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Mechanisms of differential toxicity between honey bee (*Apis mellifera* L.) castes with an
emphasis on coumaphos

by

Lizette A. P. Dahlgren

A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska

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Under the Supervision of Professors Marion Ellis & Blair Siegfried

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Mechanisms of differential toxin sensitivity between honey bee (*Apis mellifera* L.) castes
with an emphasis on coumaphos

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University of Nebraska, 2014

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Abstract: Acaricides are used to treat honey bee (*Apis mellifera* L.) colonies to control the parasitic Varroa mite (*Varroa destructor* Anderson & Trueman), a worldwide threat to honey bee health. As such, these compounds act as drugs to mitigate bee losses but may also stress the bees. This dissertation quantifies differences between queen and worker tolerance of five acaricides and clarifies the honey bee mechanism of tolerance for coumaphos.

Selected acaricides were topically applied to adult queen and worker bees to generate dose-response curves and LD₅₀s. Twenty-four hours after treatment, queens were 3-times more tolerant of tau-fluvalinate and 6-times more tolerant of thymol than workers when adjusted for body weight differences between workers (108 mg) and queens (180 mg). Queens survived the highest administered doses of fenpyroximate (1620 µg/g) and coumaphos (2700 µg/g) indicating that queens are at least 12-fold more tolerant of coumaphos and at least 40-fold more tolerant of fenpyroximate than workers. However, queens treated with as little as 54 µg/g of fenpyroximate exhibited reduced survival over 6 weeks following treatment. Amitraz was the only acaricide tested for which queens were not more tolerant than workers.

Differences between queen and worker tolerance of coumaphos was explored further by topical bioassays with the metabolite coumaphos oxon. Coumaphos oxon was only half as toxic as the parent compound to *Apis mellifera*. This insensitivity to both coumaphos and coumaphos oxon is unique as both castes were highly susceptible to chlorpyrifos, with chlorpyrifos oxon being twice as toxic as the parent compound.

Using Ellman's assay, honey bee and house fly acetylcholinesterase were all shown to be similarly inhibited by both chlorpyrifos oxon and coumaphos oxon. Target site insensitivity is therefore not the mechanism of honey bee tolerance.

Finally, bee metabolism was compared using GC/MS and LC-MS/MS at 24 hr time intervals for 5 days. Most notably, coumaphos oxon was not found. Queen and worker acetone rinse and worker internal recovered coumaphos concentrations decreased over time. Therefore, coumaphos is likely being metabolized to a less toxic coumaphos metabolite. Caste differences are likely due to metabolism, but further studies are needed to determine specific mechanisms.

DEDICATION

I dedicate this dissertation to my mother, one of many strong women in my life. She instilled in me a love of nature, a determination to succeed, and a curiosity that continues to drive my aspirations.

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CHAPTER 1: LITERATURE REVIEW

Approximately 30% of the human diet consists of plants pollinated by honey bees (*Apis mellifera* L.) (McGregor 1976). The most recent estimates of the value of pollination services to U.S. agriculture is \$16.4 billion annually for honey bees alone (Morse and Calderone 2000, Losey and Vaughan 2006). While Colony Collapse Disorder (Oldroyd 2007) has resulted in global media attention during the past 7 years, scientists have documented declining colony numbers for at least 30 years.

Decline in Honey Bee Populations and the Introduction of Parasitic Bee Mites:

Beginning in the mid-80s, pollinators around the world began experiencing declines in diversity and abundance (Williams 1986, Buchmann and Nabhan 1996, Matheson et al. 1996, Allen-Wardell et al. 1998, Kearns et al. 1998). The United States honey bee decline from 1985 to 1996 was correlated to the introduction of 2 parasitic mites, the tracheal mite, *Acarapis woodi* (1984) and the *Varroa* mite, *Varroa destructor* (1987) (de Guzman and Rinderer 1999). Tracheal mite infected apiaries initially exhibited substantial winter mortality (Eischen 1987), but today are not considered a major pest problem for honey bees. However, *Varroa* populations are still a major factor in colony health and performance (Guzmán-Novoa et al. 2010).

The widespread distribution of *Varroa* mites poses a global threat to honey bee colony health (Le Conte et al. 2010). *Varroa* mites weaken the hive by consuming hemolymph from pupal and adult bees and by vectoring viruses that may increase colony susceptibility to both abiotic and biotic environmental stressors (Shen et al. 2005). *Varroa destructor* was first detected in the U.S.S.R. in 1952 after *A. mellifera* colonies were transported to Eastern Russia.

Varroa likely transferred from *A. cerana* to *A. mellifera* and then quickly disseminated across Asia and into South America and Germany (Rosenkranz et al. 2010). Today the only continent devoid of *Varroa* is Australia (Rosenkranz et al. 2010). Many honey bee colonies infested with *Varroa* mites perish without beekeeper intervention (Kraus and Page 1995, National Research Council 2007).

Honey Bee Biology

The eusocial honey bee (*Apis mellifera* L.) colony consists of a single mated queen, the mother of tens of thousands of semi-sterile female workers and up to a few hundred haploid drones (male reproductives). Adult worker bees exhibit a temporal polyethism (age related division of labor). Nursing, wax building, nectar processing, foraging and guarding are examples of tasks that, in general, progress with age from the interior to the exterior of the hive (Winston 1987). As a result, those tasks considered the most dangerous (foraging, guarding) are usually performed by the oldest bees in the hive. Queen mandibular pheromone (QMP) (Pankiw et al. 1998), and brood pheromone (BP) (Pankiw 2004), as well as juvenile hormone (JH) (Schulz et al. 2002), octopamine (Schulz et al. 2002), and vitellogenin (Vg) (Nelson et al. 2007), have all been shown to affect the temporal polyethism or the age dependent division of labor that contributes to collective brood rearing.

Female eggs may develop into either a queen or a worker. Queens and worker castes differentiate due to the diet provided within the first 3 days of larval life (Beetsma 1979). Queen larvae receive a blend of larval food including the protein royalactin resulting in a faster growth rate, larger body size, and increased ovary development (Reginato and Cruz-Lamdim 2003, Kamakura 2011). Queens are reared by workers to replace an aging queen, in the event of a

queen death, and in times of overabundance of resources and crowded worker population for whole colony reproduction or fission (Winston 1987). Adult workers and queens differ physiologically in their energetic and metabolic requirements, their susceptibility to pathogens, and the circulating proteins found in the hemolymph (Chan et al. 2006). In essence, workers and queens are separated by their reproductive capabilities with workers being denied critical proteins necessary to become fully reproductive queens (Kamakura 2011). In the same respect, workers may differ from each other because of differences in nutritional environments (more pollen diversity and availability) leading to increased immunocompetence (Alaux et al. 2010). In addition, pollen availability is known to affect longevity, the development of hypopharyngeal glands (glands which are used to feed brood) (Pernal and Currie 2000), and pathogen susceptibility (Rinderer et al. 1974, Rinderer and Dell Elliott 1977). In spite of pronounced differences between the castes, there is little information on how queens and workers differ in their tolerance to pesticides in general or to the in-hive acaricides used by beekeepers to control *Varroa destructor*.

Acaricides:

While labor-intensive alternatives to chemical control exist (Sammataro et al. 2000, Charrière et al. 2003, Imdorf et al. 2003), most beekeepers rely on acaricides to protect their colonies. Acaricides used to control *Varroa* include compounds with many different modes of action. Examples include a cholinesterase inhibitor (coumaphos), a Na⁺ channel agonist (tau-fluvalinate), an octopamine agonist (amitraz), and a mitochondrial electron transport inhibitor (fenpyroximate). A variety of plant produced products are also used as acaricides including monoterpenoids (such as thymol) (Johnson et al. 2010).

The widespread detection of acaricide residues in beeswax (Mullin et al. 2010) gives evidence to the large scale use of acaricides and the potential for their persistence in beeswax comb long after acaricide treatment has ceased. In addition to stress from *Varroa* mites, the acaricides used to control them may also be contributing to honey bee losses individually or synergistically with pesticides applied to crops (Johnson et al. 2009, Mullin et al. 2010). Pesticides have also been shown to act synergistically with pathogens such as *Nosema* and increase disease incidence (Alaux et al. 2010, Pettis et al. 2012, Pettis et al. 2013).

As the queen is the sole egg layer, the effect of a toxin on the queen can potentially be much more important to colony success than the effects on her worker offspring. Only a few studies have examined the effects of acaricide exposure on queens (Haarmann et al. 2002, Collins et al. 2004, Pettis et al. 2004). Beginning in the late 1980s, beekeepers began introducing acaricides into beehives to control both tracheal and *Varroa* mites. Shortly afterwards, anecdotal reports of premature queen failure and supersedure increased (Tew 1996, Pettis et al. 2004). Camazine *et al.* (1998) and Tew (1996) suggested that queen failure (supersedure, drone-laying, queen-less colonies) rates had increased due to the presence of bee parasites. The co-occurrence of bee parasites and the introduction of acaricides into beehives make it difficult to establish the relative importance of the two events. It is possible, that once acaricides have been applied to the hive, their persistence within the hive wax may not be entirely stagnant (Kochansky et al. 2001). Mite treatments including tau-fluvalinate and coumaphos can negatively affect developing queens by causing developmental defects, rejection of queen larvae, and premature mortality (Haarmann et al. 2002, Pettis et al. 2004). Furthermore, chronic effects of tau-fluvalinate have been reported including decreased queen

fecundity and life span (Sokol 1996). My research aims to further explore the effects of acaricides on honey bee health.

Biotransformation in Bees:

As bees have co-evolved with plants, they possess the ability to tolerate multiple xenobiotic compounds. However, it has been speculated that due to their specialization on nectar and pollen, bees possess far fewer xenobiotic detoxifying enzymes than *Drosophila melanogaster* and *Anopheles gambiae* (Claudianos et al. 2006). Biotransformation in bees initially may include oxidation (usually by cytochrome P450s), hydrolysis (by carboxyl/cholinesterases) and subsequently glutathione catalyzed detoxification reactions by glutathione S-transferases.

Susceptibility to xenobiotics may change with sequestration or detoxification and clearance ability. The insect fat body functions in insect immune response and is analogous to the mammalian liver and adipose tissue (Canavoso et al. 2001), serving functions of xenobiotic metabolism, sequestration, and energy storage (Arrese and Soulages 2010). Additionally, it is possible the fat body plays a vital role in longevity as longevity mechanisms (dFOXO activation) have been shown to occur within the adult fruit fly (*Drosophila*) head fat body (Hwangbo et al. 2004). Winter worker bees have greater dry weight, protein, and fat contents (Kunert and Crailsheim. 1988) and live significantly longer (3-5 mo) than summer workers (30 d). Queens, which can live several years, may possibly have greater capacity to sequester or metabolize acaricides.

One way to move lipophilic xenobiotics towards elimination from the body is oxidation. This is primarily carried out by the cytochrome P450 microsomal monooxygenases (P450s) where, in general, these enzymes catalyze the nicotinamide adenine dinucleotide phosphate

(NADPH)- dependent reductive cleavage of oxygen with products including a functionalized product and water (Mao et al. 2009). In insects, cytochrome P450s are expressed in the midgut, fat body, Malpighian tubules, nervous tissue, corpora allata, glands, and antennae, with the midgut often expressing the highest level of activity. Using heterologously expressed honey bee cytochrome P450s, Mao et al. (2011) found both coumaphos and tau-fluvalinate are metabolized by at least three midgut cytochrome P450 enzymes in workers: CYP9Q1, CYP9Q2, and CYP9Q3 (Mao et al. 2011). CYP306a1 and CYP6a514's, detoxification enzymes, were down- and up-regulated respectively in workers collected from colonies treated with Apiguard (thymol) and CYP306 was down-regulated in workers collected from colonies treated with Checkmite+ (coumaphos) (Boncristiani et al. 2012). Queens were not compared in either of the two previous studies (Mao et al. 2011, Boncristiani et al. 2012). Cockroaches have been demonstrated to have age-dependent changes in levels of cytochrome P450s (Turnquist and Brindley 1975). Paralleling this hypothesis, Valles *et al.* (1994, 1996), demonstrated that male cockroaches were more susceptible to insecticides than older male and female nymphs. Adult males were found to have decreased cytochrome P450 content and methoxyresorufin O-demethylase and ethoxyresorufin O-deethylase activities in comparison with the more tolerant nymphs (Valles et al. 1996). Likewise, it is possible that bees could have caste-dependent changes in levels of cytochrome P450s, with queens having higher titers than workers.

Susceptibility to xenobiotics in insects has been shown to change with nutritional state, caste, age, and sex (Yu 2008). Susceptibility in bees has been shown to change with diet (Wahl and Ulm. 1983, Alaux et al. 2010), caste (Graves and Mackensen 1965, Corona et al. 2007), and age (van Buren et al. 1992). Feeding on high quality pollen from diverse plant sources is known

to decrease the susceptibility of adult bees to pesticides (Wahl and Ulm. 1983) and diseases (Alaux et al. 2010).

Susceptibility to xenobiotics may change with tolerance of xenobiotic induced stress. Honey bee queens have been shown to tolerate higher levels of pesticide-induced oxidative stress than workers bees (Corona et al. 2007). One potential commonality between coumaphos, tau-fluvalinte, fenpyroximate, and thymol is that all may cause oxidative stress. Oxidative stress induced by pesticides is characterized by oxygen free radical formation, induction of cytochrome P450s, alteration in antioxidant enzymes, and a glutathione redox system (Banerjee et al. 2001). Fenpyroximate functions as an inhibitor of energy metabolism in the mitochondria through interactions with complex I (Motoba et al. 2000). In mammalian neuroblastoma cells, fenpyroximate and other complex I mitochondrial inhibitors cause ATP depletion, and cell death, possibly through generation of reactive oxygen species (Sherer et al. 2007). Queens and workers differ in the fatty acid composition of their respective membranes (Haddad et al. 2007). Newly emerged workers and queens, which have less polyunsaturated and more monounsaturated fatty acids, were found to be more resistant to peroxidation than other workers (Haddad et al. 2007). Monounsaturated phospholipids are less susceptible to peroxidation than polyunsaturated phospholipids. Queens may maintain a low ratio of peroxidation-susceptible polyunsaturated fats throughout life through consumption of a filtered diet of jelly, which has been shown to contain little to no polyunsaturated fats (Haddad et al. 2007).

As postulated by Amdam and Omholt (2002), it is possible that vitellogenin plays a role in the tolerance of xenobiotics. Worker lifespan is known to be influenced by the synthesis of vitellogenin, a glyco-lipoprotein produced in the fat body (Seehuus et al. 2006). Vitellogenin is a female-specific yolk precursor in many oviparous species and in honey bees functions in brood

food production (Seehuus et al. 2007), regulation of foraging behavior, longevity independent of behavior (Nelson et al. 2007), as an anti-oxidant (Seehuus et al. 2006) and in enhancing innate immunity (Amdam et al. 2004, Amdam et al. 2005). Vitellogenin has been shown to affect adult longevity of both queens and workers (Amdam et al. 2009), partially because of its free-radical scavenging capacity (Seehuus et al. 2006). In addition, vitellogenin comprises up to 50% of the total hemolymph protein in queens (Engels et al. 1990), a 20-fold higher concentration than in summer workers (Engels et al. 1990, Corona et al. 2007). If acaricide exposure causes oxidative stress in bees, as pesticide exposure does in mammals (Abdollahi et al. 2004), then the greater tolerance to acaricides exhibited by queens may be mediated by the anti-oxidant capacity of vitellogenin.

Evidence of this possible hormone-related susceptibility to acaricides has been shown numerous times. Younger bees have been shown to tolerate more coumaphos than older bees (van Buren et al. 1992). The shorter lived summer bees were shown to be more sensitive to imadacloprid than the long lived winter bees (Decourtye et al. 2003). Summer bees appeared to be approximately eightfold more susceptible than winter bees to the synergistic action of prochloraz and deltamethrin (Meled et al. 1998). As stressed bees are more likely to forage earlier in life leading to the likelihood of a reduced overall lifespan, it is possible that pesticides are stressing bees. Evidence for this is shown in MacKenzie and Winston (1989), when workers treated with diazaron showed a decreased time to foraging and a decreased lifespan. Guez *et al.* (2005) found an increased frequency of foraging after application of methyl parathion. More recently, an average four day lifespan reduction of worker bees reared in pesticide contaminated waxcomb was observed (Wu et al. 2011).

Finally, by comparing gene expression between queens and workers it may be possible to pinpoint differences between the two castes. To date, there are many genes of unknown function that are upregulated in queen castes of hymenoptera. Studies comparing gene expression between worker and queen castes have found multiple up-regulated genes in queens associated with oxidative pathways, mitochondrial genes, and insulin signaling (Grozinger et al. 2007). Honey bee larval studies showed queens express significantly higher Locus 1CB6, including hexamerins and arylphorins (Evans and Wheeler 1999). When compared to workers, Corona et al. (1999) found more AmIF-2_{mt} mRNA in developing queens. This increased translation initiation factor may result in enhanced mitochondrial activity (Corona et al. 1999). Honey bee caste gene family expression differences included oxidoreductases (metabolism), peptidases (immune function), mitochondrial (metabolism), and defensins (immune function) (Grozinger et al. 2007). Studies comparing gene expression between worker and queen castes in ants found significant upregulation in queen genes hypothesized to be involved with immune defense (Graff et al. 2007); however, this was not the case in bumble bees (Pereboom et al. 2005). Pereboom et al. (2005) did find significant upregulation of electron transport genes in queens. Apparently absent from all of these findings are genes encoding detoxification functions, which may indicate that the mechanism of queen tolerance is not linked to the capacity of carboxylesterases or cytochrome P450s.

Rational and Significance:

The intentional introduction of acaricides into beehives has resulted in a high incidence of detection of these pesticides in hive products, especially beeswax combs (Bogdanov 2006, Mullin et al. 2010). A recent survey of hives and hive products found 121 different pesticides

and metabolites in 887 wax, pollen, bee and associated hive samples (Mullin et al. 2010). Although coumaphos and tau-fluvalinate have largely been abandoned by beekeepers, tau-fluvalinate, coumaphos and its metabolite, coumaphos oxon, are still present in greater than 89% of wax samples from North America (Mullin et al. 2010).

Honey bee colonies can be overwhelmed by parasitic bee mites and bee diseases that can negatively affect the health of the colony. Beekeepers necessarily add chemicals to colonies to prevent colonies from dwindling or perishing. While miticides effectively control *Varroa* infestations, their effects on the bees themselves are not as well-established. Most pesticide research has focused on the worker caste due to the time and expense involved in raising large numbers of queens. Unfortunately, both the parasites and the chemicals used to treat parasites may be affecting the success of queens, both in developmental stages and as adults. The possibility that honey bees may be stressed by miticide accumulation within the hive and by the likely addition of future chemicals leaves beekeepers with tough choices regarding the treatment of their hives. Scientists also have problems finding a chemical that does not accumulate somewhere in the hive and lead to the rapid selection of tolerant parasitism and microsporidia. Now is the time to create an establishment of known LD50s for a variety of pesticides to queens that would aid in the search to provide a chemical with minimal sublethal effects to queens. This dissertation describes research defining differences in acaricide toxicity between queen and worker honey bees and provides a detailed examination of coumaphos metabolism.

Objectives:

Chapter 2: Establish queens and worker LD50s for five acaricides (amitraz, coumaphos, fenpyroximate, tau-fluvalinate, thymol)

Chapter 3: Compare topical contact toxicity of two phosphorothioate insecticides and their active metabolites to honey bee queens, workers, and the non-*Apis* species, *Musca domestica*.

Chapter 4: Compare phosphorothioate target sites sensitivity in honey bee queens, workers, and a non-*Apis* species, *Musca domestica*.

Chapter 5: Establish whether honey bee queens and workers metabolize coumaphos to the activated metabolite coumaphos oxon.

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**CHAPTER 2: COMPARATIVE TOXICITY OF ACARICIDES TO HONEY BEE
(HYMENOPTERA: APIDAE) WORKERS AND QUEENS**

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Abstract:

Acaricides are used to treat honey bee (*Apis mellifera* L.) colonies to control the varroa mite (*Varroa destructor* Anderson & Trueman), a worldwide threat to honey bee health. Although acaricides control a serious honey bee parasite and mitigate bee loss, they may cause harm to bees as well. We topically applied five acaricides, each with a different mode of action, to young adult queen and worker bees to generate dose-response curves and LD₅₀. Twenty-four hours after treatment, queens were found to be three-times more tolerant of tau-fluvalinate and six-times more tolerant of thymol than workers when adjusted for body weight differences between workers (108 mg) and queens (180 mg). Queens survived the highest administered doses of fenpyroximate (1620 µg/g) and coumaphos (2700 µg/g) indicating that queens are at least 11-fold more tolerant of coumaphos and at least 54-fold more tolerant of fenpyroximate than workers. However, queens treated with as little as 54 µg/g of fenpyroximate exhibited reduced survival over 6 wk after treatment. Amitraz was the only acaricide tested for which queens were not more tolerant than workers. The striking difference in acaricide tolerance of queen and worker honey bees suggests physiological differences in how the two castes are affected by xenobiotics.

Introduction:

The eusocial honey bee lives in a colony in which a single mated queen is mother to tens of thousands of semi-sterile female workers. Genetically identical female eggs may develop into either a queen or a worker depending only on their diet. Queen and worker larvae receive different diets (Beetsma 1979), with queen larvae receiving a blend of larval food that includes the protein royalactin that results in a faster growth rate, larger body size, and increased ovary development (Reginato and Cruz-Lamdim 2003, Kamakura 2011). Adult workers and queens differ physiologically in their energetic and metabolic requirements, their susceptibility to pathogens, and the circulating proteins found in the hemolymph (Chan et al. 2006). Despite pronounced differences between the castes, there is little information on how queens and workers differ in their tolerance of pesticides in general, or to the in-hive acaricides used by beekeepers to control the ectoparasitic mite, *Varroa destructor* Anderson & Trueman.

The widespread distribution of *Varroa* mites poses a global threat to honey bee colony health (Sammataro et al. 2000). *Varroa* mites weaken the hive by consuming hemolymph from pupal and adult bees and by transmitting viruses that may increase colony susceptibility to both abiotic and biotic environmental stressors (Shen et al. 2005). Many honey bee colonies infested with *Varroa* mites perish without beekeeper intervention (Kraus and Page 1995, National Research Council 2007). While labor-intensive alternatives to chemical control exist (Sammataro et al. 2000, Charrière et al. 2003, Imdorf et al. 2003) many beekeepers rely on acaricides to protect their colonies. The widespread detection of acaricide residues in beeswax (Mullin et al. 2010) serves to demonstrate the widespread use of acaricides and indicates that the exposure of bees to these compounds may continue long after acaricide treatment has ceased.

Acaricides used to control *Varroa* include compounds with different modes of action including a cholinesterase inhibitor (coumaphos), a Na-channel agonist (tau-fluvalinate), an octopaminergic agonist (amitraz), and a mitochondrial electron transport inhibitor (fenpyroximate). Natural products have been successfully used as acaricides as well, such as blends of essential oils including the monoterpenoid thymol (Johnson et al. 2010) that acts as a GABA modulator in humans and flies (*Drosophila melanogaster* (Meigen) and *Phaenicia sericata* (Meigen) (Waliwitiya et al. 2010, Priestley et al. 2003).

A vast majority of laboratory-based research on honey bee toxicology has been performed using worker bees, as mandated for pesticide registration requirements (U.S. Environmental Protection Agency 1996, Fischer and Moriarty 2011), with only a few studies examining the field effects of acaricide exposure on queens (Haarmann et al. 2002, Pettis et al. 2004). We sought to generate LD₅₀ statistics for queens that would be comparable to those commonly generated for workers in laboratory assays using five different acaricides: amitraz, coumaphos, fenpyroximate, tau-fluvalinate, and thymol. Additionally, unmated treated queens were monitored for 6 wk after treatment to assess the effects of single-dose acaricide exposure on queen longevity.

Materials and Methods:

Honey bee colonies and queen banks were located at the University of Nebraska-Lincoln East Campus (Lincoln, NE). The same colonies supplied larvae selected through grafting to become queens, were used for rearing queen cells and supplied adult workers for parallel bioassays conducted from April through September 2010. All colonies were requeened in April 2010 with Italian queens obtained from C. F. Koehnen & Sons, Inc., Glenn, CA. Bacterial brood

diseases and the gut pathogen *Nosema* were prevented with oxytetracycline as Terramycin (Pfizer, New York, NY) and fumagillin as Fumadil B (Medivet Pharmaceuticals, High River, Alberta, Canada) applied according to label instructions in March 2010. Thymol as Apiguard (Vita, Basingstoke, United Kingdom) and oxalic acid (Fischer Scientific, Rochester, NY) were the only acaricides used in the apiary to control *Varroa* populations during the 4 yr prior, with the last application in the fall of 2009.

Queens were reared using methods described by Doolittle (Laidlaw and Page 1997) in which individual 12-24 h old worker larvae were grafted into plastic cups (JZ BZ Base Mount Cell Cups, Mann Lake Supply, Ltd., Hackensack, MN). Ripe capped queen cells were placed into queen confinement cages (QC-115, Mann Lake Supply) and a small drop of queen candy (1:1 honey and confectioners' sugar) was placed in the base of each individual cage to ensure that newly emerged virgins had access to food until they became attractive to workers (~4 d). As many as 60 caged queen cells were placed in a queen bank to emerge. Queen banks consisted of four or five frame nucleus colonies stocked with 1 kg of worker bees (Laidlaw and Page 1997). Frames of larvae were added weekly to maintain colony strength and frames were inspected twice per week to remove any uncaged queen cells.

Worker bees were emerged from frames filled with late-stage capped brood in a dark 32-34°C incubator. Newly eclosed bees were brushed from frames daily and placed inside an 8-mesh screen-wooden cage (15.25 cm: 10.15 cm: 17.75 cm) (l:w:h). Caged worker bees were provided 1:1 sugar-water, stored in an incubator at 32-34°C, and maintained for 3-4 d before pesticide application. Technical grade chemicals were used for all trials. Amitraz, coumaphos, fenpyroximate, and tau-fluvalinate were purchased from Chem Services Inc. (West Chester, PA).

Thymol was purchased from Aldrich (Milwaukee, WI). ACS grade acetone (Fisher, Pittsburg, PA) was used to dilute all chemicals and was applied to bees in pure form as a solvent control.

Bioassays were conducted using methods described by Johnson et al. (2006). A range of acaricide concentrations were made through serial dilutions in acetone on the day of treatment for queen bioassays, and up to a month prior, with storage at -20°C , for worker bioassays.

Queen bioassays were performed on a minimum of 15, 2- to 5-d old adult virgin queens removed from queen banks for no ≥ 1 h. Queens were briefly anesthetized with CO_2 and treated topically on the thoracic notum in two $1\ \mu\text{l}$ applications using a $50\ \mu\text{l}$ syringe fitted to a repeating microapplicator (PB-600, Hamilton, Reno, NV). Controls were treated with two $1\ \mu\text{l}$ doses of acetone. When it was possible, acaricide treatment series included doses causing 0 and 100% mortality. Each treatment was replicated at least three times using queens from different source colonies for each treatment date. After treatment queens were weighed to the nearest milligram using an analytical balance (Sartorius AG R160P, Goettingen, Germany). Virgin queens were then placed back into the same emergence cage with a new cap (a brown cell cup; QC-110 Mann Lake Supply) and yellow cell cup holder (QC-125, Mann Lake Supply) and returned to a queen bank. Mortality was assessed at 24 h, 48 h, and then every week for 6 wk after treatment. Immobile queens were scored as dead and removed from queen banks.

Worker bioassays were conducted similarly to queens, but bees were treated with a single microliter of acaricide or acetone and were kept in groups of 17-23 inside wax-coated paper cups ($177\ \text{cm}^3$) covered with bleached cotton cheesecloth. Groups of bees were fed 1:1 sucrose-water in a punctured 1.5 ml Eppendorf tube and returned to the incubator.

Acute mortality in both workers and queens, defined as mortality recorded at 24 or 48 h after treatment, was analyzed by log-probit analysis in the R statistical environment (The R

Development Core Team 2010) with MASS libraries (Venables and Ripley 2002). Lethal dose (LD) values with 95% CIs were calculated using Fieller's method with correction for heterogeneity where appropriate (Finney 1971). Treatment series with >5% control mortality were removed from analyses. Fenpyroximate treatment was compared at the 48 h time point, because of the relatively slow toxicity this compound shows in worker bees, while all other acaricides were compared at the 24 h time point.

A comparison of long-term queen survival of treated queens, using only those queens surviving beyond 48 h, was performed against control queens by pairwise exact log rank tests using the "coin" library in R (Everitt and Hothorn 2006), which allow for comparisons between groups of different sizes. Significance cutoffs were corrected for multiple comparisons using the Bonferroni method.

Results:

Toxicological statistics are reported as microgram toxin per gram of body weight to facilitate comparisons between castes with different masses (virgin queens 180 mg and workers 108 mg). The relative toxicities of the five acaricides to workers were amitraz fenpyroximate tau-fluvalinate coumaphos thymol (Table 1). The relative toxicities of acaricides to queens were amitraz tau-fluvalinate thymol. No significant differences in mortality occurred between 24 and 48 h for each acaricide except fenpyroximate; therefore, only one value is reported in Table 1 for both workers and queens. Dose-response curves for queens could not be determined for fenpyroximate or coumaphos as both compounds caused little mortality at 24 and 48 h time points at the highest doses that could be tested (1620 µg/g for fenpyroximate and 2700 µg/g for coumaphos).

Twenty-four hours after exposure queens were six times more tolerant of thymol and three times more tolerant of tau-fluvalinate than were workers (Table 1). Queens were at least 54 times more tolerant of fenpyroximate than workers, based on a comparison of the highest dose administered to queens and the 48 h worker LD50. Queens were at least 11 times more tolerant than workers of coumaphos based on the highest dose administered to queens and the 24 h worker LD50. No difference in susceptibility to amitraz was observed between queens and workers.

Kaplan-Meier survival estimates were plotted for each acaricide treatment level, as well as the control treatment for each group, with right-censoring of queens surviving beyond the 6-wk observation window (Fig. 1). No longevity data were collected for workers. Queens that had survived the initial 48 h after treatment with any dose of amitraz, coumaphos, or tau-fluvalinate were as likely as control-treated queens to survive to 6 wk (Fig. 1A-C). Queens receiving 2700 $\mu\text{g/g}$ thymol were significantly less likely ($P=0.011$) to survive from 48 h to 6 wk than controls, but other doses did not significantly affect the longevity of queens (Fig. 1D). Queens receiving fenpyroximate at any dose level were as likely as control queens to survive to 48 h ($P=0.17$; Fisher Exact Test). However, queens receiving any dose of fenpyroximate 27 $\mu\text{g/g}$ were significantly less likely than controls to survive the period from 48 h to 6wk ($P=0.0071$; Exact Logrank Test) (Fig. 1E).

Discussion:

This study showed that, when adjusted for differences in body weight, queens and workers differ substantially in their tolerance of most acaricides, with queens tolerating higher doses of coumaphos, fenpyroximate, thymol, and tau-fluvalinate. This is not the first time that

queens have been found to be more tolerant of pesticides than workers: the queen LD50 for the organochlorine DDT was found to be 6.6 times higher than that of honey bee workers (Graves and Mackensen 1965). These acaricides are presumed to kill arthropods through different modes of action (Johnson et al. 2010) and yet queens were found to be more tolerant of all acaricides, except amitraz, suggesting that queens possess a nonspecific mechanism of acaricide tolerance.

In contrast to all other acaricides tested, queens and workers tolerate equal amounts of amitraz, indicating that any mechanism of nonspecific pesticide tolerance with which queens are endowed is not effective against this compound. Amitraz is an octopaminergic agonist in most arthropods. Octopamine is important for honey bee dance language (Barron et al. 2007), division of labor (Schulz and Robinson 2001, Barron and Robinson 2005), and kin recognition (Robinson et al. 1999), yet there are no reports that the use of amitraz as an acaricide alters social behavior in bees. The mode of action of amitraz against honey bees is unknown, however, amitraz has been shown to increase the frequency of cell death in larval midgut cells (Gregorc and Bowen. 2000, Gregorc and Ellis 2011). In addition, unlike most insecticides that possess an ester bond, amitraz has been suggested to be bioactivated by hydrolysis (Knowles and Gayden 1983, Schuntner and Thompson 1978). As a consequence, differences in hydrolytic enzyme activity between queen and worker castes may contribute to differential sensitivity among the compounds tested. Nonspecific mechanisms of pesticide tolerance may include nutritional state, sequestration, detoxification and clearance, or tolerance of pesticide induced stress. Each possibility is explored in the next few paragraphs.

Feeding on high quality pollen from diverse plant sources is known to decrease the susceptibility of adult bees to pesticides (Wahl and Ulm. 1983) and diseases (Alaux et al. 2010). Some of the difference between workers' and queens' susceptibility to acaricides may be

because workers in this experiment were maintained in an incubator, without access to any protein source, while queens were maintained in queen banks and were likely fed protein by attending worker bees. Thus, the superior queen adult diet may be responsible for some of the reduced acaricide susceptibility observed in queens.

In insects, the fat body serves many vital functions (Arrese and Soulages 2010) and it is therefore not surprising that longevity mechanisms occur within the fat body (Hwangbo et al. 2004). Winter worker bees have greater dry weight, protein, and fat contents (Kunert and Crailsheim. 1988) and live significantly longer (3-5 mo) than summer workers (30 d). Queens, which can live several years, are generally more tolerant of acaricides, and it is possible that queens have greater capacity to sequester or metabolize these acaricides. The insect fat body functions in insect immune response and is analogous to the mammalian liver and adipose tissue (Canavoso et al. 2001), serving functions of xenobiotic metabolism, sequestration, and energy storage.

Nonspecific mechanisms that may allow queens to tolerate higher levels of coumaphos, tau-fluvalinate, thymol, and fenpyroximate may be elucidated through finding similarities in how these compounds are managed in vivo in honey bees. Coumaphos, tau-fluvalinate, and possibly thymol all upregulate cytochrome P450 genes within the honey bee (Mao et al. 2011, Boncristiani et al. 2012). Coumaphos is an organophosphate. Organophosphates as a class of insecticides are dependent on cytochrome P450s for activation. Johnson et al. (2009) suggested that both coumaphos and tau-fluvalinate are detoxified by cytochrome P450 and carboxylesterases in honey bees. Using heterologously expressed honey bee cytochrome P450s, Mao et al. (2011) found both coumaphos and tau-fluvalinate are indeed metabolized. In insects, cytochrome P450s are expressed in the midgut, fat body, Malpighian tubules, nervous tissue,

corpora allata, glands, and antennae with the midgut often expressing the highest level of activity. There are at least three midgut cytochrome P450 enzymes responsible for the detoxification reactions catalyzed in workers: CYP9Q1, CYP9Q2, and CYP9Q3 (Mao et al. 2011). CYP6S14 upregulation was triggered in workers by thymol treatment with Apistan (Boncristiani et al. 2012). Cockroaches have been demonstrated to have age-dependent changes in levels of cytochrome P450s (Turnquist and Brindley 1975). Paralleling this hypothesis, Valles et al. (1994, 1996), demonstrated that male cockroaches were more susceptible to insecticides than older male and female nymphs. Adult males were found to have decreased cytochrome P450 content and methoxyresorufin O-demethylase and ethoxyresorufin O-deethylase activities in comparison with the more tolerant nymphs (Valles et al. 1996). Likewise, it is possible that bees could have caste-dependent changes in levels of cytochrome P450s, with queens having higher titers than workers.

Finally, differences between queen and worker tolerance may be because of a greater tolerance of pesticide induced stress. Honey bee queens have been shown to tolerate higher levels of pesticide-induced oxidative stress than workers bees (Corona et al. 2007). One potential commonality between coumaphos, tau-fluvalinte, fenpyroximate, and thymol is that all may cause oxidative stress. Oxidative stress induced by pesticides is characterized by oxygen free radical formation, induction of cytochrome P450s, alteration in antioxidant enzymes, and a glutathione redox system (Banerjee et al. 2001). Therefore, one hypothesis is that queens are able to tolerate greater amounts of each of these pesticides because of their intrinsic ability to tolerate higher levels of reactive oxygen species. The ability for queens to tolerate greater oxidative stress than workers may come from differences in the fatty acid composition of their respective membranes (Haddad et al. 2007). Newly emerged workers and queens, which have

less polyunsaturated and more monounsaturated fatty acids, were found to be more resistant to peroxidation than other workers (Haddad et al. 2007). Monounsaturated phospholipids are less susceptible to peroxidation than polyunsaturated phospholipids. It may be that queens maintain a low ratio of peroxidation-susceptible polyunsaturated fats throughout life through consumption of a filtered diet of jelly, which has been shown to contain little to no polyunsaturated fats, provided to them by workers (Haddad et al. 2007).

As postulated by Amdam and Omholt (2002), it is possible that vitellogenin plays a role in the tolerance of xenobiotics. Worker lifespan is known to be influenced by the synthesis of vitellogenin, a glyco-lipoprotein produced in the fat body (Seehuus et al. 2006). Vitellogenin is a female-specific yolk precursor in many oviparous species that functions in honey bees in brood food production (Seehuus et al. 2007), regulation of foraging behavior, longevity independent of behavior (Nelson et al. 2007), as an anti-oxidant (Seehuus et al. 2006) and in enhancing innate immunity (Amdam et al. 2004, Amdam et al. 2005). Vitellogenin has been shown to affect adult longevity of both queens and workers (Amdam et al. 2009), partially because of its free-radical scavenging capacity (Seehuus et al. 2006). In addition, vitellogenin comprises up to 50% of the total hemolymph protein in queens (Engels et al. 1990), a 20-fold higher concentration than in summer workers (Engels et al. 1990, Corona et al. 2007). If acaricide exposure causes oxidative stress in bees, as pesticide exposure does in mammals (Abdollahi et al. 2004), then the greater tolerance to acaricides exhibited by queens may be mediated by the anti-oxidant capacity of vitellogenin.

Fenpyroximate administered at doses as high as 2,700 $\mu\text{g/g}$ caused little mortality in queens observed for 48 h. However, fenpyroximate treatment at doses as low as 54 $\mu\text{g/g}$, or 9.7 $\mu\text{g/queen}$, caused a significant reduction in queen survival over the subsequent 6 wk. This

acaricide functions as an inhibitor of energy metabolism in the mitochondria through interactions with complex I (Motoba et al. 2000). In mammalian neuroblastoma cells, fenpyroximate and other complex I mitochondrial inhibitors cause ATP depletion, and cell death, possibly through generation of reactive oxygen species (Sherer et al. 2007). The effects of fenpyroximate on queen longevity is of particular practical concern as it is quite plausible that queens could encounter fenpyroximate at this level if the formulated product, Hivastan, which contains 67.5 mg fenpyroximate in a patty formulation, is used.

Finally, clues to the high xenobiotic tolerance of queens as compared with workers may be elucidated through comparing gene expression between the two castes. To date, there are many genes of unknown function that are upregulated in queen castes of hymenoptera. Here we discuss those genes with known or hypothesized functions. Studies comparing gene expression between worker and queen castes have found multiple up-regulated genes in queens associated with oxidative pathways, mitochondrial genes, and insulin signaling (Grozinger et al. 2007). Honey bee larval studies showed queens express significantly higher Locus 1CB6, including hexamerins and arylphorins (Evans and Wheeler 1999) and increased AmIF-2mt mRNA, suggesting increased mitochondrial translation in queens (Corona et al. 1999). Honey bee caste gene family expression differences included oxidoreductases (metabolism), peptidases (immune function), mitochondria (metabolism), and defensins (immune function) (Grozinger et al. 2007). Studies comparing gene expression between worker and queen castes in ants found significant upregulation in queen genes hypothesized to be involved with immune defense (Graff et al. 2007); however, this was not the case in bumble bees (Pereboom et al. 2005). Pereboom et al. (2005) did find significant upregulation of electron transport genes in queens. Apparently absent from all of these findings are genes encoding detoxification functions, which may indicate that

the mechanism of queen tolerance is not linked to the capacity of carboxylesterases or cytochrome P450s.

Understanding the differences in how queens and workers respond to toxins may facilitate the design and discovery of acaricides that are tolerated by bees. Reciprocally, it may also provide insight into target traits for selecting bees that are better able to tolerate exposure to toxins. The assessment of the response of individual virgin queens to toxicants requires more time and resources than assessing worker bee responses, but is still far easier than assessing queen survival in a whole-colony setting. Our results indicate that this is a valuable field of inquiry and will likely increase our understanding of molecular, metabolic, and endocrine processes of how honey bees and social insects deal with chemical stressors in their environment. The increased understanding of pesticide interactions within the honey bee may also lead to alleviation of current health problems seen in honey bees around the world.

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Table 1:

Acaricide	Time (hours)	Queen LD₅₀ µg/g (95 % C.I.)	Queen N	Worker LD₅₀ µg/g (95% C.I.)	Worker N	Queen Toxicity Relative to Workers
Amitraz	24	21.8 (17.8-29.4)	36	26.7 (17.8-36.6)	552	0.816
Coumaphos	24	>2700	14	222 (144.4-362.9)	328	>12.2*
Fenpyroximate	48	>1620	16	39.9 (33.3-47.4)	771	>40.6*
Tau-fluvalinate	24	586 (439-837)	90	188 (155-242)	528	3.11*
Thymol	24	3240 (2680-4520)	74	524 (401-666)	902	6.18*

* Differences between castes considered significant if confidence intervals did not overlap

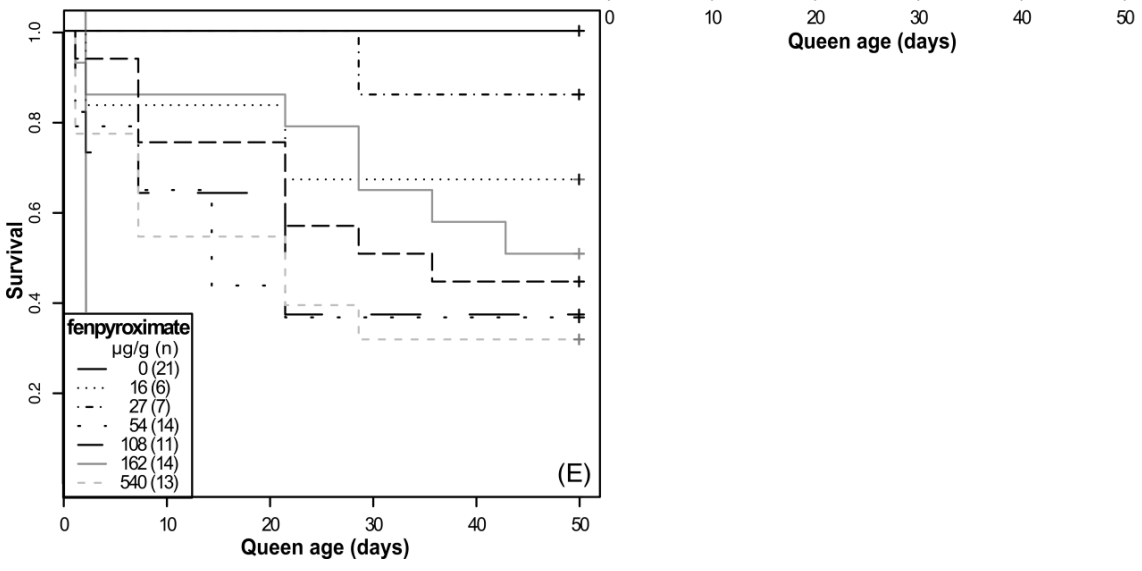
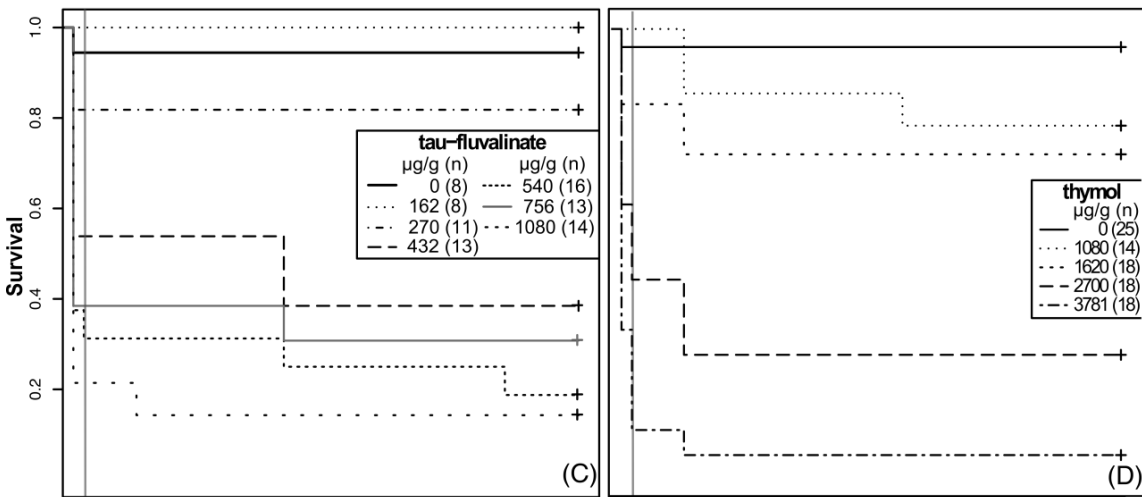
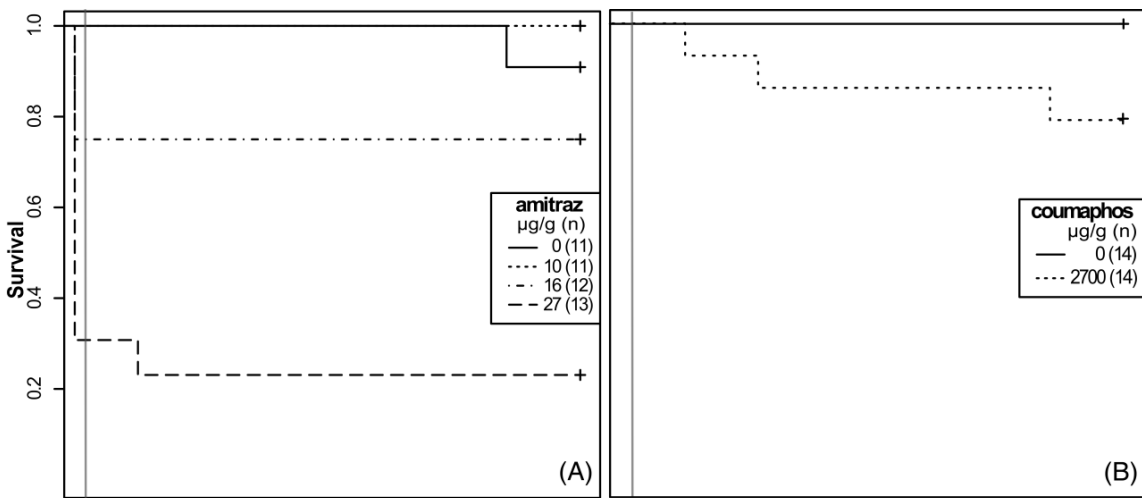


Figure 1. Kaplan-Meier survival curves for five acaricides applied to 3-d-old virgin queens honey bees. Acaricides included (A) amitraz, (B) coumaphos, (C) tau-fluvalinate, (D) fenpyroximate, and (E) thymol. Vertical lines indicate the 48 h timepoint. Line styles represent queen mortality for acaricide dose ($\mu\text{g/g}$). The number of queens treated for each dose is given in parenthesis after the dose $\mu\text{g/g}$. In general, mortality increased with increased acaricide dose except when treated with (D) fenpyroximate.

CHAPTER 3: COMPARATIVE TOXICITY OF THIOPHOSPHATES AND ACTIVATED METABOLITES IN QUEEN AND WORKER HONEY BEES

Abstract:

Acaricides are used to treat honey bee (*Apis mellifera* L.) colonies to control the varroa mite (*Varroa destructor* Anderson & Trueman), a worldwide threat to honey bee health. Although acaricides are used successfully to control a serious honey bee parasite and mitigate bee loss, it is not clear exactly how bees tolerate these drugs. To better understand coumaphos tolerance, we compared the contact toxicity of coumaphos and its activated metabolite, coumaphos oxon, as well as a comparable phosphorothioate, chlorpyrifos, and its active metabolite chlorpyrifos oxon to queens, workers, and to a non *Apis* species, the common housefly, *Musca domestica*. Twenty-four hours after treatment, workers and queens were seen to be highly susceptible to chlorpyrifos (>500-fold more susceptible relative to coumaphos), with chlorpyrifos oxon being more than twice as toxic as the parent compound. In contrast, coumaphos was >2- and >4-fold more toxic than coumaphos oxon to workers and queens, respectively. Additionally, house flies were equally susceptible to both the parent and activated metabolite for both phosphorothioates highlighting the uniqueness of coumaphos oxon tolerance in honey bees which were only 7-fold more tolerant of coumaphos than chlorpyrifos. The results strongly suggest that the mechanism by which honey bees tolerate high doses of coumaphos is unique to this molecule and is unique to honey bees as flies did not exhibit the same tolerance.

Introduction:

Organophosphate (OP) insecticides were discovered in Germany during World War II while developing nerve gas agents. All OPs are phosphoric acid derivatives with substitutions of hydrogen atoms with organic radicals such as methyl, ethyl or phenyl and the hydroxyl group substituted with sulfur, nitrogen, or carbon (Yu 2008). Thiophosphates, defined by a phosphorous double bonded to sulfur ($P = S$) in place of oxygen, are one of six subclasses of OPs (Yu 2008). Thiophosphates are weak inhibitors of acetylcholinesterase, but when activated *in vivo* to the active phosphate ester or oxon ($P = O$), become a potent inhibitor of acetylcholinesterase.

The thiophosphate acaricide, coumaphos, is a lipophilic compound first registered in 1958, to control parasites and biting flies of cattle, goats, horses, sheep, and swine such as cattle grubs, screwworms, lice, scabies, flies, and ticks (Yu 2008). The recommended use has been amended by the manufacturer to no longer include control for biting flies in sheep and goats (U.S. Environmental Protection Agency 2006).

In 1999, coumaphos became the third acaricide (following tau-fluvalinate and amitraz) registered for use in beehives in the U.S.A. to control the parasitic mite, *Varroa destructor*, and more recently to control the small hive beetle (U.S. Environmental Protection Agency 2006). Widespread application across U.S. beehives led to the rapid development of coumaphos resistance in *Varroa* by 2001 (Elzen and Westervelt 2002). The acaricide is still available for purchase and is used to reduce populations of the small hive beetle (Johnson et al. 2010). In a recent survey of pesticide contaminants of bee hives, the top three most common pesticides found in wax samples included coumaphos and coumaphos oxon (Mullin et al. 2010). The high

detection rate parallels the widespread use of this pesticide in beehives and the potential for its persistence in beeswax comb long after acaricide treatment has ceased (Johnson et al. 2010).

The mode of action of organophosphates (OP) insecticides is to inhibit acetylcholinesterase (AChE), an enzyme responsible for hydrolysis of the excitatory neurotransmitter, acetylcholine, at neuromuscular and neuronal synapses. AChE first forms a reversible complex with the OP, followed by hydrolysis and phosphorylation. This results in inhibition due to the stability of the phosphorylated AChE. The phosphorylated enzyme can no longer bind to acetylcholine resulting in its accumulation at the synapse. The excess acetylcholine binds to acetylcholine receptors on the post synaptic membrane, resulting in excitation, paralysis, and with a high enough dose, eventual death of the organism (Perry et al. 1998).

For thiophosphates, cytochrome P450s are known to catalyze sulfoxidation of the parent compound resulting in activation to the more toxic oxon metabolite (Perry et al. 1998, Siegfried and Scharf 2001). P450s convert phosphorothioates to the sulfoxides which inhibit AChE (Figure 1; reviewed in Perry et al. (1998)). Activation of coumaphos to the more potent cholinesterase inhibitor, coumaphos oxon, has been demonstrated *in vitro* in a number of arthropods including the common house fly *Musca domestica*, third instar cattle grub *Hypoderma bovis* (L.) (O'brien and Wolfe 1959), and cockroaches (Vickery and Arthur 1960).

Model of Interest:

In order for coumaphos to be effective as an acaricide for controlling *Varroa* mites with minimal effects on bee health, bees must be capable of tolerating high levels of coumaphos. However, it is unknown whether bees are also capable of tolerating equally high doses of the

coumaphos oxon metabolite. Differences in toxicity of the activated metabolite and the parent thiophosphate may shed light on the role of activation of coumaphos within bees as a mechanism conferring high levels of tolerance. In this study, the LD₅₀ (lethal dose at which 50% die) of coumaphos oxon for queens, workers and a positive control of a susceptible non-*Apis* species, the common house fly, *Musca domestica*, were estimated. In addition, the LD₅₀s of chlorpyrifos and chlorpyrifos oxon were compared to determine whether honey bees' tolerance of coumaphos is unique to that compound or if honey bees are tolerant of phosphorothioates in general. Chlorpyrifos was chosen as a comparable phosphorothioate because of its similarity to coumaphos in structure (Figure 1) and physical properties. The partition coefficient of coumaphos is 4.1 while the solubility in water is 1.5 mg/L at 20 °C (Finizio et al. 1997). Chlorpyrifos' partition coefficient is 4.7 while the solubility in water is 1.4 mg/L at 25 °C (Tomlin 2006).

Coumaphos oxon and chlorpyrifos oxon represent the activated metabolites of coumaphos and chlorpyrifos, respectively. If bees are more susceptible to the activated metabolites, the mechanism of tolerance may be associated with reduced rates of activation. In other words, bees and flies may tolerate less oxon metabolite than the parent compound because the metabolites are already in the active state. In addition, comparing the ratio of toxicity for the parent phosphorothioates and the respective oxon metabolites between honey bees and house flies will provide insight to the mechanism by which bees tolerate higher doses of coumaphos.

Materials and Methods:

Insects. Honey bee colonies and queen banks (Laidlaw and Page 1997) were located at the University of Nebraska-Lincoln East Campus (Lincoln, NE). The same apiary supplied larvae

grafted to become queens and adult workers for parallel bioassays conducted from May through August 2011. All colonies were requeened in April 2011 with Italian queens obtained from C. F. Koehnen & Sons, Inc., Glenn, CA. Bacterial brood diseases and the gut pathogen *Nosema* were prevented with oxytetracycline (Duramycine-10, Durvet Inc., Blue Springs, MO) and Fumagillin-B (Medivet Pharmaceuticals Ltd., High River, Alberta, Canada) respectively, applied according to label instructions in March 2011 (Medivet Pharmaceuticals Ltd. 2010). Thymol as Apiguard® (Vita, Basingstoke, United Kingdom) and oxalic acid (Fischer Scientific, Rochester, NY) were the only acaricides used in the apiary to control *Varroa* populations during the 5 yr prior to the bioassays, with the last application in the fall of 2010.

Queens were reared using methods described by Doolittle (Laidlaw and Page 1997) in which individual 12-24 h old worker larvae were grafted into plastic cups (JZ BZ Base Mount Cell Cups, Mann Lake Supply, Ltd., Hackensack, MN). Ripe capped queen cells were placed into queen confinement cages (QC-115, Mann Lake Supply) and a small drop of queen candy (2:1 confectioners' sugar and honey) was placed in the base of each individual cage to ensure that newly emerged virgins had access to food until they became attractive to workers (~4 d). As many as 60 caged queen cells were placed in a queen bank to emerge. Queen banks consisted of colonies in 5-frame nucleus boxes (KD-120, Mann Lake Supply) stocked with 1 kg of worker bees (Laidlaw and Page 1997). Frames of larvae were added weekly to maintain colony strength and frames were inspected twice per week to remove any queen cells started on the brood combs.

Worker bees emerged from frames filled with late-stage capped brood in a dark 32-34°C incubator. Newly eclosed bees were brushed from frames daily and placed inside an 8-mesh screen-wooden cage (15.25 x 10.15 x 17.75 cm). Caged worker bees were provided a 1:1 by

weight sugar-water solution, stored in an incubator at 32-34°C, and maintained for 3-4 d before pesticide application.

House fly colonies maintained by the USDA-ARS Agroecosystem Management Research Unit (Lincoln, NE) were used for bioassays conducted in October 2011. All stages were reared in the same rearing room at 25 °C, 50% RH, 12:12 L:D. Adult house flies were transferred to a small cage (30 x 30 x 30 cm) (All Aluminum Window Co., Lincoln, NE) and provided a 10% sugar-water solution via a 125 mL bottle (Nalgene, 312088-0004) with a dental wick. Adult flies were aged 3-4 d before pesticide application.

Chemicals. Technical grade chemicals were used for all trials. Coumaphos, coumaphos oxon, chlorpyrifos, and chlorpyrifos oxon were purchased from Chem Services Inc. (West Chester, PA). ACS grade acetone (Fisher, Pittsburg, PA) was used to dilute all chemicals and was applied to bees in pure form as a solvent control.

Bioassays. Bioassays were conducted using methods described by Johnson et al. (2006). A range of concentrations was prepared as serial dilutions in acetone on the day of treatment for queen bioassays, and up to a week prior, with storage at -20°C, for worker and house fly bioassays.

Queen bioassays were performed on a minimum of three replications of 15, two- to five- d old adult virgin queens removed from queen banks and handled at room temperature for \leq 1 hr. Queens were briefly anesthetized with CO₂ and treated topically on the thoracic notum in two or four 1 μ l applications (coumaphos/oxon at 250 μ g/ μ L) using a 50 μ l syringe fitted to a repeating microapplicator (PB-600, Hamilton, Reno, NV). Controls were treated with two or four 1 μ l

doses of acetone. Doses were as follows ($\mu\text{g}/\text{bee}$): chlorpyrifos (0, 0.14, 0.2, 0.3, 0.4); chlorpyrifos oxon (0, 0.04, 0.06, 0.08, 0.12, 0.18); coumaphos (0, 1000); coumaphos oxon (0, 1000). As coumaphos does not cause mortality at the highest dose tested (Dahlgren et al. 2012), and no significant mortality (2 of 12 queens) was seen at the same dose with coumaphos oxon, only a single dose was tested for each compound to confirm the high level of tolerance. Each treatment was replicated at least three times using queens from different source colonies for each treatment date. After treatment, a single virgin queen was maintained with 10 untreated worker attendants inside wax-coated paper cups (177 cm^3) covered with bleached cotton cheesecloth. Groups of bees were fed 1:1 by weight sucrose-water in 2 1.5 mL Eppendorf tubes perforated with 2 holes at the tip end and returned to the incubator.

Worker bioassays were conducted with methods similar to those used for queen bioassays, except that workers were treated with a single microliter of acaricide or acetone and were kept in groups of 17-23 inside wax-coated paper cups (177 cm^3) covered with bleached cotton cheesecloth. Groups of bees were fed 1:1 sucrose-water in 2 punctured 1.5 ml Eppendorf tubes perforated with 2 holes at the tip end and maintained at $33^\circ\text{C} \pm 1^\circ\text{C}$ in an environmental chamber (H024, Darwin Chambers Co, St. Louis, MO). Acaricide treatment dilutions included doses causing 0 and 100% mortality when possible. Doses were as follows ($\mu\text{g}/\text{bee}$): chlorpyrifos (0, 0.03, 0.04, 0.05, 0.1); chlorpyrifos oxon (0, 0.01, 0.013, 0.17, 0.2, .025); coumaphos (0, 3, 20, 50, 100); coumaphos oxon (0, 5, 50, 75, 150).

Adult *Musca domestica* of mixed sexes were anesthetized with CO_2 and treated with 0.5 μL of acaricide as previously described. Treated flies were maintained in groups of 17-23 inside wax-coated paper cups (177 cm^3) covered with bleached cotton cheesecloth. Doses were as follows ($\mu\text{g}/\text{fly}$): chlorpyrifos (0, 0.005, 0.015, 0.05, 0.15, 0.5); chlorpyrifos oxon (0, 0.005,

0.015, 0.05, 0.15, 0.5); coumaphos (0, 0.05, 0.15, 0.5, 1.5, 5); coumaphos oxon (0, 0.015, 0.05, 0.15, 0.5, 5, 20). Each cup of flies received a 10% sucrose-water solution on a soaked dental wick and were maintained at room temperature with natural daylight (10.5-11.75 : 12.25-13.5 L:D October 2011).

Statistical analysis:

Acute mortality of both workers and queens was recorded at 24 h after treatment, and analyzed by log-probit analysis in the R statistical environment (The R Development Core Team 2010) with MASS libraries (Venables and Ripley 2002). Lethal dose (LD) values with 95% confidence intervals were calculated using Fieller's method with correction for heterogeneity where appropriate (Finney 1971). Bioassays with >5% mortality in the control group were not included in analyses. All acaricides were compared at the 24 h time point.

Results:

The relative toxicity of topically applied phosphorothioates and their oxon metabolites to worker and queen bees is described in Table 1. Queens were 2, 2, >12, and >5 times more tolerant of chlorpyrifos, chlorpyrifos oxon, coumaphos, and coumaphos oxon, respectively, than workers which is consistent with the generally higher tolerance of queens to a variety of modes of action noted previously (Dahlgren et al. 2012). Chlorpyrifos oxon was 2-fold more toxic to both workers and queens than chlorpyrifos while coumaphos was >2- and >4-fold more toxic than coumaphos oxon to workers and queens, respectively.

House flies were equally susceptible to both the oxon metabolite and the parent compound for both coumaphos and chlorpyrifos and were 7-fold more tolerant of coumaphos

than chlorpyrifos. House flies were 3- and 9-fold more tolerant of chlorpyrifos and 9- and 25-fold more tolerant of chlorpyrifos oxon than queen and worker bees, respectively. In contrast, house flies were >108- and 8-fold less tolerant of coumaphos and >220- and >40-fold less tolerant of the oxon than queen and worker bees respectively. House fly LD₅₀s for coumaphos and coumaphos oxon were approximately twice that reported in Vickery and Arthur (1960). However, the ratio of the values remained the same with house flies being equally susceptible to both coumaphos and the oxon.

Discussion:

This study shows that, when adjusted for body weight, honey bees are extremely tolerant of coumaphos, with the active metabolite, coumaphos oxon, being only half as toxic as the parent compound (Table 1). To reiterate, this apparent insensitivity to both coumaphos and coumaphos oxon is unique as both workers and queens were highly susceptible to chlorpyrifos (>500-fold more susceptible relative to coumaphos), with chlorpyrifos oxon being more than twice as toxic as the parent compound. Furthermore, house flies were equally susceptible to both the parent and metabolite for both phosphorothioates highlighting the uniqueness of coumaphos oxon tolerance in honey bees. As in previous publications comparing queen and worker susceptibility (Graves and Mackensen 1965, Dahlgren et al. 2012), queens were more tolerant of all chemicals tested than were workers.

The results in Table 1 strongly suggest that the mechanism by which honey bees tolerate high doses of coumaphos is unique to this molecule and is unique to honey bees as flies did not exhibit the same tolerance. Pesticide tolerance mechanisms include penetration barriers, target site insensitivity, increased rate of detoxification, and a reduced rate of activation (Yu 2008).

The insect cuticle makes up the first barrier preventing topically applied chemicals from reaching their target site. The cuticle is made up of an outer epicuticle comprised of lipids, lipoprotein and proteins, and the inner procuticle (exo- and endo-cuticle) made of chitin-protein layers. The outermost layer, the epicuticle, is lipophilic, while the inner layers of the cuticle are more hydrophilic (Yu 2008). House fly strains resistant to organophosphates and carbamates known to show reduced penetration as a resistance mechanism had higher total lipids, monoglycerides, diglycerides, fatty acids, sterols, and phospholipids in the cuticle (Patil and Guthrie 1979). As queens may have greater total lipids than workers, reduced penetration and/or sequestration may partly explain why queens tolerate higher doses of insecticides relative to workers. However, as queens and workers were equally susceptible to amitraz, and amitraz has an intermediate partition coefficient of chemicals tested in Chapter 2 (Dahlgren et al. 2012), it is unlikely that penetration is the only tolerance mechanism bees may possess. In addition, honey bees are generally not more tolerant of organophosphates than house flies based on results with chlorpyrifos and also reported by Hardstone and Scott (2010).

Target site insensitivity may also be a contributing factor to coumaphos tolerance in honey bees. Resistance to phosphorothioate organophosphates may be due, at least in part, to insensitive AChE (Siegfried and Ono 1993, Moores et al. 1996, Siegfried and Scharf 2001). Considering honey bees tolerated coumaphos oxon, the active metabolite of coumaphos, in greater quantity than the parent compound (Table 1), it is possible honey bee AChE is insensitive to coumaphos oxon. If honey bee acetylcholinesterase does not easily bind to coumaphos oxon, differences between workers and queens may be explained by differences in target site interactions. Increased tolerance of coumaphos and chlorpyrifos in queens may be due to a reduced affinity of queen AChE to the phosphorothioate metabolites or to an increased activity

of queen AChE in comparison to workers and flies. This possibility will be explored in the next chapter.

Differences in biotransformation including an increased rate of detoxification or a reduced rate of activation could also play a part in coumaphos tolerance. Piperonyl butoxide (PBO) is a model inhibitor of microsomal oxidation performed by cytochrome P450s (Hodgson and Levi 1998) and is often used as a synergist in insecticide bioassays. However, as cytochrome P450s activate organophosphates, when PBO is applied with an organophosphate, antagonism is often observed. Antagonism of PBO with the organophosphates malathion and parathion has been shown in *Daphnia magna* (Rider and LeBlanc 2005), chlorpyrifos in the German cockroach (Siegfried et al. 1990), parathion in the greenbug (Siegfried and Ono 1993) and methyl parathion in the corn rootworm (Miota et al. 1998). Dose-dependent antagonism has been shown when PBO was applied at high doses in combination with diazinon in both resistant and susceptible strains of horn fly *Haematobia irritans irritans* (Li et al. 2007a). In contrast, PBO has also been observed to synergize toxicity of some phosphorothioates providing evidence that P450s also play a role in metabolic detoxification. Synergism of PBO with coumaphos has been observed in resistant strains of cattle ticks *Boophilus microplus* (Li et al. 2003) and at low concentrations of PBO in combination with diazinon with resistant strains of horn flies *Haematobia irritans irritans* (Li et al. 2007a). The honey bees' mechanism of tolerance to coumaphos has yet to be determined (Johnson et al. 2009), but may involve detoxification via cytochrome P450s (Mao et al. 2011). In worker bees, piperonyl butoxide (PBO) synergizes coumaphos toxicity indicating the involvement of cytochrome P450s in coumaphos metabolism and detoxification (Johnson et al. 2009).

Within the honey bee, coumaphos is likely detoxified by cytochrome P450s based on topical bioassays to workers where synergism was seen between coumaphos and PBO (known to inhibit P450s) (Johnson et al. 2009, Johnson et al. 2013). This was further confirmed when Mao et al. (2011) heterologously expressed honey bee midgut P450s *in vitro*, demonstrating that CYP9Q1, CYP9Q2, and CYP9Q3 enzymes metabolized coumaphos. Differences seen between queens and workers may be due in part to expression differences between these particular P450s. Differences seen between bees and flies may also be due to differences between P450 detoxification capacity. In *Musca domestica* L., resistance to diazinon is due to overexpression of CYP6A1 which detoxifies diazinon faster than it catalyzes oxidation of the parent thiophosphate to diazoxon (Sabourault et al. 2001). Detoxification of diazinon, a phosphorothioate, is also mediated by CYP12A1 in the house fly (Guzov et al. 1998) and in *Drosophila melanogaster*, CYP6A2 (Dunkov et al. 1997).

Hydrolytic detoxification can also play a role in metabolism of phosphorothioates and can be demonstrated with synergism by DEF (S,S,S-tributyl phosphorotrithioate), a known inhibitor of hydrolytic enzymes. Synergism by DEF has been observed with chlorpyrifos in German cockroaches (Siegfried et al. 1990) and a resistant *Choristoneura rosaceana*, *Choristoneura rosaceana* with azinphosmethyl (Ahmad and Hollingworth 2004), and with parathion in greenbugs (Siegfried and Ono 1993). Based on synergistic interactions of DEF and coumaphos in topical bioassays of honey bees, it is also likely that coumaphos or its metabolite(s) interact with honey bee carboxylesterases (Johnson et al. 2009, Mao et al. 2011, Johnson et al. 2013). Carboxylesterases can lead to insecticide resistance through either esterase gene amplification or esterase mutation (Li et al. 2007b). Overexpression (Siegfried and Zera 1994) and modification of esterases have been associated with resistance to organophosphates in

greenbugs (Siegfried et al. 1997). Again, differences seen between queens and workers, and bees and flies may be due in part to expression differences between these particular enzymes. The possibility of differences between workers and queen metabolism will be explored further in Chapter 5.

In this study of topical bioassays, honey bees, even after adjusted for body weight, tolerate much higher doses of coumaphos than *Musca domestica* (Table 1). This side by side comparison of house flies and honey bees shows that in fact, honey bees exhibit extreme tolerance of coumaphos and are uniquely more tolerant of its active metabolite coumaphos oxon. Further clarification of how honey bees tolerate significantly higher doses of coumaphos and coumaphos oxon than a similar thiophosphate, chlorpyrifos and its metabolite chlorpyrifos oxon is needed. Future experiments exploring penetration rates, target site insensitivity, and metabolism properties will provide a better understanding of the differences in how both honey bee queens and workers respond to toxins. This in turn may facilitate the design and discovery of acaricides that are easily tolerated by bees. Reciprocally, it may also provide insight into target traits for selecting bees that are better able to tolerate exposure to toxins. The increased understanding of pesticide interactions within the honey bee may also lead to alleviation of current health problems seen in honey bees around the world.

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Table 1. Parent and metabolite LD50s for Queens, Workers, and Flies

Chemical	<i>A.mel.</i> Queen			<i>A.mel.</i> Worker ^a			Queen Toxicity Relative to Worker ^e	<i>M. domestica</i>		
	LD ₅₀ (µg/g) ^b	C.I. ^c	N ^d	LD ₅₀ (µg/g) ^b	C.I. ^c	N		LD ₅₀ (µg/g) ^b	C.I. ^c	N
Coumaphos	>2700		14	221	(172-284)	792	>12.2*	25	(20-32)	783
Coumaphos oxon	>5500		12	>1000		755	>5.5*	25	(17-37)	921
Chlorpyrifos	1.06	(0.9-1.2)	83	0.40	(0.36-0.46)	654	2.7*	3.4	(2.7-4.1)	808
Chlorpyrifos oxon	0.47	(0.38-0.58)	83	0.17	(0.15-0.19)	605	2.8*	4.3	(3.5-5.2)	756

^aWorker data originates from within the same lab from the same apiary as queens throughout the summer 2011.

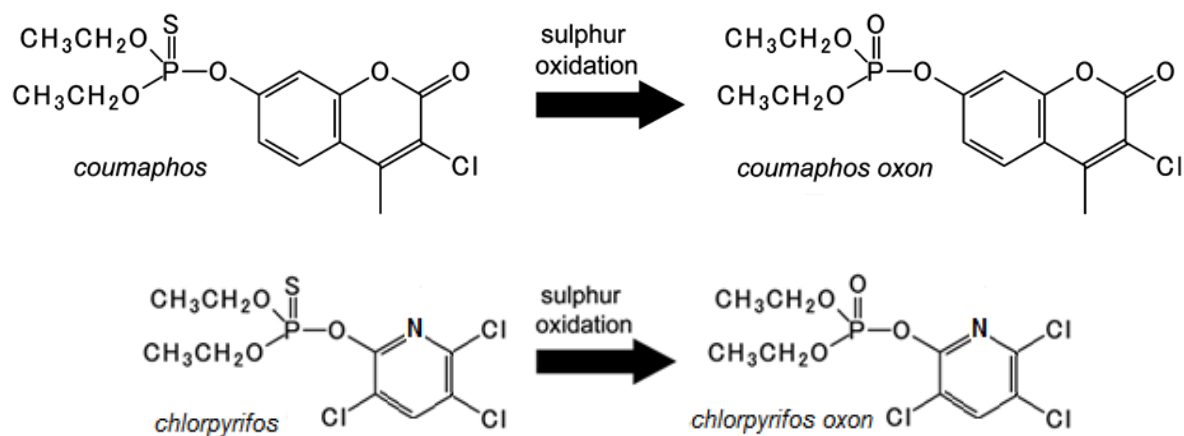
^bTwenty-four hour LD₅₀ for four organophosphates to 3-4 day-old virgin queens, worker honey bees, and house flies shown.

^cConfidence intervals (95%) indicated with LD₅₀ dose curve when possible.

^dA minimum of 3 queens for a single dose and a minimum of 3 replications were performed.

^eWorker and queen LD₅₀s with non-overlapping confidence intervals are considered significantly different as indicated with an asterisk (*).

Figure 1. Coumaphos (Right Top, [*O*-(3-Chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) *O,O*-diethyl Phosphorothioate]) and Coumaphos oxon. Chlorpyrifos (Right bottom, [*O,O*-Diethyl *O*-(3,5,6-trichloro-2-pyridyl) Phosphorothioate]) and Chlorpyrifos oxon structures. Oxon analogs are a substitution of an oxygen atom for the sulfur atom in both cases. This sulphur oxidation is aided by cytochrome P450s.



CHAPTER 4: ACETYLCHOLINESTERASE INHIBITION WITHIN QUEENS AND WORKERS

Abstract:

Honey bees are extremely tolerant of both coumaphos and its activated metabolite coumaphos oxon. The target site for organophosphate insecticides is the enzyme, acetylcholinesterase. Acetylcholinesterase (AChE) hydrolyzes acetylcholine to acetate and choline at neuromuscular and neuronal synapses aiding in regulation of synaptic transmission. AChE is inhibited by organophosphates (OP) in a dose dependent manner, and it is not uncommon for insects to develop resistance to OP insecticides through AChE modification rendering the AChE insensitive to OP inhibition. Acetylcholinesterase activity can be measured using Ellman's assay. In this study queen, worker, and house fly acetylcholinesterase were all similarly inhibited by both coumaphos oxon and chlorpyrifos oxon although in general, chlorpyrifos oxon was a more potent inhibitor of AChE activity for workers and queen honey bees and house flies. These results suggest that the mechanism by which honey bees tolerate high doses of coumaphos is likely due to something other than target site insensitivity.

Introduction:

Coumaphos is a phosphorothioate organophosphate that is well tolerated by honey bees. In fact, a recent attempt to produce reliable dose-responses in queens failed as mortality was not observed at the highest concentration tested (2700 $\mu\text{g/g}$) (Dahlgren et al. 2012). In addition, queens were 12 times less susceptible to coumaphos than workers at 24 h. This is not the first time that queens have been found to be more tolerant of pesticides than workers: the queen LD_{50}

for the organochlorine DDT was found to be 6.6 times higher than that of honey bee workers (Graves and Mackensen 1965). Even more interesting than the tolerance to coumaphos is that the activated metabolite of coumaphos, coumaphos oxon was only half as toxic to queens and only a fourth as toxic to workers as coumaphos when topically applied (Chapter 3). The mechanism responsible for the large differences between the LD_{50} s for queens and workers and the reduced toxicity of the activated metabolite is unclear. One possible reason for the observed tolerance is target site insensitivity.

The target site for organophosphate insecticides is the enzyme, acetylcholinesterase. Acetylcholinesterase (AChE) removes the excitatory neurotransmitter acetylcholine from neuromuscular and neuronal synapses and thus acts as an important regulatory enzyme for synaptic transmission of nerve impulses (Perry et al. 1998). AChE is inhibited by organophosphates in a dose dependent manner. It is not uncommon for insects to develop resistance to OP insecticides through AChE modification rendering the AChE insensitive to OP (Siegfried and Scharf 2001).

Model of Interest:

AChE activity can be measured with a simple spectrophotometric activity assay known as Ellman's assay (Ellman et al. 1961). The compound, acetyl-thio-choline iodide (ACTHi), acts as a model substrate for acetylcholine and as AChE breaks ACTHi down into acetate and thiocholine, the thiocholine reacts with dithiobis nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoate (TNB). TNB is a yellow-colored compound with a peak absorbance at 412 nm. The rate that TNB is produced during the reaction is a function of AChE activity. With the addition of inhibitors, an estimate of the *in vitro* availability of AChE after inhibition is obtained

(Ellman et al. 1961). In this investigation, queen, worker, and house fly AChE preparations were incubated with oxon metabolites of coumaphos and another representative phosphorothioate (chlorpyrifos) to determine AChE insensitivity.

It is possible the honey bee target site is insensitive, and honey bee AChE does not easily bind to coumaphos oxon. If *Apis mellifera* acetylcholinesterase is insensitive to coumaphos oxon, this mechanism of tolerance could explain the toxicity difference between chlorpyrifos and coumaphos, as chlorpyrifos was >500 fold more toxic than coumaphos to bees (Chapter 3). Additionally, it is possible that the mechanism by which queens are able to tolerate more coumaphos than workers may be explained by differences in the affinity of coumaphos oxon to the queen and worker bee AChE. Clarifying the honey bee's mechanism of tolerance to coumaphos may provide direction in the development of novel acaricides that do not negatively impact the honey bee.

Materials and Methods:

Honey Bee and House Fly Sources: Honey bee colonies and queen banks were located at the University of Nebraska-Lincoln East Campus (Lincoln, NE). The same apiary supplied larvae grafted to become queens and adult workers for parallel assays conducted from May through August 2011. All colonies were requeened in April 2011 with Italian queens obtained from C. F. Koehnen & Sons, Inc., Glenn, CA. Colonies were treated to prevent bacterial brood diseases and gut pathogen *Nosema* with oxytetracycline (Duramycine-10, Durvet Inc., Blue Springs, MO) and fumagillin (Fumagilin-B, Medivet Pharmaceuticals Ltd., High River, Alberta) respectively, applied according to label instructions in March 2011. Thymol as Apiguard (Vita, Basingstoke, United Kingdom) and oxalic acid (Fischer Scientific, Rochester, NY) were the only acaricides

used in the apiary to control *Varroa* populations during the 5 yr prior to the bioassays, with the last application in the fall of 2010.

Queens were reared using methods described by Doolittle (Laidlaw and Page 1997) in which individual 12-24 h old worker larvae were grafted into plastic cups (JZ BZ Base Mount Cell Cups, Mann Lake Supply, Ltd., Hackensack, MN). Ripe capped queen cells were placed into queen confinement cages (QC-115, Mann Lake) and a small drop of queen candy (1:1 honey and confectioners' sugar) was placed in the base of each individual cage to ensure that newly emerged virgins had access to food until they became attractive to workers (~4 d). As many as 60 caged queen cells were placed in a queen bank to emerge. Queen banks consisted of four or five frame nucleus colonies stocked with 1 kg of worker bees (Laidlaw and Page 1997). Frames of larvae were added weekly to maintain colony strength and frames were inspected twice per week to remove any uncaged queen cells. Queens were freeze killed, 14-15 d after the initial grafting date with an estimated adult age of 3-4 days. Queens were stored at -20 °C up to 10 months prior to enzyme collection.

Worker bees were emerged from frames filled with late-stage capped brood in a dark 32-34°C incubator. Newly eclosed bees were brushed from frames daily and placed inside an 8-mesh screen-wooden cage (15.25 x 10.15 x 17.75 cm). Caged worker bees were provided 1:1 sugar-water, stored in an incubator at 32-34°C, and maintained for 3-4 d before being freeze killed for AChE analysis. Workers were stored at -20 °C up to 10 months prior to enzyme collection.

House flies (*Musca domestica*) were obtained from colonies maintained by the USDA-ARS Lincoln, NE and were used for bioassays conducted October 2011. All stages were reared in the same rearing room at 25 °C, 50% RH, 12:12 L:D. Adult house flies were maintained in a

small cage (30 x 30 x 30 cm) (All Aluminum Window Co., Lincoln, NE) and provided with a 10% sugar-water solution via a 125 mL bottle (Nalgene, 312088-0004) with a dental wick.

Adult flies were aged 3-4 d before storage at -20 °C up to 11 months prior to enzyme preparation.

Chemicals: Technical grade chemicals were used for all trials. Coumaphos, coumaphos oxon, chlorpyrifos, and chlorpyrifos oxon were purchased from Chem Services Inc. (West Chester, PA). Ethanol from Sigma Aldrich (St. Louis, MO) was used to dilute all phosphorothioates. Acetylthiocholine iodide (ATCh) and 5, 5' – dithionitrobenzoic acid (DTNB) were purchased from Sigma Aldrich (St. Louis, MO). All other buffers and solvents were of reagent grade or better.

AChE preparation: Ten frozen bee heads or forty fly heads (aged 3-4 d) were homogenized in a 2 mL Teflon glass Potter-Elvehjem homogenizer attached to a Elas-col stirrer (500-11000 RPM) at setting 2 for 30 sec in 1 mL ice-cold buffer (0.1M phosphate buffer, pH 7.5 containing 1% Triton X-100) and then centrifuged at 10,000 g for 20 min in an Eppendorf Centrifuge 5415C refrigerated at 4°C. The supernatant was analyzed for protein content and then diluted to 1 mg/mL protein and stored in 100 µL aliquots into as many as 10 separate 1.5 mL labeled microcentrifuge tubes at -80 °C until used for assays no more than 3 months following preparation. Protein concentrations were determined using the Thermo Scientific Bicinchronic acid (BCA) assay kit (Waltham, MA).

Inhibition Studies: AChE activity was determined by the spectrophotometric method of Ellman et al. (1961), and followed the inhibition methods adapted from Siegfried and Scott (1990). For the AChE assay, diluted enzyme preparations were analyzed by incubating with a control of absolute ethanol or the inhibitor 5×10^{-6} M coumaphos oxon or 5×10^{-8} M chlorpyrifos oxon at 37°C for 5 time intervals as described by Obrien and Wolfe's (1959). Enzyme (11 μL) was incubated with 320 μL of 0.1 M phosphate buffer (pH 7.5) and 5 μL of inhibitor (coumaphos oxon or chlorpyrifos oxon) in absolute ethanol for 5 time intervals (3, 5, 7, 9, and 11 min with coumaphos oxon and 1, 2, 3, 4, and 5 min with chlorpyrifos oxon). After incubation at 37°C , the assay was initiated with 25 μL of inhibitor-enzyme solution to 3 of 4 wells containing 50 μL DTNB (1.25 mM), 20 μL ATCH-I (1 mM), and 105 μL phosphate buffer. Final concentrations for coumaphos oxon and chlorpyrifos oxon were 9×10^{-9} and 9×10^{-11} M, respectively. In place of enzyme, 25 μL phosphate buffer was added to the control well. The change in absorbance was recorded at 21 second intervals for 7 minutes at 412 nm with a Bio-tek Powerwave Spectrophotometer. Slopes and standard errors of inhibition curves were calculated by linear regression and converted to bimolecular rate constants as described by Main and Iverson (1966). All results comprise the mean values of three separate preparations with three determinations for each time point. Differences among mean bimolecular rate constants for workers, queens, and flies were determined by analysis of variance.

Results:

Bimolecular rate constants of coumaphos oxon and chlorpyrifos oxon for workers, queens, and house flies are given in Table 1. Queen, worker, and house fly acetylcholinesterase

were all similarly inhibited by both chlorpyrifos oxon and coumaphos oxon (Figure 1) although in general, chlorpyrifos oxon was a more potent inhibitor of AchE activity for workers and queen honey bees and house flies. All slopes were significantly different from zero ($P > 0.05$). Both workers and queens differed in their response to both inhibitors in comparison to flies ($P > 0.05$). No significant differences were detected for queens and workers for either inhibitor ($P < 0.05$).

Discussion:

This study indicates that target site insensitivity is not a contributing factor to coumaphos tolerance in honey bees and that other factors are responsible. Honey bee queen and worker acetylcholinesterase, like susceptible house fly acetylcholinesterase, is inhibited *in vitro* by organophosphates including oxon metabolites of both coumaphos and chlorpyrifos.

Previous results strongly suggest that the mechanism by which honey bees tolerate high doses of coumaphos is unique to coumaphos and is unique to honey bees as house flies do not exhibit the same tolerance and bees are not uniquely tolerant to other organophosphate insecticides such as chlorpyrifos (Chapter 3). Additionally, the higher tolerance of queens relative to workers is unrelated to target sensitivity since both queen and worker AChE exhibited equal sensitivity to inhibition by both organophosphates. Pesticide tolerance mechanisms apart from the target site insensitivity not yet examined include penetration barriers, increased rate of detoxification, and a reduced rate of activation (Yu 2008). Each of these mechanisms will be further evaluated in the next chapter.

In this study of acetylcholinesterase activity, honey bees exhibited similar AChE activity when compared with *Musca domestica* (Table 1). This side-by-side comparison of house flies and honey bees demonstrates conclusively that honey bee acetylcholinesterase is inhibited by

both chlorpyrifos oxon and coumaphos oxon *in vitro*. These results indicate that coumaphos oxon is not able to reach the acetylcholinesterase target site *in vivo* in honey bee workers and to a greater extent, honey bee queens. Further clarification of the mechanism by which honey bees tolerate significantly higher doses of coumaphos and coumaphos oxon than a similar thiophosphate, chlorpyrifos and its metabolite chlorpyrifos oxon is needed. Future experiments exploring penetration rates and metabolism will provide a better understanding of the differences in how both honey bee queens and workers respond to toxins. This in turn may facilitate the design and discovery of acaricides that are tolerated by bees and selectively toxic to varroa mites. Reciprocally, it may also provide insight into traits for selecting bees that are better able to tolerate exposure to toxins. The increased understanding of pesticide interactions within the honey bee may also lead to alleviation of current health problems seen in honey bees around the world.

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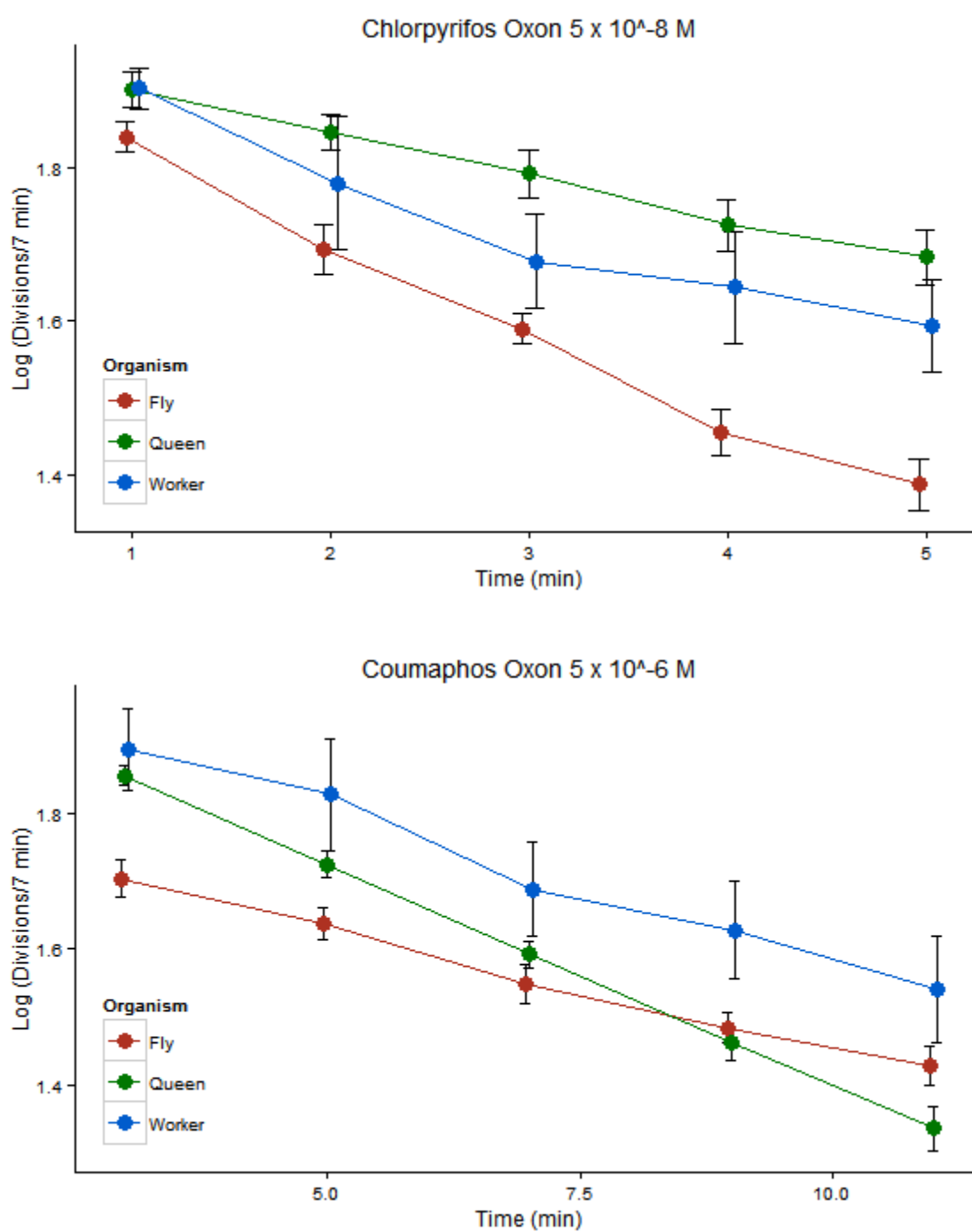
Table 1:

Organism ^a	Inhibitor	$10^{-3} \times k_i$ ($M^{-1} \text{ min}^{-1}$) ^b	Final concentration of inhibitor (M)	Slope
Queen	Coumaphos oxon	14	7×10^{-8}	-.001
Worker	Coumaphos oxon	11	7×10^{-8}	-.0008
House fly	Coumaphos oxon	14	7×10^{-8}	-.001
Queen	Chlorpyrifos oxon	1400	7×10^{-10}	-.0010
Worker	Chlorpyrifos oxon	2000	7×10^{-10}	-.0014
House fly	Chlorpyrifos oxon	3000	7×10^{-10}	-.0021

^aAcetylcholinesterase preparations from 3-4 d old adult queen or worker honey bees (10 heads/mL buffer), and 3-4 d old male and female house flies (40 heads/mL buffer)

^b k_i calculated as described by Main and Iverson (1966).

Figure 1:



CHAPTER 5: METABOLISM OF ORGANOPHOSPHATES BY QUEENS AND WORKER HONEY BEES

Abstract:

Pesticide tolerance mechanisms include penetration barriers, target site insensitivity, increased rate of detoxification, and a reduced rate of activation. Honey bees are extremely tolerant of both coumaphos and even more so of its activated metabolite coumaphos oxon. Honey bee acetylcholinesterase, the target site for organophosphate insecticides, was inhibited by both chlorpyrifos oxon and coumaphos oxon. The means by which honey bees tolerate coumaphos could be due to penetration barriers, an increased rate of detoxification or a reduced rate of activation. Additionally, differences in toxicity of coumaphos between queens and workers may be due to differences in metabolism. In this study, bee metabolism was compared using GC/MS and LC-MS/MS at 24 hr time intervals for 5 days. Most notably, coumaphos oxon was not identified from any of the samples. Both queen and worker acetone rinse recovered coumaphos concentrations decreased over time, although less so in queens. Additionally, worker internal recovered coumaphos concentrations decreased over time. Therefore, coumaphos is likely being metabolized to a less toxic coumaphos metabolite. Caste differences are likely due to metabolism, but further studies are needed to determine specific mechanisms.

Introduction:

The eusocial honey bee lives in a colony in which a single mated queen is mother to tens of thousands of semi-sterile female workers. Fertilized eggs may develop into either a queen or a worker depending on the diet provisioned during the 72 hrs following egg eclosion. Queens receive a 1:1 hypopharyngeal (water/protein): mandibular (sugar) gland secretion, while worker

larvae receive 3-4:1 (Haydak 1970, Beetsma 1979). Queen larvae also receive the protein royalectin responsible for the increased growth rate, body size, and ovary development (Reginato and Cruz-Lamdim 2003, Kamakura 2011). Adult workers and queens differ physiologically in their energetic and metabolic requirements, in their susceptibility to pathogens, and in the circulating proteins found in the hemolymph (Chan et al. 2006).

Coumaphos, a phosphorothioate organophosphate, is well tolerated by honey bees and has been used as an acaricide for controlling the parasitic *Varroa destructor* mite beginning in 1999 (U.S. Environmental Protection Agency 2006). A recent attempt to produce reliable dose-responses to coumaphos in queens failed as mortality was not observed at the highest concentration tested (2700 $\mu\text{g/g}$) (Dahlgren et al. 2012). In general, coumaphos is not acutely toxic to bees, and queens are even less susceptible to coumaphos than workers at 24 h. This is not the first time that queens have been found to be more tolerant of pesticides than workers: the queen LD_{50} for the organochlorine DDT was found to be 6.6 times higher than that of honey bee workers (Graves and Mackensen 1965). Even more interesting than honey bee tolerance of coumaphos is that the activated metabolite of coumaphos, coumaphos oxon was at least half as toxic to queens and at least a fourth as toxic to workers relative to coumaphos when topically applied (Chapter 3).

The large differences between the LD_{50} s for queens and workers and the reduced toxicity of the activated metabolite suggests that coumaphos metabolism by honey bees may be unique relative to other organophosphate insecticides. The activated oxon metabolite is usually more toxic than the parent compound in susceptible populations (Yu 2008). This pattern was seen in house flies in response to both coumaphos and chlorpyrifos oxons, and in honey bees in response to chlorpyrifos oxon (Chapter 3). Based on results described in Chapter 4, neither the queen nor

the worker target site is insensitive, with honey bee acetylcholinesterase being readily inhibited by coumaphos oxon. Therefore, it is possible that honey bee metabolism does not follow established metabolic pathways of phosphorothioates. Additionally, differences in toxicity of coumaphos between queens and workers may be due to differences in metabolism.

Model of Interest:

Previously, coumaphos metabolites have been identified in dead honey bees collected from several hive locations (Portolés et al. 2009, Mullin et al. 2010). Interestingly, Portolés *et al.* (2009) did not detect coumaphos oxon in sampled bees and Mullin *et al.* (2010) only detected coumaphos oxon in 1.4% of bee samples (2 of 140). However, it was not determined whether coumaphos oxon was present as a result of oxidative metabolism by living bees or through uptake from contaminated hive environments.

To test whether workers or queens with known exposures to coumaphos metabolize coumaphos to coumaphos oxon *in vivo*, bees were topically treated with a single dose of coumaphos, and live bees were sampled at 24 hour time intervals over a period of 5 days. Samples were then analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS/MS) and gas chromatography-mass spectrometry (GC/MS).

Materials and Methods

Insects. Honey bee colonies and queen banks were located at the University of Nebraska-Lincoln East Campus (Lincoln, NE). The same apiary supplied larvae grafted to become queens and adult workers for parallel bioassays conducted in 2012. All colonies were requeened in April 2011 with Italian queens obtained from C. F. Koehnen & Sons, Inc., Glenn, CA. Bacterial brood

diseases and gut pathogen *Nosema* were prevented with oxytetracycline (Duramycine-10, Durvet Inc., Blue Springs, MO) and fumagillin (Fumagilin-B, Medivet Pharmaceuticals Ltd., High River, Alberta, Canada) respectively, which were applied according to label instructions in March 2011 (Medivet Pharmaceuticals Ltd. 2010). Thymol as Apiguard® (Vita, Basingstoke, United Kingdom) and oxalic acid (Fischer Scientific, Rochester, NY) were the only acaricides used in the apiary to control *Varroa* populations during the 5 yr prior to the bioassays, with the last application in the fall of 2010.

Queens were reared using methods described by Doolittle (Laidlaw and Page 1997) in which individual 12-24 h old worker larvae were grafted into plastic cups (JZ BZ Base Mount Cell Cups, Mann Lake Supply, Ltd., Hackensack, MN). Ripe capped queen cells were placed into queen confinement cages (QC-115, Mann Lake Supply) and a small drop of queen candy (2:1 confectioners' sugar and honey) was placed at the base of each individual cage to ensure that newly emerged virgins had access to food until they became attractive to workers (~4 d). As many as 60 caged queen cells were placed in a queen bank to emerge. Queen banks consisted of four or five frame nucleus colonies stocked with 1 kg of worker bees (Laidlaw and Page 1997). Frames of larvae were added weekly to maintain colony strength and frames were inspected twice per week to remove any queen cells started on the brood combs.

Worker bees emerged from frames filled with late-stage capped brood in a dark 32-34°C incubator. Newly eclosed bees were brushed from frames daily and placed inside an 8-mesh screen-wooden cage (15.25 x 10.15 x 17.75 cm). Caged worker bees were provided a 1:1 by weight sugar-water solution, stored in an incubator at 32-34°C, and maintained for 3-4 d before pesticide application.

Chemicals. Technical grade chemicals were used for all trials. Reference standards for coumaphos, coumaphos oxon, and terbutylazine were purchased from Chem Services Inc. (West Chester, PA). Labelled [$^{13}\text{C}_3$] atrazine was purchased from Merck Frost Canada Ltd. (Kirkland, QC). ACS grade acetone (Fisher, Pittsburg, PA) was used to dilute coumaphos for bioassays. Ethanol (dehydrated, 200 proof) obtained from Pharmcoproducts, Inc. (Brookfield, CT) was used to dilute calibration standards. Liquid nitrogen and helium (Ultra-Pure Carrier grade) and nitrogen gas used for drying samples were obtained from Matheson Tri-Gas (Lincoln, NE). Stock solutions, calibration standards, and spiking solutions were prepared in 12-ml amber salinized vials (National Scientific, Lawrenceville, GA).

In vivo metabolism. Exposure to coumaphos was conducted using methods described by Johnson et al. (2006). A single concentration solution was prepared in acetone on the day of treatment for queen and worker exposure with storage at -20°C prior to exposure. To quantify coumaphos and metabolites through time, queens and workers were CO_2 anesthetized, treated topically on the thoracic notum with one $1\ \mu\text{l}$ application (coumaphos at $5\ \mu\text{g}/\mu\text{L}$; $5\ \mu\text{g}/\text{bee}$) using a $50\ \mu\text{l}$ syringe fitted to a repeating microapplicator (PB-600, Hamilton, Reno, NV). The $5\ \mu\text{g}/\text{bee}$ dose corresponded to the worker LD_{10} .

A minimum of 180 treated worker bees were maintained in groups of 20 ± 3 inside wax-coated paper cups ($177\ \text{cm}^3$) covered with bleached cotton cheesecloth. Groups of bees were provided with a sucrose solution (1:1 by weight) in two $1.5\ \text{mL}$ Eppendorf tubes with two perforations in the tip end and maintained at $33\ ^\circ\text{C} \pm 1^\circ\text{C}$ in an environmental chamber (H024, Darwin Chambers Co, St. Louis, MO) until checked for mortality. All live workers in a single randomly selected cup were sampled at each designated time point (0, 24, 48, 72, 96, and 120 h).

At the time of sampling, mortality was checked for all cups. Selected living worker bees were placed into a 1 mL acetone rinse individually and vortexed for 10 sec to remove any unpenetrated insecticide from the cuticle. Bees were then allowed to air dry on ice and stored in individual vials at -80 °C until further analysis. The acetone rinse was stored in corresponding individual vials at -20 °C until further analysis.

Queen exposure to coumaphos was performed on a minimum of sixty two- to five-d old adult virgin queens removed from queen banks and handled at room temperature for ≤ 1 hr. Singly caged queens in groups of three to five were briefly anesthetized with CO₂ and treated topically on the thoracic notum in one 1 μ l application (coumaphos at 5 μ g/ μ L; 5 μ g/bee) using a 50 μ l syringe fitted to a repeating microapplicator (PB-600, Hamilton, Reno, NV). After treatment, virgin queens were maintained singly with 10 untreated worker attendants inside wax-coated paper cups (177 cm³) covered with bleached cotton cheesecloth. Groups of bees were provided with a sucrose solution (1:1 by weight) in 2-1.5 mL Eppendorf tubes with two perforations in the tip end and maintained at 33 °C \pm 1 °C in an environmental chamber (H024, Darwin Chambers Co, St. Louis, MO) until checked for mortality and a minimum of three individuals were sampled at each designated time point (0, 24, 48, 72, 96, and 120 h). At the time of sampling, mortality was checked for all cups. Selected live queens were gently vortexed in 1 mL acetone for 10 seconds and then stored at -80°C until further analysis by LC-MS/MS. The acetone wash was stored at -20°C until further analysis by GC/MS.

Quantification methods were modified from Portolés *et al.* (2009) and Mullin *et al.* (2010). A single honey bee was homogenized with 1.5 g anhydrous sodium sulfate, 4.5 mL acetone, 5000 ng terbutylazine and 5000 ng [¹³C₃] atrazine as an internal standard with a POLYTRON® drive unit PT-45/50, PCU-Triac speed variator, and dispersing aggregate PTA 7

EC (Kinematica, Inc., Bohemia, NY) for 2 min at speed 7 (less than 8 m/sec) in a 9 mL 13 x 100 mm disposable culture tube (47729-572, VWR, Radnor, PA). After the sodium sulfate settled, a 2.5 mL aliquot was moved to a Corning Pyrex 15 mL reusable rimless culture tube (16 mm x 125 mm) (9820-16X, Corning, Inc., Corning, NY) and diluted with 5.0 mL of 2% aqueous NaCl (w/v). The aliquot was then extracted twice with 2.5 mL of dichloromethane, vortexed for 30 seconds and centrifuged at 1000 RPM (150 g-force) for 2 minutes before organic extracts were moved into a 9 mL disposable culture tube. Organic extracts were evaporated to dryness under a gentle nitrogen stream. The final residue was then dissolved in 1.8 mL 50/50 methanol/distilled, de-ionized, organic free water and moved to an autosampler vial using a disposable Pasteur pipette (Fisher Scientific, 13-678-20D).

To the acetone rinses of individual bees, 5000 ng terbutylazine and 5000 ng [$^{13}\text{C}_3$] atrazine were added to each sample and evaporated to dryness under a gentle nitrogen stream. The final residue was dissolved in 200 μL ethyl acetate prior to analysis.

Quantification of metabolites was performed at the University of Nebraska Water Center Water Science Laboratory. Coumaphos metabolites were analyzed using GC/MS for surface rinses while whole bee homogenates were analyzed using LC-MS/MS. GC/MS standard concentrations of coumaphos, coumaphos oxon, and terbutylazine were 0, 1, 5, 10, and 30 $\mu\text{g}/\mu\text{L}$. [$^{13}\text{C}_3$] atrazine was 25 $\mu\text{g}/\mu\text{L}$ for all standards. LC-MS/MS standard concentrations of coumaphos, coumaphos oxon, and terbutylazine were 10, 200, 600, 2000, 3000, and 6000 $\text{pg}/\mu\text{L}$. [$^{13}\text{C}_3$] atrazine internal standard was 25 $\mu\text{g}/\mu\text{L}$ for all solutions.

Instrumentation. GC/MS. A MSD 5973 mass spectrometer and a GC 6890 gas chromatograph from Agilent technologies (Santa Clara, CA) was equipped with a LEAP CombiPAL

autosampler (Carrboro, NC). The GC separation was performed using a fused silica DB-1 capillary column with a length of 30 m, 0.25 mm i.d. and a film thickness of 0.25 μm (Agilent technologies, Santa Clara, CA). The oven temperature was programmed as follows: 80°C (.75 min); 40 °C/min to 170°C; 2.5°C/min to 236°C; 40°C/min to 75°C (hold 9.6 min). Splitless injections of 1 μL samples with helium as carrier gas at 1 mL/min were used for all GC/MS analyses. The injection temperature was set to 280°C, interface temperature to 300°C, source temperature to 230 °C, and a solvent delay of 4 min was selected. The instrument detection limit (IDL) for coumaphos and coumaphos oxon was 68.6 pg and 29.2 pg injected respectively.

LC-MS/MS. Samples were analyzed on a 2695 Separation Module (Waters Corporation, Milford, MA) and model LCQ ion trap mass spectrometer (Finnigan Corp., San Jose, CA) operated in positive ESI (electrospray ion) mode while scanning from 400-100 amu. Separation was performed using a Thermo HyPURITY C18 bonded silica 5 μm particle size analytical column 2.1 mm (Thermo Fisher Scientific Inc., Waltham, MA) at a flow rate of 200 $\mu\text{L}/\text{min}$. The mobile phase used was a time programmed gradient using H₂O and MeOH, both containing 1% formic acid. The percentage of organic modifier changed from 5% MeOH for 3 minutes, then 20% MeOH mixture linearly from 20 to 95 % MeOH mixture in 23 min, held for 9 minutes, and then 5% MeOH mixture for 8 minutes. The injection volume was 5 μL . The desolvation gas was nitrogen, obtained from a nitrogen tank with liquid nitrogen headspace. Spray voltages of 4.5 kV were used in positive ionization modes. A cone voltage of 14 V was selected. The capillary temperature was set to 200°C. The LCQ used MS/MS for quantitation. The parent ions and quantitation daughter ions were as follows: [¹³C₃] atrazine [219 at 35% with mass range (m/z) 176-178]; terbutylazine [230 at 35% with mass range (m/z) 173-175]; coumaphos [363 at 35%

with mass range (m/z) 306-308 with an expected retention time (min) 25.80 and ICIS peak integration baseline window of 25]; coumaphos oxon [347 at 35% with mass range (m/z) 318-320]; coumaphos metabolite 1 [363 at collision energy 35% with mass range (m/z) 334-336 with an expected retention time (min) 23.00]; coumaphos metabolite 2 [363 at collision energy 35% with mass range (m/z) 306-308 with an expected retention time (min) 19.87 and ICIS peak integration baseline window of 40]. The instrument detection limit (IDL), determined by repeated analysis of the low standards for coumaphos and coumaphos oxon were: 3.8 pg and 4.5 pg injected respectively.

Results:

Coumaphos oxon was just above the lowest standard for one of three queens at the 0 hr (estimated concentration of 38 ng/g) and one of three queens at the 24 hr (estimated concentration of 60 ng/g) internal metabolites. It was not found above detectable limits for any of the other queens (16 total) or any of the workers (18 total).

The coumaphos recovered in the acetone rinse varied between time points and when comparing queens and workers (Figure 1). Initial concentration recovered in workers was above the 5000 ng (~50,000 ng/g workers; 25,000 ng/g queens) treatment given to each bee, and is depicted as a percent of time zero (Figure 1). The calculated coumaphos concentration measured for worker bees in the acetone rinse was estimated as low as 5% (2500 ng/g) at the 120 hr time point while queen acetone rinse did not average less than 20% (5000 ng/g) (Figure 2). In general, the recovery of coumaphos for workers declined over time, while queen acetone rinse did so to a lesser degree. In fact, no apparent differences exist between the 24 and 120 hr time points for queens (Figure 1).

The calculated concentration (ng/g) of coumaphos internally metabolized varied in both queens and workers (Figure 2). In general, the calculated recovery of coumaphos (ng/g) in internal extracts decreased over time, with queen recovery at 120 hrs being 1500 ng/g less than the zero time point and worker recovery being 2300 ng/g less than the zero time point (Figure 2). Notably, calculated concentration (ng/g) of coumaphos in internal extracts of queens was twice that of workers (Figure 2).

In addition to coumaphos and coumaphos oxon, another two metabolites ($C_{14}H_{17}O_7PCl$, Figure 3) were noted with the same nominal mass as coumaphos (m/z 363). It is unclear which metabolite represents the structures shown in Figure 3. Based on the expected retention time (23 and 20 respectively), it is possible metabolite 1 has an alcohol attached to the phenyl ring, and metabolite 2 has an alcohol attached to the methyl. Metabolite 1 is shown to increase over time in both queens and workers, from 200 to 1000 ng/g in queens and varies from 2000 to 4000 ng/g in workers (Figure 2). Metabolite 2 did not occur in concentrations above the lowest coumaphos oxon standard in any of the samples and is therefore not reported.

Discussion:

Coumaphos applied topically to queens and workers clearly penetrates the cuticle within 24 hours, though possibly less so in queens. Concentrations of coumaphos in the acetone surface rinse declined by greater than 50% between the 0 and 24 hour time points in both workers and queens (Figure 1). Additionally, metabolite 1 ($C_{14}H_{17}O_7PCl$, Figure 3) increased over time in both workers and queens (Figure 2). Together, this indicates that penetration is occurring in both castes, although perhaps less so in queens. As not all of the applied coumaphos was accounted for in metabolites measured or recovered coumaphos, bees may additionally be removing

coumaphos from the cuticle by a mechanical means (such as grooming) causing the decreased recovery over time. Alternatively, worker bees may be exuding coumaphos through their brood food glands and their proboscis into the bioassay environment (sugar water, wax of the paper cups, and/or bleached cheesecloth). Bees were shown to pass coumaphos through trophallaxis as little as 15 minutes after consumption of coumaphos as Perizin® (Bayer AG) (van Buren et al. 1992). In the hive, coumaphos was shown to accumulate in wax and to a lesser degree in honey in as little as a day after treatment (Tremolada et al. 2004).

The large decrease (>50%) of recovered coumaphos from the acetone rinse between the 0 and 24 hr time points for both queens and workers suggests that the mechanism for coumaphos tolerance involves rapid metabolism of coumaphos (Figure 1). This is in agreement with ¹⁴C labelled coumaphos fed to bees as Perizin, which peaked in the haemolymph at 4 hours after ingestion, and by 15 minutes 45% had passed the proventriculus (van Buren et al. 1992). Internally, coumaphos was at its highest at the 0 and 24 hour time points in workers, and with no significant change over time in queens. Tremolada et al. (2004) also found coumaphos peaked in bees 24 hours after treatment at levels as high as 1000 ng/g treated in a hive environment.

Importantly, it is clear that coumaphos is not readily metabolized to coumaphos oxon in honey bees. We recovered coumaphos oxon in only two of thirty samples at just above the limits of detection. It is possible that bees rapidly metabolize coumaphos into coumaphos oxon prior to the 24 hour time point. However, analysis of a worker at 3 hr and 6 hr and a queen at 6 hr and 12 hr after treatment (one bee for each time point) also indicated that coumaphos oxon was not detectable (data not shown). As coumaphos oxon inhibits honey bee acetylcholinesterase (Chapter 4), the lack of or slow coumaphos oxon production is likely one mechanism by which bees tolerate coumaphos exposure. It is also possible that bees quickly metabolize coumaphos

oxon after its initial oxidation from coumaphos. The ability to quickly metabolize coumaphos oxon could explain why in topical bioassays, coumaphos oxon was less toxic than coumaphos (Chapter 3).

As surface rinsed coumaphos decreased over time, it is likely coumaphos is metabolized into something other than coumaphos oxon. There is an increase in internal metabolite 1 seen in both queens and workers over time (Figure 2). Quantified metabolite 1 at its highest detection at 120 hours in queens is only half of the initial concentration measured in workers at 24 hrs. Adding metabolite 1 and coumaphos recovered both internally and in the acetone rinse does not account for all applied coumaphos (51% accounted for in workers at 24 hours; 44% accounted for in queens at 120 hours). Other likely metabolites not quantified in this study include potasan, 4-methylumbelliferone, 3-chloro-7-hydroxy-4-methyl-2H-chromen-2-one, O,O-Diethyl phosphorothioate, diethyl hydrogen phosphate, and the hydroxylation product of coumaphos ($C_{14}H_{17}O_6PSCl$) (Portolés et al. 2009).

This study lends further evidence to the hypothesis that coumaphos is likely detoxified by cytochrome P450s. Previous evidence includes topical bioassays to workers where synergism between coumaphos and PBO (known to inhibit P450s) (Johnson et al. 2009, Johnson et al. 2013) suggests that P450s are involved with detoxification reactions rather than activation. This was further confirmed when Mao et al. (2011) heterologously expressed honey bee midgut P450s *in vitro*, demonstrating that CYP9Q1, CYP9Q2, and CYP9Q3 enzymes metabolized coumaphos to non-toxic metabolites. Differences in susceptibility between queens and workers may therefore be due in part to expression differences of P450s between the two castes.

The side-by-side comparison of honey bee queens and workers shows that in fact, honey bees do metabolize coumaphos over time, and a portion of the queens' greater tolerance may be

due in part to reduced penetration and speed of metabolism within the first 24 hours (as queens had an average of 6,000 ng/g (under 40%) coumaphos recovered in the acetone surface rinse while workers had an average of 30,000 ng/g (over 40%) at the 24 hour time point) (Figure 1). In addition, it appears that coumaphos not metabolized within the first 24 hours in queens remains constant on the cuticle at about a fifth of the applied dose (Figure 1). Internally, coumaphos does not appear to change throughout the five day experiment in queens (Figure 2), lending more weight to the hypothesis bees do not readily convert coumaphos to coumaphos oxon.

Future experiments exploring production of metabolites other than coumaphos oxon and penetration rates between the zero and twenty-four hour time points will provide a better understanding of the differences in how both honey bee queens and workers respond to toxins. This in turn may facilitate the design and discovery of acaricides that are easily tolerated by bees. These results may also provide insight into traits for selecting bees that are better able to tolerate exposure to toxins. The increased understanding of pesticide interactions within the honey bee may also lead to alleviation of current health problems seen in honey bees around the world.

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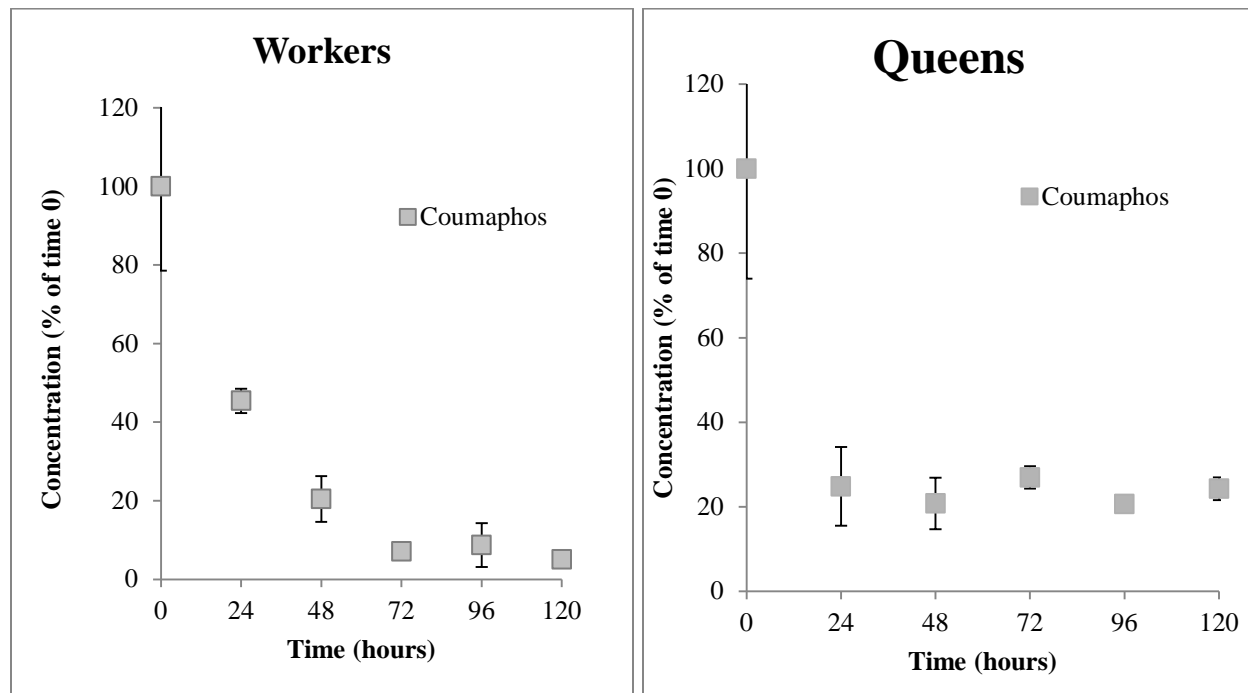
Figure 1. Acetone Surface Rinse

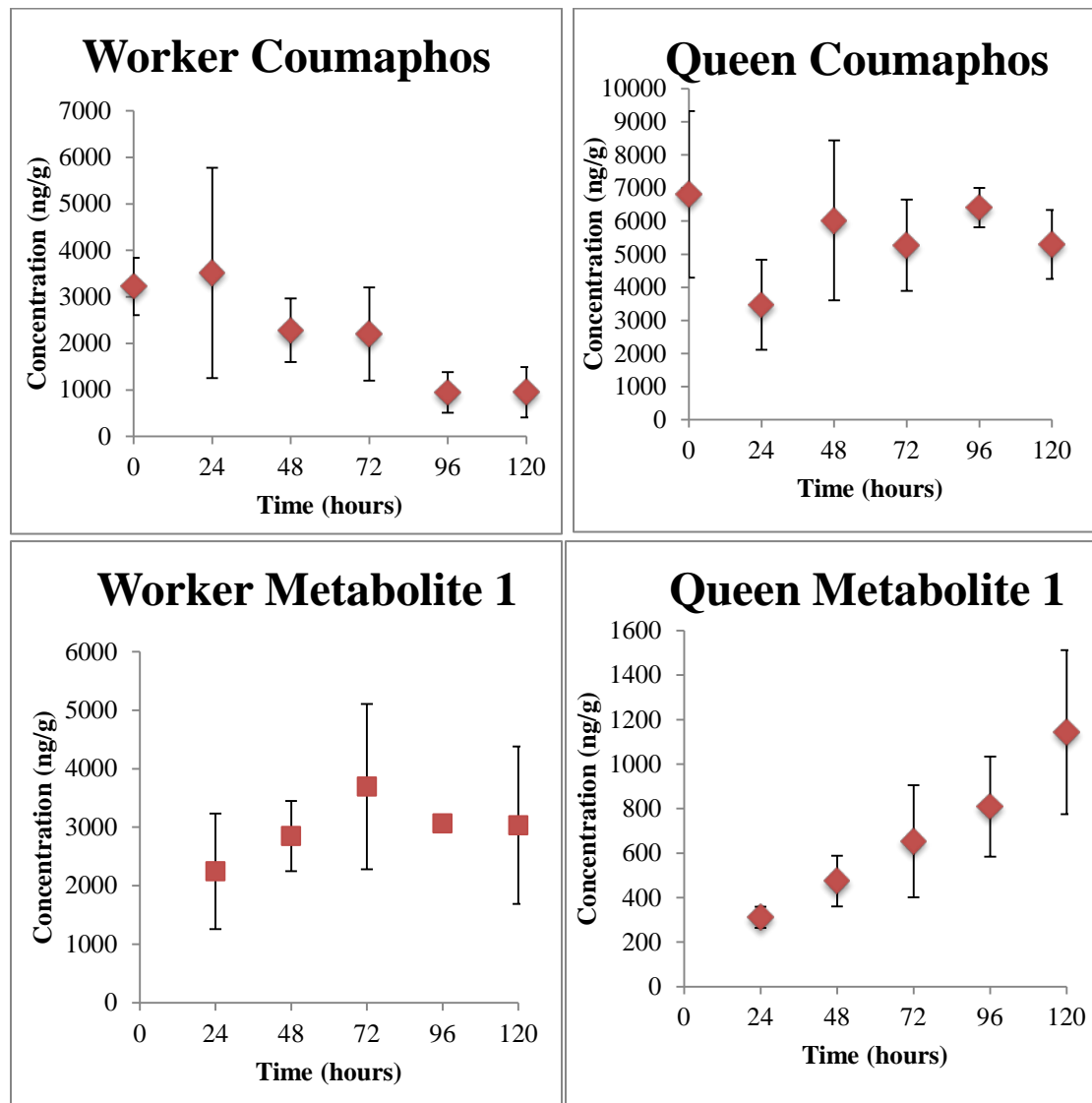
Figure 2. Internal Metabolites

Figure 3. Metabolite 1 and 2, the hydroxylation and sulphur oxidation product of coumaphos, or the hydroxylation product of coumaphos oxon.

