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Toxic and Repellent Effects of Pyrethroids Used in Orchards on the Honey Bee, *Apis mellifera* L. (HYMENOPTERA: APIDAE)

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TOXIC AND REPELLENT EFFECTS OF PYRETHROIDS USED IN ORCHARDS ON THE HONEY BEE, *Apis mellifera* L. (HYMENOPTERA:APIDAE)

By

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Managed honey bee colonies provide valuable pollination services and are rented by fruit orchards to improve fruit quality and yield. The placement of colonies in this agricultural setting increases the possibility of exposure to pyrethroids used for broad-spectrum pest control in orchards. Although highly toxic to bees, pyrethroids are believed to pose a relatively low hazard due to their low application rates in the field as well as their contact repellent properties. Previous studies have noted a decrease in foraging visits following pyrethroid application possibly preventing bees from acquiring a lethal dose in the field.

This research quantified behaviors associated with sub-lethal exposure to orchard-applied pyrethroids, lambda-cyhalothrin, esfenvalerate, and permethrin, using video tracking software, Ethovision XT (Noldus Information Technologies). Bee locomotion, social interaction, and time spent near a food source were measured over a 24-hour period. This project also evaluated the repellency of pyrethroids currently used in orchard production to foraging worker bees under artificial feeding conditions and in a field setting. The objective of this study was to achieve a better understanding of

behavioral effects associated with sub-lethal pyrethroid exposures in the laboratory and determine if a field-relevant exposure would result in repellency of foragers. This research will aid in the development of better-informed management decisions made by both growers and beekeepers and provide risk assessment tools and protocols to regulatory agencies seeking to quantify sub-lethal pesticide effects on pollinators.

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CHAPTER 1: LITERATURE REVIEW

Honey bees and orchard pollination services

The western honey bee, *Apis mellifera* L., is the most dependable orchard pollinator due to relative ease of hive management, mobility, large foraging populations throughout the year, wide foraging range, and polylectic nature (Mayer et al. 1986, Calderone 2012). Honey bees in the U.S. are credited with adding an estimated $19.2 billion annually to agricultural production in the United States through increased crop quality and yield (Calderone 2012). The value added to U.S. orchard crops including almonds, apples, apricots, avocado, sweet and tart cherry, kiwifruit, macadamia nut, nectarine, olive, peach, plum, prune and plum/prune hybrid is estimated to be $6.56 billion of this total (Calderone 2012). Of these orchard crops, almonds are of particular importance to the beekeeping industry; the large number of acres in production and the need for 100% insect-facilitated cross-pollination fuel the demand for managed honey bee pollination services. While some orchard crops require a 5-10% fruit set for a successful crop, almonds require 30-60% fruit set and growers are paid by the pound (Delaplane and Mayer 2000). Not every pollinated flower will yield fruit; therefore, nearly 100% of almond flowers need to be pollinated to achieve a profitable crop (Delaplane and Mayer 2000).

Since 1996, almond-bearing acres have increased 46% from 418,000 acres in 1995 to 780,000 acres in 2012 and continue to expand as 75,000 non-bearing acres reach fruit-bearing age (National Agricultural Statistics Service 2012a). Additionally, almonds have replaced alfalfa and cotton acres as almond prices remain steady and higher relative values are realized ($5,088 per acre for almond versus $1,969 per acre for cotton and

$1,635 per acre for alfalfa calculated by dividing total production value by acres harvested in 2011) (National Agricultural Statistics Service, California Field Office 2012). With the continued expansion of the almond industry, the demand for pollination services cannot be met by the estimated 370,000 resident colonies located within the state of California (National Agricultural Statistics Service 1999). The 780,000 almond-bearing acres in California currently in production require an average of 2 to 2.5 colonies per acre for adequate pollination (Delaplane and Mayer 2000, National Agricultural Statistics Service 2012a). In 2012, this single orchard crop required 1.5 – 1.9 million colonies for pollination, or nearly 70% of all managed colonies accounted for in the U.S. The high demand for honey bee colonies coupled with a decrease in colony supply due to recent colony losses has caused a rapid rise in colony rental fees. The average rental price for a single colony pollinating almonds increased 108% from $72.58 in 2005 to $150.79 in 2010 (Carmen 2011). In addition to almonds, colonies are rented for the pollination of tree fruits, blueberries, cranberries, cucurbits, berries, vegetable and clover (seed production) and oil seed crops including canola and meadowfoam (Caron 2011, Caron et al. 2012). This has created a market in which many commercial beekeepers earn more annual income from pollination rental fees than from honey sales (*California County Commissioners' Report 2011*).

Fortunately, with both orchardists and beekeepers benefitting financially from strong colonies, a common goal is maintaining colony health (Riedl 2006). Cooperating with beekeepers, fruit and nut growers seek to balance management of arthropod pests with the need to protect valuable managed pollinators, both of which are necessary to produce a profitable orchard crop. Beekeepers rely on strong and productive colonies for

their livelihoods, and measures to reduce both biotic and abiotic stress are essential to their success. Many factors can negatively impact colony health including exposure to insecticides commonly applied to fruit and nut trees (Riedl 2006).

Pesticides in orchard settings

Orchardists must strive to produce a high-quality, cosmetically-appealing crop, free from signs of pest injury to attain a premium price in the commercial fruit market (Jackson and Looney 1999). Meeting these strict standards requires adequate pollination as well as management of arthropod pests. Adequate pollination during bloom ensures fruit set and a plentiful crop of fully developed fruit. Balancing pollination requirements and the control of orchard pests is a critical aspect of profitable fruit production.

Proper orchard management calls for control of arthropod pests prior to and throughout the growing season. In many cases, insect pests are controlled by chemical means to minimize cosmetic damage or infestation (Jackson and Looney 1999). While these treatments are sometimes a necessary component in orchard management (Jackson and Looney 1999), exposure to these chemicals can be harmful and possibly lethal to honey bees (Johansen and Mayer 1990, Riedl 2006).

Chemical pest control options available to orchardists have gone through significant changes over the past 17 years. The Food Quality Protection Act passed in 1996 called for a phasing out of pesticides with high human toxicity, including cholinesterase-inhibiting compounds such as organophosphates and carbamates that once were commonly used in fruit production. Use of these pesticides has steadily declined since 1995 (*Summary of Pesticide Use Report Data* 2011) as these compounds have been

replaced by insecticides with alternative modes of action including pyrethroids (Spurlock and Lee 2008).

Pyrethroids became an attractive replacement for organophosphates due to their broad-spectrum insecticidal activity, low avian and mammalian toxicity and comparatively low rates of application (Oros and Werner 2005, Spurlock and Lee 2008). In California, pyrethroid application amounts doubled between 1994 and 2005 (Spurlock and Lee 2008). Current reports indicate pyrethroid use is still prevalent in orchards (Table 1.1) with nearly 1 million acres of various orchard crops including almonds, apples, apricots, sweet and tart cherries, peaches, plums, prunes, pears, and nectarines in the U.S. receiving various pyrethroid applications (*Summary of Pesticide Use Report Data* 2011, National Agricultural Statistics Service 2011, 2012b).

Pyrethroids in orchards

Pyrethroids are currently being used in orchard management to control a wide variety of fruit pest insects (Lewis et al. 2012). The heaviest use of pyrethroids in orchards occurs in almond orchards when comparing pounds applied to almonds versus other orchard crops in 2011 (*Summary of Pesticide Use Report Data* 2011, National Agricultural Statistics Service 2012b) (Table 1.2). Almond growers decreased their use of permethrin, an older pyrethroid formulation, by 39 percent in 2011. However, use of two newer formulations, bifenthrin and esfenvalerate increased by 32 and 26 percent, respectively, to treat for navel orangeworm and peach twig borer, two key almond pests (*Summary of Pesticide Use Report Data* 2011). Frequently, pyrethroids targeting the peach tree borer are applied during bloom, increasing the likelihood that honey bee

colonies placed in orchards for pollination could be exposed to pyrethroids (*Summary of Pesticide Use Report Data* 2011).

Compared to previous organophosphates or carbamates used in fruit production, pyrethroids are believed to be less hazardous to bees due to their low application rates and evidence of repellent properties in some cases (Smart and Stevenson 1982, US EPA 2013a). However, with the exceptions of fluvalinate and flumethrin, miticides used widely by beekeepers to control for the parasitic varroa mite within bee hives, all pyrethroids are highly toxic when topically applied to bees under laboratory conditions. Following pyrethroid exposure, voltage-gated sodium channels are blocked open causing an influx of sodium ions. This results in depolarization of the membrane and subsequent loss of membrane potential leading to eventual death of the insect (Soderlund and Bloomquist 1989). To protect non-target insects, such as honey bees, label directions indicate pyrethroids are not to be applied to blooming crops or weeds; however, at very low application rates, some pyrethroids including esfenvalerate and lambda-cyhalothrin are suggested for application during late evening on bloom (when foragers are not present) if the amount of active ingredient is no greater than 0.025 pounds per acre for esfenvalerate or 0.02 pounds per acre for lambda-cyhalothrin (Riedl 2006).

Despite labeling efforts to minimize honey bee exposure to pyrethroids, Mullin et al. (2010) detected fourteen different pyrethroids inside hives. Pyrethroids are lipophilic in nature (Davies 1985), and it is not surprising that these compounds were found in non-polar hive components including wax, pollen, and bee samples. Three pyrethroids of interest for this research project, cyhalothrin, esfenvalerate, and permethrin, were recently detected in wax (at maximum levels of 17 ppb, 56 ppb, and 372 ppb, respectively), pollen

(at maximum levels of 28 ppb, 60 ppb, and 92 ppb), and bee samples (at maximum levels of 2 ppb, 9 ppb, and 19600 ppb) (Mullin et al. 2010). In addition, eight different pyrethroids have been detected at ppb levels in pollen gathered during various crop pollination events including maximum levels of 131 ppb of cyhalothrin and 216 ppb of esfenvalerate in apple pollen (Pettis et al. 2013).

The presence of orchard-applied pyrethroids within the hive would suggest that foraging honey bees are exposed to pyrethroids in the field, and in some cases, return to contaminate the hive. Pyrethroid-treated orchards can expose foraging bees to contaminated pollen, and the exposure extends to non-foraging colony members including adults, nurse bees and brood coming into contact with contaminated pollen.

Behavioral shifts associated with sub-lethal pyrethroid exposure

Previous studies have reported behavioral changes in honey bees in response to pyrethroid exposure including decreased foraging (cypermethrin: Fries and Wibran 1987; Reith and Levin 1988; Shires et al. 1984; deltamethrin: Decourtye et al. 2004); permethrin: Reith and Levin 1988), altered homing flight (deltamethrin: Vandame et al. 1995), and increased non-foraging behaviors including grooming, abdomen tucking, rotation, and trembling (permethrin: Cox and Wilson 1984) under experimental, field, and semi-field conditions (e.g. normal hive state is altered (i.e. colony is housed in an observation hive) or foraging conditions are limited (i.e. hives are placed in a glasshouse setting) to allow for observation).

In some cases, pyrethroids have been described as “repellent” when foraging activity was reduced (Delabie et al. 1985, Fries and Wibran 1987, Rieth and Levin 1988,

Decourtye et al. 2004). A variety of repellent compounds have been researched for their potential to protect bees from pesticide poisoning (Atkins et al. 1975). Generally, these compounds are inert ingredients added to a tank mix with insecticides preventing foragers from making contact with a toxic compound in the field in order to prevent or reduce mortality (Johansen and Mayer 1990). Pyrethroids lie outside the realm of typical repellents, as these compounds are highly toxic insecticides when they make direct contact with bees. However, an unknown mechanism of repellency likely plays a role in preventing forager mortality under field conditions (Rieth and Levin 1987).

It is of note that the term "repellent" in this case does not refer to olfactory deterrence. Instead, pyrethroid repellency is the result of behavioral changes in response to contact exposure. More precisely, the effect may be described as the result of two sub-lethal toxic responses—an irritation effect paired with a period of transitory inhibition of activity (Rieth and Levin 1988, Thompson 2003). First, the irritation associated with contact exposure appears to induce excessive grooming (e.g. cleaning of the proboscis and antennae) causing disruption of normal foraging behaviors. In addition, affected foragers may return to the hive to recover from symptoms of irritation and experience a subsequent period of inactivity (Rieth and Levin 1988). However, it is important to note that pyrethroid toxicity increases with lower temperatures (Blum and Kearns 1956). In the case of a sub-lethal exposure, foragers capable of returning to the hive experience decreased toxicity due to the higher temperatures maintained within the hive (30-35^{o}C; Winston 1991) and are protected from predation during recovery (Rieth and Levin 1987, 1988). However, low field temperatures causing increased toxicity may expose foragers to a lethal rather than sub-lethal dose. The negative temperature

coefficient associated with pyrethroids is important as orchardists apply these compounds at various temperatures that can impact their potential toxicity to honey bees.

Behaviors observed following pesticide exposure are difficult to quantify and laboratory or field experiments that include behavioral observations are often not quantified and are often difficult to statistically analyze (Desneux et al. 2007). Lack of precise measurements makes it difficult to estimate the effect of sub-lethal pyrethroid exposure on honey bee colony health. However, sub-lethal exposure could have a variety of negative impacts on a colony. A decrease in foraging due to homing failure or inactivity following exposure may lead to reduced visitation to a treated crop. In turn, this may lead to reduced pollination efficiency or a disruption in the colony's food supply limiting colony growth. Foragers returning to the colony may contaminate the hive with tainted food stores, which are ingested by workers, brood, drones, and queen. Chronic exposure to pyrethroids at the colony level could have serious impacts on colony health. Colonies fed bifenthrin- or deltamethrin-tainted (LC_5) sucrose solution showed reduced fecundity and slower brood development times (Dai et al. 2010). In addition, colonies fed low doses of cypermethrin in sucrose solution showed a decrease in brood area and an increase in queen supercedure rate and colony death when compared to controls (Bendahou et al. 1999).

In addition to the difficulty of quantitatively measuring behavioral effects, it is especially challenging to examine the effect of sub-lethal exposures under field conditions. A honey bee colony's foraging range can be highly variable with studies indicating mean foraging distances of < 1 km, 2.3 km, and 6.1 km (Visscher and Seeley 1982, Beekman and Ratnieks 2000) making it difficult for researchers to assure that test

hives are foraging only within the experimentally treated area (Brady 2011). Additionally, honey bee colonies have been shown to contain a multitude of pesticides within the wax matrix of their comb (Mullin et al. 2010) making it challenging for researchers to preclude previous colony exposure to pesticides (Brady 2011). Field experiments are also confounded by natural variability of colony health due to factors such as parasitic mite loads, viruses, bacterial and fungal diseases and the impact of differing nutrition on immune response (Brady 2011). Finally, variability of insecticide application, weather conditions during and after application, and attractiveness of the crop bloom being tested can impact colony exposure to pesticides and make replication of field experiments exceedingly difficult (Brady 2011). These factors likely play significant roles in determining the extent of honey bee pyrethroid exposure injury and associated behavioral effects in an orchard setting.

Risk assessment for honey bees

In the interest of public health and environmental protection, all pesticides are regulated at both the state and federal levels (Yu 2008). The Environmental Protection Agency (EPA) is responsible for enforcing the Federal Insecticide Fungicide and Rodenticide Act (FIFRA), which sets forth the registration process for pesticides (Yu 2008). Prior to registration of a product, an assessment of lethal risk to non-target species, including the honey bee, is required to determine if the product and its intended uses comply with the law (Yu 2008).

Currently, the EPA is focused on minimizing unintended exposure to honey bees by using a three-tiered risk assessment model with a focus on acute toxicity. Any

outdoor-use product that would likely be contacted by honey bees must first be tested in an acute toxicity assay to establish a lethal dose of pesticide causing 50% mortality (LD_{50}) in a test bee population (US EPA 2011a). If this dose is <11 µg per bee, the pesticide moves on to the second tier in which a foliar application of pesticide is then exposed to normal weather conditions and the toxicity of residues are measured (US EPA 2011a). Finally, field pollinator testing may be required if the first- or second-tier tests indicate adverse effects on colonies, prolonged residual toxicity, or potential chronic, reproductive, or behavioral effects (US EPA 2011a).

This approach to risk assessment is being re-examined as honey bee colony survival continues to be an issue for beekeepers (National Research Council 2007) and an expanding body of research provides evidence that the impacts of sub-lethal pesticide exposure to individual bees and the colony as a whole are not fully understood (Thompson 2003, Desneux et al. 2007, Johnson et al. 2010). Currently, the EPA is pursuing an updated pollinator protection program examining sub-lethal and chronic effects to all life stages of pollinators, and effects of different routes of exposure (US EPA 2013b).

In the pursuit of a more comprehensive risk assessment approach, Brady (2011) has noted that the acute contact toxicity assay does not provide an endpoint other than mortality of young adult worker bees and that signs of intoxication (i.e. sub-lethal effects) are not typically quantified even though guidelines state a need for this information to be reported. The EPA currently reviews registration for many pesticides each year, and the development of effective and efficient tools to quantify sub-lethal behavioral effects are

needed to screen for those pesticides with the highest likelihood of causing harm to non-target species (Ellis and Teeters 2011, Teeters et al. 2012).

RESEARCH OBJECTIVES

General objective

To examine the toxic and repellent effects of lambda-cyhalothrin, esfenvalerate, and permethrin on the honey bee, both in the laboratory and in a semi-field and orchard setting.

Specific objectives

1. Identify and quantify sub-lethal behavioral effects of topical application of pyrethroids on three- to four-day-old worker honey bees under lab conditions using Ethovision XT to record behaviors.
2. Conduct repellency test under semi-field conditions using artificial feeding stations with a solution of sucrose and a volatile essential oil to attract honey bee foragers. The stations were outfitted with either treated or control styrofoam floats for the bees to land on while gathering sucrose. Photographs of each float were taken at 10-minute intervals over a 1.5-hour period. Mean forager counts were calculated for control and pyrethroid-treated floats and compared to assess repellency of pyrethroid compounds.
3. Conduct repellency test under field conditions using apple blossoms sprayed with either pyrethroid formulations or a water control at rates recommended for orchards. Forager visits to each bouquet of blossoms

were counted in 10-minute intervals at three time points. Mean forager counts at control- and treated-bouquets were compared to assess repellency.

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TABLES

Table 1.1: Percent of select U.S. orchard acres treated with esfenvalerate, lambda-cyhalothrin, and permethrin (*Summary of Pesticide Use Report Data* 2011, National Agricultural Statistics Service 2011, 2012a, 2012b)

	Percent (%) of Total U.S. Acres Treated		
	Esfenvalerate	Lambda-cyhalothrin	Permethrin
Almonds	33	18	6
Apples	7	15	1
Peaches	31	19	7
Tart Cherries	25	7	12
Plums	25	Not available	0
Apricots	16	Not available	0
Nectarines	25	0	0
Prunes	11	Not available	0
Pears	Not available	36	Not available
Sweet Cherries	8	14	4

Table 1.2: Total pounds of esfenvalerate, lambda-cyhalothrin, and permethrin applied to select U.S. orchard acres (*Summary of Pesticide Use Report Data* 2011, National Agricultural Statistics Service 2012b).

	Total Pounds Applied to Select U.S. Orchard Acres		
	Esfenvalerate	Lambda-cyhalothrin	Permethrin
Almonds	14729	4109	9936
Apples	1200	2700	500
Peaches	3200	1300	4700
Tart Cherries	400	100	700
Plums	300	Not available	0
Apricots	100	Not available	0
Nectarines	500	0	0
Prunes	400	Not available	0
Pears	Not available	900	Not available
Sweet Cherries	400	700	700

CHAPTER 2: USING VIDEO TRACKING TO QUANTIFY BEHAVIOR FOLLOWING SUB-LETHAL EXPOSURE TO ORCHARD PYRETHROIDS

INTRODUCTION

Despite their status as important pollinators of agricultural crops and wild plants, U.S. honey bee colonies have suffered a steady decline from 5.9 million colonies in 1947 to 2.6 million in 2012 (National Research Council 2007, National Agricultural Statistics Service 2012c). Colony numbers dramatically declined from 4.2 million to 2.4 million from 1981 to 2005 (National Research Council 2007) due, in part, to how colony numbers were estimated (Johnson et al. 2010). Research has supported the hypothesis that numerous factors likely contribute to colony losses including parasites, pathogens, beekeeping management practices, inadequate nutrition and exposure to pesticides (vanEngelsdorp et al. 2009, CCD Steering Committee 2012).

Colonies are exposed to a variety of pesticides including commercial agricultural formulations and in-hive pesticides used for the control of various honey bee pests (Johnson et al. 2010). Mullin et al. (2010) reported the presence of 121 different pesticides within hive components including wax, pollen, and bee samples with pyrethroids being the most commonly detected insecticide class. Frazier et al. (2011) reported 312 of 340 in-hive wax samples tested positive for pyrethroid residue content. While fluvalinate, a beekeeper-applied acaricide used to control the parasitic bee mite, *Varroa destructor*, was most prevalent with 307 detections, agricultural and horticultural pyrethroids including esfenvalerate, fenpropathrin, bifenthrin, cypermethrin, cyfluthrin, pyrethrin, cyhalothrin, deltamethrin, and permethrin were commonly found (229 detections in all).

Pyrethroids are known to be highly toxic to honey bees (Smart and Stevenson 1982), and they have been linked to honey bee mortality incidents (Mineau et al. 2008). Less obvious effects on foraging behavior and colony health due to sub-lethal exposure to pyrethroids and other pesticides are also a concern (Desneux et al. 2007, Johnson et al. 2010, Frazier et al. 2011). The presence of various pyrethroids in the hive matrix suggests that some foragers are exposed to sub-lethal doses in the field and are capable of returning to the hive where contamination of the colony's combs and food supply may occur. Sub-lethal exposure to these compounds has been associated with reduced foraging or hive activity (Shires et al. 1984, Fries and Wibran 1987, Rieth and Levin 1988), disorientation and homing failure (Cox and Wilson 1984, Vandame et al. 1995), and impaired learning function (Taylor et al. 1987, Mamood and Waller 1990, Decourtye et al. 2004, 2005).

Various methods have been used to qualitatively and quantitatively measure changes in behavior when honey bees are exposed to pyrethroids. Honey bee olfactory learning ability has been examined via the proboscis extension response assay (PER) (Taylor et al. 1987, Mamood and Waller 1990, Decourtye et al. 2004, 2005). Cox & Wilson (1984) manually tracked bees and characterized behaviors qualitatively following sub-lethal permethrin exposure and recorded the amount of time spent performing selected behaviors. Other studies have used the number of active foragers or amount of pollen gathered to assess foraging activity (Shires et al. 1984, Delabie et al. 1985, Fries and Wibran 1987). Teeters et al. (2012) used video tracking software, Ethovision XT**,** to quantify bee behavior following sub-lethal topical exposure to the in-hive miticide, tau-fluvalinate.

This study aims to quantify effects of three pyrethroids currently used in orchard pest control, esfenvalerate, lambda-cyhalothrin, and permethrin, on worker honey bee behavior. Behavioral effects of pyrethroids were examined in the 1980's with compounds registered at that time including permethrin which was first registered by the EPA in 1979 (US EPA 2011b). However, esfenvalerate and lambda-cyhalothrin were registered by the EPA in 1986 and 1989, respectively (US EPA 2009, 2010), and information on their sub-lethal and behavioral impacts to honey bees is limited. In recent years, newer pyrethroids have replaced older compounds, and they are extensively used on numerous orchard crops and over large acreages to control a wide range of insect pests (*Summary of Pesticide Use Report Data* 2011). My research provides data on behavioral effects of more recently developed pyrethroids that are currently applied to orchard crops.

In addition, this study uses a new tool to analyze bee behavior (Ethovision XT) and to assess sub-lethal effects of pesticides on non-target organisms including honey bees. The current EPA risk assessment model takes acute honey bee toxicity into account during the process of pesticide registration. However, this all-or-nothing approach does not consider sub-lethal effects of pesticide exposure despite mounting evidence suggesting that more thorough measures of behavioral changes resulting from pesticide exposure should be evaluated (Desneux et al. 2007). The EPA has been advised by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) to consider additional endpoints other than mortality during pollinator risk assessment including "measurement of motility, social interactions, and other behavioral changes" (Bailey 2012). Following methods described by Teeters et al. (2012), this study uses video tracking to quantify distance moved in a 24-hour period, time spent in social

interaction with another worker bee, and time spent in a food source area under laboratory conditions.

MATERIALS AND METHODS

Acute contact toxicity bioassay

Prior to behavioral assays, a lethal dose for 50% of an adult honey bee test population (LD_{50}) was calculated for each pyrethroid—esfenvalerate, lambda-cyhalothrin, and permethrin (Robertson et al. 2007).

Source of chemicals

Technical-grade 40:60 cis:trans permethrin (CAS# 52645-53-1), esfenvalerate (CAS# 66230-04-4), and lambda-cyhalothrin (CAS# 91465-08-6) were obtained from Chem Service (West Chester, PA).

Source of honey bees

The test population of honey bees was selected from 2 of 16 colonies maintained in the University of Nebraska-Lincoln East Campus apiary. Frames of emerging brood, or sealed brood within 24 hours of eclosion were selected and moved to a laboratory incubator (model H024; Darwin Chambers) maintained at $33^{o}C \pm 2\ ^{o}C$, with high humidity and in the absence of light except during treatment and observation times. Adult bees were brushed from the frames daily after emergence into screened wooden boxes (9 cm × 10 cm × 18 cm) and fed a 50% sucrose/water solution *ad libitum*. Caged bees were then returned to the incubator to mature for 2 to 3 days prior to treatment.

Bioassay methods

Three- to four-day-old bees in cages were anesthetized with carbon dioxide. For each chemical tested, eight groups of 17-23 bees were distributed to waxed paper dessert cups and covered with cheesecloth and a rubber band. Bees in each cup were again anesthetized with CO_2 and treatments of a 1 µl acetone (Sigma-Aldrich Co. LLC, St. Louis, MO) solution of 0.0, 0.25, 0.75, 1.25, 1.75, 2.50, 5.00 or 10.00 µg of esfenvalerate; and 0.0, 0.10, 0.33, 0.50, 0.75, 1.00, 2.50, or 10.00 µg of lambda-cyhalothrin; 0.0, 1.00, 1.25, 1.75, 2.50, 3.33, and 10.00 µg of permethrin were applied with a microapplicator (PB-600, Hamilton) to 17-23 bees. Treated bees were fed a 50% sucrose/water solution *ad libitum* and returned to the incubator. Mortality was recorded at 24-and 48-hours. Three replicates of each dose-range were tested on bees sourced from two hives. Data were analyzed to establish a dose-response curve for each insecticide by Probit analysis (Finney 1971) using PoloPlus software (LeOra Software 1987).

Video tracking

Using data from the acute contact toxicity bioassays, the LD_{10} was estimated and used as the highest sub-lethal dose to be applied for video tracking experiments. A low dose (equal to a 1:10 dilution of the LD_{10} in acetone) and an intermediate dose (equal to a 1:1 dilution of the LD_{10} in acetone) were also tested.

Following topical treatment methods similar to those of the acute contact toxicity bioassay and video tracking methods set forth in Teeters et al. (2012), adult honey bee locomotion, time spent feeding and time spent in social interactions were measured.

Honey bees were housed, fed, and anesthetized in cages as in the toxicity assay. Four groups of approximately 12 anesthetized bees were randomly selected and placed into waxed paper dessert cups. Each cup of bees was anesthetized and 8 bees were randomly selected and treated with one of the four treatments (control, low, intermediate, or high sub-lethal dose of pyrethroid). Following topical treatment of all dose levels, bees were placed in 16 polystyrene petri dishes (Figure 2.1). Two bees were placed in each dish, with both bees belonging to the same treatment group. A 3 mm wire mesh bisected each dish to allow for interaction while maintaining separation of the two bees. Food and moisture were provided via a 0.5 cm × 1.0 cm cube of sucrose agar for each bee. Sucrose agar cubes were comprised of 8 g of granulated sugar, 0.17 g of agar powder, and 20 mL of distilled water. Each dish was partially sealed with a plastic paraffin film to prevent desiccation while allowing for air exchange.

The 16 dishes were arranged on a translucent Plexiglass platform under a video camera and lit from below with an infrared light bulb housed within a 45.72 × 53.34-cm plywood box (Figure 2.2). Visible light was excluded by encasing the unit in a PVC-pipe framework with a black plastic wrapping. An ultrasonic humidifier (model V5100NS; Kas, Inc., Hudson, NY) with automated humidity gauge maintained 80% relative humidity within the unit.

Twenty-six hours of video were recorded via an MPEG recorder within the Ethovision XT (Version 7.0; Noldus Information Technology, Wageningen, Netherlands) software package. The first and last hour were excluded from analysis to allow the bees to recover from handling and anesthetization and to allow for 24-hour continuity across all recordings. A screen capture of the video was used as a guide during the manual

defining of the 32 arenas of locomotion, consisting of half the petri dish, and the "food zone" designated as a 30-pixel × 15-pixel rectangle surrounding the location of the sucrose cube (Figure 2.1). Ethovision XT software used these designations as well as manually defined visual contrast settings to detect the location of all 32 subjects simultaneously. For every second of the 24-hour video, a series of 15 (*x, y*) coordinates and frequency of bee visits to the food zone were recorded for each subject. By calibrating the arena size to the corresponding 9 cm diameter of the dish, coordinates were then translated to actual distance and compiled to calculate total distance traveled by each bee. Distance between two subjects was calculated using each bee's (*x, y*) coordinates. When two bees were within 1.5 cm of one another, these time points were tallied to determine time spent in social interaction. It is important to note that calculation of social interaction time relies on the assumption that social behavior of bees correlates to physical proximity (Sams-Dodd 1995). In the same way, time spent in the food zone is not a direct measure of food consumption; however, this method assumes that time spent in the food zone correlates to feeding behavior.

All response variables were log transformed to normalize the data and eliminate variance heterogeneity (Dowdy et al. 2011). Statistical comparisons were made using a one-way analysis of variance (ANOVA) in SAS 9.2 statistical software (Cary, NC). When the overall effect of treatment was significant, pair-wise mean comparisons of each dose level and control were performed using Dunnett's post-hoc test (two-tailed) (Teeters et al. 2012).

RESULTS

Acute contact toxicity bioassay

Results for the toxicity bioassays appear in Table 2.1. Data are also represented as dose-response curves in Figure 2.3. Relative toxicity of the three pyrethroids tested was lambda-cyhalothrin > esfenvalerate > permethrin based on non-overlapping 95% confidence limits.

In the case of permethrin, the LD_{10} was estimated to be 104.6 ng/bee; however, preliminary video tracking experiments using this high dose level resulted in 70% mortality. In order to produce a sub-lethal rather than lethal response, a 1:1 dilution of the LD_{10} was selected as an alternative high dose. The amended permethrin high dose used in subsequent video tracking experiments was 52.3 ng/bee.

Video tracking

Distance moved

Movement of honey bees was significantly affected by exposure to all tested pyrethroids. Total distance moved was significantly reduced by exposure to all three pyrethroids tested at some of the doses tested [lambda-cyhalothrin ($p=0.0331$, $F_{3,104}=3.02$), esfenvalerate ($p<0.0001$, $F_{3,118}=20.07$), and permethrin ($p=0.0016$, $F_{3,122}=5.38$)] (Table 2.2a).

All dose levels of lambda-cyhalothrin treatment resulted in reduced bee movement, although these differences were only detectable at the moderate dose level. Bees receiving a low dose (1.28 ng) or high dose (12.78 ng) of lambda-cyhalothrin moved an average distance of 186.6 ± 17.8 m and 202.8 ± 40.0 m, which did not differ significantly from control bees (255.5 ± 39.7 m). However, bees receiving a moderate

dose (6.39 ng) traveled 37% less (p=0.0107) on average than control bees (159.7 ± 25.7 m) (Figure 2.4a).

Bees exposed to esfenvalerate showed a decrease in total distance moved with increasing exposure. While the statistical analysis did not detect a significant decrease in distance moved at the lowest dose level (2.60 ng), this group traveled 41% less than control bees (254.6 ± 33.2 m and 359.5 ± 45.8 m, respectively). Significant differences were detected at moderate (12.98 ng) and high dose (25.96 ng) levels of esfenvalerate in which bees moved 61% and 71% less on average (136.9 ± 16.1 m; p<.0001 and 103.7 ± 14.8 m; p<.0001, respectively) than control bees (Figure 2.5a).

Permethrin treatment did not significantly affect bee movement at low (5.23 ng) and moderate (26.15 ng) doses (246.4 ± 25.6 m and 258.6 ± 40.6 m, respectively). However, a 30% decrease in distance moved was detected (p=0.0064) at the high dose of 52.29 ng (161.9 ± 28.5 m) compared to the control bees (232.1 ± 28.0 m) (Figure 2.6a).

Time spent in social interaction

Social interaction time was significantly impacted by exposure to esfenvalerate (p=0.0183, $F_{3,54}$=3.64) and permethrin (p=0.0002, $F_{3,59}$=7.69) (Table 2.2b). Bees spent less time interacting when treated with the highest sub-lethal dose of esfenvalerate and permethrin. However, there was no evidence of a treatment effect of lambda-cyhalothrin (p=0.6621, $F_{3,49}$=0.53) and no pair-wise comparisons were significant relative to control bees.

Interaction time for control bees averaged 418.33 ± 109.33 min while bees treated with a low (1.28 ng), moderate (6.39 ng), or high dose (12.78 ng) of lambda-cyhalothrin

averaged 359.33 ± 74.77 min, 398.60 ± 100.76 min, and 248.06 ± 72.78 min, respectively (Figure 2.4b).

Bees treated with low (2.60 ng) and moderate (12.98 ng) doses of esfenvalerate did not differ in time spent in social interaction compared to control bees (472.70 ± 70.17 min and 531.68 ± 100.76 min compared to 377.76 ± 85.24 min). Social interaction time decreased (p=0.0390) at the highest dose level of 25.96 ng (212.96 ± 76.83 min) (Figure 2.5b).

Bees treated with a low (5.23 ng) and moderate (26.15 ng) dose of permethrin did not differ significantly in time spent in social interaction compared to control bees (284.97 ± 48.95 min and 275.55± 44.38 min compared to 444.99 ± 74.33 min). A significant decrease (p=0.0004) was observed in social interaction time at the highest dose level, 25.96 ng (146.65 ± 39.89 min) compared to control bees (Figure 2.6b).

Time spent in food zone

Pyrethroid exposure had a significant effect on the amount of time spent in the food zone for all three compounds [lambda-cyhalothrin (p=0.0228, $F_{3,103}$=3.32), esfenvalerate (p=0.0043, $F_{3,118}$=4.62), permethrin (p=0.0019, $F_{3,122}$=5.28)] (Table 2.2c). Pair-wise comparisons of control versus treated groups indicated differences at some exposures for all three compounds; however, average time decreased in some instances and increased in others.

For lambda-cyhalothrin, bees treated with a low (1.28 ng) or high dose (12.78 ng) did not differ from control bees in time spent in the food zone (45.45 ± 19.23 min and 22.92 ± 8.23 min compared to 33.92 ± 7.87 min for the control). Bees treated with a

moderate dose (6.39 ng) spent significantly less time (15.83 ± 4.02 min) in the food zone than control bees (p=0.0185) (Figure 2.4c).

Time spent in the food zone did not differ between bees treated with a low dose (2.60 ng) of esfenvalerate and control bees (10.87 ± 2.35 min and 22.53 ± 3.92 min, respectively). Average time spent in the food zone decreased for the moderate (12.98 ng) dose treatment group (10.23 ± 3.07 min; p=0.0032) (Figure 2.5c). One outlying data point in the high dose treatment was eliminated due to an experimental error in which a bee became stuck to the sucrose cube for a prolonged period. At the high (25.96 ng) dose treatment level, bees spent less time in the food zone (16.16 ± 5.49 min; p=0.0013) compared to control bees.

Bees treated with a low dose (5.23 ng) or moderate dose (26.15 ng) of permethrin did not differ significantly in time spent near the sucrose food cube compared to control bees (14.10 ± 3.46 min, 37.25 ± 13.99 min, and 19.25 ± 6.48 min, respectively) However, bees treated with the high dose (52.29 ng) spent a significantly longer period in the food area (100.94± 30.28 min; p=0.0020) relative to control bees (Figure 2.6c).

DISCUSSION

Three orchard-applied pyrethroids were tested at sub-lethal doses to determine if effects on honey bee behavior could be detected using video tracking software. This study was able to identify differences in bee movement, time spent in social interaction, and time spend in the food zone following exposure to a moderate or high sub-lethal dose of pyrethroids. These results provide evidence for the development of Ethovision XT as a

potential screening tool for evaluation of pesticide risk to non-target insects including the honey bee.

Bees treated with the highest dose level of permethrin exhibited decreased locomotion compared to control bees confirming results of previous manual tracking reported by Cox and Wilson (1984) in which bees treated with permethrin spent less time walking than untreated bees. Teeters et al. (2012) also observed a decrease in total distance moved in response to a sub-lethal dose of tau-fluvalinate, the pyrethroid found in Apistan®, a beekeeper-applied acaricide for control of Varroa parasites. Our study noted a similar trend of decreasing locomotion following pyrethroid exposure for lambda-cyhalothrin and esfenvalerate. As these compounds are largely missing from previous behavioral studies, this work provides evidence that these pyrethoroids elicit a similar effect on locomotion as described for permethrin and tau-fluvalinate. Decreased movement may be the result of treated bees experiencing typical symptoms of pyrethroid exposure including loss of coordinated movement, convulsions, and paralysis (Soderlund and Bloomquist 1989). Additionally, honey bees may be replacing typical exploratory movement in the arena with relatively stationary grooming behaviors as noted by Cox and Wilson (1984).

Cox and Wilson (1984) also reported that bees treated with permethrin spent less time in antennal probing and food giving behavior than control bees. Our findings support this observation as bees treated with the highest dose of permethrin spent less time in social interaction with another bee than control bees. The highest dose level of esfenvalerate elicited a similar response in treated bees; however, no effect on social

interaction was observed for lambda-cyhalothrin indicating that not all pyrethroids have the same degree of behavioral impact on social interaction.

Additionally, time spent in the food zone was variable in response across chemicals. Our results indicated that at the highest dose level of permethrin, treated bees spent more time in the food zone than untreated bees. As the social interaction area and food zones were in opposing locations within the petri dish, it is not surprising that these response variables were inversely correlated. Conversely, this trend was not observed with esfenvalerate- and lambda-cyhalothrin-treated bees, which spent less time in the food zone than untreated bees. Typically, pyrethroids are classified as Type I or II compounds based on differences in structure and intoxication symptoms (Soderlund and Bloomquist 1989, Yu 2008). Permethrin is classified as a Type I compound while esfenvalerate and lambda-cyhalothrin are grouped with Type II compounds. While differing symptoms in insects are less clearly defined than in mammals (Soderlund and Bloomquist 1989), differences in intoxication to Type I versus Type II pyrethroids may explain inconsistencies in behavioral response to food across the three pyrethroids tested.

It is possible that genetic differences between hives may account for differences in pyrethroid tolerance. Additionally, a variety of hive conditions including time of year, temperature, presence of pests, parasites, or diseases, and nutritional state may impact the ability of bees to tolerate exposure to chemicals encountered in their environment. These differences may influence their level of intoxication, and in turn, their behavioral response following pesticide exposure. The scope of this study was limited to two hives, and further testing with additional hives could provide a better sense of colony variation in response to pyrethroid exposure.

It is worth noting that pyrethroids have been detected in apple pollen at maximum levels of 131 ppb of cyhalothrin and 216 ppb of esfenvalerate (Pettis et al. 2013). These levels are greater than the highest doses tested for these compounds validating that the sub-lethal dose range tested with this video tracking system is within the scope of potential exposure in the field. While this study attempts to represent realistic field doses, the topical treatment protocol used in this assay requires the test subjects to unavoidably encounter a known dose of purified toxin in an acetone solution, and behavior is tracked under conditions that do not simulate a hive environment. As a consequence, the laboratory assay used in this investigation does not equate to a field evaluation of pyrethroid exposure but rather should be considered a worst-case scenario of exposure (Decourtye and Pham-Delegue 2002). Despite these limitations, video tracking under laboratory conditions allows for greater control over application of treatment and environmental conditions. In addition, video tracking quantifies bee behavior providing objective, precise, and predictive data on the effects of pesticide exposure. With numerous chemicals requiring risk assessment prior to registration with the EPA or similar regulatory agencies, Ethovision XT could offer a high-throughput screening tool to identify compounds which have sub-lethal effects on bee behavior and warrant further testing early in the risk assessment process.

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Table 2.1: Acute contact toxicity of lambda-cyhalothrin, esfenvalerate, and permethrin to 3-4 day older worker honey bee

Chemical	No. Replicates[a]	*N*	χ^2	Mortality		
				Slope ± SE	LD_{50} (95% CI) [b]	LD_{10} (95% CI) [b]
Lambda-cyhalothrin	3	829	3.57	2.778 ± 0.300	36.99 (25.87-45.52)	12.79 (4.99-19.94)
Esfenvalerate	3	949	5.74	2.369 ± 0.171	90.24 (73.57-106.80)	25.963 (16.49-35.59)
Permethrin	3	825	4.28	4.225 ± 0.306	210.276 (192.02-231.98)	104.59 (86.43-119.71)

[a] Three replicates of each chemical were tested on bees sourced from 2 hives with 17-23 bees tested at each dose level.

[b] Nanograms of a.i. per bee

Table 2.2: One-way ANOVA p-values associated with (a) distance moved, (b) time spent in social interaction, and (c) time spent in the food zone. Significant differences are denoted with an asterisk.

a

	Overall effect of treatment	Pair-wise comparisons (versus Control)		
		Low Dose	Moderate Dose	High Dose
Lambda-cyhalothrin	0.0331*	0.6178	0.0107*	0.5595
Esfenvalerate	<.0001*	0.1975	<.0001*	<.0001*
Permethrin	0.0016*	0.943	0.9999	0.0064*

b

	Overall effect of treatment	Pair-wise comparisons (versus Control)		
		Low Dose	Moderate Dose	High Dose
Lambda-cyhalothrin	0.6621	0.9297	0.8363	0.9859
Esfenvalerate	0.0183*	0.9486	0.9999	0.039*
Permethrin	0.0002*	0.9946	0.9862	0.0004*

c

	Overall effect of treatment	Pair-wise comparisons (versus Control)		
		Low Dose	Moderate Dose	High Dose
Lambda-cyhalothrin	0.0228*	0.9269	0.0185*	0.1666
Esfenvalerate	0.0043*	0.1421	0.0032*	0.0073*
Permethrin	0.0019*	0.9997	0.7487	0.002*

FIGURES

Figure 2.1: Petri dish consisting of two arenas with food zones indicated. Social interaction occurs along the wire mesh divider. Photo: Bethany Teeters.

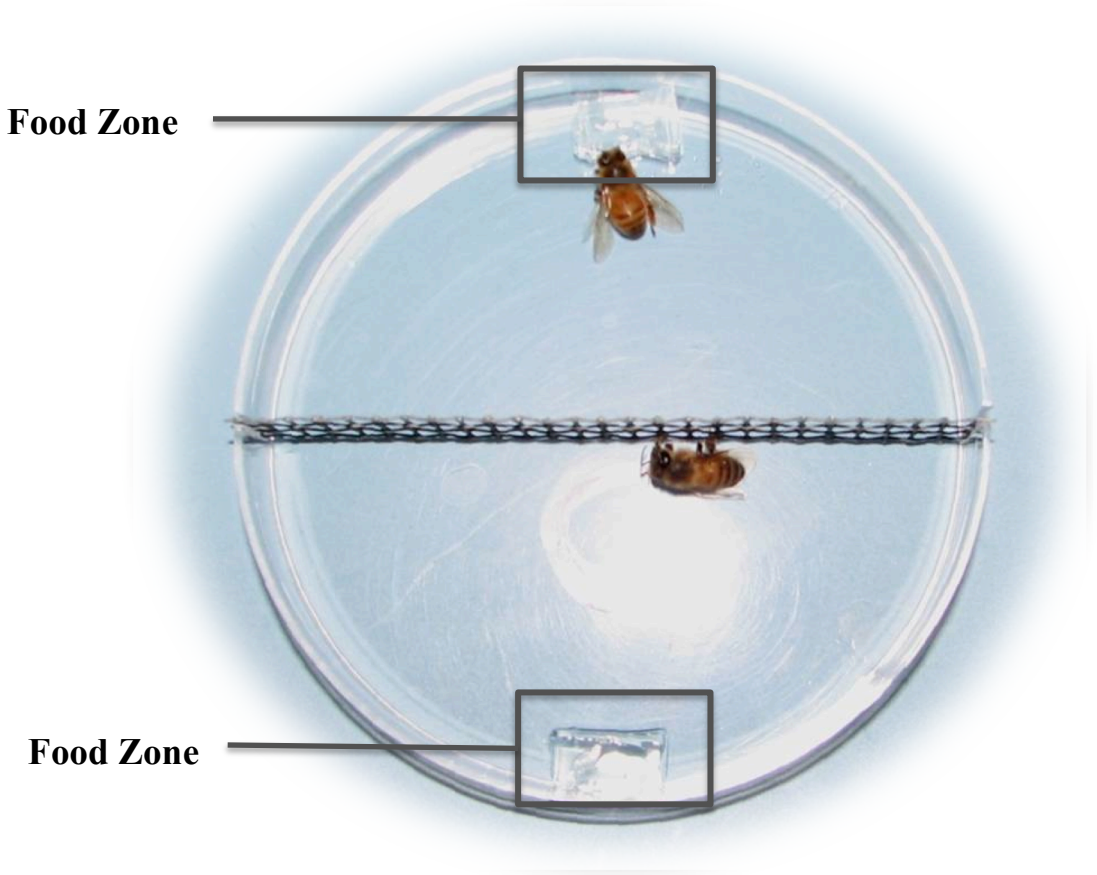


Figure 2.2: Experimental setup for laboratory video tracking experiment.

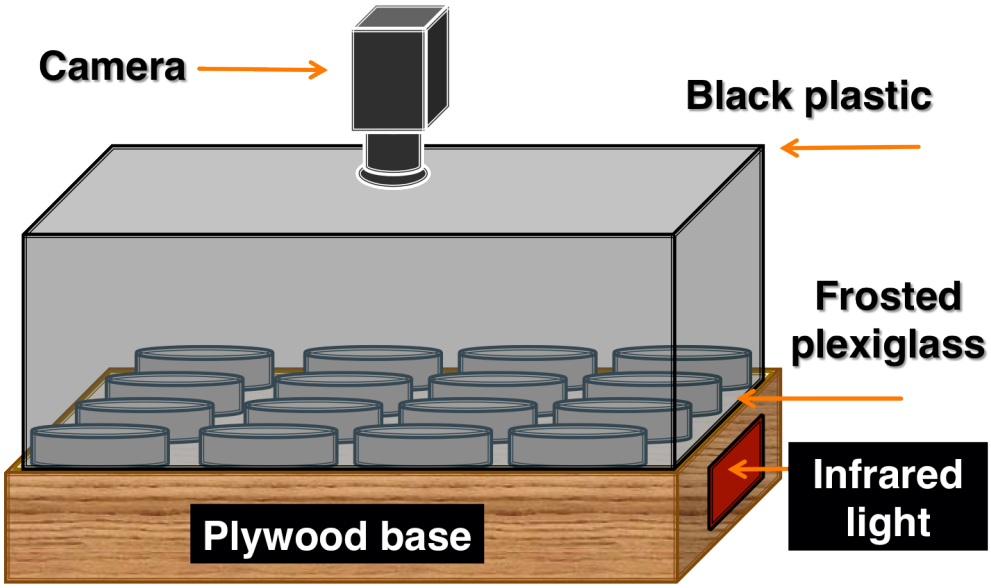


Figure 2.3: Dose-response curves for (a) lambda-cyhalothrin, and (b) esfenvalerate. Error bars represent standard error of the mean mortality at each dose.

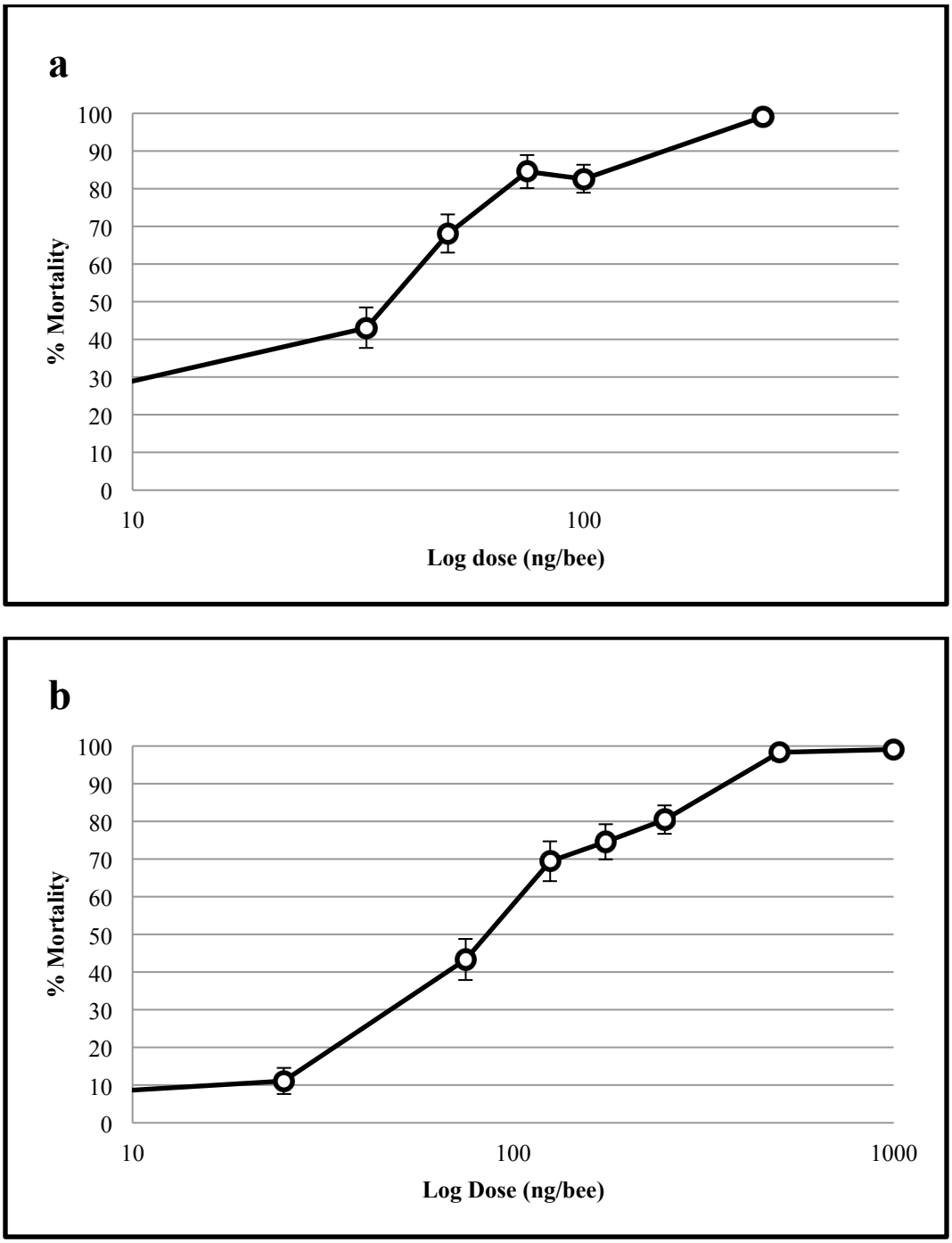


Figure 2.3: Dose-response curve for (c) permethrin. Error bars represent standard error of the mean mortality at each dose.

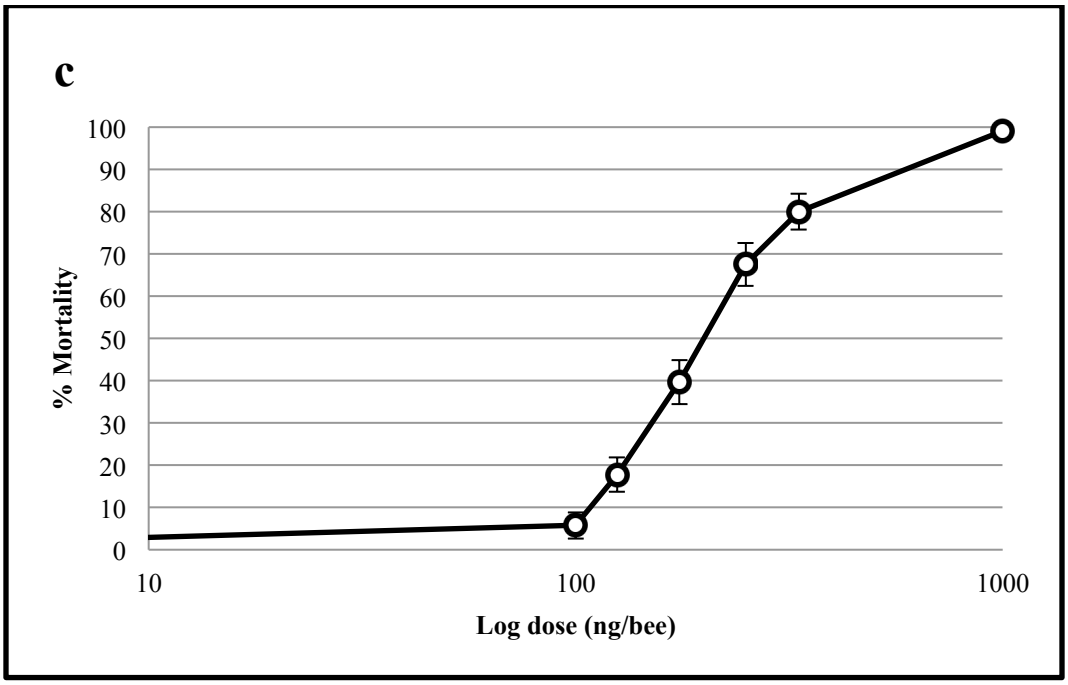


Figure 2.4: Effect of a single topical application of lambda-cyhalothrin on (a) distance moved, (b) time spent interacting with another bee, and (c) time spent in the food zone in a 24-hour period. Doses tested: vehicle control (n = 31); 1.28 (n = 32); 6.39 (n = 30); and 12.79 ng/bee (n = 15). Dose levels significantly different from control indicated by *p<0.05; **p<0.01; ***p<0.001.

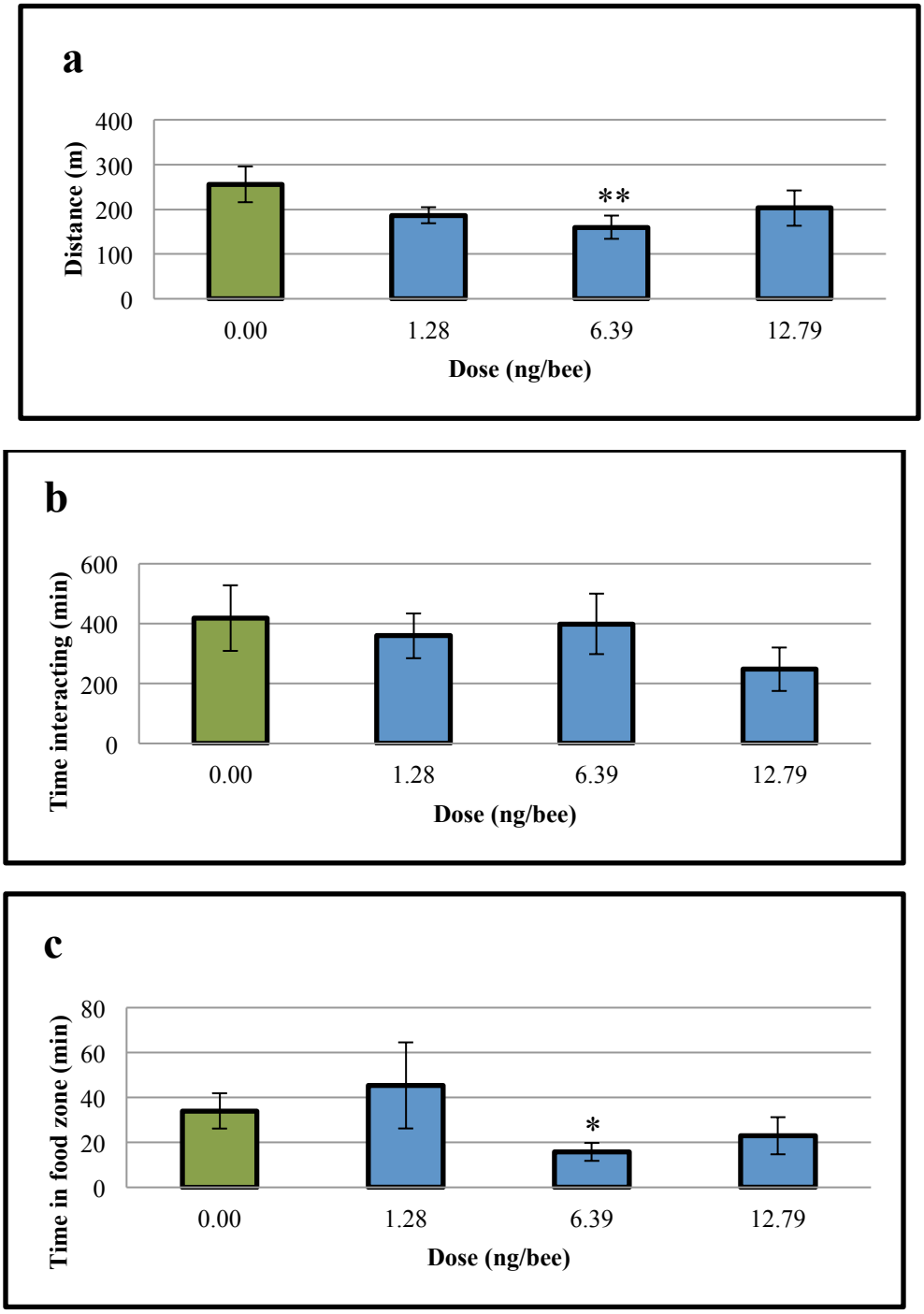


Figure 2.5: Effect of a single topical application of esfenvalerate on (a) distance moved, (b) time spent interacting with another bee, and (c) time spent in the food zone in a 24-hour period. Doses tested: vehicle control (n = 30); 2.60 (n = 31); 12.98 (n = 32); and 25.96 ng/bee (n = 29). Dose levels significantly different from control indicated by *p<0.05; **p<0.01; ***p<0.001.

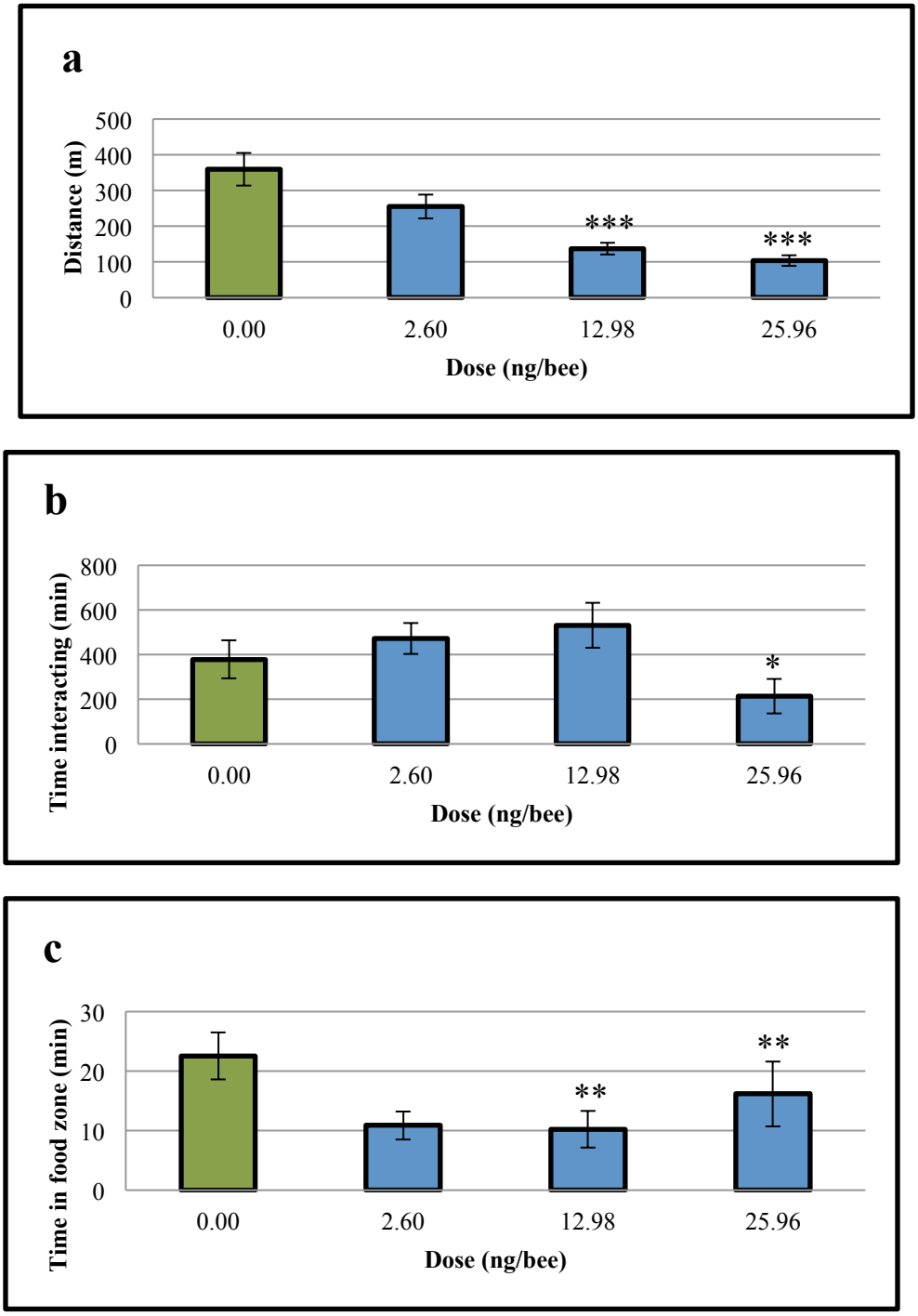
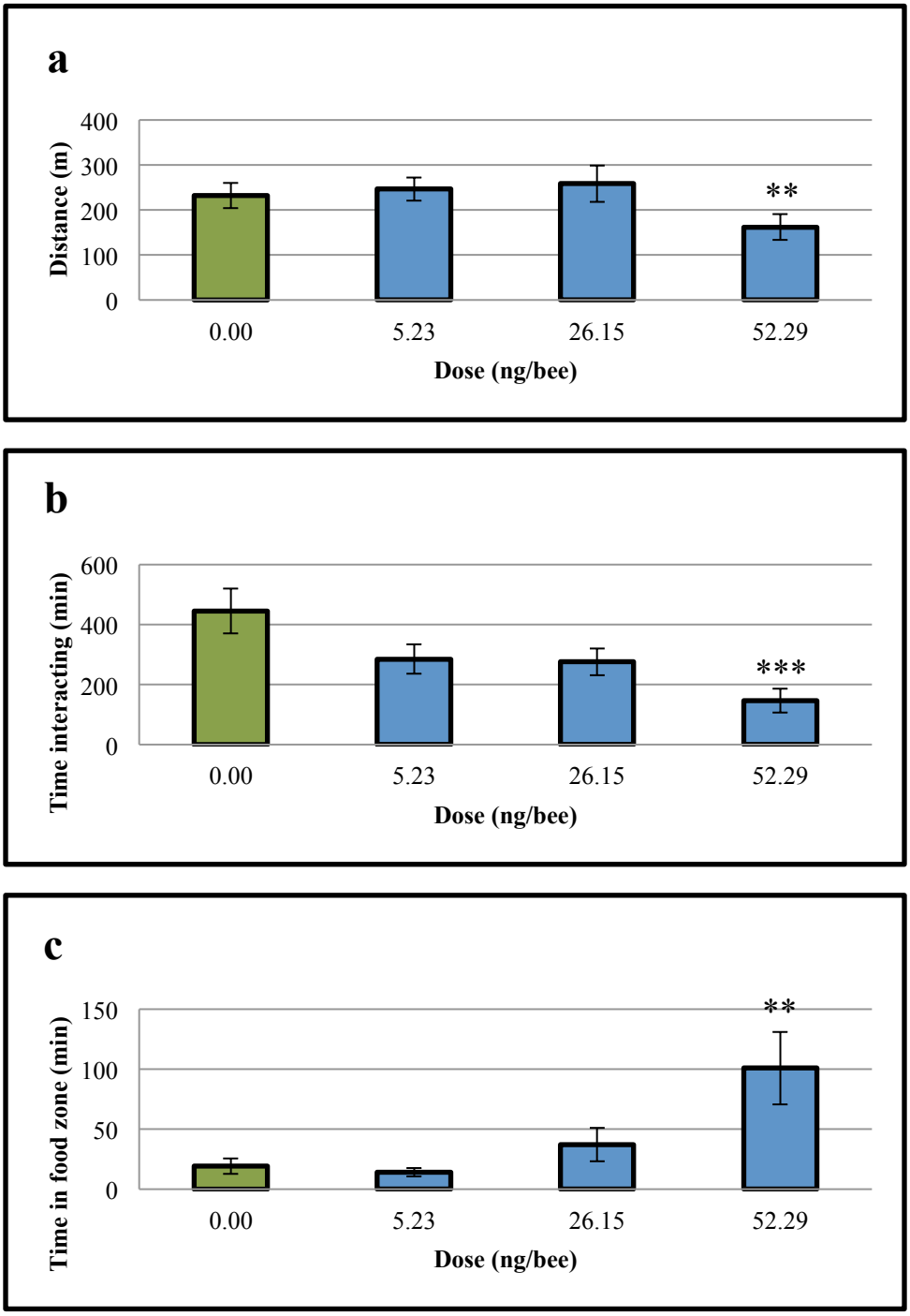


Figure 2.6: Effect of a single topical application of permethrin on (a) distance moved, (b) time spent interacting with another bee, and (c) time spent in the food zone in a 24-hour period. Doses tested: vehicle control (n = 32); 5.23 (n = 32); 26.15 (n = 32); and 52.29 ng/bee (n = 30). Dose levels significantly different from control indicated by *p<0.05; **p<0.01; ***p<0.001.



CHAPTER 3: REPELLENT EFFECT OF PYRETHROIDS ON THE HONEY BEE AT ARTIFICIAL FEEDING STATIONS

INTRODUCTION

Since the early 1900's, repellents have been examined for their ability to prevent or reduce accidental injury to honey bees (Johansen 1977). Bee repellency became especially important when beekeepers suffered serious colony losses due to pesticide poisoning in California between 1966 and 1979. Insecticides were responsible for over 1 million colony deaths (47%) and an overall loss of 10% of the U.S. bee population (National Academy of Sciences 2007). The usefulness of repellent compounds in mitigating risk is debatable as many of these chemicals have shown efficacy in laboratory settings but limited success in field situations (Anderson and Atkins 1968).

Traditionally, screening for repellent compounds requires finding a chemical that is long-lived, cost-effective to manufacture and apply, and does no damage to bees or crops (Jay 1986). Ideally, applying a repellent in conjunction with a highly toxic insecticide would prevent bee poisoning by deterring forager visitation to the treated crop (Johansen and Mayer 1990). Unlike inert repellent compounds, pyrethroids are distinctive in that they serve as both a repellent and an insecticide. Most pyrethroids are highly toxic to bees when applied directly (Smart and Stevenson 1982); however, in field situations, pyrethroids are thought to pose a relatively low hazard to bees based on their low application rates and suspected repellent properties (Rieth and Levin 1988). Because these chemicals have been detected within the hive (Mullin et al. 2010, Frazier et al. 2011, Pettis et al. 2013) foragers may not completely repelled by their presence on foraging resources.

In the case of pyrethroids, it is important to emphasize that "repellency" is not accomplished through disruption of olfactory perception, as is the case with mosquito repellents (Rieth and Levin 1987). Traditional repellents rely on volatile compounds capable of targeting insect odorant receptors. In contrast, pyrethroid repellency is dependent on contact exposure, generally with the tarsi and ventral abdomen, producing an apparent irritant response that results in avoidance of the pyrethroid (Rieth and Levin 1988, Mamood and Waller 1990). These compounds are then transferred to the proboscis and antennae via the tarsi during grooming resulting in additional irritation (Rieth and Levin 1988). Grooming may also serve as a route for additional exposure as a pyrethroid is passed from appendages to the proboscis allowing for ingestion (Wiles and Jepson 1994). If exposure occurs during the course of food gathering, normal foraging behaviors may be replaced by abnormal, excessive grooming and a period of inactivity (Cox and Wilson 1984, Rieth and Levin 1988). As a consequence of decreased foraging, growers may see a reduction in pollination of treated crops as bees could leave flowers without pollinating them. The behavioral shift away from foraging has been termed a repellent effect as the result is forager absence in a treated area, but the mechanism for this repellency has not been determined (Rieth and Levin 1989, Hall and Thacker 1993).

Exposure to pyrethroids has been shown to reduce foraging in artificial feeding settings (Delabie et al. 1985, Rieth and Levin 1988, 1989, Decourtye et al. 2004) and under more realistic field settings on flowering oilseed rape or white mustard seed (Shires et al. 1984, Delabie et al. 1985, Fries and Wibran 1987). As a benefit, forager avoidance of a treated crop may limit exposure to a sub-lethal dose and allow for recovery of exposed individuals (Rieth and Levin 1987, Thompson 2003). However, sub-lethal

exposure may have adverse impacts at the individual or colony level. Chronic, low-level doses of pyrethroids fed to colonies in sucrose solution have resulted in reduced fecundity, decreased rate of successful development and longer developmental times (Dai et al. 2010). Also, increased queen supercedure and reduced brood area have been observed in colonies fed sucrose tainted with Cymbush (cypermethrin) (Bendahou et al. 1999).

Assessing repellency can be challenging as numerous factors may influence foraging behavior including genetics, geography, availability and attractiveness of resources, needs of the hive and environmental conditions. Therefore, in order to control for environmental factors while simulating a field environment, this study was carried out under semi-field conditions (Delabie et al. 1985). The purpose of the study was to evaluate the repellent effects of three pyrethroids currently applied in orchards, lambda-cyhalothrin, esfenvalerate, and permethrin, on the honey bee. While permethrin is an older pyrethroid with a history of repellency testing (Pike et al. 1982, Cox and Wilson 1984, Rieth and Levin 1988), information on the repellent effects of two newer pyrethroids, esfenvalerate and lambda-cyhalothrin, is limited (Fries and Wibran 1987, Mayer et al. 1990). Using an artificial feeding setup with treated floats designed to mimic a flowering resource, foraging activity of honey bee workers was quantified to assess repellency.

MATERIALS AND METHODS

Source of chemicals

Technical-grade 40:60 cis:trans permethrin (CAS# 52645-53-1), esfenvalerate (CAS# 66230-04-4), and lambda-cyhalothrin (CAS# 91465-08-6) were obtained from Chem Service (West Chester, PA).

Source of honey bees

Foraging bees came from a population of 16 honey bee colonies located at the University of Nebraska East Campus apiary. Colonies were headed by Italian queens obtained from C.F. Koehnen and Sons of Glenn, California.

Semi-field setup and treatment

Artificial bait stations similar to those in Rieth (1986) were used to gather bee foraging data in September and October 2012. Each bait station consisted a 1.1 L plastic dish filled with approximately 600 mL of 20% (w:w) sucrose:water solution and placed on top of an inverted 5-gallon bucket. Sucrose solution was scented with peppermint essential oil at 30 ppm. Honey bee colonies were trained to forage at feeding stations located 3.6 meters (12 feet) in front of the hives (Figure 3.1). Sucrose solution was provided from 9:30 am to 12:00 pm *ad libitum* with untreated floats for three days prior to starting the experiment.

Treatment was administered to polystyrene floats covered by a treated, 9cm filter paper allowing for contact exposure to the tarsi and abdomen as bees landed on the floats. The 63.58 cm^2 filter paper received acetone alone or an active ingredient dissolved in acetone. From 9:00-9:30 am prior to the trial, filter papers were treated at rates of 14.2 µg per mL (0.22 µg/cm^2) with lambda-cyhalothrin, 53.5 µg per mL (0.83 µg/cm^2) with esfenvalerate, and 282.9 µg per mL (4.45 µg/cm^2) with permethrin using a 1000 µL

pipette. These rates correspond to a field-relevant dose as they corresponded to the maximum amount of active ingredient (a.i.) allowed per acre on apple according to label specifications. Rates were scaled-down to the surface area of the filter paper (63.59 cm^2) by converting lbs. of a.i. per acre to µg of a.i. per cm^2.

Data were gathered for 15 days in the fall when weather conditions were favorable for foraging (i.e. average temperature range 9.4-21.7^oC, wind < 24 kph, no precipitation). Pairs of control and treatment stations were used for each pyrethroid chemical, esfenvalerate, lambda-cyhalothrin, and permethrin. Following sucrose solution and float distribution at 9:30 (Figure 3.2a, 3.2b) , foraging was allowed to normalize for 30 minutes. After this time, the 6 floats were photographed once every 10 minutes over a 1.5-hour period (10:00 – 11:30) using a digital camera (Figure 3.2c). Using Image J software (Version 1.47; Bethesda, MD) to identify each bee on the float in the photographs, honey bee forager counts were determined for each time point (Figure 3.2d).

Statistical comparisons of the mean forager counts on control and treated floats at each time point were performed using PROC GLIMMIX (version 9.22; SAS Institute 2011) with repeated measures to look for significant differences in foraging intensity over time. The Pearson chi-square divided by degrees of freedom was approximately 1, therefore, a Poisson distribution was assumed for foraging count data (Gbur et al. 2012). The null hypothesis tested was that pyrethroids would not produce a repellent effect at field-relevant doses in an artificial feeder setting.

RESULTS

Of the three chemicals tested, permethrin was the only compound with reduced forager visitation relative to controls confirming a repellent effect for this compound (Figure 3.3c). Fewer foragers were observed on treated floats relative to control floats at all time points and this difference was significantly different for 50 minutes (Table 3.1).

Although significantly increased visitation was detected at one time point for lambda-cyhlaothrin treatment compared to the control (Table 3.1), the overall trend for this compound indicated no difference in forager intensity and thus no repellency (Figure 3.3b). Esfenvalerate showed no significant repellent effect (Figure 3.3a) at any time point.

DISCUSSION

In this study, field-relevant exposure to three pyrethroids used for orchard pest control were applied at artificial feeding stations to assess their repellency based on forager visitation. Decreased foraging was detected for permethrin which is consistent with the report of Rieth and Levin (1988) who reported a decrease in foraging at permethrin-treated feeders, although a higher dose of 8 $\mu g/cm^2$ was tested relative to the 4.45 $\mu g/cm^2$ used in the present study. Additionally, Cox and Wilson (1984) observed that bees topically treated with 0.001 μg of permethrin made fewer foraging trips.

Conversely, no detectable decrease in foraging was observed for lambda-cyhalothrin or esfenvalerate in this study. While little has been reported concerning the repellency of these pyrethroids to honey bees, our findings are in contrast to previous observations. Fries and Wibran (1987) reported reduced forager presence in flowering oilseed rape treated with formulated lambda-cyhalothrin indicating that this

chemical may exhibit repellency for 4-5 hours following treatment. In addition, Mayer et al. (1990) observed a ≥ 86% reduction in bee visitation to sucrose solution tainted with the active ingredient, esfenvalerate, at rates of 50 ppm or more. Lastly, Rieth and Levin (1989) examined the active ingredient, fenvalerate, a compound related to esfenvalerate, and reported repellency at 96 μg a.i./cm^2 on treated floats.

Differences in our methods compared to previous studies may explain contrasting repellency findings. Our method of exposing foragers to a treated float was different from Mayer et al. (1990), who provided esfenvalerate-treated sucrose for consumption, and Fries and Wibran (1987), which examined foragers on lambda-cyhalothrin-treated oilseed rape. Alternative routes of exposure likely influence forager response though all tested methods represent potential means of intoxication. In the case of Rieth and Levin (1989), the route of exposure was consistent with our study; however, our rate of application for esfenvalerate, 0.834 μg/cm^2, was 115-fold lower than the fenvalerate application rate, 96 μg a.i./cm^2, associated with repellency which could explain differences in repellency detection.

This study was designed to simulate field exposure to pyrethroids by testing field-relevant doses to pyrethroids via contact exposure at an attractive, scented food source. Variation was minimized in numerous ways. Treated floats allowed for contact exposure, mimicking a foliar application in the field while maintaining controlled delivery. Bees were trained to forage at artificial feeding stations prior to testing to ensure forager visitation. Additionally, this experiment was carried out during a dearth period, when no other nectar-producing plants were blooming, eliminating the presence of competing nectar sources with possible pesticide contamination. While weather

conditions were not artificially controlled in a greenhouse or tunnel setup, data were only gathered when foraging conditions were optimized. Finally, using technical grade chemicals dissolved in an acetone carrier rather than commercial formulations allowed for a known dose of active ingredient to be tested. Commercial formulation ingredients may have repellent properties aside from the active ingredient which could influence repellency findings (Delabie et al. 1985, Hall and Thacker 1993).

While semi-field testing allows for control of environmental factors, assessing pyrethroid exposure based solely on findings under these conditions is inadvisable, as they may not translate to behavior observed on natural foraging resources (Thompson 2003). Our feeding stations did not incorporate UV fluorescence, an important cue used by bees to locate food resources (Srinivasan 2010). The presence of such signals could increase bees' biological imperative to forage on a crop regardless of pyrethroid application. Previous discrepancies have been observed between semi-field and field repellency findings. Reduced bee visitation was observed on esfenvalerate-treated sucrose but not on blooming red raspberry treated with esfenvalerate compared to untreated plots (Mayer et al. 1990). This may be the result of floral attractiveness negating the repellent effect (Thompson 2003). Additional research conducted under field conditions may provide more definitive repellency evidence for lambda-cyhalothrin, esfenvalerate, and permethrin.

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TABLES

Table 3.1: Mean forager counts and p-values for lambda-cyhalothrin, esfenvalerate, and permethrin. Asterisk indicates significance at α=0.05.

		Mean forager counts		
	Time point	Control	Treated	P-value
Lambda-cyhalothrin	1	65	51	0.4639
	2	77	66	0.4952
	3	93	81	0.2611
	4	91	91	0.9580
	5	86	100	0.3381
	6	90	98	0.5734
	7	98	106	0.4724
	8	91	110	0.1334
	9	105	122	*0.0301
	10	104	120	0.1630

		Mean forager counts		
	Time point	Control	Treated	P-value
Esfenvalerate	1	41	40	0.9571
	2	63	61	0.9388
	3	78	79	0.9633
	4	97	88	0.5055
	5	96	93	0.8343
	6	103	97	0.5946
	7	103	101	0.8869
	8	106	100	0.5801
	9	112	113	0.8603
	10	108	110	0.8096

		Mean forager counts		
	Time point	Control	Treated	P-value
Permethrin	1	57	43	0.3771
	2	69	49	0.1452
	3	85	54	*0.0151
	4	95	63	*0.0250
	5	95	66	*0.0400
	6	94	60	*0.0106
	7	98	65	*0.0238
	8	103	68	*0.0201
	9	112	77	0.0756
	10	109	74	0.0651

FIGURES

Figure 3.1: Experimental setup for artificial feeding station experiment including 16 honey bee colonies and 6 artificial feeding stations (3 chemicals tested in control + treatment pairs).

Figure 3.2: Process of repellency testing including (a) providing 20% sucrose solution in feeder, (b) placing treated or control float, (c) photographing floats at 10-minute intervals for 1.5 hours, and (d) calculating forager visitation with Image J software.

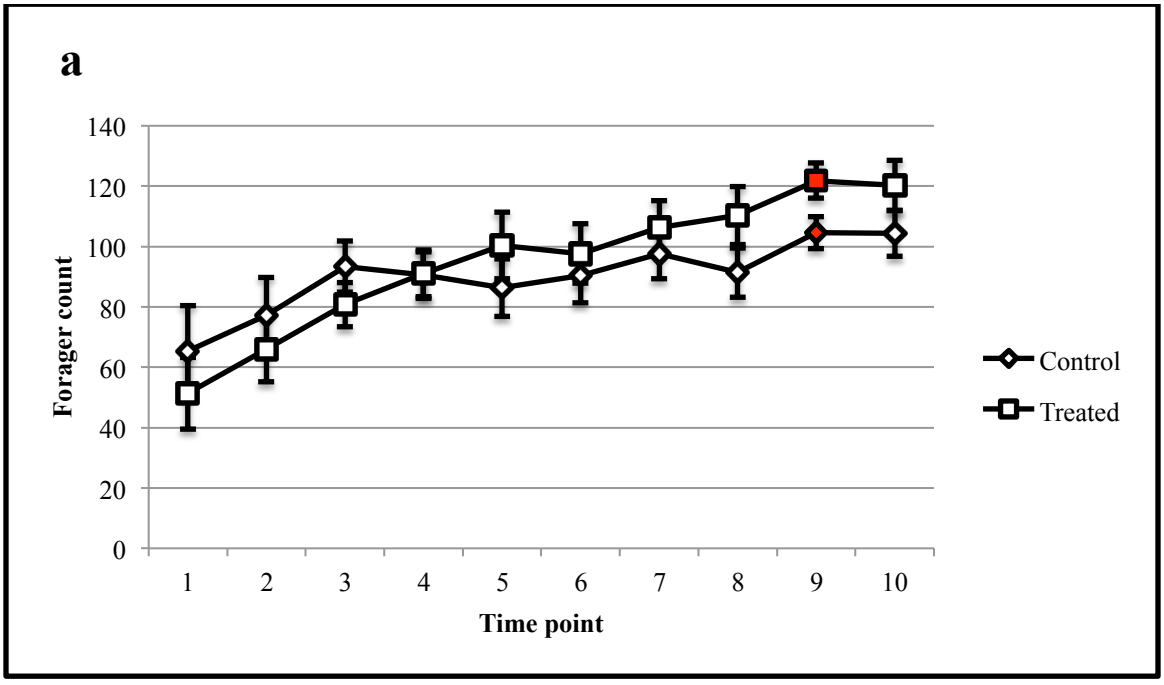
a

b

c

d

Figure 3.3: Comparison of mean forager counts on control and treated floats for (a) lambda-cyhalothrin and (b) esfenvalerate. Error bars indicate the standard error of the mean. Treated means significantly different from control indicated by colored data points.



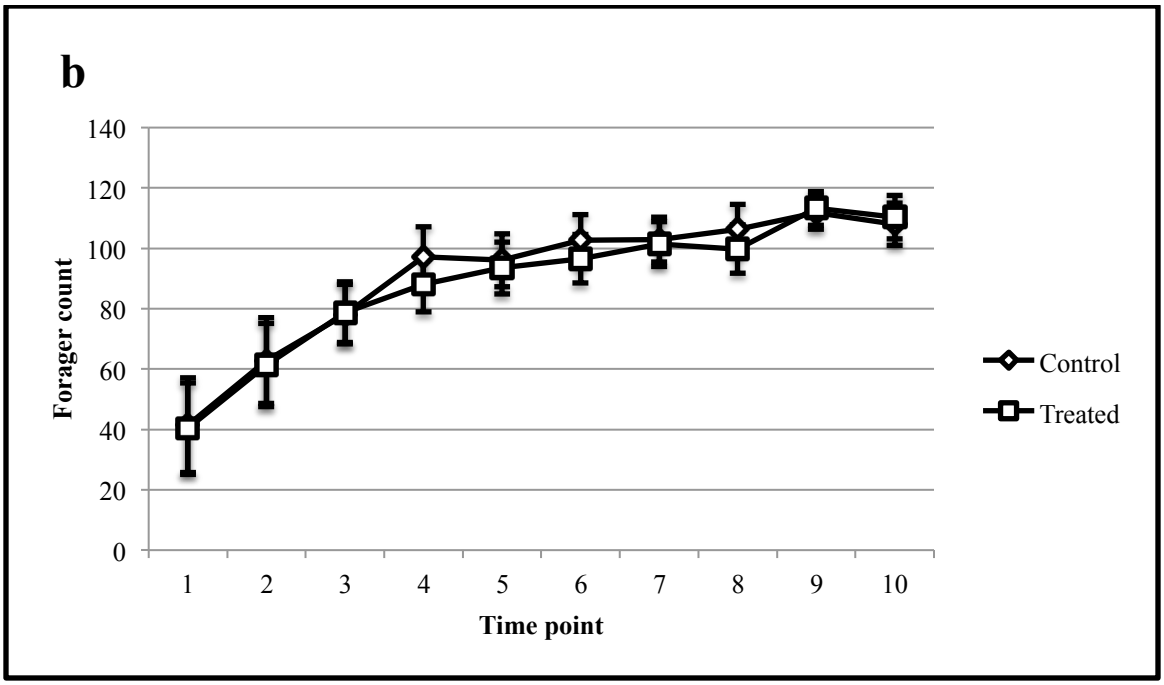
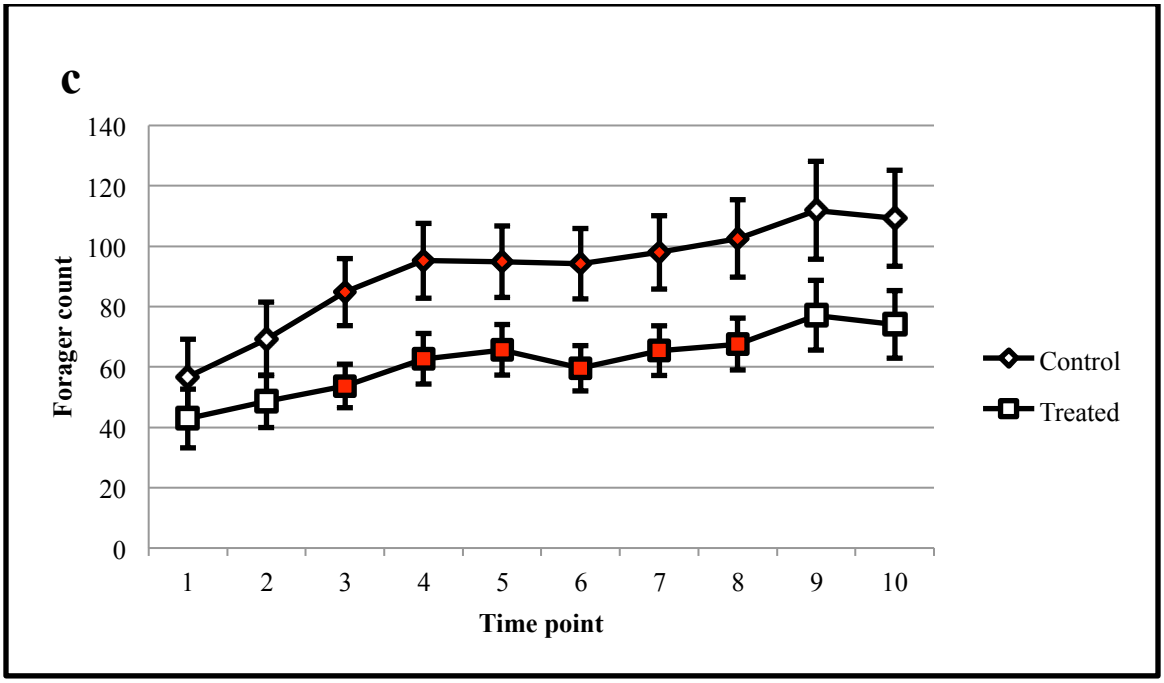


Figure 3.3: Comparison of mean forager counts on control and treated floats for (c) permethrin. Error bars indicate the standard error of the mean. Treated means significantly different from control indicated by colored data points.



CHAPTER 4: ASSESSMENT OF REPELLENCY OF PYRETHROIDS ON THE HONEY BEE IN AN ORCHARD SETTING

INTRODUCTION

Assessing pesticide risk to honey bees requires laboratory toxicity assays as well as an investigation of factors that influence exposure and toxicity in the field. Field application factors that merit investigation include rate and timing of application, residual activity, weather conditions before, during, and after application, the attractiveness of the treated crop or competing crops in the immediate area, and potential repellent effects of the pesticide (Smart and Stevenson 1982). As a first step in risk assessment, pesticides are screened for toxicity using laboratory bioassays (US EPA 2011a). If a pesticide's $LD_{50,}$ or lethal dose required to kill 50% of a test population, is < 11 μg/bee, higher-tier testing is carried out to establish residual toxicity and adverse effects that may result from acute or chronic field exposure (US EPA 2011a). Without higher-tier testing, it is impossible to ascertain the true hazard that pesticides pose to honey bees.

Prior to the development of photostable pyrethroids, most insecticides were applied at approximately equal rates (Smart and Stevenson 1982). Risks posed to bees in the field were generally assumed to correlate to bioassay results (Smart and Stevenson 1982). Acute contact toxicity bioassays for three pyrethroids, lambda-cyhalothrin, esfenvalerate, and permethrin, (see chapter 2) indicate that these compounds are highly toxic to honey bees when exposed by contact or by residues on treated crops (US EPA 2012). However, under field conditions environmental factors including lower rates of application and reported repellency may reduce the potential hazard of these insecticides to honey bees (Smart and Stevenson 1982).

Previous studies have examined pyrethroid repellency under field conditions. Pike et al. (1982) noted decreased forager visitation on permethrin-treated sweet corn. Delabie et al. (1985) examined total pollen gathered by foraging bees and observed a smaller proportion of oilseed rape pollen collected from cypermethrin-treated fields indicating a reduction in foraging activity. Fewer bees were counted on oilseed rape fields treated with lambda-cyhalothrin compared to untreated fields (Fries and Wibran 1987). In contrast to studies reporting repellency, Mayer et al. (1990) did not see a reduction in honey bee foraging activity on blooming red raspberry treated with esfenvalerate.

Field testing for pesticide effects on honey bees presents multiple challenges. Delabie et al. (1985) reported that "bad climatic conditions" did not allow for reliable data collection when measuring forager visitation to cypermethrin-treated oilseed rape. In Delabie's study, the ratio of oilseed rape pollen to total pollen collection was assessed in lieu of forager counts. Rieth (1986) tested repellency of Pounce® (a permethrin formulation) on flowering buckwheat in a greenhouse. This approach was abandoned because: application of formulated product was determined to be inconsistent; commercial formulation did not allow for a known dose of permethrin to be applied; and bees freely moved from untreated to treated buckwheat plants and subsequently avoided all buckwheat regardless of treatment. Additional issues may include limited time, space and resources to carry out successful field studies.

As a result of the numerous challenges facing realistic test conditions, field experiments should be designed to address specific questions unanswered by lower-tier testing (Brady 2011). In the case of pyrethroids applied to field crops, determining repellency is an important part of assessing potential risk in the field. Therefore, as a

follow-up to semi-field repellency testing (see chapter 3), this study was carried out under field conditions using commercial formulations of lambda-cyhalothrin, esfenvalerate, and permethrin to gain a better understanding of potential repellency of pyrethroids used in orchards.

MATERIALS AND METHODS

Source of chemicals

A commercial formulation of Warrior II with Zeon Technology® (lambda-cyhalothrin; Syngenta Crop Protection, Inc., Greensboro, NC) was provided by the manufacturer. Commercial formulations of Asana® XL (esfenvalerate; E.I duPont de Nemours and Company, Wilmington, DE) and Permastar AG (permethrin; LG International, Inc., Englewood Cliffs, NJ) were purchased from Farmers Cooperative Company, Waverly, NE.

Source of honey bees

Sixteen honey bee colonies located at the University of Nebraska East Campus apiary provided the honey bee population for this experiment. Colonies were headed by Italian queens obtained from C.F. Koehnen and Sons of Glenn, California.

Field setup and treatment

Repellency testing was carried out in spring 2013 using lambda-cyhalothrin, esfenvalerate, and permethrin in an orchard setting. In order to approximate field conditions, we used commercial formulations of the three pyrethroids rather than

technical grade insecticide dissolved in acetone as previously used in laboratory and artificial feeding station experiments.

Rather than attempt to count foragers on whole apple trees, branches with open apple blossoms were removed from trees, grouped into bouquets, and placed into 5-gallon buckets with water. This made it easier to observe foraging accurately and apply treatment as uniformly as possible. Treatment was applied using a separate hand sprayer (RL Flo-Master polymer funnel top sprayer, 3.8L capacity; Root-Lowell Manufacturing Co., Lowell, MI) for each pyrethroid tested. Each sprayer was calibrated to apply 19.27 mL per sq. ft. To approximate a field-relevant dose, each pyrethroid was applied at the maximum amount allowed for application on apple trees according to label specifications (lambda-cyhalothrin at 2.56 oz. per acre, esfenvalerate at 14.5 oz. per acre and permethrin at 16 oz. per acre) or with water as a control. Application rates were converted from oz. per acre to mL per sq. ft. to account for the small scale application to bouquets. Commercial formulation was thoroughly mixed with 1L of water and applied to bouquets between 17:30 and 18:00 the evening prior to forager observation. Treated plant material was exposed to outdoor weather conditions for approximately 16 hours prior to starting the experiment.

Preliminary observations indicated that foraging on apple blossoms began at approximately 10:30. In preparation for foraging, 16 treated bouquets were arranged in front of the colonies at 9:30 in a 4×4 randomized complete block with each of the four treatments represented in each row (Figure 4.1).

Forager visits were manually counted in 10-minute intervals. Counts were recorded for two parameters: (1) foragers landing on a blossom for < 3 sec indicating repellency and (2) foragers remaining on a blossom for ≥ 3 sec indicating attraction. Multiple visits would be counted if a single forager landed on multiple blossoms as each blossom visit offered another opportunity for a forager to be exposed to the treatment.

Four individuals simultaneously observed the treatments in a single row beginning with row 1 and continuing through row 4 (as seen in Figure 4.1). This allowed for visitation to be counted for all sixteen bouquets once within 40-45 minutes. This process was repeated at three time points (10:30, 11:15, and 12:00). Data were gathered for as long as apple blossoms were viable and when weather did not prevent foraging behavior (temperature > 12.8^{o}C, wind speed < 24.1 km/hour, and no precipitation). Observations were made on three days in which weather conditions and bloom were sufficient to attract foragers.

Following data collection, statistical comparisons of the mean control and treatment counts at each time point were performed using PROC GLIMMIX (version 9.22; SAS Institute 2011) with repeated measures to look for differences in foraging intensity over time. The alternative hypothesis tested was that the presence of the applied pyrethroid would repel bees from apple blossoms.

Results and Discussion

Assuming adequate bloom and weather, our goal was to gather data over four dates which would provide 16 experimental units for each treatment. Unfortunately, poor weather, petal drop, and low forager counts allowed observations on only three days.

Limited foragers were observed during the narrow bloom period with 23, 11, and 2 foragers visiting the bouquets. Consequently, a high percentage of bouquets received no visitation at any point in the experiment (85%, 73%, and 75% of bouquets lacked foraging at time points 1, 2, and 3, respectively). The extreme lack of foragers and their limited visitation to experimental units did not allow for confident analysis of these data. Raw data were pooled and are presented in Figure 4.2 with total foraging divided into counts of visitation lasting less than 3 sec indicating repellency and visitation lasting greater than or equal to 3 sec indicating attraction. While statistical analysis could not be carried out, variable foraging behavior was noted at time points 1 and 2, but observations indicated that attraction outweighed repellency at all treatments by time point 3.

Multiple factors likely contributed to lack of usable data for this experiment including competing floral resources, poor weather, difficulty in handling apple blossom, and high variability of forager visitation. Limited forager counts could be the result of bees attracted to competing floral resources in the immediate area. Dandelion (*Taraxacum officinale*), henbit (*Lamium amplexicaule*) and apple trees (*Malus domestica*) were in bloom and found in close proximity to our experimental apiary (Figure 4.3). The small size of our bouquets likely attracted fewer foragers than full trees in bloom or fields of dandelion and henbit.

In addition, spring weather is often volatile and may negatively impact honey bees' ability to forage. A combination of heavy cloud cover, high humidity, low temperatures, and high wind speeds prevented foraging during the first days of open bloom. A narrow window of good weather from May 13 – 15 allowed for foraging observations; however, by the final day of observations, forager counts were low with

only two foragers observed, and petal fall was considerable. In this instance, weather proved to be a challenge to gathering usable data under field conditions providing evidence for the usefulness of artificial feeding station trials in place of field trials. Sheltered conditions, such as those found in cage and greenhouse experiments, could be used to increase forager visits.

Using treated apple blossom rather than artificial feeding stations presented another challenge to this experiment. The stage of bloom maturity was highly variable on a single branch leading to a wide range of 10 – 202 open blossoms within a bouquet over the three testing dates. As petal fall increased, usable bloom was available but spread out over many branches making it difficult to equalize bouquets. Additionally, apple blossoms became delicate after they were cut from the tree and were difficult to treat and arrange without damaging their structure. For our experiment, apple blossoms were used as they were representative of a realistic, floral orchard source with attractive volatiles and UV fluorescence; however, the challenges inherent in timing of apple blossom and handling of this plant material may indicate that cuttings of apple blossom are not a suitable test material. Blooming red raspberry and oilseed rape may be better suited for repellency testing as they are still attractive to bees and have been used successfully in previous studies (Delabie et al. 1985, Fries and Wibran 1987, Mayer et al. 1990).

Finally, the act of observation may have contributed to minimal or variable foraging behavior. Disruptions to foraging were minimized by locating observers out of the flight path between the hives and bouquets, dressing observers in white, veiled jackets; and limiting unnecessary movements. However, the presence of four individuals at the test bouquets cannot be discounted. Digital video recording may provide an

alternative method of observation for future work. Cameras placed on tripods would eliminate all extraneous movement, bees could be trained to their presence, and footage could be reviewed at a future date.

In conclusion, field repellency testing did not result in usable data for analysis. Therefore, artificial feeding station results (see Chapter 3) were more useful in estimating repellency of lambda-cyhalothrin, esfenvalerate, and permethrin in the absence of reliable field data.

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FIGURES

Figure 4.1: Randomized complete block experimental design for field experiment with sample treatment assignments.

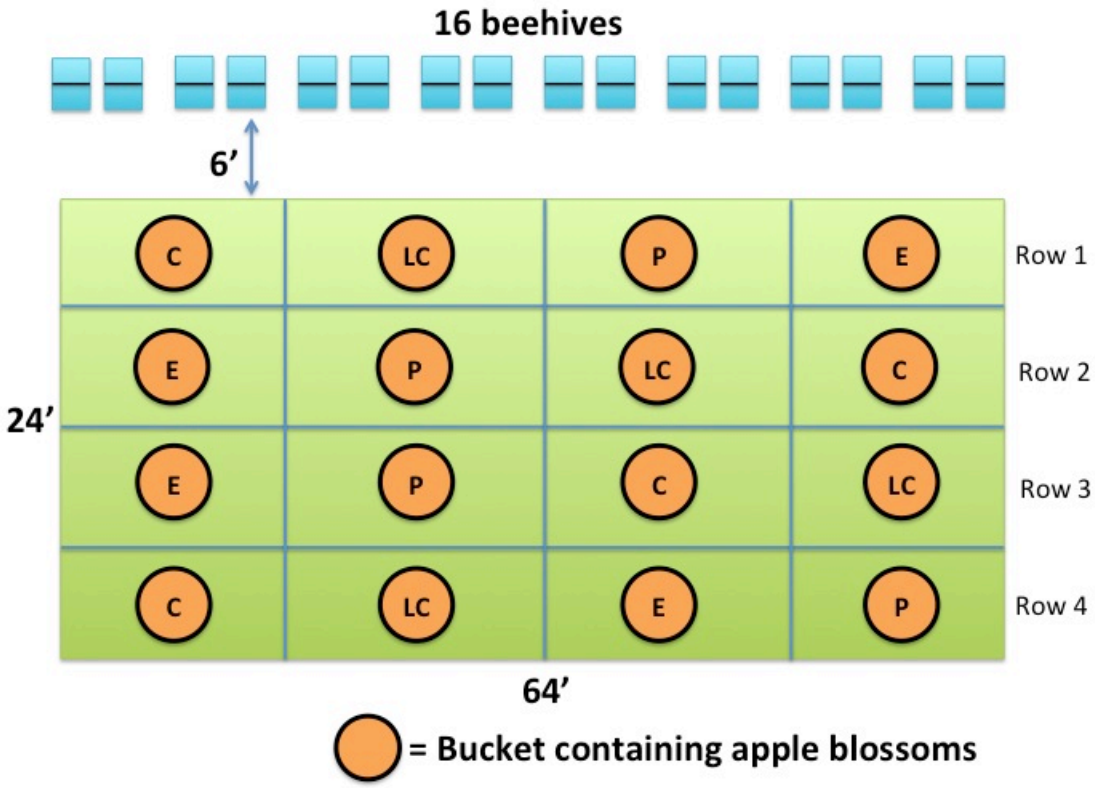

Figure 4.2: Total forager visitation to apple blossom bouquets treated with (C) control, (LC) lambda-cyhalothrin, (ES) esfenvalerate, and (PER) permethrin.

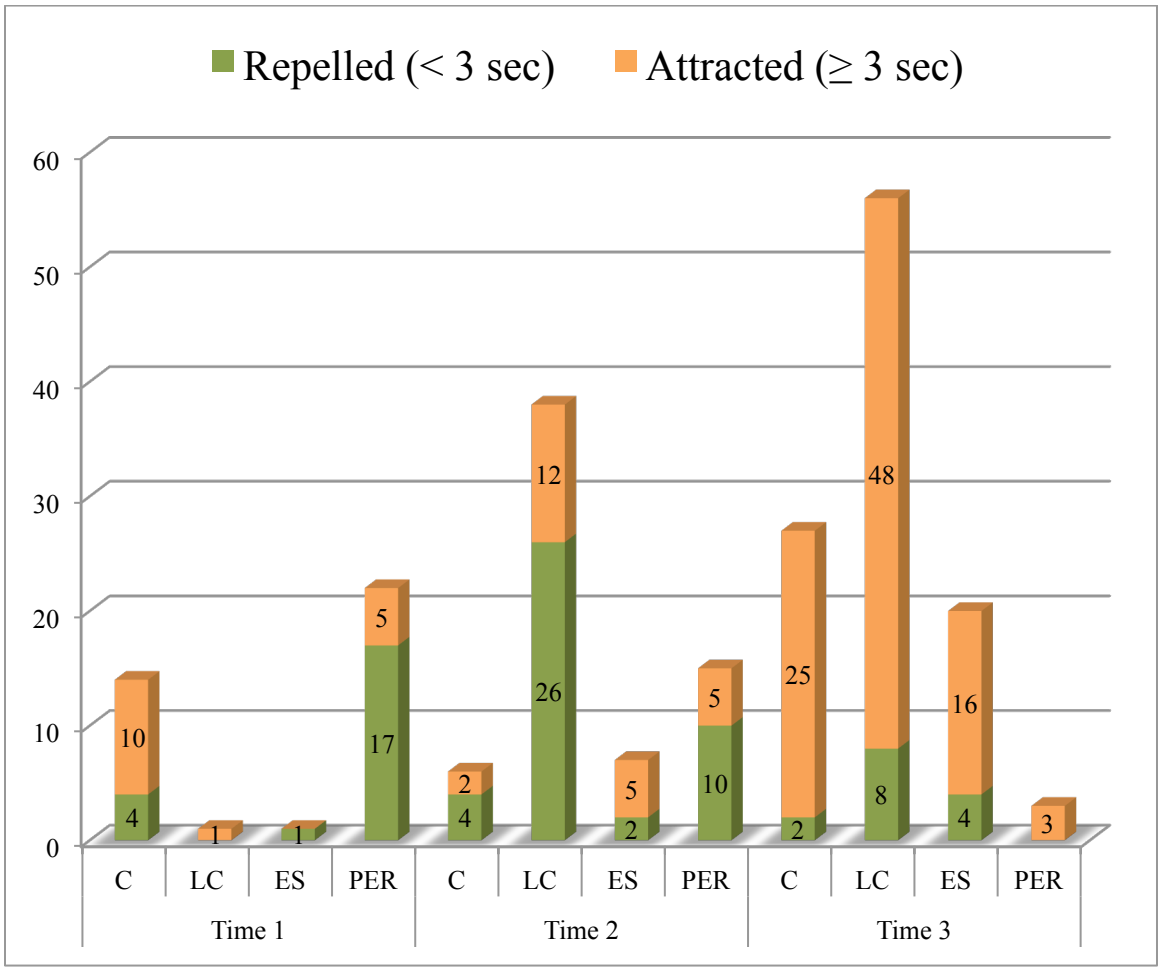

Figure 4.3: Dandelion, henbit, and apple trees blooming in proximity to field experiment on May 8, 2013.