Effects of Pesticide Exposures on the Nutritional and Immune Health of the Honey Bee, *Apis mellifera* L.

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ABSTRACT

The honey bee is a widely managed crop pollinator that provides the agricultural industry with the sustainability and economic viability needed to satisfy the food and fiber needs of our society. Excessive use of agrochemicals such as the acaricides coumaphos and *tau*-fluvalinate, and the fungicide, chlorothalonil is implicated in the reduced number of managed bee colonies available for crop pollination services. Here, I report the effects of pesticide exposures on the nutritional and immune health of the honey bee. Total protein concentration was significantly reduced in the coumaphos- and chlorothalonil-treated individuals compared to the pesticide-untreated bees. Total carbohydrate concentration was significantly lower in the *tau*-fluvalinate, coumaphos-, and chlorothalonil-treated individuals compared to the pesticide-untreated bees. Total lipid concentration was significantly decreased in the chlorothalonil-treated individuals compared to the pesticide-untreated bees. Body weight was significantly reduced for the *tau*-fluvalinate-, coumaphos-, and chlorothalonil-treated individuals, compared to the pesticide-untreated bees. Wing length was significantly reduced for the coumaphos and chlorothalonil-treated individuals, compared to the pesticide-untreated bees. Phenoloxidase activity was significantly increased in the coumaphos-treated individuals compared to the pesticide-untreated bees. Glucose oxidase activity was significantly increased in the chlorothalonil-treated individuals compared to the pesticide-untreated bees. While more research is needed to verify the observed effects of the pesticides on the nutritional and immunity health of the honey bee, it is important for beekeepers to consider alternative methods for control of varroa mites and the use of fungicides near their colonies.

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CHAPTER 1

INTRODUCTION

Honey bees provide important food and fiber needs for human society. It is estimated that one third of our diet comes from insect pollinators, and of that 80% is from honey bees (Thapa, 2006). Worldwide, honey bees provide $223 billion per year in agricultural benefits and contribute 9.5% total value of human food production (Gallai et al., 2009). In the US, pollinator services are estimated at $14.6 billion per year (Stokstad, 2007).

However, in the US, the benefits provided by honey bees are being threatened by a decline in the number of managed hives. Virginia has been affected by a loss of honey bees and managed hives at a similar rate to the national average. Honey bee colonies have declined by about 50% since the 1980s, and over the past ten years the annual loss of colonies has been about 30% a year (Fell and Cobb, 2009). Based on the average colony loss, reduced honey sales, loss of pollination fees, and cost of colony replacement, honey bee decline costs Virginia beekeepers $1.3-1.8 million per year (Fell and Cobb, 2009).

Initial colony losses in the 1980s were due to two introduced parasitic mites, *Varroa destructor* (1987) and *Acarpis woodi* (1984) (Johnson et al., 2010). The introduction of these mite pests was devastating for beekeepers, but has been managed by miticides. The two of the most common miticides found in hives are the pyrethroid *tau*-fluvalinate and the organophosphate coumaphos (vanEngelsdorp et al., 2008; Mullin et al., 2010). These two miticides are often found in combination with the agricultural fungicide chlorothalonil (Mullin et al., 2010). Chlorothalonil is picked up by foraging honey bees and brought back to the hive in pollen (Mullin et al., 2010).

Both *tau*-fluvalinate and coumaphos are lipophilic compounds that are absorbed in beeswax in the hive with an estimated half-life of five years (Bogdanov, 2004). *tau*-Fluvalinate and coumaphos have been shown to impair reproductive physiology, reduce the ability to reproduce queens, reduce sperm viability in drones, and increase queen failure (Burley et al., 2008; Fell, 2001). Chlorothalonil is the third most common agricultural fungicide used in the US, only after copper and sulfur (Mullin et al., 2010). In some hives sampled, chlorothalonil has been found in pollen at concentrations as high as 99 parts per billion. The contamination of pollen is reported to correspond with concentrations in wax and bee brood samples from the hive (Mullin et al., 2010). The high presence of this fungicide in hives may be related to the fact that there are no label restrictions for spraying chlorothalonil in areas pollinated by insects (Mullin et al., 2010).

Here, I report the effects of pesticide exposures on the nutritional and immune health of the honey bee, including: 1) the concentration of total proteins, 2) the concentration of total carbohydrates, 3) the concentration of total lipids, 4) the morphometric measurements, 5) the phenoloxidase activity, and chlorothalonil-treated colonies, and 6) the glucose oxidase activity in brood and forager honey bees in in *tau*-fluvalinate, coumaphos, and chlorothalonil-treated colonies.

CHAPTER 2

LITERARURE REVIEW

2.1 THE BASIC BIOLOGY OF THE HONEY BEE

The European honey bee, *Apis mellifera*, was first described by Linnaeus in 1758 (Day, 1979). The honey bee is a eusocial insect in the order Hymenoptera which includes other insects such as the wasps and ants (Delaplane and Mayer, 2000). The superfamily Apoidea has an estimated 25,000 species of bees, including the honey bee (O'Toole and Raw, 1999). The herbivorous larvae and the pollen-collecting extremities of the adults are some characteristics that make the family Apidae unique from other hymenopterans (Delaplane and Mayer, 2000).

The natural range of *A. mellifera* extends from the southern tip of Africa through Northern Europe and Scandinavia. The vast amount of geography covered by this one species has resulted in many subspecies of *A. mellifera*. The most common subspecies for commercial beekeepers are; *A. mellifera mellifera, A. mellifera carnica*, *A. mellifera ligustica*, and *A. mellifera caucasica* (Winston, 1991).

The honey bee is a holometabolous insect that completes a metamorphic transition from egg to larva, pupa, and adult. Honey bee eggs are laid in a wax cell by the queen bee, which is the only individual responsible for sexual reproduction within the hive. The eggs hatch into larvae, which are fed by adult worker bees. After feeding, wax cell in which the larva is contained is capped over to allow the pupae to molt into an adult. The length of time spent within the capped brood cell depends on the sex and caste of the honey bee. The female queen will remain in the capped cell for eight days whereas the female worker and male drone bees will remain in the brood cell for 12.5 and 14.5 days, respectively (Delaplane and Mayer, 2000; Winston, 1991).

There are three physiological divisions of the honey bee, including queen, workers, and drones, which form the basis for the social roles of the colony. The queen starts as a fertilized egg and larvae. The developmental difference between the two castes depends on the type of brood food fed to the individual. The queen is fed a diet of royal jelly and the workers are fed worker jelly. The brood cells that are occupied by the queen bee for development are raised from the comb and hang downwards. Once a queen bee emerges from her cell, she must make a mating flight to fertilize her eggs. Approximately two days after fertilization, the queen bee will begin to lay her eggs. The queen bee can live and be sexually reproductive for several years (Winston, 1991). woe

The male (drone) bee is produced from an unfertilized egg typically produced by the queen bee or as a result of worker laid eggs. Drones bees are produced in the spring and summer, with production stopping in the fall. The cells of drone bees are slightly larger than those of a worker bee cell. A drone also takes a longer period of time to emerge from the pupal cell compared to a worker bee. The main role of a drone bee is to mate with a queen, after which it dies (Winston, 1991).

The female worker bee undertakes different tasks within the hive hierarchy as she becomes older or the needs change in the hive. The primary responsibilities of a worker bee include brood-nest bee care, queen bee attendance, nest construction, nest hygiene, food handling, guard duty, hive maintenance, and food gathering. These responsibilities are regulated as the physiological development of the worker bee changes with age. However, if the needs of the bee colony change, the worker bee can reverse its physiology to accommodate the changing hive condition. The responsibilities of a worker bee are regulated by a variety of

processes, such as, the queen bee through the production of specific pheromone, gene regulation, and food intake (Ament et al., 2008; Delaplane and Mayer, 2000; Winston, 1991).

2.2 THE IMPORTANCE OF THE HONEY BEE

The flowering plants evolved more than 225 million years ago and, along with this came the development of pollinators for these plants (Thapa, 2006). Today, much of our food comes from flowering plants and their products. It is estimated that over 80% of all pollination comes from insects and, of this, about 80% is from bee species (Thapa, 2006). The honey bee provides about $223 billion per year in worldwide agricultural benefits (Gallai et al., 2009). In the United States, the pollination services provided the honey bee are estimated at $14.6 billion per year (Stokstad, 2007).

The Virginia Department of Agriculture and Consumer Services estimates 9,000 commercial hives and 15,000 hobbyist hives in the Commonwealth of Virginia (VDAC, 2006). The price of wholesale honey can be $1 to $4 per pound, in bulk, depending on the type of honey. The honey bee also provides pollination services for many of Virginia's commercial crops, including cucumbers, watermelons, cotton, and apples (VDAC, 2006). In Virginia, the loss of managed honey bee colonies is similar to that across the United States. Since the 1980s, the number of managed honey bee colonies has declined by about 50%, with annual losses continuing at about 30% per year over the past ten years (Fell and Cobb, 2009). These honey bee colony losses are costing Virginia beekeepers $1.3 to $1.8 million per year as a result of reduced honey sales, loss of pollination fees, and cost of colony replacement (Fell and Cobb, 2009).

2.3 THE NUTRITIONAL HEALTH OF THE HONEY BEE

The honey bee requires essential nutrients to survive, including protein, carbohydrates, lipids, amino acids, minerals, and water (Huang, 2010). These essential nutrients are gathered by the honey bee from its major food sources of pollen and nectar. The foraging honey bee will collect and deliver pollen and nectar to the hive and distribute each of these food sources to the colony (von Frisch, 1967).

Pollen and nectar are the major source of protein and carbohydrates, respectively (Huang, 2010). To maintain the nutrition requirements of the honey bee colony, there must be between 10 to 26 kg of pollen collected by the foraging honey bees per year (Wille, 1985). A foraging honey bee will collect pollen from flowering plants and bring the pollen back to the hive to store. The protein content of pollen is dependent upon the plant from which it is collected by the foraging honey bee (Roulston and Cane, 2000). Once the pollen is back in the hive, it is mixed with regurgitated nectar, honey, microorganisms (ex. lactic acid and bacteria), and glandular secretions (Brodschneider and Crailsheim, 2010). This mixture is called bee bread and has a lower pH and less starch than freshly collected pollen (Knox et al., 1971). There are ten amino acid proteins that are critical to honey bee health and any deficiencies of these amino acids can limit the development, productivity, and maintenance of the honey bee colony (Brodschneider and Crailsheim, 2010).

The protein content of a honey bee can change throughout life and can by affected seasonal changes and the availability of pollen (Kleinschimdt, 1977). If honey bees in a colony do not receive the proper amounts of protein in their diet, the resulting protein deficiencies can dramatically affect the production of honey bee brood, nourishment of adult honey bees, and longevity of honey bees (Kleinschimdt, 1977; Knox et al., 1971). In addition, an insufficient

amount of protein within the honey bee colony can lead to malnourished adult bees and reduction of bee brood (Imdorf, 1998; Jay, 1964). The pollen collected by a foraging honey bee is an important source of lipids (Brodschneider and Crailsheim, 2010), which can vary in concentration from 0.8 to 19% depending on the plant from which it is collected by the foraging honey bee (Roulston and Cane, 2000). These lipids are not only important for honey bee brood development (Herbert, 1980), but are also an important source of sterols for the production and regulation of growth hormones in the adult honey bee (Haydak, 1970; Huang, 2010).

The primary source of carbohydrates for the honey bee is nectar, which provide for energy intensive tasks such as flight (Huang, 2010). The foraging honey bee collects nectar with its proboscis and stores the nectar in a specialized crop called a honey stomach. The nectar is brought back to the colony and the moisture content is reduced to 16 to 20% (Brodschneider and Crailsheim, 2010). The enzymes invertase, diatase, and glucose oxidase are added to the nectar, these enzymes breakdown the sucrose into glucose, fructose and other saccharides (Brodschneider and Crailsheim, 2010). The adult forager honey bee will consume up to 15.6 mg of carbohydrates per day to survive, and a honey bee larva can consume about 60 mg of carbohydrates throughout its development cycle (Rortais et al., 2005).

In addition to proteins, lipids, and carbohydrates, the honey bee requires essential vitamins and minerals (Huang, 2010), which are important for the production, maintenance, and development of honey bee brood. These vitamins include vitamin B, vitamin C, and pyridoxine (Brodschneider and Crailsheim, 2010; Huang, 2010). The honey bee can consume minerals from proteins, nectar, or water (Imdorf, 1998; Knox et al., 1971).

2.4 THE IMMUNE HEALTH OF THE HONEY BEE

There are multiple honey bee pathogens, parasites, and pests that have been identified and reported by the apiculture industry to be primary causes for honey bee colony failures in the US (Engelsdorp et al., 2010). Therefore, the honey bee must maintain an efficient immune system to cope with the stress of pathogen infections as well as parasite and pest infestations within the honey bee colony. There are multiple levels of pathogen, parasite, and pest defense strategies in an individual honey bee and a population of honey bees, which begin with the physical structure of hive. The next level of pathogen, parasite, and pest defense can include the utilization of plant resins and the reduced entry of pathogens, parasites, and pests. In addition to the above physical and mechanical defense strategies; the honey bee can provide both individual and social immune responses to minimize the introduction of pathogens, parasites, and pests into the honey bee colony (Evans and Pettis, 2005).

The honey bee can provide social immune responses in the form of behavioral and organizational tasks to reduce the introduction of pathogens, parasites, and pests into the hive. These tasks can include hygienic behaviors, removal of dead bees, division of hive tasks, nest architecture, and use of propolis (Cremer et al., 2007; Wilson-Rich et al., 2008; Evans et al., 2006; Traniello, 2002). In addition, the honey bee can produce glucose oxidase as a social immune response to defend the colony against pathogens (Alaux et al., 2010; Evans et al., 2006). The honey bee produces glucose oxidase in the hypopharyngeal glands, and adds this enzyme to brood food or honey. Glucose oxidase catalyzes the glucose reaction to gluconic acid and hydrogen peroxide, which have antiseptic properties that sterilize honey and hive products (Alaux, et al., 2010; Alaux et al., 2012).

The honey bee can provide individual immune responses in the form of the phenoloxidase activity, release of reactive oxygen species, or production of antimicrobial peptides (vanEngelsdorp et al., 2009; Laughton et. al., 2011). These individual immune responses originate in the hemolymph and organs of the honey bee in order to target pathogen infections, parasite attacks, heal wounds, and in response to other immune challenges (Evans and Spivak, 2010). For example, phenoloxidase is an enzyme produced in the pro-phenoloxidase cascade and is responsible for humoral immune responses. The phenoloxidase cascade is a series of interactions between enzymes that ultimately provide the formation of melanin (Laughton et al., 2011). Melanin is important for wound healing and cellular response such as coagulation, encapsulation, and phagocytosis (Chan et al., 2009). The phenoloxidase activity of the honey bee is correlated to the level of pathogen tolerance and susceptibility in insects (Chan et al., 2009; Laughton and Siva-Jothy, 2011; Scherfer et al., 2008).

Reactive oxygen species (ROS) are reactive oxygen molecules that are used by animals and plants as molecular messengers (Dowling and Simmons, 2009). These ROS are not only important in the longevity, and sexual selection of insects, but also for the maintenance of a healthy immune system (Dowling & Simmons, 2009; Diaz-Albiter et al., 2012).

In response to pathogen infections, the honey bee can produce specific antimicrobial peptides (AMPs) (Laughton et al., 2011; Schmid-Hempel, 2005). These AMPs are specific proteins that are released into the hemolymph to target and eliminate pathogens (Bulet et al., 1999). While the mechanism with which these AMPs target and eliminate pathogens is unknown, the current theories include AMP-lipid interaction and receptor recognition of invading pathogens (Bulet et al., 1999).

Alaux et al. (2010) report increased levels of phenoloxidase and glucose oxidase activity in honey bees provided with a protein-rich diet. However, another study reported no significant association between the amount of pollen fed to honey bees and their phenoloxidase activity (Wilson-Rich et al., 2008). Kohler et al. (2012) observed a detrimental synergistic effect with honey bees exposed to a combination of dietary toxicant and pathogen . Furthermore, Huang et al. (2012) report that pollen-deficient honey bees are less tolerant to the fungal pathogen *Nosema apis* and exhibit higher titer levels of viral infection .

2.5 FACTORS THAT AFFECT HONEY BEE HEALTH

The transmission and consequences of honey bee viruses were first discovered by G.F White (1913). Today, there are more than 18 viruses that can infect the honey bee (Chen and Siede, 2007). The social nature of the honey bee colony, including the interaction between generations, maintenance of hive homeostasis, and storage of food, makes the hive environment an ideal place for the transmission of pathogens (Evans and Pettis, 2005). The most common honey bee viruses include, but are not limited to, deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood (SBV), Kashmir bee virus (KBV), acute bee paralysis (ABPV), and chronic bee paralysis (CBPV) (Chen and Siede, 2007).

The hematophagous mite, *Varroa destuctor*, (Anderson and Trueman, 2000) is currently the most damaging parasite to honey bee colonies (vanEngelsdorp and Meixner, 2010). The varroa mite was originally a parasite of the Asian bee, *Apis cerana*, but was later introduced to European honey bee colonies as these colonies were imported from Asia. In the Asian honey bee colony, the varroa mite-bee interaction is managed by the fact that the varroa mite only reproduces in the drone cells and from grooming behavior. In the European honey bee colony,

the varroa mite has adapted to reproduce in all developing brood cells (Wallner and Fries, 2003). Today, the varroa mite has spread to almost all continents that manage the European honey bee (vanEngelsdorp and Meixner, 2010). The life cycle of the varroa mite starts with an adult female laying an egg in a capped brood cell. The female usually lays about 4 to 5 eggs per honey bee brood cell at intervals of 30 hours. Usually one male emerges to mate with his sisters. As a honey bee brood cell is uncapped, the mated female varroa mite will emerge to complete the reproduction cycle (Wallner and Fries, 2003). The honey bee brood is reared in a honey bee colony from the spring to fall season, with a sharp declined in brood production in the winter season. The varroa mite life cycle follows the production pattern of brood. In addition, the varroa mite prefers the drone brood as a result of the longer period of capped brood development (Francis et al., 2013).

The varroa mite is an infectious-disease vector in honey bee colonies, where it transmits several viruses (Francis et al., 2013). To control varroa mite populations and virus transmission, chemical control has been used extensively. These chemicals include formic acid, oxalic acid, amitraz, coumaphos, and *tau*-fluvalinate. Formic and oxalic acid are both water-soluble and, therefore, do not leave a residue in beeswax, although they can be absorbed by honey. Coumaphos and *tau*-fluvalinate are both lipid soluble and can be absorbed in hive products such as beeswax (Wallner & Fries, 2003). Both *tau*-fluvalinate and coumaphos have an estimated half-life of five years (Bogdanov, 2004).

The genus *Nosema* contains microsporidia that are important intercellular parasites typically found in the gut of insects (Traver and Fell, 2011). For honey bees, there are two species of concern *Nosema ceranae* and *Nosema apis* (Dussaubat et al., 2012; Fries, 1993, 2010). Both *Nosema* species affect the epithelial cells of the honey bee midgut. The visible signs of

Nosema infection include fecal streaking on the front of hives and high winter mortality; however, these are not diagnostic symptom, particularly with *N. ceranae* (Fries, 1993, 2010).

American (AFB) and European (EFB) foulbrood are the most significant bacterial diseases of honey bees (vanEngelsdorp & Meixner, 2010). The AFB disease affects the honey bee brood which causing them to become a dark brown color and having a ropey consistency with scales in the cells that are hard to remove (Ashiralieva and Genersch, 2006). The AFB disease is highly contagious in the spore form and, therefore, a high concern to beekeepers (vanEngelsdorp and Meixner, 2010). The EFB disease will cause the larva to be yellowish and attached to the side of the cell with a pasty consistency (Bailey, 1961; Tomkies et al., 2009).

In addition to the above mentioned parasites and pathogens, honey bee colony pests can include mammals such as mice, bear, skunks, and birds. The primary concerns associated with mice and bears involve the structural damage of hive bodies and food stores, whereas skunks and birds are predators of the honey bees (vanEngelsdorp and Meixner, 2010). The two top invertebrate pests in honey bee colonies are the wax moth and small hive beetle (vanEngelsdorp and Meixner, 2010). The wax moth cause the most damage to wax and comb; however, the damage of wax moth to a honey bee hive may indirectly lead to the failure of a weak honey bee colony (vanEngelsdorp et al., 2008). The small hive beetle is a native parasite to African bees, and an introduced pest to honey bee colonies elsewhere. The adult small hive beetle feeds on honey bee eggs, honey, and pollen. The small hive beetle is an emerging pest and the exact pressure on honey bee hives remains unclear (Ellis and Hepburn, 2006).

The loss of farmland and the reduction of honey bee forage can have significant effects on honey bee colony health (Bohan et al., 2005). In addition, climate alterations have been reported to affect honey bee colony health, with severe winter weather contributing to a

significant number of annual honey bee colony failures in the US (vanEngelsdorp et al., 2008). There have been unexplained cycles of bee decline and growth reported for many years (vanEngelsdorp et al., 2009), with many prominent cases including the Isle of Wight in England, Australia in 1910, Portland Oregon 1915, the US in the 1960s, and France in the 1990s (Underwood, 2007).

2.6 PESTICIDES EXPOSURES IN HONEY BEE HIVES

The effects of pesticide exposures on honey bee colony health have been a major concern for several years. The most direct effect that can be seen from pesticides is poisoning. These pesticide exposures are often reduced as the result of label restrictions that protect non-target insects from the unintentional exposures to pesticides (vanEngelsdorp and Meixner, 2010). However, there are occasional honey bee deaths that occur from the improper use of pesticides and the direct exposure of these pesticides to honey bee colonies. In addition to direct poisoning, there are more subtle non-lethal side effects of pesticide usage on honey bee colonies. Because this type of effect is less apparent, it is harder to quantify and understand.

Recent studies have examined the effects of systemic pesticides, fungicides with no label restrictions, and the pesticide residues in hive products (Mullin et al., 2010; Pettis et al., 2013; vanEngelsdorp et al., 2009; vanEngelsdorp and Meixner, 2010). These studies have found surprisingly high pesticide residues in colonies and colony products (Mullin et al., 2010), interactions between pathogens, immune reactions and pesticide exposure (Pettis et al., 2013), and developmental changes correlated with pesticide exposure (vanEngelsdorp and Meixner, 2010).

In recent decades our agricultural system has depended more and more on pesticides for pest control, thus increasing the risks of honey bee hives to pesticide exposure. Recent studies report the presence of more than 120 pesticide residues and metabolites in honey bee hives across North America (Mullin et al., 2010). The most common pesticides found in these honey bee hives are the beekeeper-applied varroacides *tau*-fluvalinate and coumaphos (vanEngelsdorp, et al., 2008; Mullin et al., 2010). These varroacides are often found in combination with the agricultural fungicide chlorothalonil (Mullin et al., 2010) residues of which can be collected from pollen by foraging honey bees and brought back to the hive (Mullin et al., 2010).

There have been conflicting reports on the effects of fungicides on honey colony health. Fell et al. (1983) reported that honey bee exposures to fungicide formulations did not affect foraging behaviors ; however, DeGrandi-Hoffman et al. (2013) report that the combined exposure of honey bee queens to a pesticide and fungicide mixture compromises immune health . Johnson et al. (2009, 2013) reported similar synergistic effects in honey bees exposed to pesticide and fungicide -combinations . In addition, Mussen et al. (2004) observed a higher mortality rate of honey bee larvae and pupae provided dietary exposures of fungicides.

2.6.1 *tau*-Fluvalinate (Apistan®), A Honey Bee Hive Miticide

The pyrethroid miticide *tau*-fluvalinate was first registered in 1983 as racemic fluvalinate. *tau*-Fluvalinate is a current-use varroacide registered for use in honey bee hives as well as a miticide for agriculture, ornamental, and urban pest management. The mode of action of *tau*-fluvalinate is to alter the gating kinetics of voltage-gated sodium channels and, thus, interfere with the propagation of action potentials in the cholinergic nervous system (E.P.A, 2005). *tau*-Fluvalinate exceeds the U.S. Environmental Protection Agency (U.S. EPA) level of

concern for acute and chronic exposure risk to aquatic organisms and mammals. Although *tau*-fluvalinate is reported as highly toxic to honey bees by the U.S. EPA, it remains a registered product for the control of varroa mite in honey bee hives (E.P.A, 2005). In 1987, *tau*-fluvalinate was the first registered varroacide for use in honey bee colonies (Ellis, 1988). The first treatments were formulated on plywood strips soaked in the agricultural product. In 1990, the first *tau*-fluvalinate impregnated plastic strips, specifically designed for use in honey bee hives, were introduced to the apiculture industry (Johnson et al., 2010). The current *tau*-fluvalinate formulation Apistan® is registered to the manufacturer Wellmark International (Zoecon). Each Apistan® strip contains 10.25% of the active ingredient *tau*-fluvalinate. The manufacturer recommends that *tau*-fluvalinate impregnated strips should be applied to the hive frames, and should not come into contact with honey in the honey bee hive. For example, one *tau*-fluvalinate impregnated strip is applied for every five hive frames and hangs near the center of a honey bee brood cluster within the honey bee hive. The manufacturer recommends the application of *tau*-fluvalinate impregnated strips in the early spring and fall season for no more than six weeks (Wellmark, 2002). The first incidences of varroa mite resistance to *tau*-fluvalinate were observed in the 1990s and later described as target-site insensitivity as a result of mutations in the voltage-gated sodium channel of the varroa mite (Elzen, et al., 1999; Lodesani, et al., 1995; Johnson, 2010; Wang, et al., 2003). Regardless of the varroa mite resistance to *tau*-fluvalinate, the varroacide continues to be used by, beekeepers to manage varroa mite populations in honey bee hives. In addition, Burley et al. (2008) report that honey bees exposed to *tau*-fluvalinate exhibit impaired reproductive physiology, reduced sperm viability of honey bee drones, and increased reproductive failure of honey bee queens.

2.6.2 Coumaphos (CheckMite+®), A Honey Bee Hive Miticide

The organophosphate coumaphos is a miticide first registered in 1958 for the management of arthropod pests. To date, there are 26 registered products that contain coumaphos as an active ingredient. Coumaphos is available in several formulations including, powders, liquids, and dusts (E.P.A, 1996). Coumaphos is an acetylcholinesterase inhibitor that prevents the breakdown of the neurotransmitter acetylcholine leading to the continual stimulation of the neuron and paralysis of the exposed arthropod pest (Brown, 2006). Coumaphos has been shown to be highly toxic by inhalation and moderately toxic dermally to humans. Coumaphos is very highly toxic to birds, if consumed and is moderately to highly toxic to aquatic organisms (E.P.A, 1996). Coumaphos has been approved for use in honey bee hives for the management of varroa mite infestations since 1999. The current coumaphos formulation CheckMite+® is registered to the manufacturer Bayer Health Care, LLC. Each CheckMite+® strip contains 10% of the active ingredient coumaphos. The manufacturer recommends that coumaphos impregnated strips should be applied to the hive frames, and should not come into contact with honey in the honey bee hive. For example, one coumaphos impregnated strip is applied to every five hive frames and hangs near the center of a honey bee brood cluster within the honey bee hive. The manufacturer recommends the application of coumaphos impregnated strips in the early spring and fall seasons for no more than six weeks (E.P.A, 1996). The use of coumaphos impregnated strips was first introduced to mitigate the *tau*-fluvalinate resistance of varroa mites in honey bee hives. Although, coumaphos resistance was observed in varroa mite populations within a few years after use in honey bee hives (Elzen and Wetervelt, 2002). Few studies exist on the mechanisms of coumaphos resistance in varroa mite populations; however, it is believed that increased metabolic detoxification activities play a significant role in varroa mite resistance

to coumaphos (Sammataro, et al., 2005). In addition, Burley et al. (2008) report that honey bees exposed to coumaphos exhibit impaired reproductive physiology, reduced sperm viability of honey bee drones, and increased reproductive failure of honey bee queens .

2.6.3 Chlorothalonil, A Crop Fungicide

The organochlorine chlorothalonil is the third most common agricultural fungicide used in the United States (Mullin et al., 2010). In a survey of pesticide residues in honey bee hives across North America, chlorothalonil residues were reported to exceed 99 parts per billion in the hive. The contamination of pollen is reported to correspond with concentrations in honey bee brood and wax samples collected from honey bee hives (Mullin et al., 2010). The high presence of chlorothalonil in honey bee hives may be related to no label restrictions for spraying chlorothalonil around insect pollinators (Mullin et al., 2010). Chlorothalonil is described by U.S. EPA as a broad spectrum, non-systemic pesticide with an unknown mode of action. Chlorothalonil has been found most useful as a fungicide and mildewicide. Alternative uses of chlorothalonil include bactericide, microbiocide, algaecide, insecticide, and acaricide. Chlorothalonil was first registered in the United States in 1966 for use in turfgrass (E.P.A, 1999). The U.S. EPA has registered chlorothalonil for use as a fungicide in fields, vegetables, orchards, and turfgrass; and as a mildewicide in surface treatments. Chlorothalonil has many different formulations that include powders, dusts, flowables, or impregnated materials. These chlorothalonil formulations can be applied in a variety of ways including a duster, granule spreader, sprayer, fogger, and chemigation (E.P.A, 1999). Chlorothalonil is labeled as an acute Toxicity Category IV for human oral exposure, as Toxicity Category II for human inhalation, and as Toxicity Category IV for human dermal effects. It is practically non-toxic to avian and

small mammal species. Chlorothalonil is also relatively non- toxic to honey bees, but highly toxic to fish and aquatic invertebrates.

CHAPTER 3

EFFECTS OF *TAU*-FLUVALINATE (APISTAN®), COUMAPHOS (CHECKMITE+®), AND CHLOROTHALONIL ON THE NUTRITIONAL HEALTH OF BROOD-NEST AND FORAGING HONEY BEES

3.1 INTRODUCTION

The honey bee requires essential nutrients to survive, including proteins, carbohydrates, lipids, minerals, and water (Huang, 2010). These nutrients are provided from the nectar and pollen collected by a foraging honey bee and distributed throughout the honey bee colony (von Frisch, 1967). The eusocial nature of the honey bee colony means that at different life stages different nutrients are required for the development of the individual honey bee and for the colony (Haydak, 1970).

A foraging honey bee will collect pollen from flowering plants and bring the pollen back to the colony to create honey bee bread To maintain the nutrition requirements of the honey colony, there must be between 10 to 26 kg of pollen collected by the foraging honey bees per year (Wille, 1985). A foraging honey bee will collect pollen from flowering plants and bring the pollen back to the hive to store. The protein content of pollen is dependent upon the plant from which it is collected by the foraging honey bee (Roulston and Cane, 2000). Once the pollen is back in the hive, it is mixed with regurgitated nectar, honey, microorganisms (ex. lactic acid and bacteria), and glandular secretions (Brodschneider and Crailsheim, 2010). This mixture is called bee bread and has a lower pH and less starch than freshly collected pollen (Knox et al., 1971).

There are ten amino acid proteins that are critical to honey bee health, any limitation of the protein source for these amino acids will limit the colony development (Brodschneider and Crailsheim, 2010).

Carbohydrates are used by honey bees as a source of energy (Huang, 2010). Honey bees use nectar as their main source for carbohydrates. Nectar is collected with their proboscis and the liquid is stored in a specialized crop called a honey stomach. The foraging honey bee collects nectar with its proboscis and stores the nectar in a specialized crop called a honey stomach. Nectar is brought back to the colony and the moisture content is reduced to 16-20% (Brodschneider and Crailsheim, 2010). The enzymes invertase, diatase, and glucose oxidase are added to the nectar, these enzymes breakdown the sucrose into glucose, fructose and other saccharides (Brodschneider and Crailsheim, 2010). Adult forager honey bee will consume up to 15.6 mg of carbohydrates per day to survive, and a honey bee larva can consume about 60 mg of carbohydrates throughout its development cycle (Rortais et al., 2005). Similar to protein, the content of carbohydrates in nectar varies among plants, from 5% to 75% (Huang, 2010). Developing honey bees require fewer carbohydrates than adults, but do require some sugars in their brood food (Rortais et al., 2005).

Lipids are obtained by honey bees through ingestion of pollen (Brodschneider and Crailsheim, 2010). Lipid concentration in the pollen depends on the source plant and can vary from 0.8%-18.9% (Roulston and Cane, 2000). Lipids have been shown to be important for the development of brood (Herbert, 1980) and as a source of sterols for adult honey bees (Haydak, 1970). Additionally, sterols are important for the regulation of growth hormone in molting (Huang, 2010).

Nutrition is the cornerstone for honey bee development. The social nature of the honey bee means that nutrition can be examined at two different levels, the individual development and the colony development (Brodschneider and Crailsheim, 2010). Honey bees store their food supply in the colony structure, which ensures that all castes in the honey bee colony have access to nutrients; however, when there is a shortage of nutrients the colony may be compromised (Brodschneider and Crailsheim, 2010; Haydak, 1970). Those honey bee colonies with a shortage of pollen will first use up stored pollen, body reserves, and eventually stop rearing bee brood (Haydak, 1970). Ultimately, the adult honey bees will cannibalize the younger larvae to feed the older larvae (Schmickl and Crailsheim, 2002). Carbohydrates are used for the metabolism of energy and performing energy intensive tasks such as flight, thermoregulation, and comb building (Brodschneider and Crailsheim, 2010). A lack of carbohydrates can affect the ability of the colony to successfully overwinter (Engelsdorp et al., 2010).

The colony structure is made up of the individual honey bees. Not only does a loss of nutrients affect the dynamics of the colony, but also the individual honey bees. (Brodschneider and Crailsheim, 2010). Those honey bee colonies that do not have access to sufficient protein or have access to lower nutrient protein have been shown to have a decrease in bee brood production (Kleinschimdt, 1977) and worker lifespan (Knox et al., 1971). Carbohydrates are used for performing energy intensive tasks and a loss of carbohydrates and proteins can affect the ability of the honey bee to develop as larvae. In addition, poor nutrition can have detrimental effects on honey bee reproduction and the immune system (Alaux, et al., 2010; Brodschneider and Crailsheim, 2010).

As our agricultural system depends on pesticides for pest control, honey bee colonies are at risk to higher pesticide exposure rates. Recent studies of pesticide residues in colonies in North America reveal over 120 different pesticides and metabolites in colonies (Mullin et al., 2010). Knowledge of the consequences of these pesticide residues and their relationship to honey bee health is limited. Two of the most common pesticides in colonies are the pyrethroid *tau*-fluvalinate and the organophosphate coumaphos (vanEngelsdorp, et al., 2008); (Mullin et al., 2010).

The varroacides *tau*-fluvalinate (Apistan®) and coumaphos (CheckMite+®) are used in the management of the parasitic varroa mite, *Varroa destructor*. The varroa mite has been a pest of the European honey bee since the 1960s and in North America since the 1980s. Problems with the varroa mite start with its parasitic behavior on developing and adult honey bees and are increased by the amount of viruses transmitted by the mite (Francis, et al., 2013). Various methods of chemical control have been used in an effort to regulate the mite populations, including *tau*-fluvalinate (Apistan®) and coumaphos (CheckMite+®) (Sammataro, et al., 2000).

Both *tau*-fluvalinate (Apistan®) and coumaphos (CheckMite+®) are often found in combination with the agricultural fungicide chlorothalonil (Mullin et al., 2010). Chlorothalonil is a popular choice to treat against mildew, bacteria, and algae in a wide variety of crops (E.P.A, 1999). Chlorothalonil is picked up by foraging honey bees and brought back to the colony through pollen (Mullin et al., 2010). The high presence of this fungicide in honey bee colonies may be related to the fact that there are no label restrictions for spraying chlorothalonil around insect pollinators (Mullin et al., 2010).

Pesticides have been linked to aspects of growth and development in honey bees (vanEngelsdorp et al., 2009). Boncristiani et al. (2012) report that exposure to different pesticides alter the expression of metabolism genes which in turn affect the development of the honey bee. Moreover, Fell (2001) reports a decrease in reproduction and development in queen and drone honey bees exposed to the varroacides *tau*-fluvalinate (Apistan®) and coumaphos (CheckMite+®). In addition, researchers observed an increase in brood-nest honey bee mortality and the delayed emergence of adult honey bees from brood cells in comb with a higher frequency of *tau*-fluvalinate (Apistan®) and coumaphos (CheckMite+®) contamination (Wu et al., 2011).

This study examined the concentrations of total proteins, carbohydrates, and lipids as well as the body weight, head width, and wing length morphometrics of brood-nest and foraging honey bees collected from colonies treated with *tau*-fluvalinate (Apistan®), coumaphos (CheckMite+®), and chlorothalonil. Here, I report: 1) the concentration of total proteins, total carbohydrates, and total lipids in brood-nest and foraging honey bees in *tau*-fluvalinate (Apistan®), coumaphos (CheckMite+®), and chlorothalonil-treated colonies, and 2) the morphometric measurements of brood-nest and foraging honey bees in *tau*-fluvalinate (Apistan®), coumaphos (CheckMite+®), and chlorothalonil-treated colonies.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Anthrone and vanillin reagents were purchased from Acros Organics (New Jersey, USA). Bicinchoninic acid, Triton X-100, bovine serum albumin, and copper sulfate were purchased from Sigma Aldrich (St. Louis, MO, USA). Chloroform and sulfuric acid were purchased from Fisher Scientific. Coumaphos (CheckMite®) purchased from Bayer CropScience (RTP, NC, USA). *tau*-Fluvalinate (Apistan®) purchased from Zoecon (Charlotte, NC, USA).

3.2.3 Experimental Honey Bee Colonies and Pesticide Treatments

The experimental honey bee colonies consisted of a single-story hive provided with a sister queen to reduce genetic variation between colonies. Each hive was constructed with new frames and foundation to reduce initial exposures to pesticides. Three colonies were used for each pesticide treatment (i.e., three replicates per treatment). These hives were located at three apiaries maintained by the Department of Entomology at Virginia Tech, including Price's Fork, Kentland Farms, and Moore Farms. Kentland Farm is 3,000 acres and is focused on small fruit and vegetable crop production. Price's Fork is a small research area that is in a more suburban environment. Moore Farm is 250 acres and has a diverse native plant population. These colonies were established in May 2012 and allowed to reach colony strength by July (i.e., six weeks after colony establishment).

The experimental honey bee colonies included a 1) control (no pesticide), 2) *tau*-fluvalinate (Apistan®, Zoecon) treatment, 3) coumaphos (CheckMite+®, Bayer CropScience) treatment, and 4) chlorothalonil treatment. For the *tau*-fluvalinate and coumaphos treatments, the honey bee colonies were treated with two *tau*-fluvalinate-impregnated or two coumaphos-impregnated strips, each containing ~ 10% active ingredient, for six weeks using the manufacturer's label recommendations. The chlorothalonil treatment (10 μg/L, or parts per billion) was provided to the honey bees in a 30% sucrose solution for six weeks. In addition, the *tau*-fluvalinate- and coumaphos-treated and -untreated honey bee colonies were also provided with a 30% sucrose solution for the six-week treatment period.

Random groups of honey bees were marked after adult emergence from the brood frame so that we could collect honey bees of known ages. Two random frames of brood from each colony were collected for marking, this allowed for approximately 100 bees to be marked. The frames were put into custom made cages in an incubation chamber (34℃) for 6-8 hours. During this incubation period adult honey bees emerged from the brood frames. These bees were marked using Testors™ model paint. Honey bees were then smoked with pine needle smoke to eliminate the paint odors. Marked honey bees were returned to their hives. Each treatment group was marked with a distinguishable color for collection.

In August 2012, a random sample of brood-nest bees and another of foraging honey bees were collected from each of the honey bee colonies prior to the addition of each pesticide treatment. The samples were used to measure the baseline macromolecule contents and morphometrics of brood-nest and foraging honey bees prior to the addition

of the pesticide treatment. Following a six week pesticide treatment period, a random sample of brood-nest honey bees was collected from the brood frames and a random sample of foraging honey bees was collected from the hive entrance.

3.2.4 Measurement of Total Proteins in Pesticide-Treated Honey Bees

The concentration of total proteins in the brood-nest and foraging honey bees from the control hives and the hives treated with *tau*-fluvalinate, coumaphos, or chlorothalonil was measured according to the method of Smith et al. with modifications (Smith et al., 1985). Brood and forager honey bees were collected from the experimental colonies and transported on ice to the laboratory for the protein assay. A glass-teflon tissue homogenizer was used to homogenize individual honey bees in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% Triton X-100. One milliliter of the homogenizing buffer was used per honey bee. Each homogenate was centrifuged at 10,000 x *g* for 10 min. at 4 °C and the supernatants were transferred to clean 1.5-ml microcentrifuge tubes. Ten microliters of the supernatants were added to the individual wells of a 96-well microplate containing 10 µl 0.1 M sodium phosphate (pH 7.8) and 180 µl bicinchoninic acid with 4% (v/v) copper sulfate. The protein samples were incubated for 30 min. at 37 °C and cooled to room temperature for 5 min. The total protein content in the honey bee samples was measured at 560 nm using a Molecular Devices SpectraMax M2 multimode microplate reader (Sunnyvale, CA). The optical densities of the protein samples were compared to those measured for the protein standard of bovine serum albumin. The R^2 value for the equation was 0.9946.

3.2.5 Measurement of Total Carbohydrates in Pesticide-Treated Honey Bees

The concentration of total carbohydrates in the brood-nest and foraging honey bees from the control hives and the hives treated with *tau*-fluvalinate, coumaphos, or chlorothalonil was measured according to the method of Van Handel and Day, with modifications (Van Handel & Day, 1988). Brood and forager honey bees were collected from the experimental colonies and transported on ice to the laboratory for the carbohydrate assay. A glass-teflon tissue homogenizer was used to homogenize individual honey bees in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% Triton X-100. One milliliter of the homogenizing buffer was used per honey bee. Each homogenate was centrifuged at 10,000 x *g* for 10 min. at 4 °C and the supernatants were transferred to clean 1.5-ml microcentrifuge tubes. Twenty microliters of the supernatants were added to a 5-ml glass centrifuge tube containing 1.98 ml anthrone reagent. The carbohydrate samples were incubated at 90 °C for 15 min and then cooled at room temperature. Two-hundred microliters of each sample were added to the individual wells of a 96-well microplate. The total carbohydrate content in the honey bee samples was measured at 625 nm using a Molecular Devices SpectraMax M2 multimode microplate reader (Sunnyvale, CA). The optical densities of the carbohydrate samples were compared to those measured for the carbohydrate standard of glucose. The R^2 value for the equation was 0.9971.

3.2.6 Measurement of Total Lipids in Pesticide-Treated Honey Bees

The concentration of total lipids in the brood-nest and foraging honey bees from the control hives and the hives treated with *tau*-fluvalinate, coumaphos, or chlorothalonil was measured according to the method of Van Handel and Day (1988), with modifications. Brood and forager honey bees were collected from the experimental colonies and transported on ice to the laboratory for the lipid assay. A glass-teflon tissue homogenizer was used to homogenize individual honey bees in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% Triton X-100. One milliliter of the homogenizing buffer was used per honey bee. Each homogenate was centrifuged at 10,000 x *g* for 10 min. at 4 °C and the supernatants were transferred to clean 1.5-ml microcentrifuge tubes. Twenty microliters of the supernatant were added to a 5-ml glass centrifuge tube containing 200 µl chloroform and 200 µl sulfuric acid. The lipid samples were incubated at 90 °C for 10 min. followed by the addition of vanillin. The lipid samples were cooled at room temperature. Two-hundred microliters of each sample were added to the individual wells of a 96-well microplate. The total lipid content in the honey bee was measured at 625 nm using a Molecular Devices SpectraMax M2 multimode microplate reader (Sunnyvale, CA). The optical densities of the lipid samples were compared to those measured for the lipid standard of soybean vegetable oil. The R^2 value for the equation was 0.9993.

3.2.7 Head Width, Wing Length, and Body Mass in Pesticide-Treated Honey Bees

The morphometrics of the brood-nest and foraging honey bees from the control hives and the hives treated with *tau*-fluvalinate, coumaphos, or chlorothalonil were

measured according to the method of Wilson-Rich et al. (2008). The total body mass (mg wet weight) of individual bees was measured to the nearest milligram using a Mettler AE 100 analytical balance (Mettler, Toledo). The head width (mm) and forewing length (mm) of individual honey bees was measured using a Dinolite Pro AM413T/AD413T Microscope with DinoCapture 2.0 analysis software (BigC, Torrance, CA). The forewing of the honey bee was measured from the connection to the body to the apex of the wing for the maximum possible distance. The head width of the honey bee was measured from the most distant points on either side of the head.

3.2.8 Statistical Analysis

The differences in total proteins, carbohydrates, lipids, and morphometrics for each pesticide treatment were statistically compared to the control treatment using a one-way analysis of variance (ANOVA) in combination with a Dunnett multiple comparison test (JMP, 1989-2007). Each set of honey bees was divided into two age groups; brood-nest bees (n = 3; total of 45 individuals) and forager bees (n = 3; total of 45 individuals).

3.3 RESULTS

3.3.1 Measurement of Total Proteins in Pesticide-Treated Honey Bees

The concentrations of total protein of the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, and chlorothalonil is shown in Figures 3.1 and 3.2. The total protein concentration in the brood-nest bees was significantly lower by 15.1% in the coumaphos-treated individuals compared to the pesticide-untreated bees (Figure 3.1). For the foraging bees, the total protein concentration were significantly reduced by 15.8% and 20.5 % in the coumaphos- and chlorothalonil-treated individuals, respectively, compared to the pesticide-untreated bees (Figure 3.2).

3.3.2 Measurement of Total Carbohydrates in Pesticide-Treated Honey Bees

The concentrations of total carbohydrates of the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, and chlorothalonil is shown in Figures 3.3 and 3.4. The total carbohydrate concentration in brood-nest bees was significantly reduced by 35.1% in the chlorothalonil-treated individuals compared to the pesticide-untreated bees (Figure 3.3). The total carbohydrate concentration of foraging bees was significantly reduced by 47.6%, 37.0%, and 43.7% in the *tau*-fluvalinate-, coumaphos-, and chlorothalonil-treated individuals, respectively, compared to the pesticide-untreated bees (Figure 3.4).

3.3.3 Measurement of Total Lipids in Pesticide-Treated Honey Bees

The concentrations of total lipid of the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, and chlorothalonil is shown in Figures 3.5 and 3.6. The total lipid concentration in brood-nest bee showed no significant differences in the pesticide-treated brood-nest bees when compared to the pesticide-untreated individuals. The total lipid concentration in forager bees was significantly decreased by 16.8% in the chlorothalonil-treated individuals compared to the pesticide-untreated bees (Figure 3.6).

3.3.4 Body Weight, Head Width, and Wing Length in Pesticide-Treated Honey Bees

The morphometric measurements of the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, or chlorothalonil are shown in Figures 3.7 - 3.12. The body weight of brood-nest honey bees was significantly reduced by 13.3% and 27.9% for the *tau*-fluvalinate- and chlorothalonil-treated individuals, respectively, compared to the pesticide-untreated bees (Figure 3.7). The body weight of foraging bees was significantly reduced by 16.8%, 14.8%, and 28.9% for the *tau*-fluvalinate-, coumaphos-, or chlorothalonil-treated individuals, respectively, compared to the pesticide-untreated bees (Figure 3.8). Wing length was significantly reduced by 2.9% and 2.0% for the coumaphos and chlorothalonil-treated individuals, respectively, compared to the pesticide-untreated bees (Figure 3.12).

3.4 DISCUSSION

Chlorothalonil is a popular choice of treatment against mildew, bacteria, and algae in a wide variety of crops (E.P.A, 1999). The high presence of this fungicide in colonies may be related to the fact that there are no label restrictions for spraying chlorothalonil around insect pollinators (Mullin et al., 2010). Our findings show that there are significant nutritional changes in honey bees exposed to chlorothalonil. Loss of nutrients has been shown to lead to developmental problems in the colony and individual level.

Chlorothalonil is picked up by foraging honey bees and brought back to the colony through pollen (Mullin et al., 2010). Pollen is used as a protein source for developing brood and for forager honey bees (Haydak, 1970). Brood-nest honey bees in the colonies exposed to chlorothalonil showed an increase in protein concentration, while the foraging bees showed lower concentration of proteins. Protein is most important for the developing honey bees and the increase in protein concentration could be a compensation related to the exposure to chlorothalonil. Other studies have shown that brood-nest honey bees fed a diet with fungicides showed a higher mortality (Mussen et al., 2004). Colonies that do not get enough protein or low nutrient protein have been shown to have a decrease in bee brood production (Kleinschimdt, 1977) and worker lifespan (Knox et al., 1971).

All ages of honey bees exposed to chlorothalonil showed a significant decrease in carbohydrate concentrations. Loss of carbohydrates can affect the ability of the honey bee to develop as larvae. Additionally, poor nutrition can have detrimental effects on reproduction and the immune system of the honey bee (Alaux et al., 2010; Brodschneider and Crailsheim, 2010). Carbohydrates are used for the metabolism and performing

energy intensive tasks such as flight, thermoregulation, and comb building (Brodschneider and Crailsheim, 2010).

Chlorothalonil is not the first fungicide to be studied in relation to honey bee health. Early formulations of several fungicides were tested and toxicity to honey bees examined in the 1950s (Anderson, et al., 1957). More recently, in the 1980s, there were several studies focusing on the effects of fungicides, especially ones used in orchards. Fell et al. (1983) report that fungicides do not affect foraging behavior in orchards. DeGrandi-Hoffman et al. (2013) report that sub-lethal exposures to a pesticide and fungicide compromised the immunity of queens. A similar synergistic effect between pesticides and fungicides have been reported for honey bees (Johnson, 2013; Johnson et al., 2009). Moreover, Mussen et al. (2004) report that honey bee larvae and pupae exhibit higher mortality when fed a diet containing various fungicides.

The effectiveness of chlorothalonil as a fungicide makes it an important part of our agricultural system. Our data suggests that chlorothalonil exposure affects the nutritional status of honey bees. It is important for both beekeepers and farmers to protect both of their investments and, thus, it is critical for both parties to work together to develop solutions that will reduced the unintentional exposure of honey bee colonies to pesticides. More label restrictions and better education about fungicides and pollinators are important to creating this change.

Various methods of chemical control have been used in an effort to regulate varroa mite populations (Sammataro et al., 2000). Coumaphos and *tau*-fluvalinate are used in the management of the parasitic varroa mite. The results in our study are similar for both coumaphos and *tau*-fluvalinate, although the two pesticides act on different parts

of the insect nervous system. As a pyrethroid insecticide, the mode of action of *tau*-fluvalinate is to alter the gating kinetics of voltage-gated sodium channels and, thus, interfere with the propagation of action potentials in the cholinergic nervous system (E.P.A, 2005). As an organophosphate, coumaphos prevents the breakdown of acetylcholine, which causes the continual stimulation of the neuron and leads to paralysis of the honey bee (Brown, 2006).

Protein concentrations in forager honey bees exposed to coumaphos and *tau*-fluvalinate were lower than the control treatments. Carbohydrate concentration in brood and forager honey bees exposed to coumaphos and *tau*-fluvalinate were lower than the control treatments. Lower nutritional concentrations have been linked to have a decrease in brood production (Kleinschimdt , 1977), worker lifespan (Knox et al., 1971) and the honey bee's ability to perform energy intensive tasks such as flight, thermoregulation, and comb building (Brodschneider and Crailsheim, 2010).

Lipids are obtained by honey bees through ingestion of pollen (Brodschneider and Crailsheim, 2010). Lipids have been shown to be important for the development of brood (Herbert , 1980) and as a source of sterols for adult bees (Haydak, 1970). Sterols are important for the regulation of growth hormone in molting (Huang, 2010). The higher concentration of lipids in the brood nest honey bees compared to the foragers could be a response of the honey bees to care for the developing brood and ensure a stronger colony.

Our control colonies and the colonies treated with chlorothalonil were not treated for mites. Because our colonies were started the same season as sampling we did not expect high mite counts. Problems with the varroa mite stem from its parasitic behavior on developing and adult bees; however, the viruses transmitted by the mite are of an even

higher concern (Francis et al., 2013). The loss of colonies due to varroa mites has been reported throughout the US (Glin´ski et al., 2012; Hunt, 1998). Coumaphos and *tau*-fluvalinate provide beekeepers with suppression of the mite populations.

However, there have been side effects shown for both chemicals. Incidences of mite resistance to *tau*-fluvalinate first began in the 1990s (Elzen et al., 1999; Lodesani et al., 1995). This resistance is thought to be a result of a mutation in the sodium channel (Johnson et al., 2010; Wang et al., 2003). Coumaphos resistant strains of mites appeared in just a few years after the pesticide was approved for use in colonies (Elzen and Wetervelt, 2002). Additionally, *tau*-fluvalinate and coumaphos have been shown to impair reproductive physiology, reduce the ability to reproduce queens, reduce sperm viability in drones, and increase queen failure (Burley et al., 2008).

The interactions between the exposure to pesticides, mite stress, limits to nutrition, and immune challenges are all factors that could contribute to colony stress and a decrease in overall health. Beekeepers have to make choices about treating their hives for mites or having mite stressed colonies. This study shows that in making this decision, colonies that were untreated for mites had less nutritional stress. Nutrition in honey bees has been linked to development of the colony and the individual as well as the ability to overcome immune challenges (Alaux et al., 2010; Huang, 2010).

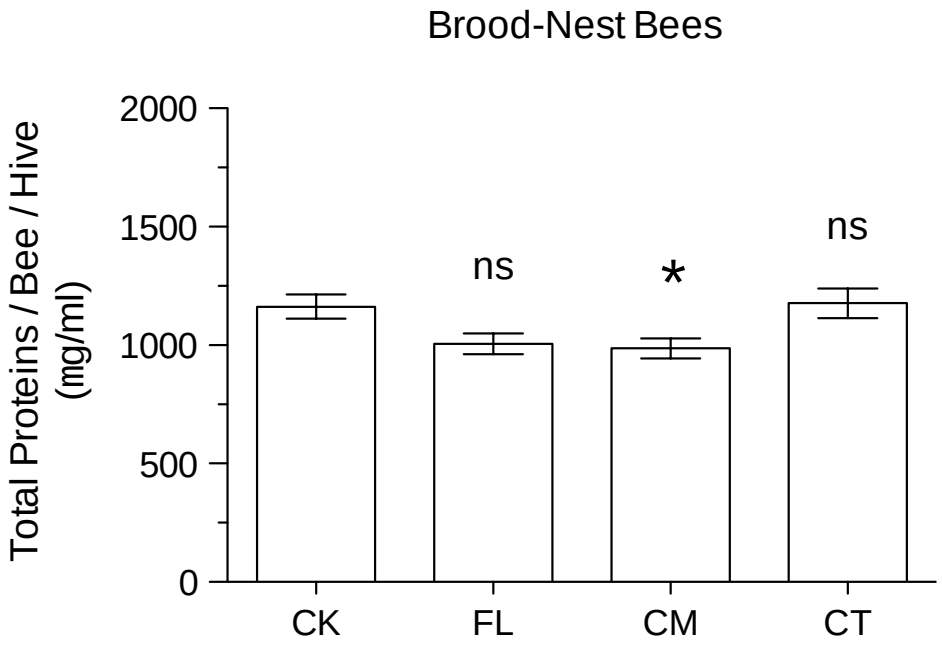

Figure 3.1. Total protein content of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean ($n = 3$). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns = not significant at the 0.05 level).

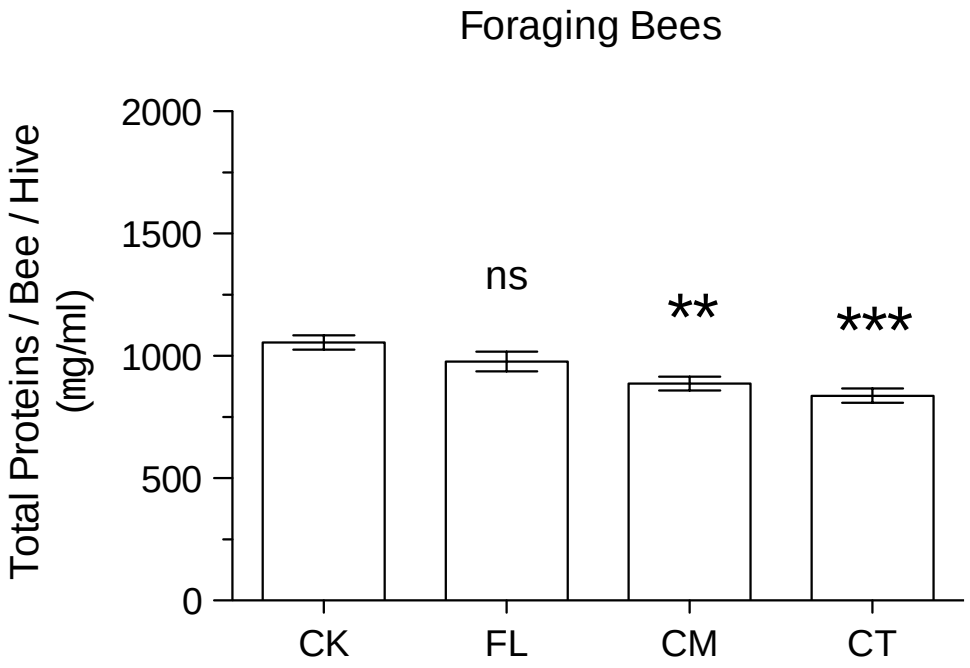

Figure 3.2 Total protein content of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

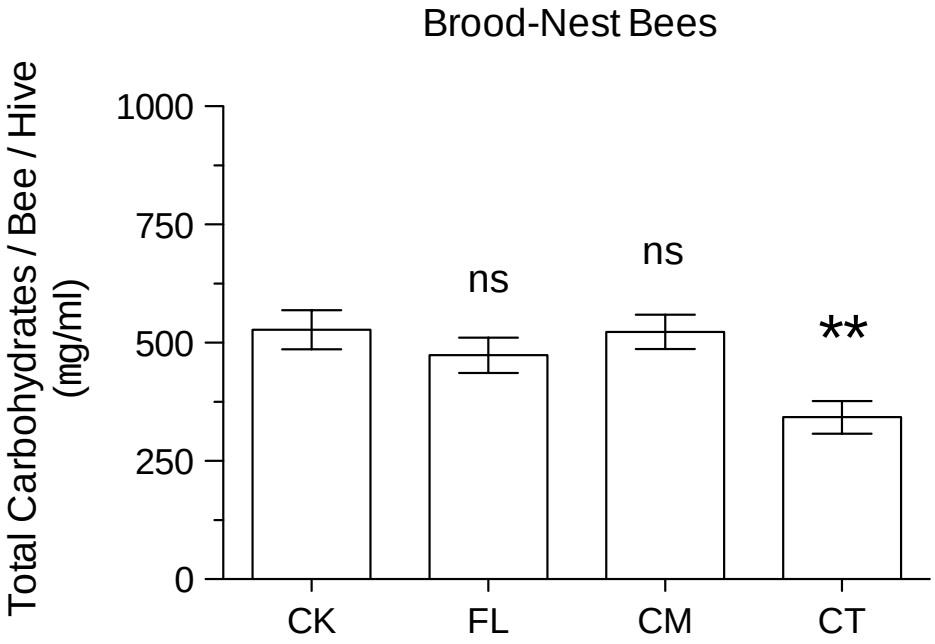

Figure 3.3. Total carbohydrate content of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

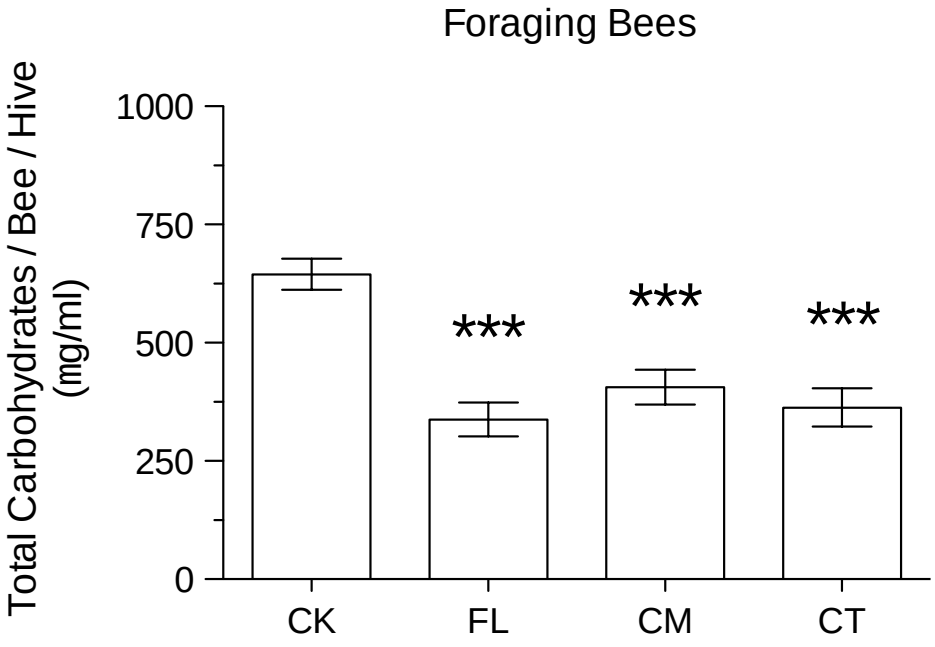

Figure 3.4. Total carbohydrate content of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

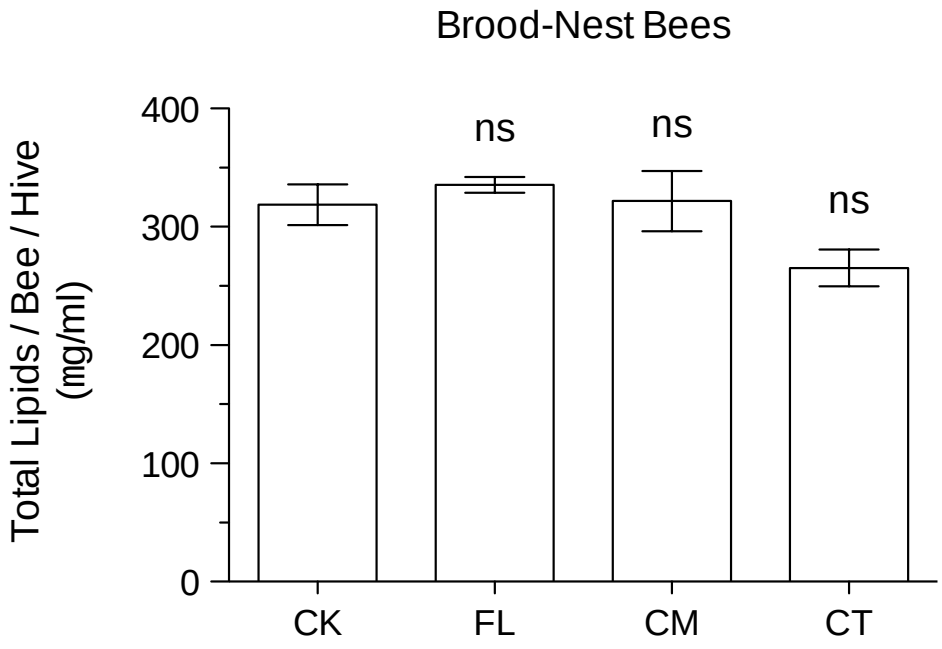

Figure 3.5. Total lipid content of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

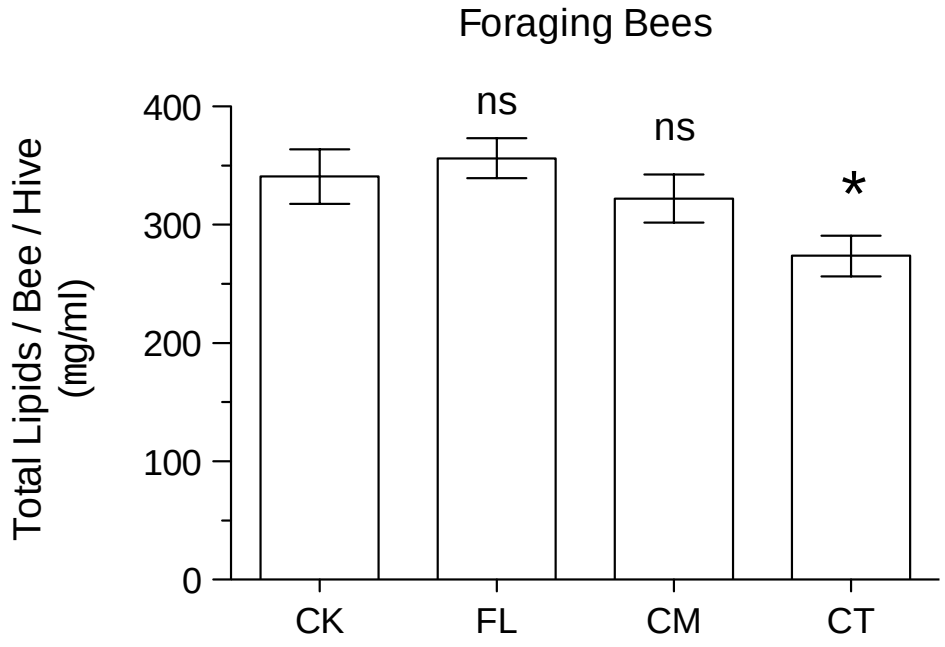

Figure 3.6. Total lipid content of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

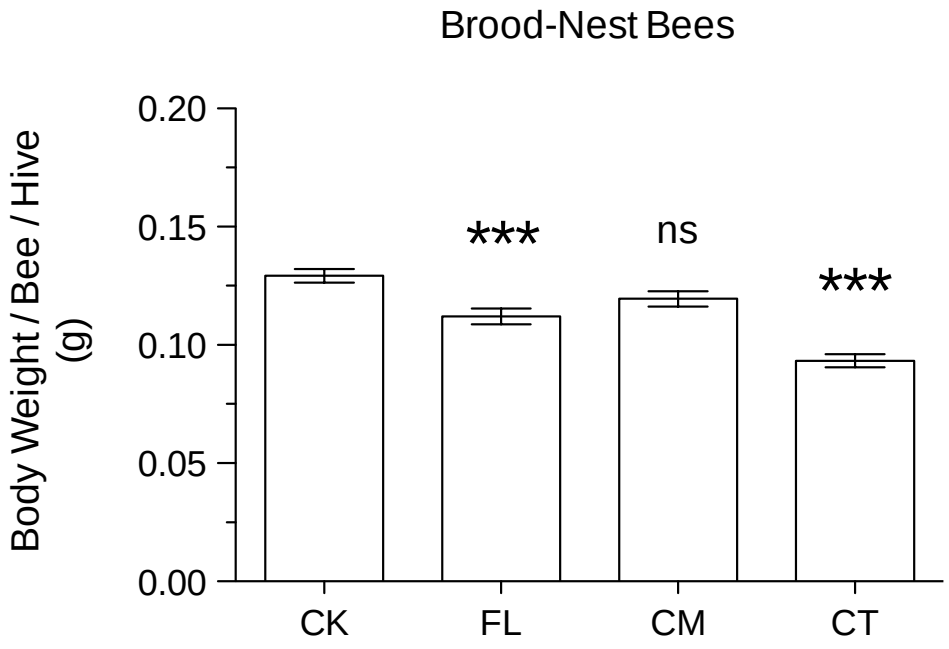

Figure 3.7. Total body weight of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 μg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

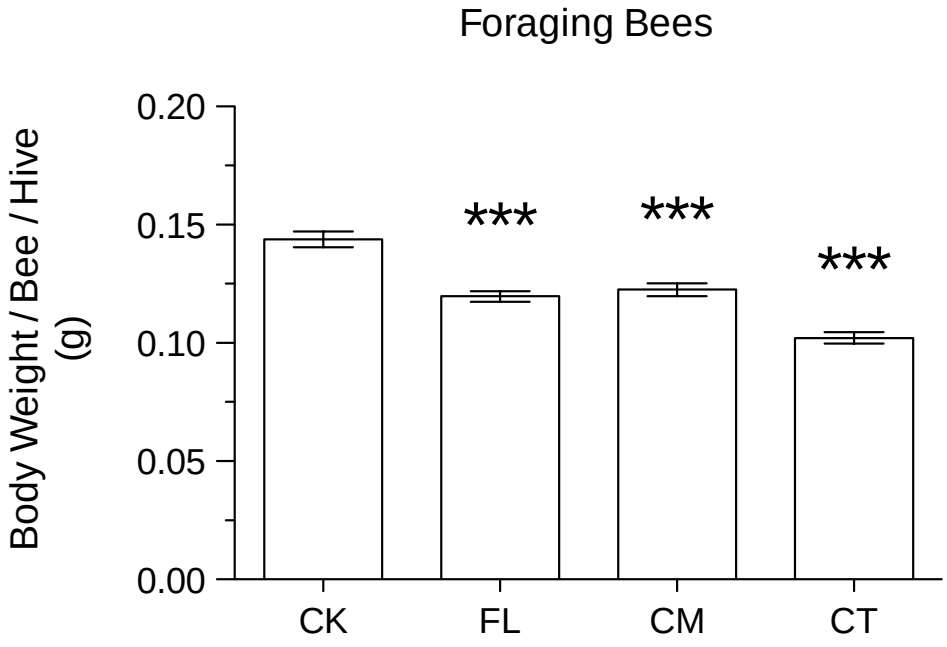

Figure 3.8. Total body weight of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 μg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

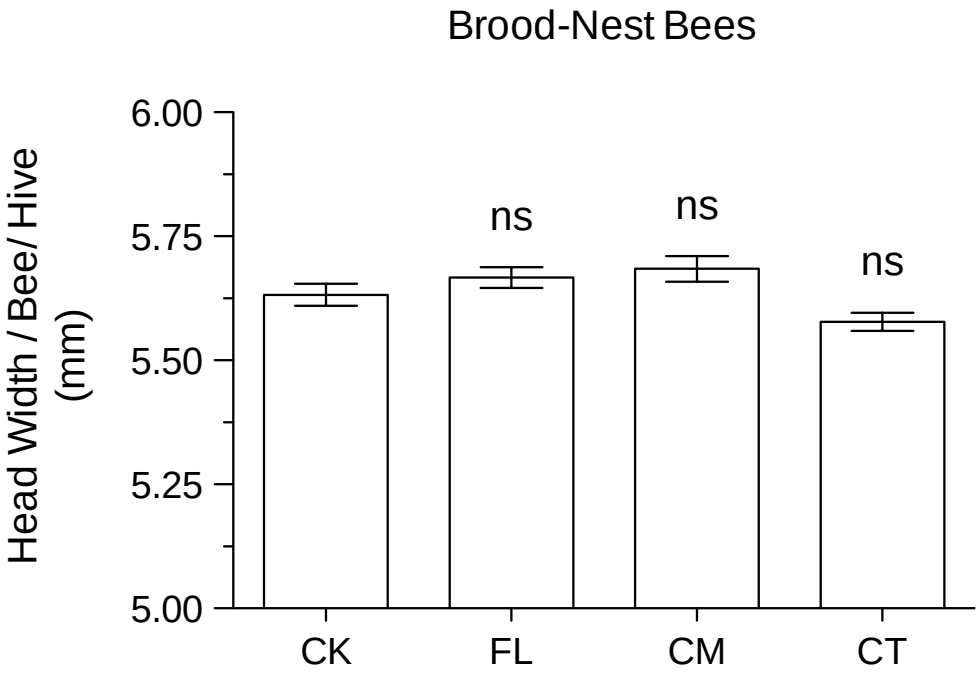

Figure 3.9. Total head width of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 μg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

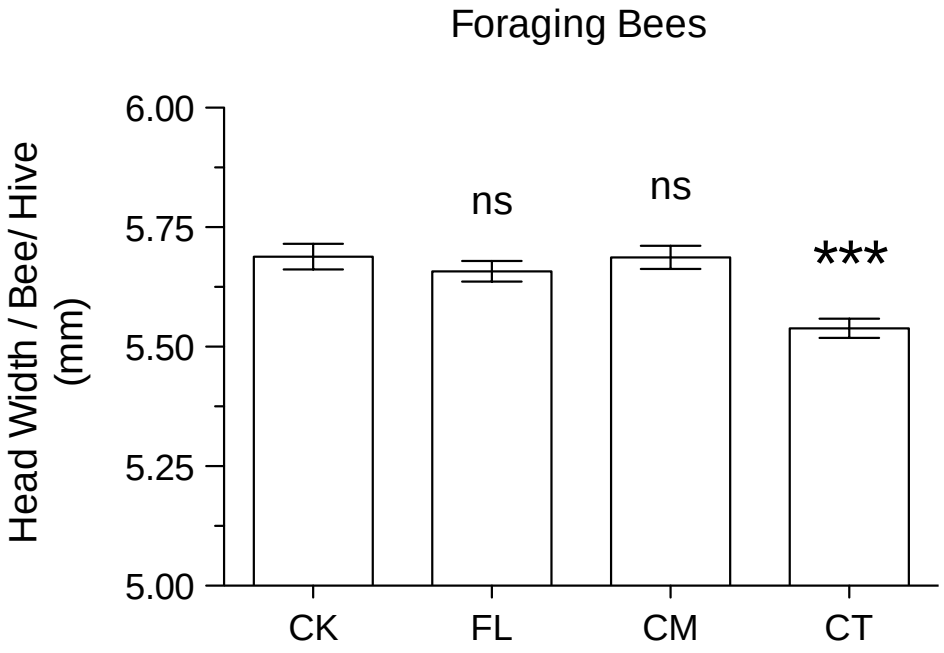

Figure 3.10. Total head width of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 μg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (*n = 3*). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

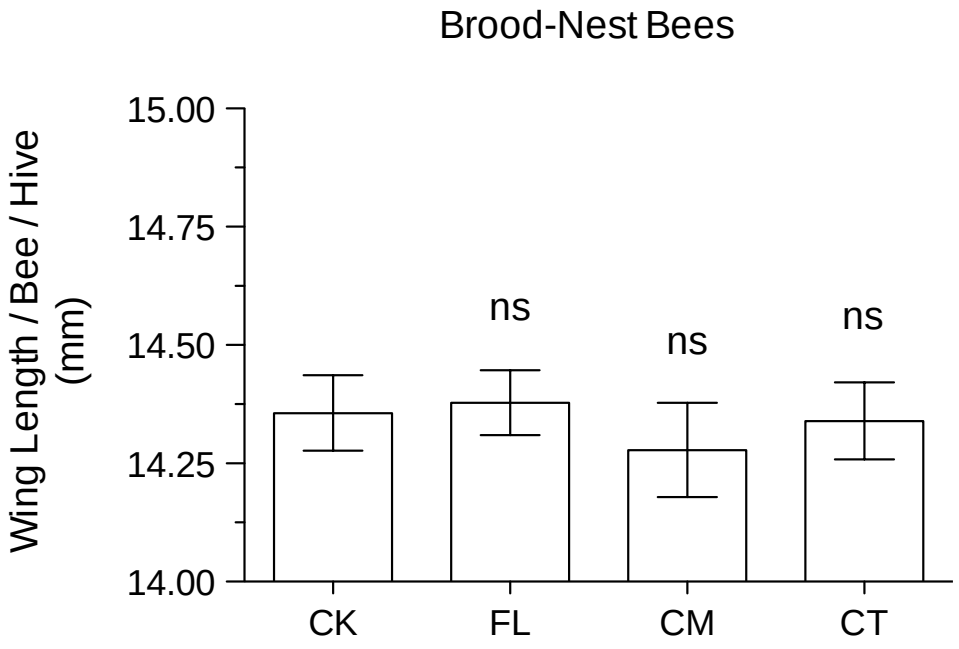

Figure 3.11. Total wing length of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 μg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

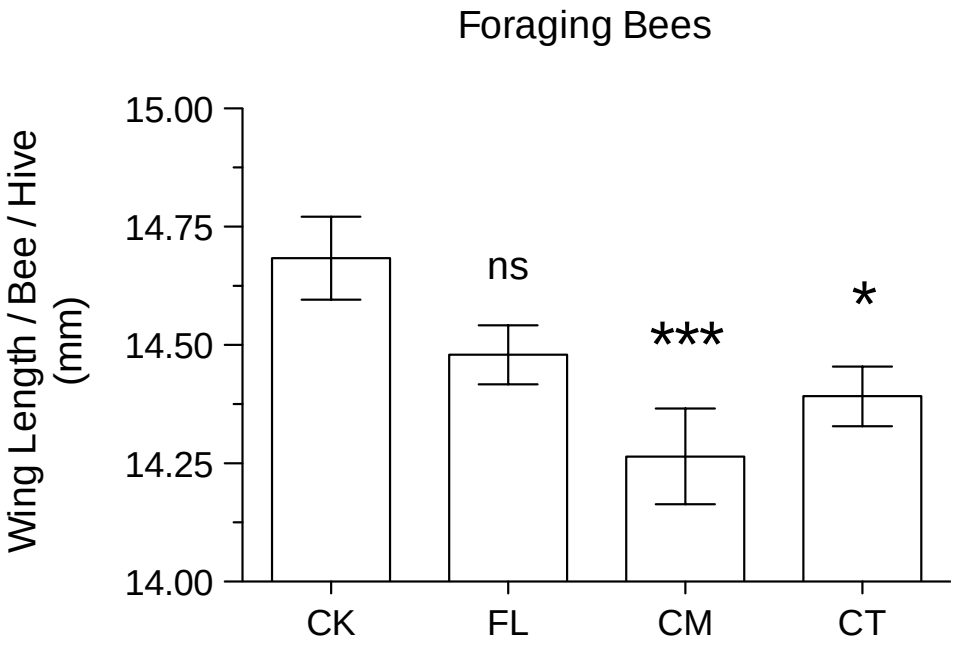

Figure 3.12. Total wing length of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 μg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

CHAPTER 4

EFFECTS OF *TAU*-FLUVALINATE (APISTAN®), COUMAPHOS (CHECKMITE+®), AND CHLOROTHALONIL ON THE IMMUNE HEALTH OF BROOD-NEST AND FORAGING HONEY BEES

4.1 INTRODUCTION

The population density within the honey bee colony, the stable nest environment, and the presence of stored food resources makes the colony the perfect setting for the transmission of pathogens and parasites (Evans et al., 2006). In a survey of beekeepers, pathogens were selected as the primary cause of colony failure (Engelsdorp et al., 2010). The honey bee individual and social immune responses are critical to the colony defense systems against these pathogens. The honey bee must maintain an efficient immune system to cope with the pathogens and parasites that are easily spread throughout the colony. The colony immune response to a challenge is a measure of both individual- and colony-level health.

In such pathogen favorable conditions, the honey bee has created immune defenses at the colony level referred to as social immunity. The mechanisms of social immunity include behavioral, physiological, and organizational adaptations (Cremer et al., 2007; Wilson-Rich et al., 2008). Examples of behavioral adaptions include hygienic behavior, nerophoric behavior, nest architecture, and the use of propolis in the colony (Evans et al., 2006; Traniello, 2002). The production of glucose oxidase and the bacterial assemblages found inside the honey bees are physiological adaptations and the division

of labor provides an example of an organizational adaptation (Alaux et al., 2010; Evans et al., 2006).

Glucose oxidase is produced in the hypopharyngeal gland and catalyzes the oxidation of glucose to gluconic acid and then to hydrogen peroxide (Alaux et al., 2010). The hydrogen peroxide product contributes to the sterilization of hive products and honey (Alaux et al., 2012). The hypopharyngeal glands in nurse honey bees are larger than those of the forager honey bees (Cascino et al., 1989), a difference due mainly because of task differentiation between the younger and older honey bees. The younger honey bees are more involved in the feeding of brood than the older honey bees, a process which requires the development of the hypopharyngeal gland (Cascino et al., 1989).

The individual immune response of the honey bee can result from either the stimulation of the phenoloxidase cascade, the release of reactive oxygen species, or the production of antimicrobial peptides (Wilson-Rich et al., 2008). Reactive oxygen species are an important part of the insect immune system that helps to maintain homeostasis (Diaz-Albiter et al., 2012). The production of antimicrobial peptides and lysosomes occurs as the result of a specific pathogen infection (Laughton et al., 2011; Schmid-Hempel, 2005). The enzyme phenoloxidase is produced from the pro-phenoloxidase (POX) cascade and is responsible for parts of the cellular immune responses.

Some of the immune responses related to the POX are melanization, wound healing, and sclerotization (Laughton et al., 2011). The phenoloxidase cascade is a series of interactions between enzymes that ends in the formation of melanin (Laughton et al., 2011). Melanin is important for wound healing and cellular response such as coagulation, encapsulation, and phagocytosis (Chan et al., 2009). POX activity in honey

bees has been shown to increase when faced with an immune challenge (Chan et al., 2009; Laughton & Siva-Jothy, 2011; Scherfer et al., 2008).

As our agricultural system depends on pesticides for pest control, colonies are at risk to high levels of pesticide exposure. Recent studies of pesticide residues in colonies across North America reveal over 120 different pesticides and metabolites in colonies (Mullin et al., 2010). However, the two most common pesticides in colonies are the pyrethroid *tau*-fluvalinate and the organophosphate coumaphos, both used by beekeepers (vanEngelsdorp, et al., 2008; Mullin et al., 2010). Coumaphos and *tau*-fluvalinate are used in the management of the parasitic varroa mite. The varroa mite has been a pest of the European honey bee since the 1960s and in North America since the late 1980s. Problems with the varroa mite begin with its parasitic behavior on developing and adult honey bees and are increased by the transmission of viruses (Francis et al., 2013). Various methods of chemical control have been used in an effort to regulate the mite populations (Sammataro et al., 2000). These chemicals include formic acid, oxalic acid, amitraz, coumaphos, and *tau*-fluvalinate.

These two miticides are often found in combination with the agricultural fungicide chlorothalonil (Mullin et al., 2010). Chlorothalonil is picked up by foraging honey bees and brought back to the colony through pollen (Pettis et al., 2013; Mullin et al., 2010). The high presence of this fungicide in colonies may be related to the fact that there are no label restrictions for spraying chlorothalonil around insect pollinators (Mullin et al., 2010). Chlorothalonil is a popular choice to treat against mildew, bacteria, and algae in a wide variety of crops (E.P.A, 1999).

There have been many studies that have shown an interaction between immune responses and pesticide exposure. Research has linked immune gene expression and exposure to miticides and fungicides (Boncristiani et al., 2012; DeGrandi-Hoffman et al., 2013; Evans et al., 2006). Additionally a gene that is involved specifically in the production of phenoloxidase has been shown to be down regulated in honey bees exposed to a pesticide and fungicide (Gregorc et al., 2012). Social immune parameters have also shown interactions with exposure to pesticides. Glucose oxidase levels have been showed to decrease in the presence of a pesticide (Alaux et al., 2010). Pesticides have also been shown to increase virus titers and pathogen loads (Alaux et al., 2010; DeGrandi-Hoffman et al., 2013; Pettis et al., 2013).

Knowledge of the consequences of these pesticide residues and their relationship to honey bee health is limited. The key to understanding these relationships requires a multifactorial assessment of honey bee health. The experimental focus of this objective is to provide an examination of the immune status of honey bees exposed to the pesticides *tau*-fluvalinate, coumaphos, and chlorothalonil. In turn, this information will serve as a prerequisite for the predictive modeling of honey bee health thresholds that can be used for the development of ecologically- and chemically-based management practices to minimize the immunodeficiencies of honey bee colonies.

To reach this objective the individual and social immunity of honey bees exposed to in-colony pesticide residues were measured. This study examined the immune factors of honey bee colonies that are affected by current-use pesticides treatments. Here, I report: the phenoloxidase and glucose oxidase activity in brood and forager honey bees in *tau*-fluvalinate, coumaphos, and chlorothalonil-treated colonies.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Bicinchoninic acid, copper sulfate, Triton X-100 , and glucose were purchased from Sigma Aldrich (St Louis, MO, USA). Chymotrypsin and *o*-dianisidine were purchased from MP Biomedicals (Solon, OH, USA). Horseradish peroxidase purchased from Novex Life Technologies (Grand Island, NY, USA). L-dopa was purchased from Acros Organics (New Jersey, USA). Coumaphos (CheckMite®) was purchased from Bayer CropScience (RTP, NC, USA). *tau*-Fluvalinate (Apistan®) was purchased from Zoecon (Charlotte, NC, USA).

4.2.3 Experimental Honey Bee Colonies and Pesticide Treatments

The experimental honey bee colonies consisted of a single-story hive provided with a sister queen to reduce genetic variation between colonies. Each hive was constructed with new frames and foundation to reduce initial exposures to pesticides. Three colonies were used for each pesticide treatment (i.e., three replicates per treatment). These hives were located at three apiaries maintained by the Department of Entomology at Virginia Tech, including Price's Fork, Kentland Farms, and Moore Farms. Kentland Farm is 3,000 acres and is focused on small fruit and vegetable crop production. Price's Fork is a small research area that is in a more suburban environment. Moore Farm is 250 acres and has a diverse native plant population. These colonies were established in May 2012 and allowed to reach colony strength by July (i.e., six weeks after colony establishment).

The experimental honey bee colonies included a 1) control (no pesticide), 2) *tau*-fluvalinate (Apistan®, Zoecon) treatment, 3) coumaphos (CheckMite+®, Bayer CropScience) treatment, and 4) chlorothalonil treatment. For the *tau*-fluvalinate and coumaphos treatments, the honey bee colonies were treated with two *tau*-fluvalinate-impregnated or two coumaphos-impregnated strips, each containing ~ 10% active ingredient, for six weeks using the manufacturer's label recommendations. The chlorothalonil treatment (10 μg/L, or parts per billion) was provided to the honey bees in a 30% sucrose solution for six weeks. In addition, the *tau*-fluvalinate- and coumaphos-treated and -untreated honey bee colonies were also provided with a 30% sucrose solution for the six week treatment period.

Random groups of honey bees were marked after adult emergence from the brood frame so that we could collect honey bees of known ages. Two random frames of brood from each colony were collected for marking, this allowed for approximately 100 bees to be marked. The frames were put into custom made cages in an incubation chamber (34℃) for 6-8 hours. During this incubation period adult honey bees emerged from the brood frames. These bees were marked using Testors™ model paint. Honey bees were then smoked with pine needle smoke to eliminate the paint odors. Marked honey bees were returned to their hives. Each treatment group was marked with a distinguishable color for collection.

In August 2012, a random sample of brood-nest bees and another of foraging honey bees were collected from each of the honey bee colonies prior to the addition of each pesticide treatment. The samples were used to measure the baseline macromolecule contents and morphometrics of brood-nest and foraging honey bees prior to the addition

of the pesticide treatment. Following a six-week pesticide treatment period, a random sample of brood-nest honey bees was collected from the brood frames and a random sample of foraging honey bees was collected from the hive entrance.

4.2.4 Measurement of Phenoloxidase in Pesticide-Treated Honey Bees

The phenoloxidase activity in the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, or chlorothalonil was measured according to the method of Laughton et al. (2011), with modifications. The brood-nest and foraging honey bees were collected from the pesticide-treated colonies and transported on ice to the laboratory for the phenoloxidase assay. The hemolymph was collected from the fourth abdominal tergite of the individual honey bees using a one-microliter capillary tube. The diluted hemolymph was added to ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% Triton X-100. Nine microliters of hemolymph were added to the individual well of a 96-well microplate containing 20 µl 0.1 M sodium phosphate (pH 7.8) and 135 µl deionized H_2O. Five microliters of chymotrypsin were added to the wells. The samples were incubated for 5 min. at 37 °C followed by the addition of 20 µl L-dopa. The phenoloxidase activity was measured at 490 nm for 60 min. at 15 sec. intervals on a Molecular Devices SpectraMax M2 multimode microplate reader. The phenoloxidase activity was recorded as Vmax and standardized using the total protein concentration for each hemolymph sample. The concentration of total protein was measured using the method of Smith et al. (1985) using bovine serum albumin as a standard. The total protein assay was carried out at 560 nm on a multimode microplate reader.

4.2.5 Measurement of Glucose Oxidase in Pesticide-Treated Honey Bees

The glucose oxidase activity in the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, or chlorothalonil was measured according to the method of Alaux et al. (2010), with modifications. The brood-nest and foraging honey bees were collected from the pesticide-treated colonies and transported on ice to the laboratory for the glucose activity assay. The heads were dissected from the individual honey bees and homogenized in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% Triton X-100. One milliliter of the homogenizing buffer was used per honey bee. The homogenate was centrifuged at 10,000 x *g* for 10 min. at 4 °C. The supernatant was transferred into a clean 1.5-ml microcentrifuge tube. Fifty microliters of the supernatant were added to the individual well of a 96-well microplate containing 0.5 M potassium phosphate (pH 7.0), 0.1 M glucose, and 2.5 U (amount of enzyme which oxidizes 1 μmol ABTS) horseradish peroxidase. The glucose oxidase samples were incubated for 10 min. at 37 °C followed by the addition of 3 mM *O*-dianisidine. The glucose oxidase activity was measured at 430 nm for 90 min. at 15 sec. intervals on a Molecular Devices SpectraMax M2 multimode microplate reader. The glucose oxidase activity was recorded as Vmax and standardized using the total protein concentration for each honey bee head sample. The concentration of total protein for the head was measured using the method of Smith et al. (1985) using bovine serum albumin as a standard. The protein assay was carried out at 560 nm on a multimode microplate reader.

4.2.6 Statistical Analysis

The phenoloxidase and glucose oxidase activity (mU/min./mg protein, milliunits per minute per milligram protein) for each pesticide treatment were statistically compared to the control treatment using a one-way analysis of variance (ANOVA) in combination with a Dunnett multiple comparison test (JMP, 1989-2007). Each set of honey bees was divided into two age groups; brood-nest bees (n = 3; total of 45 individuals) and forager bees (n = 3; total of 45 individuals).

4.3 RESULTS

4.3.1 Measurement of Phenoloxidase in Pesticide-Treated Honey Bees

The phenoloxidase activity of the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, and chlorothalonil is shown in Figures 4.1 and 4.2. The phenoloxidase activity in brood-nest bees was significantly increased by 54.7% in the coumaphos-treated individuals compared to the pesticide-untreated bees (Figure 4.1). The phenoloxidase activity in foraging bees was not significantly different in individuals treated with *tau*-fluvalinate, coumaphos, and chlorothalonil, compared to the pesticide-untreated forager bees (Figure 4.2).

4.3.2 Measurement of Glucose Oxidase in Pesticide-Treated Honey Bees

The glucose oxidase activity of the brood-nest and foraging honey bees treated with *tau*-fluvalinate, coumaphos, and chlorothalonil is shown in Figures 4.3-4.4. The glucose oxidase activity in brood-nest was significantly increased by 45.1% the chlorothalonil-treated individuals compared to the pesticide-untreated bees (Figure 4.3).

The glucose oxidase activity in foraging bees was significantly increased by 41.3% in chlorothalonil-treated individuals, compared to the pesticide-untreated foraging bees (Figure 4.4).

4.4 DISCUSSION

The results from the immunity assays show several patterns that merit further investigation. Brood nest honey bees exposed to coumaphos were the only group that showed differences in POX activity, when compared to the control. In both brood nest and forager age bees, chlorothalonil was the only pesticide that showed significant differences in GOX activity, when compared to the control.

Hives were exposed to chlorothalonil through a sugar solution. This was planned to mirror the indirect exposure to the fungicide that honey bees would undergo naturally. It could be possible that the increase in GOX is because it is produced in the hypopharyngeal gland, which helps in the breakdown of sucrose for honey production. GOX production could be stimulated because the honey bee perceives the fungicide as a threat that warrants an increase in hive and food sterilization. A recent study showed that in honey bees exposed to high levels of fungicides, there was an increased susceptibility to the gut pathogen *Nosema* (Pettis et al., 2013).

Social immune parameters have shown interactions with exposure to pesticides. Glucose oxidase levels have been shown to decrease in the presence of a neonicitinoid pesticide (Alaux et al., 2010). Hydrogen peroxide, the by-product of glucose oxidase, has been positively correlated with the suppression of pathogens in the hive (Alaux et al., 2010). The amount of hydrogen peroxide in honey has been shown to be a strong

positive predictor of the antimicrobial activity in honey (Brudzynski, 2006; Taormina et al., 2001).

Compared to solitary insects, honey bees have one third the immune response genes, which suggests the importance of the social immune defenses (Evans et al., 2006). Alaux et al., (2010) theorized that in a weakened honey bee, increased microsporidia could be using glucose and thus reducing the amount of GOX activity. The differences that we see in the age groups could be related to the different sizes of hypopharyngeal glands and the different jobs of the age groups (Alaux et al., 2010; Cascino et al., 1989). An increase in the glucose oxidase activity in honey bees could indicate two things. One, that there is no suppression by the pesticides of the honey bee's social immune system. Two, there is increased activity in the social immune system due to a perceived immune threat by the pesticides. More studies will need to be done to tease out the meaning of the increase.

We measured the POX activity by photometrically measuring the conversion of L-Dopa to Dopachrome using the honey bee hemolymph (Laughton & Siva-Jothy, 2011). This measurement is used to evaluate an insect's individual defense ability (Wilson-Rich et al., 2008). An increase in POX activity shows stimulation in the individual immune system. Phenoloxidase requires an immune challenge for activation, which follows where there were significant increases in POX, immune stress is harming bee health. POX activity has been shown to be correlated with pathogen susceptibility in insects (Chan et al., 2009; Laughton and Siva-Jothy, 2011; Scherfer et al., 2008).

There could be other reasons for the suppression and rise of POX activity. For example, a gene that specifically is involved in the production of phenoloxidase has been

shown to be down-regulated in honey bee colonies exposed to a pesticide and fungicide (Gregorc et al., 2012). Past studies have shown that POX activity increases in forager bees compared to brood bees (Schmid-Hempel, 2005). Our study showed an opposite correlation. This could be because there was an increased pesticide exposure to the brood honey bees that were limited to the colony environment.

Overall, the immune responses in the hives exposed to pesticides show that further investigation is needed for the effects of the two miticides and fungicides. Future studies could include an analysis of virus titers and pathogen load. Additional fungicides and synergistic interactions would also expand knowledge of the role of pesticide interactions and immunity. Educating beekeepers and farmers on the effects of fungicides and ways to minimize exposure would benefit the beekeeping community.

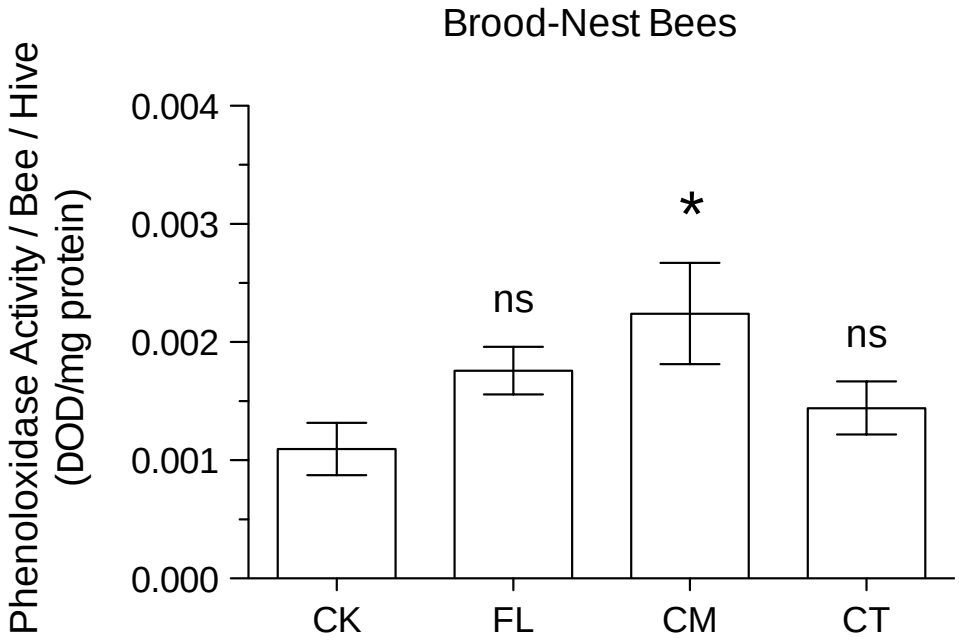

Figure 4.1. Phenoloxidase activity of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

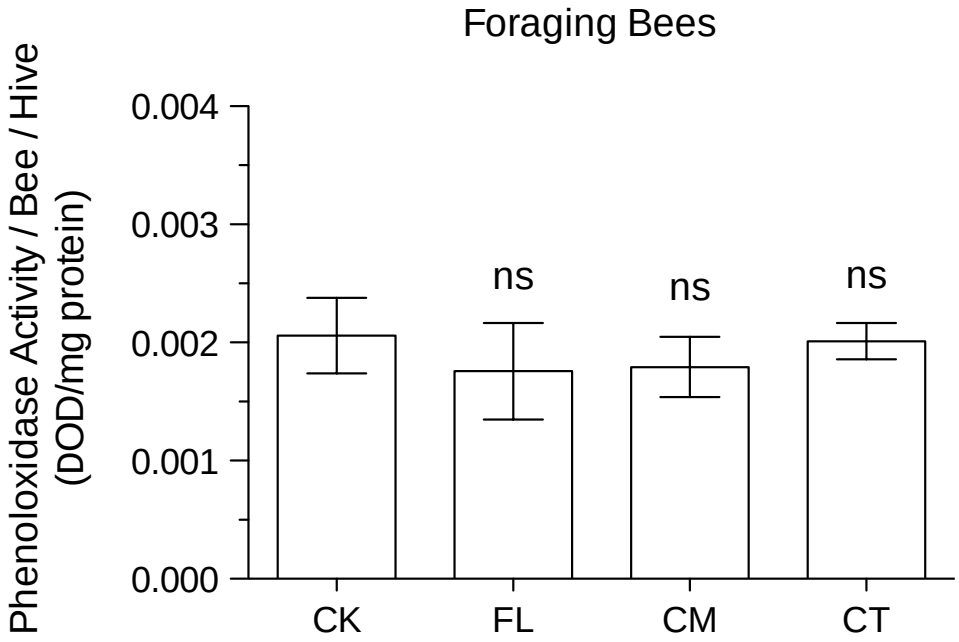

Figure 4.2. Phenoloxidase activity of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

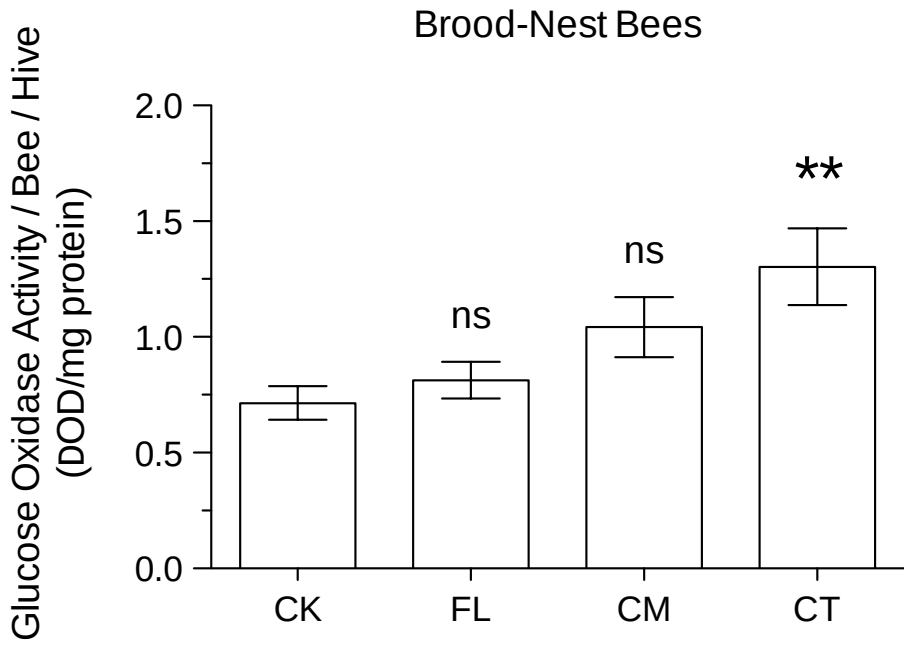

Figure 4.3. Glucose oxidase activity of brood-nest honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

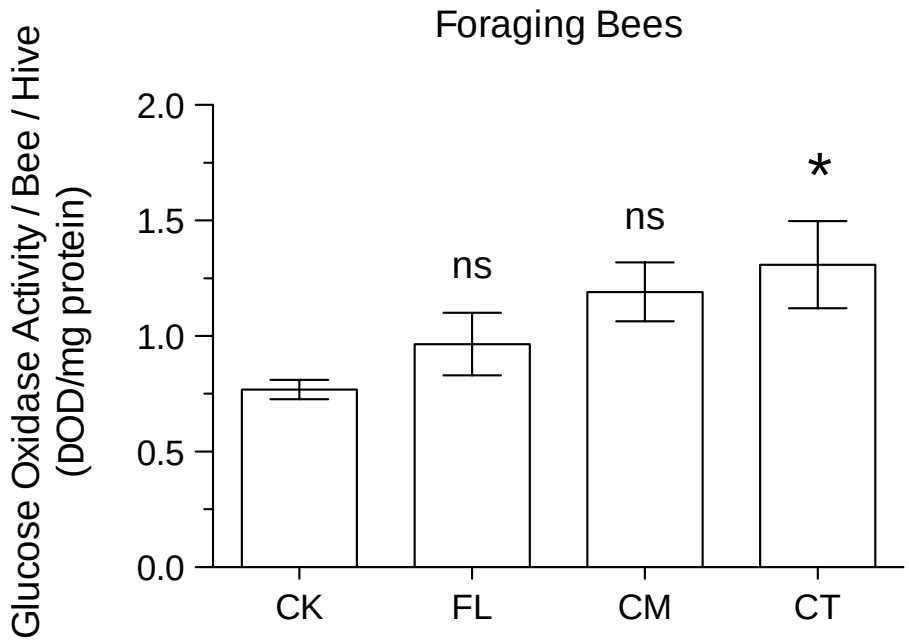

Figure 4.4. Glucose oxidase activity of foraging honey bees exposed to *tau*-fluvalinate (Apistan®, 10.25% a.i.; FL), coumaphos (CheckMite+®, 10% a.i.; CM), and chlorothalonil (10 µg/L, or parts per billion; CT). Vertical bars indicate standard errors of the mean (n = 3). Asterisks denote that the means are significantly different from the control as determined with a Dunnett's multiple comparison test after a one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant at the 0.05 level).

SUMMARY

The honey bee is a widely managed crop pollinator that provides the agricultural industry with the sustainability and economic viability needed to satisfy the food and fiber needs of our society. The excessive use of agrochemicals is implicated in the reduced number of managed bee colonies available for crop pollination services. There are several gaps in our knowledge with respect to agrochemical exposures and the health status of managed bee colonies. Thus, it is necessary to gather information relevant to the areas where knowledge is lacking in order to enhance our ability to predict conditions that are either favorable or unfavorable for bee colony health.

Chlorothalonil is one of the most commonly used fungicides in the agricultural market (Mullin et al., 2010). It is also the most commonly found fungicide in honey bee hives (Mullin et al., 2010). Many of the crops that the fungicide is used for are also pollinated by honey bees. Chlorothalonil, like many fungicides, has no label restriction for use around honey bee colonies (E.P.A, 1999).

Our study shows some correlations between exposure to this fungicide and significant differences in nutritional concentrations and immune reactions. While more research remains to make these correlations clear, it is important for beekeepers to consider the use of fungicides near their colonies. One option is to decrease the amount of synthetic fungicides used and replace them with alternatives such as antagonist microorganisms, naturally occurring bioactive compounds, and resistant plant varieties (Ghaouth, Wilson, & Wisniewski, 2004). Another option is to spray the compounds in the evening or early morning when the honey bees are not foraging.

Since the introduction of the varroa mite in the 1980s, there has been a 50% loss of colonies across the US (vanEngelsdorp et al., 2009). To combat this loss, beekeepers have turned to the use of synthetic varroacides to protect their colonies. However, resistance and sub-lethal effects are just a few of the side problems that have arisen from the use of these varroacides (P. Elzen, J. et al., 1999). Our study adds to this growing body of research that is examining honey bee health and synthetic varroacides usage.

Beekeepers that treat for varroa mites using *tau*-fluvalinate and coumaphos might consider using alternative treatments. This can include mechanical control such as drone-brood trapping, inert dusts, and the use screen bottom boards in the warmer months (Wallner & Fries, 2003). Additionally mite-tolerant bee stocks that are selected for their varroa sensitive hygienic behavior can reduce the numbers of mites in the colony (Fagan et al., 2012). Biopesticides, naturally occurring organisms and their derivatives, can be used in lieu of synthetic chemicals as well. This includes essential oils like thymol eucalyptol, and menthol; such as are found in the product Apilife VAR (Skinner, Parkman, & Studer, 2001). Formic acid is another biopesticide that can be used to control varroa mites.

In addition to finding alternatives to the miticides and fungicides, this study highlights the importance of providing honey bees with access to good nutrition. Having forage that blooms year-round and a water source is helpful in supplying the colony with sources of supplemental nutrition. Checking the hives occasionally during the winter for food stores and providing supplemental food such as protein patties and fondant can also encourage overwintering and lessen colony stress.

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