number of Eggs Laid. Graph illustrates the average number of eggs laid in each treated comb section (acetone, untreated control, and compound). Compounds were applied at low, medium, or high dose levels (0.01, 0.1, and 1 mg/L for amitraz and 0.1, 1, and 10 mg/L for chlorothalonil). When queens laid eggs in frames, there were generally more eggs in amitraz trials, particularly at low doses, than compared to chlorothalonil, however, no statistical differences were observed in egg deposition for either treatment (amitraz ($F_{2,10}$=3.7 p=0.06); chlorothalonil ($F_{2,10}$=1.25 p=0.33)) or dose levels. Although the proportion of frames with successful egg deposition was lowest in the low dose trials and equally poor among acetone, untreated, and amitraz treated combs (figure x), when queens did lay it yielded the highest number of eggs in untreated (132) and amitraz (144) treated comb sections. However, there were insufficient replicates to show significance.

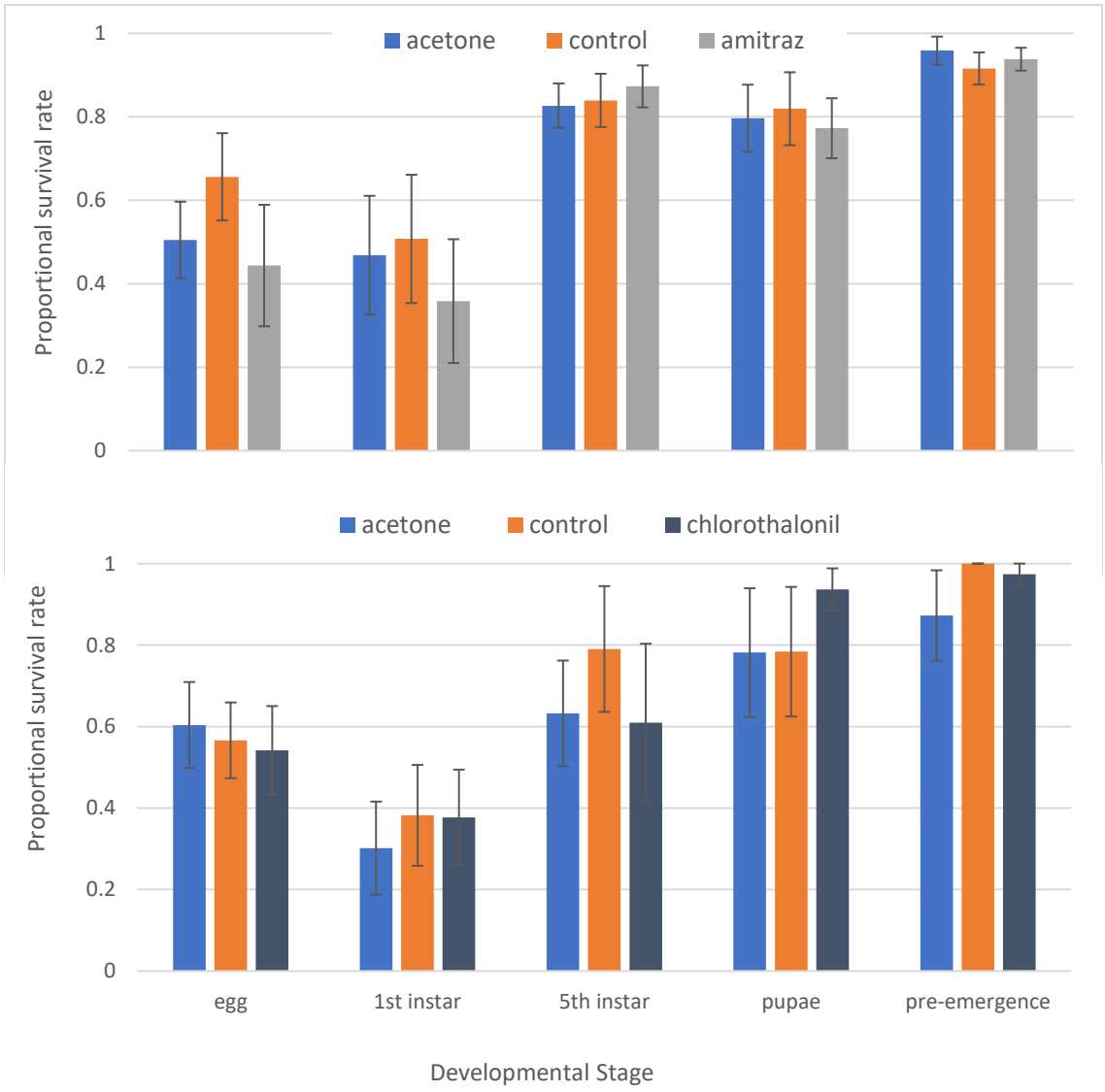


Figure 2.6.3. Proportional Survival During Larval Development. **Graph illustrates the proportional number of brood that survived to the next developmental stage (eggs (day 1), 1st instar larvae (day 4), 5th instar larvae (day 8), early pupae (day 12), late or pre-emergence pupae (day 19) in brood developing from treated comb sections (acetone, untreated control, and compound). Compounds were applied to combs at low, medium, or high dose levels ((0.01, 0.1, and 1 mg/L for amitraz (top) and 0.1, 1, and 10 mg/L for chlorothalonil (bottom)). The data suggests mortality was highest among the eggs and early 1st instar larvae (day 4) for both amitraz and chlorothalonil. Sample size was insufficient for further statistical analysis.**

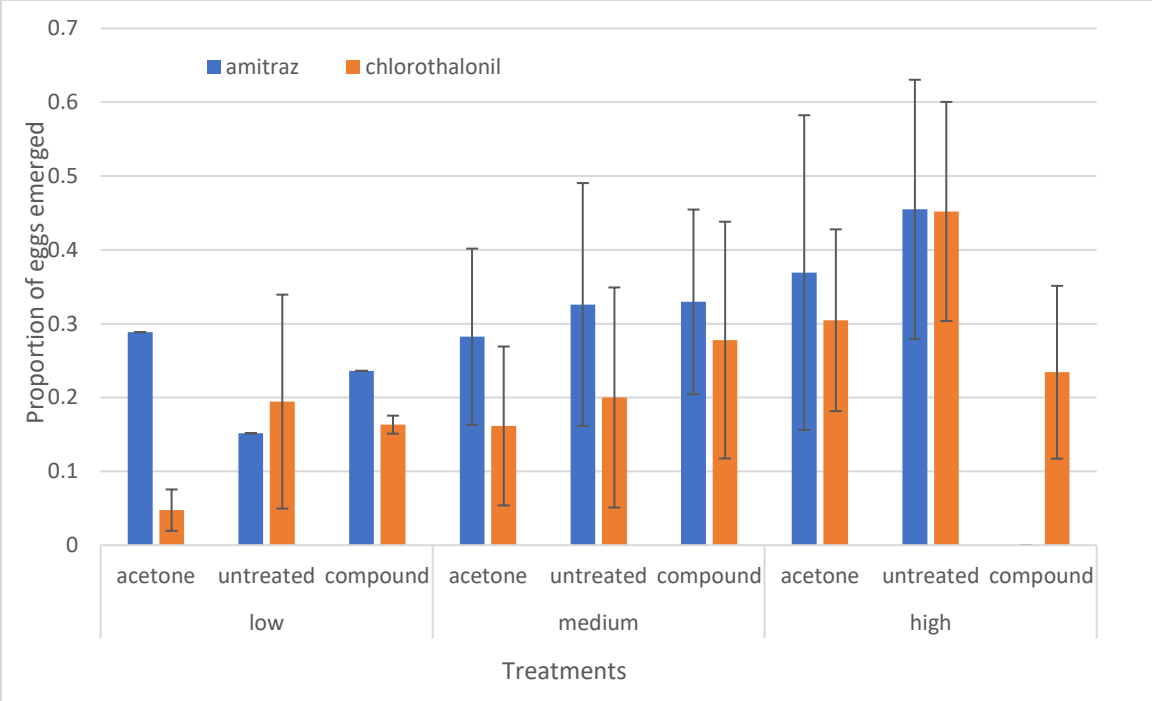


Figure 2.6.4 Proportion of Eggs that Survived to Adult Emergence. This graph illustrates the proportion of eggs that survived to emerge as adult bees from development in treated comb sections (acetone, untreated control, and compound). Compounds were applied to combs at low, medium, or high dose levels ((0.01, 0.1, and 1 mg/L for amitraz (blue) and 0.1, 1, and 10 mg/L for chlorothalonil (orange)). The data for amitraz showed that there was not a significant difference ($F_{2,9}$=0.03 p=0.9692) between treatment sections. Though there seems to be a lower level of survival for bees developing in comb with 1 mg/L amitraz, there was an insufficient sample size to show significance. The data for chlorothalonil showed that there was not a significant difference ($F_{2,9}$=0.61 p=0.56) between treatment sections.

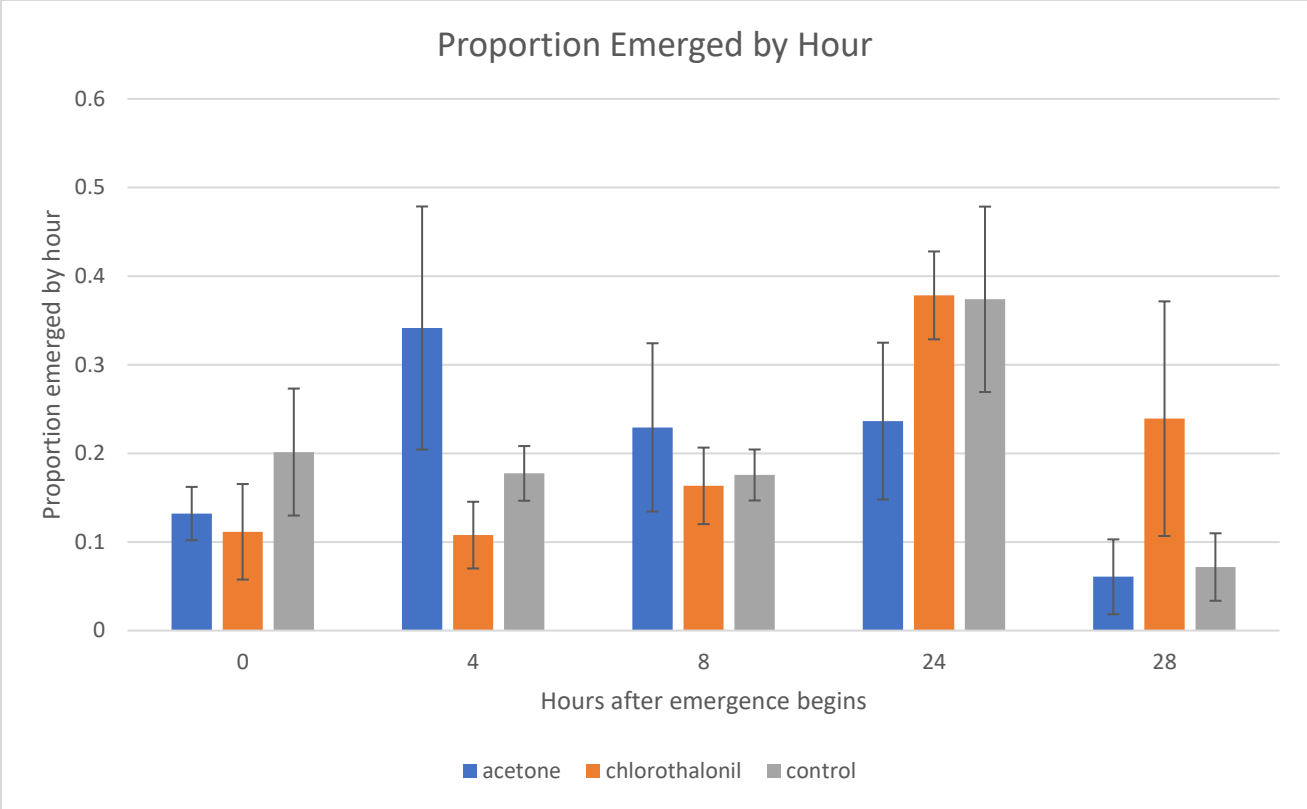


Figure 2.6.5 The Emergence Times of Adult Bees in Treated Comb. The proportion of bees emerging by hour segments until all bees had emerged from frames treated with acetone solvent, untreated control, or chlorothalonil (0.1, 1, and 10 mg/L). Data were pooled across dose levels to increase sample size. Though there were no observed delays in emergence from the 21 day emergence typically associated with honey bee development, the 0 hour indicates exactly 20 days from the time the queen was first excluded and could begin laying. We saw a trend of later emergence for comb with a treated level. Based on the average(±SE) proportion of bees in the control comb(control and acetone) that emerged when compared the the average(±SE) propotion of the bees that emerged in comb treated with chlorothalonil, the queen may have laid in control sections before laying in the section treated with chlorothalonil. The proportion of bees that emerged at 24 hours was 37.8±4% and at 28 hours was 23.9±13%. from the treated comb. On average 61.7% of the bees reared in comb treated with chlorothalonil emerged at the later hours whereas comparatively, acetone and control had a combined proportional emergence of 29.3±11% and 44.4%, respectively, before the 24 hour time mark This was not analyzed but could indicate preferential egg laying patterns by queens.

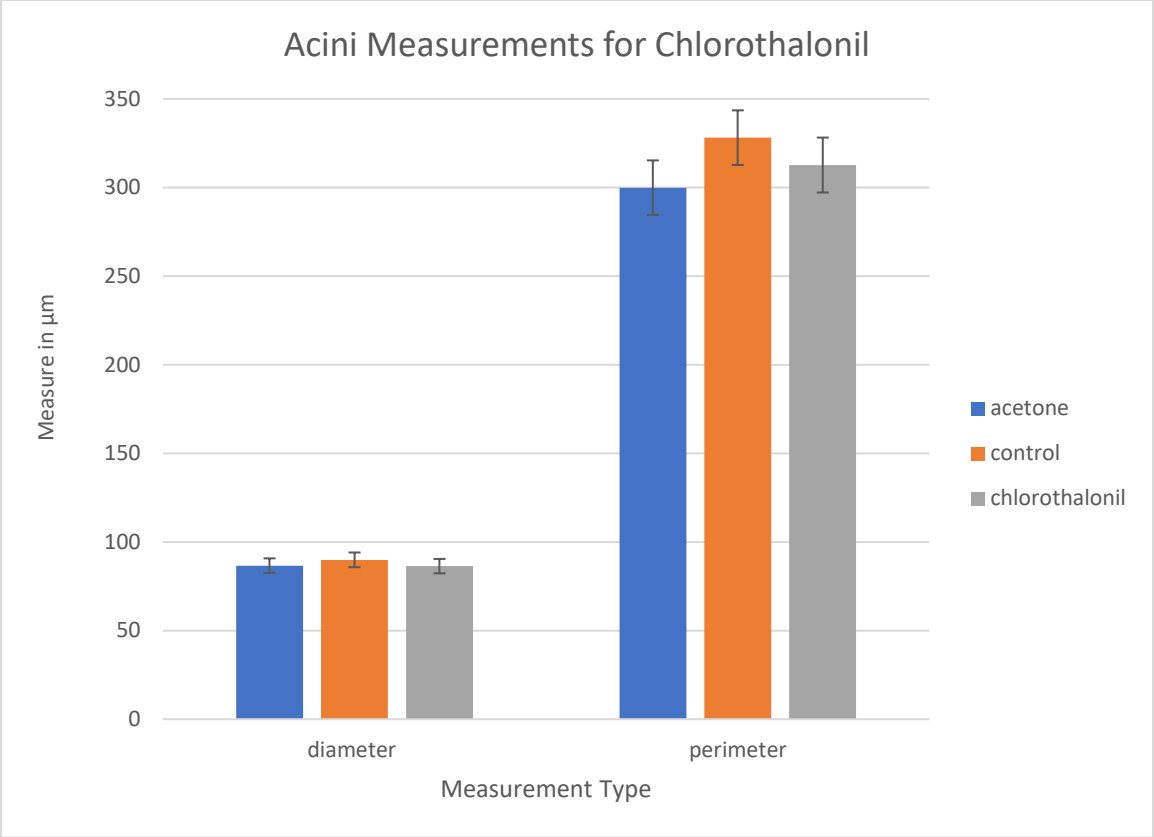


Figure 2.6.6. Average Acini Measurements for Bees in Chlorothalonil Frames. This Graph illustrates the measurements of individual acini in bees that developed in treated comb sections (acetone, control, chlorothalonil). Compounds were applied to combs at low, medium, or high dose levels of (0.1, 1, and 10 mg/L) for chlorothalonil. To increase power dose levels were combined and averaged. Measurements assessed were the diameter and perimeter. Data showed similar perimeters for all three treatments, though acetone and chlorothalonil were slightly lower than the control, and similar diameters for all three treatments. The measurements of acini were not significant for diameter ($F_{2,5}$=0.68 p=0.55) or perimeter ($F_{2,5}$=2.88 p=0.15)

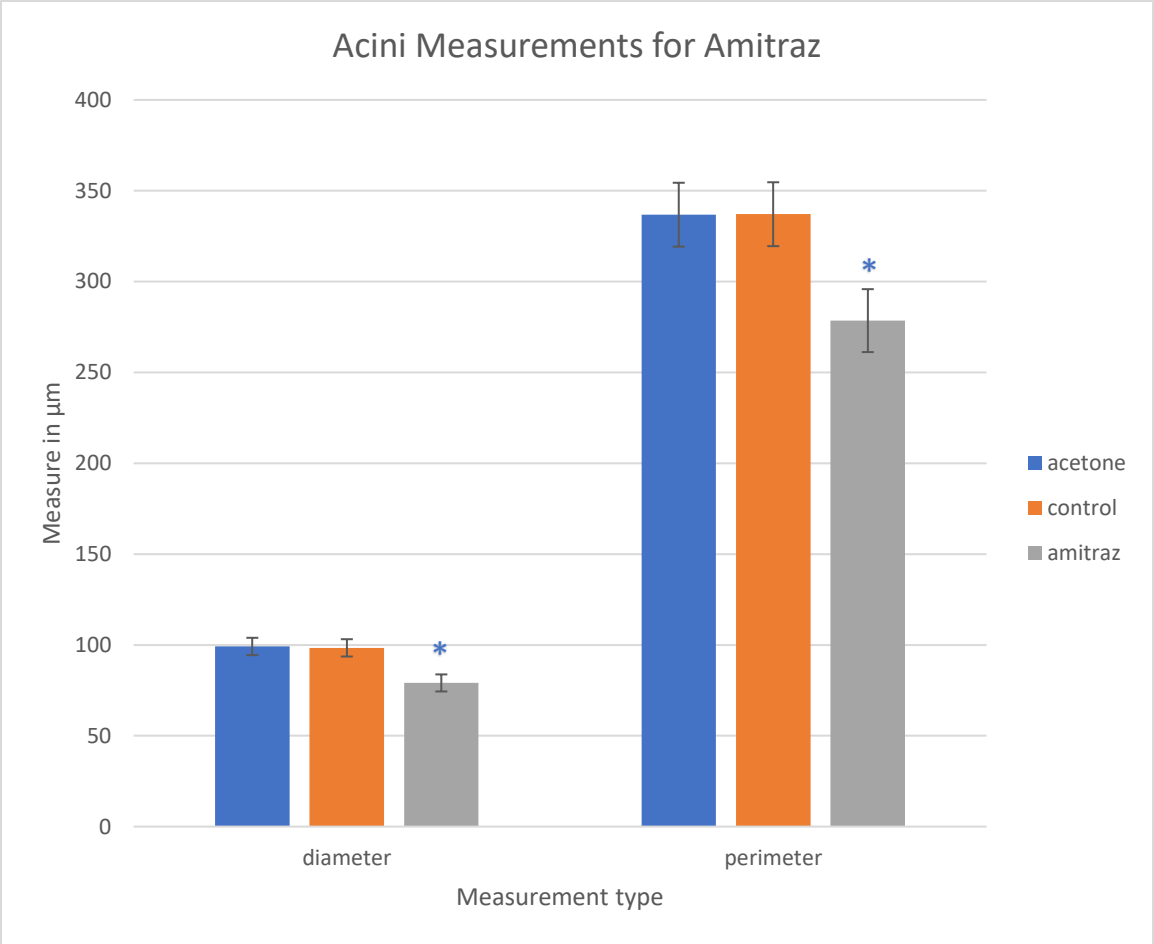


Figure 2.6.7. Average Acini Measurements for Bees in Amitraz Frames. This Graph illustrates the measurements of individual acini in bees that developed in treated comb sections (acetone, control, amitraz). Compounds were applied to combs at low, medium, or high dose levels of 0.01, 0.1, and 1 mg/L ppb for amitraz. To increase power the dose levels were added together and averaged for all three treatment types. Measurements assessed were the diameter and perimeter. Diameter of acini resulted in the bees that emerged from comb treated with amitraz had significantly smaller acini. Data also showed that the perimeter of bees that emerged from comb treated with amitraz were significantly smaller than bees from acetone and control. The measurements of acini were significant for diameter ($F_{2,5}$=9.14 p=0.02) or perimeter ($F_{2,5}$=6.55 p=0.04)

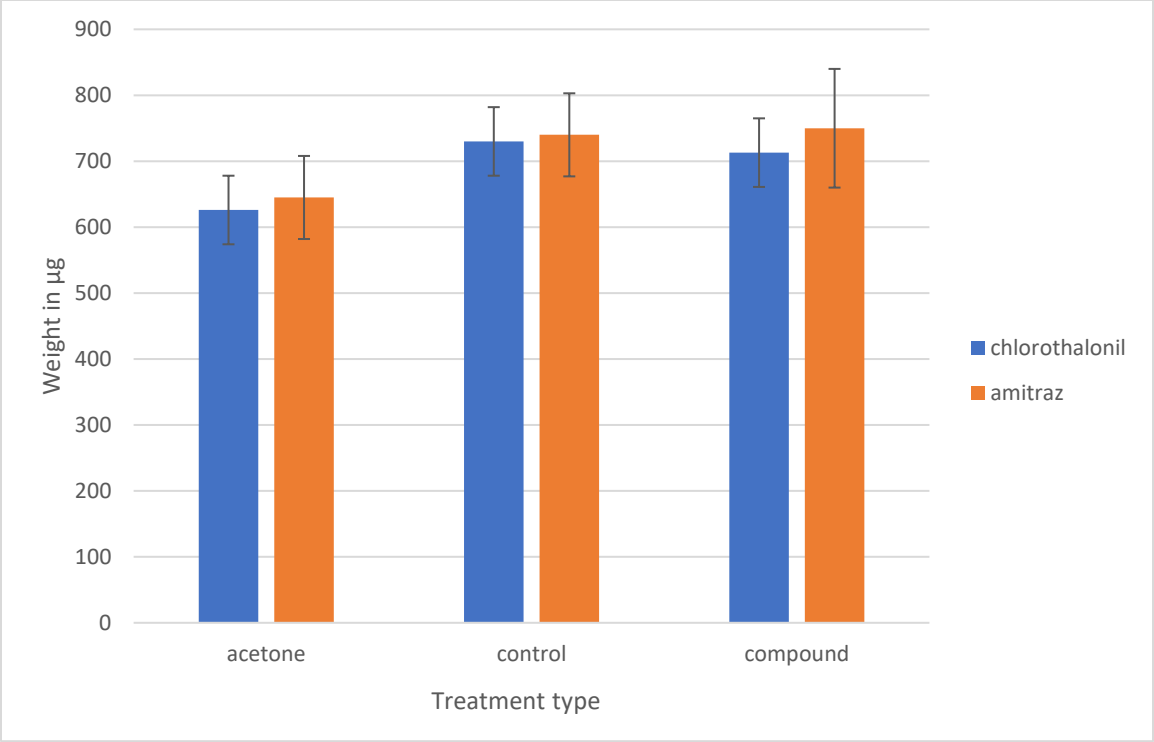


Figure 2.6.8 Average Weight of Fat Body for Bees. **Experimental frames consisted of three comb sections; one section treated with a compound (amitraz or chlorothalonil), one section treated with acetone solvent and the other left untreated. The average weight of the fat body in bees emerging from treatment type by compound. Dose levels (0.01, 0.1, and 1 mg/L for amitraz and 0.1, 1, and 10 mg/L for chlorothalonil) were combined to increase sample size and statistical power. Data shows a lower average fat body weight in acetone, however, the control comb sections and compound comb were similar average weights. No statistical differences in fat body weights were observed for either treatment (amitraz ($F_{2,5}$=0.76 p=0.51); chlorothalonil ($F_{2,5}$=1.23 p=0.37)) or dose levels.**

Chapter 3: An Evaluation of Dead Bee Traps for Monitoring Pesticide Incidents in Honey Bee Colonies.

3.1 Introduction

In the United States, the national average for honey bee (*Apis mellifera L.)* colony losses are about 40%, however some states are reporting devastating losses as high as 70%. This level of losses has been reported by beekeepers over the past decade and are considerable higher than the widely accepted typical annual loss of 15-20% (vanEngelsdorp et al. 2012; Lee et al. 2015; Seitz et al. 2016; Kulhanek et al. 2017). The loss of colonies at such high levels is an important discussion because of the pollination services that honey bees provide. Over one third of the foods we eat are pollinated by insects, and commercially managed honey bees contribute more than 80% of that pollination service. The pollination provided by honey bees contributes roughly $15 billion USD in added crop value annually to numerous bee-dependent crops, like almonds, broccoli, blueberries, and many other fruits, vegetables, and nuts (Thapa 2006, Klein 2007).

Many crops do not require insect pollination but obtain additional production benefits in crop yield, uniformity, and even taste, however, others are completely bee-dependent and would fail without the pollination services provided by honey bees. For example, over 2 million hives are transported across the US to meet pollination service demands for almond production in California. California is the largest global exporter of almonds and the state currently has 1.2 million acres of bearing almond trees that are highly dependent on honey bee pollination for successful crop yield. In fact, it's estimated that the 1.2 million acres in 2020 will require approximately 2.4 million

colonies, however, in 2019, 1.17 million bearing acres only received 1.86 million colonies for pollination which was down from 1.93 million colonies contracted the previous year (Goodrich 2020) and well below the ideal number to obtain full pollination potential. This and studies in other pollinator-dependent crops show that the number of available colonies currently does not meet the demand for pollination which has risen by 300% in the last 50 years (Aizen and Lawrence 2009; Ellis et al. 2010). The increase in need for pollination, however, is not paralleled by increases in the number of available colonies but rather colonies in the US have decreased from 6 million in 1948 to current estimates of 2.6 million (Ellis et al. 2010). The increases in crop production paired with high annual losses of colonies continues to strain the beekeeping industry and beekeepers struggle to maintain pollination contracts to meet growing demands.

3.1.1 Factors in Bee Decline

Bees are affected by several factors that can decrease their ability to survive such as infestation by pests, infection from pathogens, poor nutrition, exposure to pesticides as well as improper management of honey bees. Approximately 8% of the total annual colony loss can be attributed to mismanagement of bees (vanEngelsdorp et al. 2008) which may be defined as a general lack of care (improperly feeding, not managing for pests or pathogens, not managing for swarming, etc). Inexperienced beekeepers may ignore recommendations to provide supplemental feed (syrup and or pollen) because they do not understand the nutritional needs and or amounts required for colony development in the spring and for sustaining populations over winter (Standifer 1980). Hives faced with a lack of nutrition often become more susceptible to

other stressors (Huang 2012). Colonies experiencing malnutrition may have a lack of proteins and amino acids vital to ward of pathogen infection, comprising their immune systems (vanEngelsdorp et al. 2008). A lack of nutrition has also been shown to decrease the instances where foragers waggle dance and cause them to be less precise when they do dance and reducing the potential for other foragers to revisit floral resources (Scofield and Mattila 2015). Another common management issue is not controlling for swarming behavior in colonies, or the natural mode of colony-level reproduction. When swarming occurs, the queen and roughly one third of nestmates leave the hive to find a new location which disrupts brood production and reduces the adult worker population resulting in a loss of productivity and honey production.

The management of pests and pathogens in the hive can also result in many improper and or inadequate control treatments and strategies. Often mismanagement occurs due to a lack of education or understanding of the biology behind the pest or pathogen and the available management strategies to control them or mitigate impacts on hive health. Beekeeping management techniques, particularly newer ones, are understudied because strategies may be highly dependent on numerous factors, such as location, season landscape type and use, all of which affect resource availability and nutrition. Additionally, there are other unquantifiable confounding factors like pesticide exposure, particularly when bees are potentially exposed through multiple routes and throughout the season. Some of the pesticide exposures occur through the migration of systemic compounds that can be applied to soil or on seeds and then translocate throughout plants leading to residues in nectar and pollen (Bonmatin et al. 2003; Stoner

and Eitzer 2012; Krischik et al. 2015; Sánchez-Hernández et al. 2016; David et al. 2016). The foraging bees may be exposed to levels that cause mortality away from the hive. To replace lost foragers, precocious maturation of younger bees into foraging roles within the hive can result in a reduction of brood care and eventually affect the population size. Many of these systemic compounds are frequently used in agriculture practices as well as urban settings across the nation and are of major concern to beekeepers.

3.1.2 Neonicotinoid insecticides and bees

Neonicotinoids are a common class of pesticides that have received a lot of media attention and who's safety to bees is currently under scrutiny and debate in many countries, including the US. They are listed as a class II or III toxicant which means they are relatively toxic to humans (US EPA 2015). Neonicotinoids are systemic insecticides that bind to nicotinic acetylcholine receptors (nAChRs) in cells and cause excitation or stimulus of the cell resulting in the overstimulation of the nervous system and eventually causing paralysis and death. They are highly selective toward insects because the compounds bind more tightly to nAChRs in insect systems than binding to muscarinic acetylcholine receptors which mammals have a higher proportion of in relation to nAChRs. Therefore, neonicotinoids are preferred by pesticide applicators and handlers over older traditional and more toxic classes of insecticides, such as organophosphates and pyrethroids. There are seven active ingredients within the class of neonicotinoids and four (imidacloprid, clothianidin, thiamethoxam, dinotefuran) are listed as "highly toxic" to bees while the other three (acetamiprid, thiacloprid, and nitenpyrum) are considered "moderately toxic" (Fishel 2005; US EPA 2015).

Imidacloprid, was the first active ingredient released on the market in 1985. Since then it has been listed as the most used insecticide in 1999 and is still used pervasively in most countries today (Yamamoto 1999). Neonicotinoid residues degrades rapidly with water and ultra violet light but may persists in plants and soil for several weeks to months depending on the species of plant, soil type, and moisture (Westwood et al. 1998; Liu et al. 2011). Neonicotinoids can be applied as seed-coat treatments, sprayed on foliage, applied to soil or added to irrigation. And due to their systemic nature and board spectrum toxicity, are used to control various insects, particularly stem/leaf boring and root feeding pests that are difficult to control with older chemistries (Yamamoto 1999). Neonicotinoids may be detected in nectar, pollen, and flowers of treated plants at levels from 5 to 218 ppb in squash (Stoner and Eitzer 2012) and 1 to 39 ppb in sunflower and wildflowers (Bonmatin et al. 2003; David et al. 2016; Sánchez-Hernández et al. 2016), even as high 660 ppb in eucalyptus nectar(Paine, et al. 2011) and as high as 6030 ppb in Mexican milkweed (Krischik et al. 2015) when applied as seed, soil, or drip irrigation treatments. Within the hive, neonicotinoid levels are highly varied and dependent on the matrices (wax, pollen, honey, bee) tested. Residues have been detected at levels as high as 206 ppb and as low as 2.4 ppb in brood comb within the hive.

Neonicotinoid exposure in bees may cause varying adverse effects from increased mortality in larvae and adults to sublethal impacts on normal colony behaviors, such as reduced foraging, egg-laying, and brood care. Imidacloprid exhibits high toxicity to honey bees and acute mortality is observed when bees come into

contact at ranges from 7.8 to 242 ng/bee (Cresswell 2011). Additionally, acute mortality of bees was observed in colonies within 9 meters of aerial powder applications of imidacloprid at levels of 199 (ng/bee) (Girolami et al. 2009) and when bees were fed syrup containing (3.75 ppm or 0.3ng/bee) of clothianidin (Laurino et al. 2011). Further, 19% acute mortality was also shown in bees when they were exposed to both low nutrition (less than 15%sucrose) and thiamethoxam at 1 ng/bee, this combination also reduced trehalose and glucose in the hemolymph which are important for energy production (Tose et al. 2017). There have also been numerous studies examining sublethal effects of imidacloprid on colony health measures including disruption in normal behaviors (worker productivity, queen egg-laying, hygienic cleaning) and colony development (brood and honey production). Several studies have noted that exposure to imidacloprid at the colony level in concentrations of 50 µg/liter can impact foraging efficiency, memory (Yang et al. 2008), and 500 ppb of imidacloprid in sugar syrup disrupted bees homing navigation (Bortolotti et al. 2003). Dively et al. (2013) also found a significant a reduction in queen fecundity and decreased winter survival in colonies fed syrup contaminated with imidacloprid (20 and 100 µg/kg). The evidence backing the sublethal and lethal impacts of imidacloprid make it an ideal candidate to begin researching methods to monitor for sublethal pesticide incidents.

3.1.3 Pesticide incidents and monitoring

Exposure to pesticides can occur outside the hive through contaminated nectar, pollen, and water, or through direct contact when flying through spray applications. (Westwood et al. 1998; Kubik et al. 1999; Stoner and Brian 2006; Liu et al. 2011; David

et al. 2016). Foragers may become exposed during foraging and or collect contaminated food sources which are brought back to the hive. However, exposure to pesticides may also occur within the hive through chemical treatments (acaricides, repellents, and antibiotics) applied by the beekeeper to manage hive pests through oral consumption of contaminated foods or through contact with pesticide-laden comb (Johnson et al. 2009; vanEngelsdorp et al. 2009; Mullin et al. 2010; Sanchez-Bayo et al. 2014; Ravoet et al. 2015). Studies show more than 121 compounds present in pollen, honey, wax, and bees (vanEngelsdorp et al. 2009; Mullin et al. 2010; Sanchez-Bayo et al. 2014) highlighting the immense chemical load within hives and the potential for interaction effects with other hive stressors.

Currently, beekeepers actively monitor and manage for queen health, malnutrition, Varroa mites, and diseases, but there are no recommendations for beekeepers regarding monitoring for pesticides. There are guidelines for protecting pollinators from pesticide exposure and reducing risk to bees, however there are no standards for how to monitor for negative effects from pesticide exposure at the onset of an exposure event rather than investigating after a "bee kill" or colony loss occurs. Acute mortality of the hive, or a classic "bee kill", can be investigated by a state apiarist or a licensed official to determine if it was the result of improper pesticide applications. Identifying which and when a pesticide kill has occurred is challenging due to the high costs of pesticide testing, and often losses do not exhibit classic "bee kill" symptoms. Classic "bee kills" exhibit high rates of mortality over a short period of time (within 24-48 hr after exposure) but beekeepers observe losses of workers over a longer extended

period. The dwindling of hive populations continues for several weeks and is not considered a pesticide “kill”, so here, we are defining these as pesticide “incidents”.

Pesticide incidents may also describe chronic, sublethal, and or indirect effects of pesticide exposure that slowly reduces the health and overall strength of a colony. Increased mortality of a few hundred bees in a colony of over 40,000 bees would not severely impact the health of the colony, however, if pesticides were disproportionately affecting bees performing vital colony roles (such as nurse bees caring for brood) then losses may have cascading indirect effects on brood production and thus affect long term colony development and productivity. Given the high prevalence and loads of pesticide residues in bees and hive products, it’s critical to better assess and monitor when and how agrochemicals are brought in and distributed within a hive. Thus, in this study we sought to evaluate dead bee traps as a monitoring tool to assess bee losses due to pesticide exposure which will inform researchers about the role pesticide incidents play in colony decline and help beekeepers mitigate adverse impacts through management.

3.2 Methods

3.2.1 Apiary Set up

Experiments took place in Nebraska at three locations with different landscape types and uses during the field season of 2019. The first location was the University of Nebraska – Lincoln East Campus (40°49'44.4"N 96°39'26.7"W) research apiary which is situated in an urban garden setting that houses roughly 20-30 research hives throughout the year and for which we will refer to as the “garden” site. The second

location was at Kimmel Orchard & Vineyard (40°42'03.3"N 95°53'37.2"W) in Nebraska City; a research and education farm that grows mainly apples, cherries, peaches, pumpkins, and many other bee-pollinated crops (referred to as “orchard” site). And lastly, the third location was at the Eastern Nebraska Research and Extension (41°09'40.1"N 96°29'18.1"W); a research and education farm that grows corn, afalfa, soybean, and many other crops (referred to as “farm” site).

Over-wintered bee colonies of equal strength and mixed Carniolan and Italian traits containing roughly 40,000 honey bees (in two brood boxes) were equipped with dead bee monitoring traps in the Spring of 2019. A total of 12 traps were set up at garden (*n* = 6), orchard (*n* = 3), and farm (*n* = 3) sites and assessed weekly for the number of dead bees ejected from hives and caught in traps. Colonies were maintained using standard beekeeping practices and assessed for health issues, such as brood diseases throughout the season. Further, no pesticide treatments were applied during the experiment. Instead, varroa mite levels were regularly monitored and managed through cultural and mechanical control tactics (breaking brood cycles and drone brood trapping). This set-up was used to assess seasonal trends of abnormal worker bee losses from all three apiaries as well as assessing the rate of recapturing paint-marked dead and pesticide-treated bees when treated bees were released into the hive and recollected from traps (only performed at the garden apiary site).

3.2.2 Dead bee trap set-up

To assess an optimal size, traps of two sizes (small 2X2ft or 0.6m^2 and large 3X3ft or 0.9m^2) were examined. Large traps were designed with 2ft X 4ft wood cut into 3ft or

0.9144 m sections and then screwed together into a square. The small traps were made with plywood and painted to protect the wood. A light-colored tarp material was then stapled to the wood frame to form the trap floor. The large trap was placed flush against the hive entrances to ensure dead bees did not fall into the grass. To remove variability between individual hive losses, the smaller traps were nested directly inside the large traps with an edge centered against the hive entrance (Figure 3.6.1). This configuration created “inner” and “outer” areas within the trap where bees collected from the “inner” area represented the capture rate of smaller traps while the bees collected from both “inner” and “outer” areas were pooled to represent the “total” bees captured from within the large trap dimensions. Here data from small traps will be referred to as “inner” and large traps will be referred to as “total” trap collections.

3.2.3 Trap Recapture Rate of Imidacloprid Treated Bees

To examine the efficiency of dead bee traps at collecting dead and dying bees, paint-marked bees topically treated with imidacloprid insecticide at low, medium, or high concentrations (0.01, 0.1, 1 mg/L or 10, 100, 1000 ppb, respectively) and freeze-killed bees (positive control) were introduced into one of six hives at the garden apiary equipped with dead bee traps. Traps were then monitored weekly and dead bees were collected from “inner” and “outer” areas from June through October, quantified, and analyzed by trap size, dose, and month.

Pesticide treatment and application: A stock solution was made by dissolving 0.005 g of imidacloprid in 5 µl of acetone. The stock solution was further diluted in acetone until solutions of low, medium, high (10, 100, 1000 mg/L or 10, 100, 1000 ppb, respectively)

imidacloprid (IMD) were obtained. The concentrations for the low and medium dose were chosen based on concentrations of imidacloprid found in the plants, nectar, pollen, and wax and the dosing range represents what bees may come into contact with (Johnson et al. 2009; vanEngelsdorp et al. 2009; Mullin et al. 2010; Sanchez-Bayo, and Goka 2014; Ravoet, et al. 2015; Stoner, and Eitzer 2012; Krischik et al. 2015; Sánchez-Hernández et al. 2016; David et al. 2016). The high dose of IMD was chosen based on previous research examining those concentrations effects on honey bee health that may be encountered through spray or drip treatments (Bortolotti, L. et al. 2003; Yang E. C. et al., 2008). Imidacloprid solutions (10, 100, 1000 mg/L) were topically applied to the dorsal side of the thorax (2 µl) of bees. To obtain bees of the same age, brood frames were removed from non-experimental donor hives on day 19 (pre-emergent) of brood development. Newly emerging adult worker bees were randomly assigned a treatment and paint-marked accordingly. For each treatment, 100 bees were topically treated with the assigned treatment and dose then marked using non-toxic Craftsmart paint markers. Bees were then fed pollen and nectar *ad libitum* for 24 hours before being placed into a hive equipped with a trap. Frozen (dead) and paint-marked bees were used as positive controls to determine percent capture rate.

3.2.4 Seasonal Apiary Capture Rate

To examine potential seasonal patterns of abnormal mortality, dead bees were collected and from inner and outer areas of traps (n = 12) weekly from all three apiaries (garden, orchard, farm) throughout the field season (April-October). Bees that were a

part of the imidacloprid recapture rate trials were excluded from the collected bees and not quantified in this assessment.

3.2.5 Citizen Science

In addition to the research hives, beekeepers volunteered 18 hives from four states (IA, NE, KS, CA) to implement and test traps in their apiaries. Beekeepers were asked to use at least three large (3" X 3" ft or 0.9m^2) traps per apiary, monitor traps weekly, and track overall health of colonies from April through October. Weekly losses were averaged for all three traps in each apiary; however, the results were not analyzed given the small sample size. Despite that, the citizen science project is an important step to begin tracking losses at the local or regional scale and identify seasonal trends to losses. Data was examined but not analyzed and is represented graphically in Figure 3.6.5.

3.2.6 Statistical Analyses

Efficacy of dead bee traps was assessed through the recapture rate of imidacloprid-treated bees at the garden apiary as well as through seasonal capture rates of colonies from all apiary sites. The average number of paint-marked imidacloprid treated bees collected from traps were analyzed by trap areas (inner, outer, total) and imidacloprid dose level (low, medium, high, positive control). Data was examined by month but not analyzed due to insufficient sampling across months and treatments. The average number of bees captured from dead bee traps in all apiaries (unmarked and not part of the imidacloprid trials) was analyzed by trap area (inner, outer, total) by apiary (garden, orchard, farm) and by month (April, May, June, July, August, September,

October) to determine if trap size, location, and season impacted the capture of dead bees. All data were assessed for normal distribution and equal variance and transformed using a generalized linear mixed model (GLMM)-(*link*-natural log function.) to account for the underlying distribution of the data. A Poisson distribution was used to fit the count response with repeated measures and statistical analyses were completed with Analysis of Variance (ANOVA) models followed by Tukey's HSD means separation tests using SAS 9.4 software program.

3.3 Results

Recapture Rate of Imidacloprid Treated Bees

A total of 21 replicated trials were performed with bees exposed to imidacloprid and released back into hive. Average weekly collections indicate more freeze-killed (positive control) treatment bees were recaptured from the inner (18.28 ± 1.36) compared to the outer (8.96 ± 1.93 bees) areas of the trap; however, roughly 27.7 ± 3.5% of paint-marked dead bees were recaptured from traps, indicating a relatively low capture efficacy. Bees treated with imidacloprid were recaptured significantly less for all doses compared to the positive control and was significantly different across all dose levels for each trap size. The average number of bees collected from the high dose (3.8 ± 0.6 bees) was significantly higher than compared to bees treated with either medium or low doses (ranging between 2.29 ± 0.42 to 1.57 ± 0.32 bees, respectively) in all three trap areas (inner ($F_{3,60}$=131.05 p=0.0001); outer ($F_{3,60}$=245.85 p=0.0001); total ($F_{3,60}$=87.67 p=0.0001))(Figure 3.6.2).

Data was divided out by month to determine if there may be seasonal differences. Due to a lack of replication within months the data was not statistical analyzed but there is a trend that shows a higher capture rate of all dose levels (low, medium, high, positive) in spring than in late summer and fall. The average(±SE) number of positive control bees recaptured in June was 45.7 ± 4.4 and numbers decreased to 24.7 ± 3.1 bees in September were recaptured out of 100. There were 1.41 less bees recaptured in the fall than in the summer and spring when treated with high imidacloprid doses. Indicating that for our examination of recapture rate the traps may be less effective in late summer and fall than they are in the spring. Further research would be necessary to reassess this and examine what may cause changes in recapture rate across the season (Figure 3.6.2).

Seasonal Apiary Capture Rate

A total of 12 traps were monitored weekly at three locations garden (*n* = 6), orchard (*n* = 3), and farm (*n* = 3) to determine average mortality over the season. Average weekly capture rates were pooled by month for each location and analyzed by trap size, apiary location, and month. The larger trap size (inner and outer measures combined) did have a higher average capture rate for all apiaries in all months (Figure 3.6.3). There were statistical differences in capture rate observed among all main factors (apiary, trap size ($F_{2,57.09}$=57.09; $p<0.0001$), and month) as well as interaction effects across apiaries and month ($F_{2,102}$=23.4; $p<0.0001$). The farm apiary location had significantly greater losses of worker bees compared to the other apiaries. The highest mortality was observed in July and the average weekly capture rate was significantly

higher in July (540.2 ± 159.2), August (416.4 ± 122.8), and September (206.6 ± 22.6) than compared to both the garden and the orchard apiaries which had losses ranging from 21.4 ± 6.8 to 67.4 ± 14.4 from July through September. There is no data for the farm for April, May, and June because hives were not moved to that location until July. The traps located in garden and orchard apiaries exhibited decreases of loss (166.7 ± 3.7 and 339.6±6.8, respectively) from April to August (Figure 3.6.4)

Citizen Science

The data collected from the citizen scientists were not analyzed due to the limited number of participants, but preliminary data suggests different patterns in abnormal mortality rates are emerging by region which could indicate possible environmental factor such as pesticide incidents. The California apiary had the highest number of traps (10) and exhibited the lowest losses observed compared to all other traps. Their weekly average mortality in June (6.4 ± 1.9) further decreased to an average of 0.79 ± 0.2. The highest weekly average capture occurred in July where the apiary experience average mortality of 29.2 ± 16.9. One of the ten traps collected 527 bees in the trap which was much higher than the average for the other weekly collections. Traps located in Nebraska collected a higher number of dead bees in the spring than they did in the fall. Traps within the state of Iowa had an increase in the average number of dead bees captured from May (28.8 ± 14.7) to August (110.2 ± 120.4) and then collection stopped because all colonies with dead bee traps died out. The Kansas apiary had an increase in capture rate as well from June (1.7 ± 0.33) to November (4 ± 1) but had overall low average numbers of bees collected. As noted earlier there may also

be differences between apiary site. There was a trend of higher mortality in the farm location than the orchard and urban location. This data is preliminary and will continue to be collected and will eventually be analyzed once there is a larger data set. (Figure 3.6.5)

3.4 Discussion

In modern agriculture the use of pesticides is a common practice and there are no indication of that use slowing. The production of crop outputs has increased by 170% (USDA 2018) and the potential exposure of pesticides to honey bees is a justifiable concern. Especially concerning are pesticides that are systemic and will translocate through the plants they are applied to. The potential of neonicotinoids to reside in nectar, pollen, and whole flowers for extended periods of time (Bonmatin et al., 2003; Stoner and Eitzer2012; Sánchez-Hernández et al. 2016; David et al. 2016; Sánchez-Hernández et al. 2016), even when applied as seed treatments, creates a unique challenge to honey bees and beekeepers alike.

Management of honey bee colonies involves monitoring for many important factors such as queen health, pest presence, and many other factors but there are no recommendations for monitoring for exposure to pesticides. Currently, there are guidelines for reducing pesticide exposure risk to bees and typically investigation of pesticide exposure occurs after a “bee kill”. Exposure to sublethal levels of pesticides through pollen and nectar may reduce survival of young nurse bees that provide essential care to brood. This effect may not kill a hive quickly, the colony population will be driven down by the inability to keep up with brood care. Our research focuses on

evaluating the use dead bee traps as monitoring tools to increase awareness of sublethal pesticide exposures and onsets of potentially lethal pesticide exposures. Dead bee traps are often used in scientific studies, particularly in pesticide field studies; however, we are suggesting the use of these traps by hobbyist, sideline, and commercial beekeeping operations to empower them to proactively monitor pesticide incidents within their own colonies. We hypothesized that using dead bee traps will allow for the proactive monitoring of pesticide incidents and will encourage beekeepers to recognize potential exposure events and mitigate its effects.

We began with the assessment of the efficacy of the dead bee traps and examined how that efficacy was impacted by the size of the trap. Our treatments included a positive control of dead bees to determine what proportion of dead bees would be captured by the traps. This resulted in the discovery of two things, the first was that the traps on average captured 27.7% of experimental bees in our positive control test group, and the second was that the number of positive control dead bees captured decreased from the spring into the fall, however, there was not enough replication of this to analyze for significance. The seasonal capture rate of dead bees for all three apiaries had similar patterns and showed significantly higher mortality in spring and early summer than late summer and fall. Previous research on undertaker bees indicates 1 to 2 percent of the hive population specializes in necrophoric behavior (Visscher 1983) and additional research indicates they may be affected behaviorally over time by trap presence (Illies et al. 2002). These undertakers typically remove the deceased bees and brood from the colony. Once the colony is strong in mid to late

summer, they may have a higher population of undertakers that are able to remove dead bees further from the hive. Moving dead bees further from the hive could be valuable to the colony health as it may deter scavengers and predators from being near the hive and eating the dead bees which previous research has indicated may be a factor (Illies et al. 2002). This is important because these scavengers may also attempt to eat living bees or steal food resources from the colony such as racoons, opossums, which was observed by one of my citizen scientists. One potential is that the undertakers are flying past the trap further to reduce the dead bees in front of the hive which previous research has indicated that dead bee traps may impact the behavior. We believe that a combination of both of the effects of behavioral changes and an increase in undertakers is the most likely scenario as during multiple replications in the late season, undertakers were witnessed flying as far as 10 feet out to drop off our positive control bees.

In this research we found evidence that the traps are significantly more effective at capturing positive control bees than bees exposed to all treatment doses of imidacloprid. Additionally, bees exposed to the high dose were captured in the trap significantly more often than bees exposed to the medium and low doses. This is consistent with previous research indicating that exposure to imidacloprid at levels of 242 ng (Cresswell 2011) can result in acute mortality and our high dose was 1 mg/L. Previous research indicates that at some levels, imidacloprid does not cause mortality but rather increases the length of time it takes to forage and decreases the ability to return home (Bortolotti, L. et al. 2003; Yang E. C. et al., 2008). Our research did not have

a way to account for bees that did not die from exposure but instead exhibited sub-lethal effects.

We also separately examined how location and season may be factors that influence capture of dead bees. Our apiaries included locations that differed in their use of agrochemicals. Areas like orchards do not always require the application of pesticides later in the season but often require applications of fungicides in early spring during bloom. Whereas areas like the urban garden and agricultural farm may have required application of pesticides at later dates to combat pest insects such as corn ear worm, or mosquitos. The three sites examined in this research were a farm, an orchard, and an urban garden area. Our expectation to see differences was met with significance. Our research indicated that the season and the location impacted the number of bees that were captured by the traps. The farm location had a significantly higher average number of dead bees for the mid summer months than the other two locations but had similar numbers to the other traps during October. This could indicate a pesticide exposure and the need for the implementation of management strategies to reduce the colony exposure and effects. Additionally, the garden apiary saw a significantly higher average number of bees in May than the orchard apiary. This location is an urban area surrounded by commercial and residential establishments and exposure to pesticides may be different during that time than in areas such as orchards where the use of pesticides is likely much lower when the trees are fruit bearing. Another significant result was the difference between season. Another possibility is that there may be less pesticide use in orchards, gardens, and farms in late summer and fall. The reduced use

of pesticides could potentially reduce the overall mortality within the colony. Though other dead bee traps describe higher capture rates of 80% (Norman 1960), our dead bee trap was designed to be an effective tool for the general public that is cheap and easy to build. This resulted in the implementation of a citizen scientist project that allowed beekeepers to utilize dead bee traps and record data from multiple locations. Due to the lack of annual replication and potential for inconsistency between citizen scientists we did not analyze this data but this preliminary data is interesting. Identifying seasonal and regional trends, using monitoring traps, may provide more information that can later be extrapolated to identify agricultural management practices, such as tank mixtures, mosquitos abatements, that may be unintentionally harming bees and or identify potentially problematic pesticide formulations. Our research sought to explore the potential of dead bee traps as beekeeper tools to assist in identification of pesticide exposure.

As with any pesticide related experiment, cost of evaluating the actual uptake of pesticides within the bees is exceedingly expensive and therefore was not conducted, this limits our knowledge of the actual exposure concentration to developing brood reared in treated combs. Making actual extrapolations from our data and the efficacy of our traps difficult. Additionally, bees are not normally exposed to acetone and traditionally exposure to imidacloprid would be from contaminated nectar or pollen and not necessarily dermal. This means that we cannot assume this capture rate is equivalent to the capture of bees that ingested imidacloprid in their diet. Previous studies documented that imidacloprid does not necessarily cause mortality but often

results in sublethal effects on bees and exposed bees exhibit impaired cognition (difficulty returning home, take longer to forage, and to some degree get “lost”). We encountered this issue in almost all replications. Paint-marked bees treated with imidacloprid and released back into the colonies could often be found a week or more later in another colony that was not associated at all with the research. Another factor that may have influenced the average capture rate is the equipment we used. Some of the frames within those hives had previously drawn comb. This may have exposed bees to one or more additional pesticides within the stored food resources or through wax. Future research could examine how mortality is affected with colonies that start with only blank frames. Though, this is not as field realistic it may clarify what beekeepers with new equipment should expect for mortality. Our dead bee traps do not have the ability to monitor for sub-lethal pesticide exposure that do not cause mortality of bees but future research could examine how sublethal levels of imidacloprid cause bees to return to hives that are not their own and potentially transfer pesticides to those colonies as well. With any colony level field research that are many variables that make the pursuit of significant results incredibly difficult, especially when it involves toxicology (Sponsler and Johnson 2016, 2017).

Overall our goal was to identify the efficacy of dead bee traps as tools to monitor for pesticide incidents and to use the information collected from the research experiments as well as from citizen scientists to begin compiling regional pesticide monitoring data. Honey bees are exposed to a wide range of chemicals inside the hive as well as outside in nectar, pollen, and flowers (Bonmatin et al. 2003; Stoner and

Eitzer 2012; Sánchez-Hernández et al. 2016; David et al. 2016; Sánchez-Hernández et al. 2016). Though we did not see high capture rates for bees exposed to imidacloprid, traps were useful in identifying times of the season and which abnormal losses of worker bees were observed in particular apiary locations. Our study found significant differences in dead bee captures between sampling sites associated with variable agrochemical use patterns. And as beekeepers implement these monitoring tools in their apiary, the information collected from individual beekeepers could be pooled together to provide baseline data to start tracking long term seasonal, regional trends that will help narrow down the potential agricultural practices that may be causing lethal and sublethal exposures. The continued collection of this data could contribute to the development of improved beekeeper management recommendations and pesticide policies that better protect the health of our critically important honey bee pollinators.

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3.6 Figures

Figure 3.6.1 Dead Bee Trap Set-up. This image shows design and placement of traps. To assess an optimal size, traps of two sizes (small 2X2ft or 0.6m^2 and large 3X3ft or 0.9m^2) were nested into one trap structure and examined for the number of bee collected in “inner” and “outer” areas. Dead bees collected from the “inner” area represented the capture rate of smaller traps while the bees collected from both “inner” and “outer” areas were pooled to represent the “total” bees captured from within the large trap dimensions. Traps were placed in front of hives in Spring and removed in mid-October.

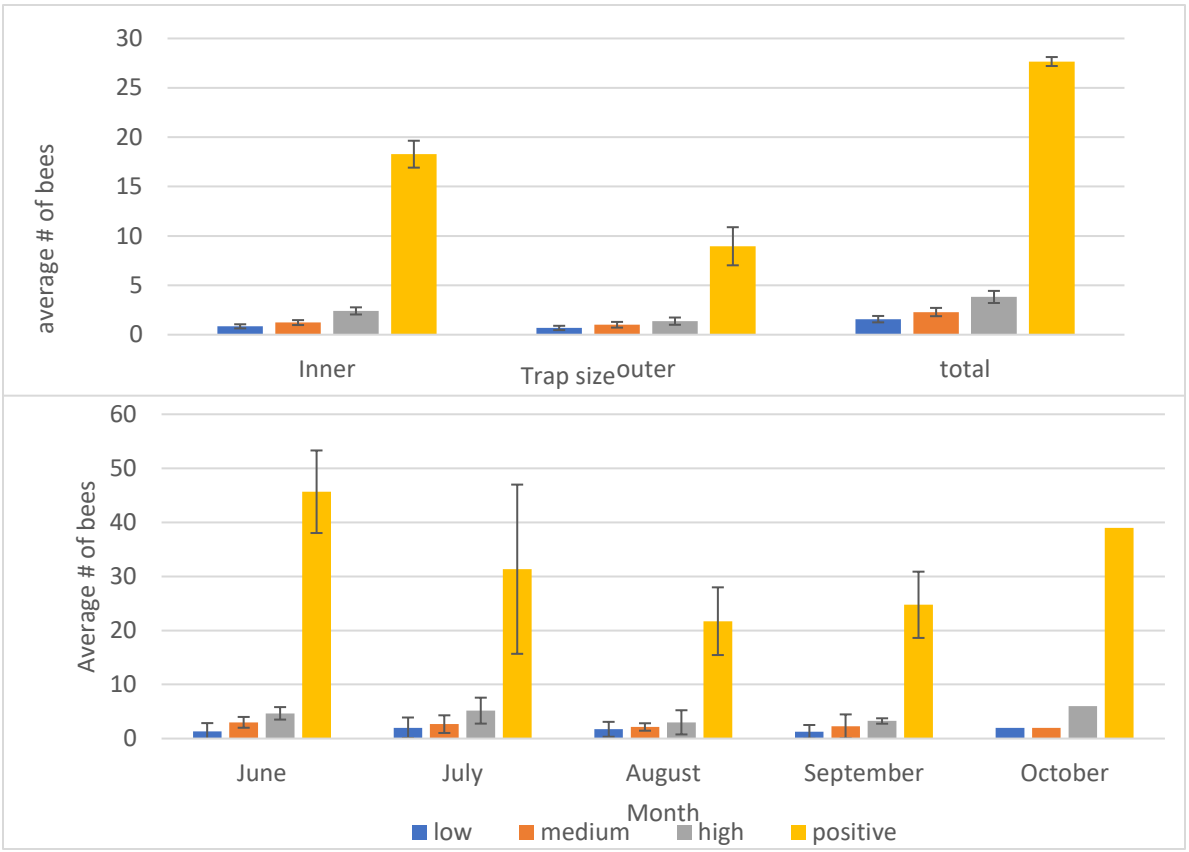


Figure 3.6.2 Efficacy of Dead Bee Traps with Bees Exposed to Imidacloprid. **Paint-marked bees topically** treated with imidacloprid insecticide at low, medium, or high concentrations (10, 100, 1000 ppb) and freeze-killed bees (positive control) were introduced into hives equipped with dead bee traps to assess the efficacy of traps to monitor for abnormal bee losses. To assess an optimal trap size, dead bees were collected weekly from the "inner" and "outer" areas of each trap from April through October. The accumulative averages from the inner and outer areas are presented as the "total" bees recaptured per trap. Weekly averages were pooled over the season and analyzed using ANOVA and Tukey-Kramer means separation tests with significance determined at alpha=0.05 and denoted with different letters. There were significantly higher recapture rates of freeze-killed dead bees (positive control) and bees treated with high doses of imidacloprid in inner ($F_{3,60}$=131.1; p= 0.0001), outer ($F_{3,60}$=87.7; p=0.0001), and total ($F_{3,60}$=245.9; p=.0001) collections compared to other doses (top graph). Data suggests that traps were more likely to recapture bees in early (June, July) and late (October) summer (bottom) and that the larger trap size ("total") was more effective at capturing dead bees removed from the hive than the smaller traps ("inner") (bottom graph).

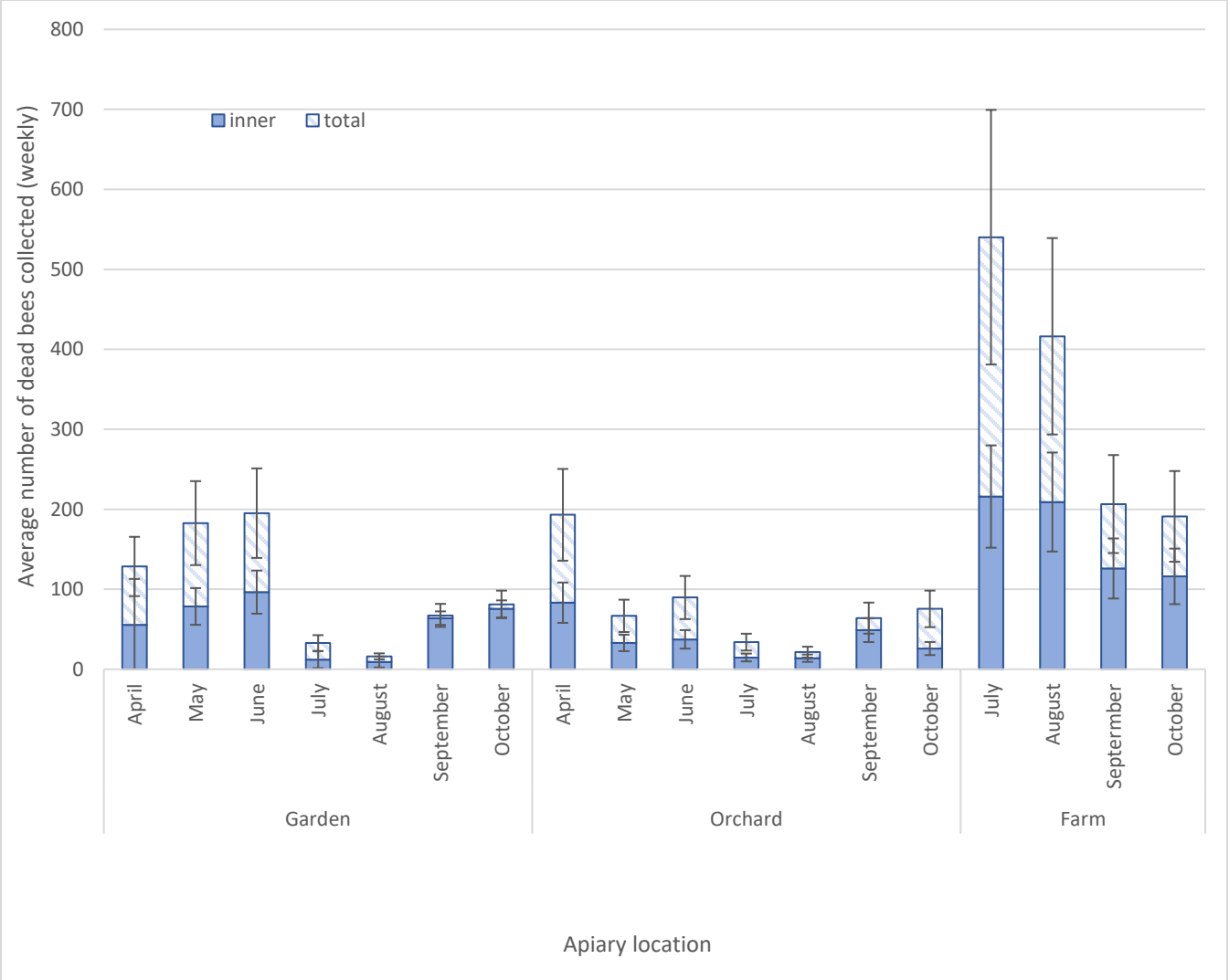
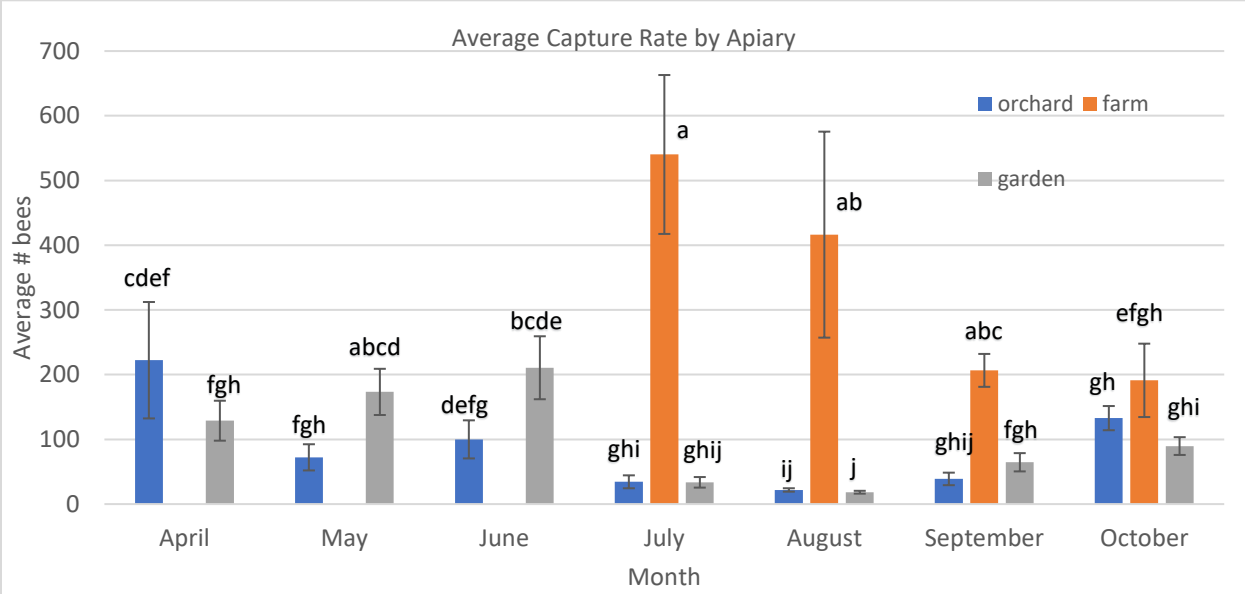
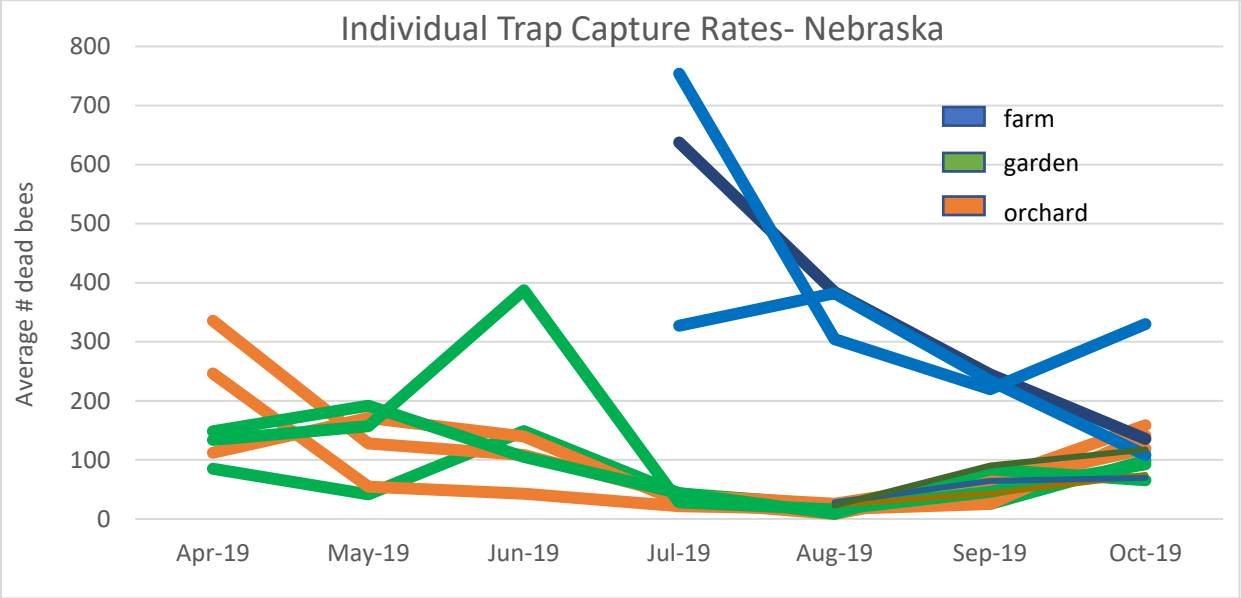


Figure 3.6.3 Trap Size Efficiency. To assess an optimal trap size, dead bees were collected weekly from the "inner" and "outer" areas of each trap from April through October at three apiary locations (garden, orchard, and farm). The average number of dead bees collected from the inner areas represent bees captured by small-sized traps (blue shaded portion) while the accumulative collection of bees in the inner and outer areas represent the "total" bees captured by large sized traps (entire bar). Weekly averages were pooled over the season and analyzed using ANOVA and Tukey-Kramer means separation tests with significance determined at alpha=0.05 and denoted with different letters. There were significant differences between trap sizes, the larger trap size does have a higher capture rate ($F_{12,50.23}=60.84$; $p=0.0001$).





(weekly) from traps placed in front of hives at three apiary sites (orchard, farm, garden) (top). A total of twelve individual traps were used to monitor abnormal losses of bees at apiaries from April through October (bottom). Weekly averages were pooled by month and analyzed using ANOVA and Tukey-Kramer means separation tests with significance determined at alpha=0.05. Interaction effects were observed between apiaries and month ($F_{2,102}$=23.4; p<0.0001) and different letters, here, denotes where observed losses were statistically different.

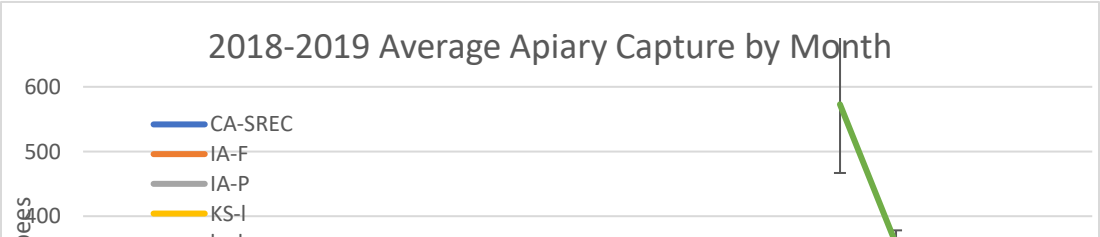


Figure 3.6.5 Citizen Science Average Monthly Mortality by Apiary and State. This graph shows a comparison of average capture rates gathered citizen scientists by region and month. This data was not analyzed but shows interesting trends for individual apiaries. The top graph examines average monthly mortality from each apiary. The apiaries are labeled by the state they are located in and then followed by the apiary name. Any data from states other Nebraska was collected by citizen scientists and compiled to begin tracking regional, seasonal mortality. The bottom graph examines each overall monthly average between all state apiaries present. This was also not analyzed due to lack of replication. Data will continue to be collected annually for eventual analysis.

Chapter 4: NebGuide

Title: Monitoring for Pesticide Incidents in Honey Bee Colonies

Introduction:

Utilizing Dead bee traps as a management tool empowers beekeepers to proactively monitor for pesticide incidents within the hive. Pesticides can cause an immediate acute death of foraging bees or they cause sublethal effects when ingested. The bees that ingest sublethal doses of pesticides return to the hive and feed the contaminated food sources to larvae, nurse bees, and house bees. The younger bees who may be more susceptible may start to consume contaminated food and slowly die off. The acute die off of older bees also causes the younger bees to forage before they are mature enough to do so. As these younger bees forage, there is a reduction in brood care. Less bees caring for brood slowly brings down the hive population and instigates a chain reaction of other health concerns. Hives experiencing a pesticide incident may take a few weeks to die off.

Dead bee traps may be effective monitoring tools in these situations. They allow beekeepers to track weekly mortality and have a unique perspective of what is happening without opening the hive. As die offs begin to occur, beekeepers may see an increase in the bees within the trap, this helps beekeepers to narrow down the window of when the pesticide exposure originally occurred. Once a time frame is recognized as the initial pesticide incident, the beekeeper can track patterns and communicate with University of Nebraska- Lincoln to assist in understanding and tracking future pesticide incidents. Currently, there are established methods to report an entire colony loss due

to pesticide exposure but there is no protocol for reporting pesticide incidents that cause partial die off and reduced colony strength. The investigation and reporting of potential sub-lethal pesticide incidents will help future beekeepers by establishing patterns that may correlate with seasonal pesticide usage and exposures. Continued efforts to track and understand what pesticide exposure does in a hive can help people create solutions to these problems.

What is a dead bee trap?

A dead bee trap is a 3’ x 3’ trap made from 2” x 4” treated wood. They are relatively easy and cheap to make but serve as a powerful tool for beekeepers. Within the university, dead bee traps are used at multiple apiaries to track how the losses change based on regional location. The traps are used not only to track weekly mortality but also to recognize other potential health issues within the hive.

Why use a dead bee trap?

One of the issues beekeepers face today is the ability to determine and investigate pesticide exposure incidents. There are no established means to report a pesticide partially because there is no easy way to determine exactly when an exposure happened and what chemical was the problem. Often, a hive will slowly die because of an exposure that was not lethal but still caused health issues. These health issues may begin with young nurse bees eating nectar or pollen with small amounts of chemicals present. This could outright kill them or just cause them to be less efficient at caring for brood. It can take several weeks for a hive to completely die and is not determined to be

a direct loss from pesticides. One of the important early signs of an exposure is the death of young bees. As bees die, they are removed from the hive by grave bees, and end up in the grass in front of the hive. Identifying how many bees and what ages they are is difficult because of the grass and dirt, so dead bee traps are a simple tool to prevent the bees from ending up on the ground. Instead they are collected in an easy to use trap where beekeepers can more closely examine them to determine issues.

When used as a pre-health check, dead bee traps can streamline the process of inspecting a hive. Health issues recognized in the trap can assist in determining what needs checked in the hive.

What is a pesticide incident?

A pesticide incident is different than an acute total kill. The only pesticide exposures currently investigated by the USDA are acute total kills where the entire hive is lost. A pesticide incident is when exposure to the hive has occurred but not at a high enough level to kill the entire colony right away. A high mortality in a hive may be an indicator that there was an exposure that did not cause a total die off but weakened the hive instead. Dead bee traps will help to track patterns of mortality in these incidents since there are no protocol for non-lethal exposures.

What can we learn from the bees in the trap?

Dead bees can tell us a lot about what is going on within a hive. When there are many dead bees it may be an indicator of a pesticide incident or health issue. Even closer examination of the dead bees can tell us a more detailed story of what is going

on. Perhaps you check your trap and noticed several pupae with deformed wing virus. This paints us a story of what may be occurring in the hive, and it is time to look for varroa mites by doing a mite check. There may even be mites in your trap on the dead bees. There are times when you may even find a dead queen. This is an immediate indicator that the hive needs some help and provides you, the beekeeper, the opportunity of trying to right the colony before a total loss.

The dead bee traps can be as helpful as we choose to make them and can serve a purpose deeper than just pesticide incidents. The great thing is that it can be combined with technology, like smart phones, to further investigate issues. Apps and online groups for beekeepers are also great tools to identify issues.

How to make a dead bee trap:

We encourage beekeepers to utilize multiple traps within an apiary to better assess impacts on individual colonies and apiaries. This will also provide us with more information for each location.

Materials:

Each trap will require:
4 - 3’ 2”X4” treated boards (we recommend a 2x4x12 board) UNITS
1 - 3’2”X3’2” section cut from white or light colored UV-resistant or outdoor material (such as tarp)
8 - 3” screws
Staple Gun

Directions:

1. Cut your board into 3 foot sections.

2. Align these boards according to the picture below.

3. Using 2 - 3” screws, screw the board together as pictured below.

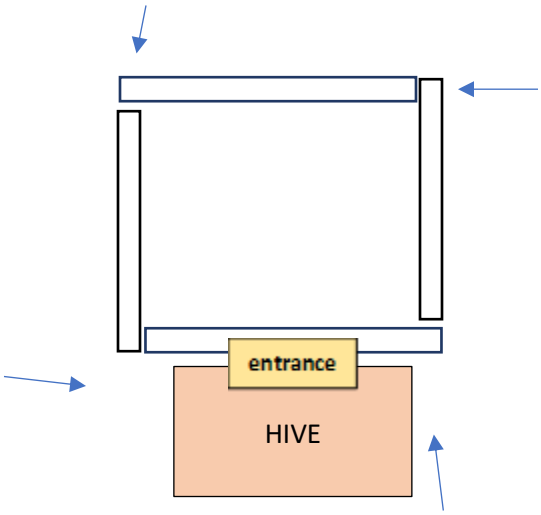
4. Repeat until you have a square. Paint or stain the wood to protect it from weather conditions.

5. Then, cut your fabric to 3 foot by 3 foot and lay on the inside of the square.

6. Staple the edges of the fabric to the inside of the boards.

7. Your 3 X 3 trap is complete.

Picture 5: This is how the trap should be placed in front of the hive.



The blue arrows indicate the locations of screws.

Recordkeeping

Recording what is happening in your hive is important. Records help beekeepers to see changes in the health of the hive and track patterns. It may not be necessary to record the exact number of dead bees in the trap but it may be helpful to have a general idea of how many there are each week. It can also be helpful to record details on what types of bees are present within the trap. Tracking a change like an increase in young bees and brood in your trap may help to recognize a colony that is crashing and allow you to take preemptive measures to get that colony back on its feet (or rather wings).

Not only is it helpful to record what is happening in the trap but also the hive itself. Many beekeepers track the number of pollen frames, brood frames, if eggs are present, number of varroa on 300 bees, if the queen was seen, etc. Records can be used to monitor how these factors fluctuate. Understanding a combination of what is going on

inside the hive as well as the trap can assist a beekeeper in catching a hive before it crashes.

To help with record keeping it is a good idea to mark each trap with a unique identifier (number, code, color, etc,). You can choose your own method to record information but we have included a template below. Using a measuring cup to estimate the total number of dead bees is a simply, effective way to track losses. A half cup of bees is approximately 300 bees. After estimating the total it is important to empty the trap. If you leave the dead bees in there you may not have accurate information about your hives health.

Sample Data entry:

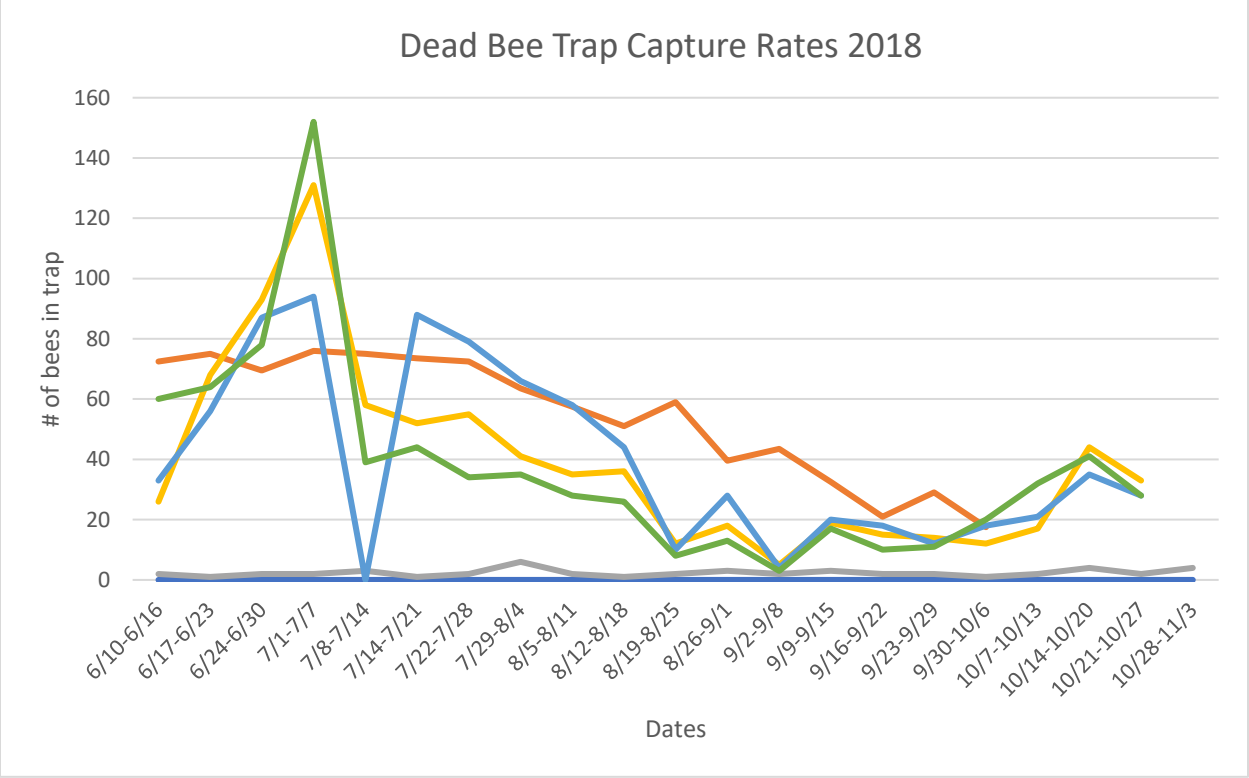
Week	Collection Date	Apiary	# of Brood Frames	# of frames of bees	Trap ID	# of dead bees in trap	# of sick or lethargic bees in trap	Notes
4/1-4/7	4/1	Pollinator Gardens	4	12	10	54	5	All old foragers
4/8-4/14	4/8	Pollinator Gardens	5	13	10	59	3	All old foragers
4/15-4/21	4/15	Pollinator Gardens	4	10	10	67	0	Saw Deformed wing, tested for varroa and found 4
4/22-4/28	4/22	Pollinator Gardens	7	14	10	255	23	Large increase in bees in trap some looked young, contacted Jennifer
4/29-5/5	4/29	Pollinator Gardens	3	9	10	45	4	Varroa test found 7 varroa. No diseases noticed
5/6-5/12	5/6	Pollinator Gardens	4	10	10	0	0	Storm last night, no bees collected

What to look for in a trap:

Determining the age of bees and identifying problems can be very difficult. There are a few things that can help determine how old the bees are and if they have obvious health issues. Young bees are the nurse bees of the colony. They are typically extra fuzzy and golden. Hives with lots of nurse bees present in a trap should be inspected thoroughly. A loss of young, nurse bees can be a sign that a pesticide incident may have happened. Old, foraging bees usually have less fuzz, have darker thorax, and sometimes tattered wings.

Previous Data

Here are some graphs showing the annual losses for traps in Nebraska and Kansas from 2018.



- This graph shows the capture for 5 traps located in Nebraska and Kansas apiaries in the summer of 2018. Data indicate a mid-summer spike in the number of dead bees

collected from traps that may be attributed to seasonal pest outbreak treatments. The drop in dead bee collections during July 8th was due to a storm in Kansas that washed out bees from traps.

When Should I Monitor?

The highest number of bees in the traps are in early spring. Monitoring early when your hive is ramping up for the season can provide a baseline for what to expect in each season and indicate when a rise in dead bees has occurred and therefore a possible pesticide exposure. Colonies that are weaker and early spring colonies tend to have higher numbers of dead bees in the trap due to fewer bees cleaning out bodies. Die offs may occur earlier in the season from an increased use of pesticides that can harm bees, though they can occur at any time. As you monitor throughout the season you may see ups and downs that can be indicative of the season. Keep in mind that certain seasons will see different types of bees in the trap. It is especially alarming in the fall to find a hive with several hundred bees only to realize many of them are drones that have been kicked out for the winter.

As you monitor your traps, it may be helpful to consider what weather events have occurred since you last checked the hive. Heavy rain, strong wind, and other factors can impact the number of bees present in the trap. Typically, the trap is helpful if checked on a weekly basis. This can be adjusted for apiaries far away or in remote locations. The best way to handle these situations is setting a schedule to

compare to traps you check more regularly. If you check one hive every two weeks and another hive every one week, the biweekly trap should be divided by two to compare it to the trap checked weekly.

I think I had a pesticide incident, now what?

Do not panic, the most important thing for you as a beekeeper is to recognize there has been an issue. The first step you should take is to document the overall hive health for your own records. Contact the University of Nebraska-Lincoln Bee Lab in the entomology department to help examine deceased bees. There is no reason to test your bees for pesticides because it will not contribute to a pesticide claim. The process is costly and cannot be included in an official investigation. If you would like to test them for your own interest you can contact the USDA Department of Agriculture, these results will not help to file a report but may assist in future monitoring for pesticide incidents. Finally, the next step is to try and right the colony if it is still alive.

Here are a few steps to boost your colony:

1. Add capped brood frames (from a healthy hive) to boost the number of nurse bees
2. Supplement by feeding pollen and nectar
3. Monitor the number of brood frames
4. Monitor frames of food in the colony
5. Monitor for varroa to prevent an added stressor to your colony

6. Combine two weak colonies

The final important thing to note is that if you have had an entire colony die from what you suspect to be an acute pesticide exposure, contacting your state USDA can start the process of an investigation into a pesticide kill.

Hive issues but not from pesticides?

In this case you do not need to contact someone to investigate a pesticide incident, but you want further guidance. The best solution is to contact a local university entomology department bee lab, entomology extension worker, or a master beekeeper. There are many issues that can arise that are not from pesticides but are important to hive health. The health of your bees can impact that health of bees nearby and getting the help you need is important. Do not hesitate to contact a knowledgeable beekeeper to find a solution.

Who can you contact

The first step is to contact a state agency that can properly investigate the issue. Below are listed a set of contacts for each state. Once you have started that process it may be good to also increase your knowledge and connections by utilizing some invaluable apps for smart phones like Beecheck, 

Driftwatch, 

and a number of others can assist in monitoring for mites and connecting with local farmers to prevent spraying of areas with apiaries. You may also consider joining a local beekeeping club or facebook group to connect with other beekeepers.

Table 4.1: List of state agencies and their contact information for reporting incidents and bee kills from suspected pesticiide exposure.

State	Agencies	Contact
Alabama	Dept. of Ag. & Industries (Pest Management Division)	(334) 240-7242
Alaska	Dept. of Environmental Conservation (Pesticide Control Program)	(800) 478-2577
Arizona	Dept. of Agriculture (Environmental Services Division)	(800) 423-8876
Arkansas	State Plant Board (Pesticide Division)	(501) 225-1598
California	CA Environmental Protection Agency (Dept. of Pesticide Regulation)	(916) 324-4100 or (877)378-5463
Colorado	Dept. of Agriculture (Division of Plant Industry)	(303) 869-9058
Connecticut	Dept. of Energy & Environmental Protection (Pesticide Management Program)	(860) 424-3369
Delaware	DE Dept. of Agriculture (Pesticide Management)	(302) 698-4571
Florida	Dept. of Agriculture & Consumer Services (Bureau of Plant and Apiary Inspection	(352)-395-4633
Georgia	Dept. of Agriculture (Plant Industry Division)	(404) 656- 4958
Hawaii	Dept. of Agriculture (Pesticides Branch)	(808) 973-9404
Idaho	State Dept. of Agriculture (Pesticides and Chemigation)	(208) 332-8613 or (208) 332-8608
Illinois	Dept. of Agriculture (Bureau of Environmental Programs)	(217) 524-7799
Indiana	Office of IN State Chemist (Pesticide Section)	(800) 893-6637 or (765)-494-1582
Iowa	Dept. of Agriculture & Land Stewardship (Pesticide Bureau)	(515) 281-8591
Kansas	Dept. of Agriculture (Pesticide & Fertilized Use)	(785) 564-6688
Kentucky	Dept. of Agriculture (Division of Environmental Services)	(502) 564-6120
Louisiana	Dept. of Agriculture & Forestry (Pesticide & Environmental Programs)	(855) 452-5323

Maine	Dept. of Agriculture (Board of Pesticides Control)	(207) 287-2731
Maryland	Dept. of Agriculture (Pesticide Regulation Section)	(410) 841-5710
Massachusetts	Dept. of Agricultural Resources (Pesticide Program)	(617) 626-1781
Michigan	Dept. of Agriculture & Rural Development (Pesticide & Plant Pest Management Div.)	(800) 292-3939
Minnesota	Dept. of Agriculture (Pesticide & Fertilizer Management Div.)	(651) 201-6333
Mississippi	Dept. of Ag & Commerce (Bureau of Plant Industry, Pesticide Program)	(662) 325-8789
Missouri	Dept. of Agriculture (Plant Industries Div., Bureau of Pesticide Control)	(573) 751-5511
Montana	Dept. of Agriculture (Pesticide Programs)	(406) 444- 5400
Nebraska	Dept. of Agriculture (Bureau of Plant Industry, Pesticide Program)	(402) 471-6882
Nevada	Dept. of Agriculture (Plant Industry Div.)	(775) 353- 3716
New Hampshire	Dept. of Agriculture (Markets & Foods, Div. of Pesticide Control)	(603) 271-3640 or (603) 271-3550
New Jersey	Dept. of Environmental Protection	(609) 984-6568
New Mexico	Dept. of Agriculture (Pesticide Compliance Section)	(575)-646-2733
New York	Dept. of Environmental Conservation (Div. of Materials Mgmt, Bureau of Pest Mgmt)	(518) 402-8727
North Carolina	Dept. of Agriculture & Consumer Services, Structural Pest Control & Pesticide Division	(919) 733-3556
North Dakota	Dept. of Agriculture (Pesticide & Fertilizer Division)	(701) 328-4922
Ohio	Dept. of Agriculture (Pesticide & Fertilizer Regulation Section)	(614) 728-6987
Oklahoma	Dept. of Agriculture (Food & Forestry, Plant Industry & Consumer Services)	(405) 522-5981
Oregon	Dept. of Agriculture (Pesticides Division)	(503) 986-4635
Pennsylvania	Dept. of Agriculture (Bureau of Plant Industry)	(717) 772-5231
Rhode Island	Dept. of Environmental Mgmt. (Div. of Agriculture)	(401) 222-2781 x4504
South Carolina	Clemson University (Dept. of Pesticide Regulation)	(864) 646-2150

South Dakota	Dept. of Agriculture (Div. of Agricultural Services, Pesticide Program)	(605) 773-4432
Tennessee	Dept. of Agriculture (Pesticides & Agriculture Inputs)	(800) 628-2631
Texas	Dept. of Agriculture (Pesticide Programs)	(800) 835-5832
Utah	Dept. of Agriculture & Food (Div. of Plant Industry)	(801) 538-4925
Vermont	Agency of Agriculture (Food & Markets, Agricultural Resource Management & Environmental Stewardship)	(802) 828-6531 or (802) 828-3482
Virginia	Dept. of Agriculture & Consumer Services, (Office of Pesticide Services)	(804) 371-6560
Washington	Dept. of Agriculture (Pesticide Management Division)	(360) 902-2040 or (360) 902-2010
West Virginia	Dept. of Agriculture (Regulatory & Environmental Affairs Division)	(304) 558-2209
Wisconsin	Dept. of Agriculture (Trade & Consumer Protection, Agricultural Resource Management Division)	(608) 224-4500 or (608) 224-4529
Wyoming	Dept. of Agriculture	(307) 777-6585
Washington D.C.	Dept. of the Environment (Environmental Programs)	(202) 535-2600