

FORAGING BEHAVIOR OF HONEYBEES EXPOSED TO MOSQUITO
INSECTICIDES

By

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ABSTRACT

ANALYSIS OF CHANGES IN FORAGING BEHAVIOR OF HONEYBEES EXPOSED TO MOSQUITO INSECTICIDES

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The main aim of this study was to quantify the effects of mosquito insecticides on honey bee (*Apis mellifera*), foraging behavior, explicitly assessing the impacts of adult mosquito control practices on honey bee health. The extent to which honey bees are exposed to pyrethrum, pyrethrins, permethrin, and pyrethroids is unknown, as are their effects on honey bee health and vitality. This study sought to answer this question by exposing honey bees to D-Phenothrin with the addition of Piperonyl butoxide (PBO), one of many synthetic pyrethroids widely used against adult mosquitoes to control mosquito-borne diseases. Honey bees were exposed to nonlethal concentrations of D-Phenothrin, and D-Phenothrin with the addition of piperonyl butoxide (PBO), and the effects on hive trips (foraging and short trips) were analyzed using a field study where foraging bees were tracked using Radio-frequency identification (RFID) tags attached to the thorax. These performance measures were selected for their relevance to bees' ability to survive in nature. Although D-Phenothrin is an axonic excitotoxin that prevents the closure of voltage-gated sodium channels of axonal membranes, foragers exposed to nonlethal doses did not show any significance in foraging behavior. With the inclusion of PBO (Primary Hypothesis), D-Phenothrin showed no significant changes in the number of hive

trips (foraging or short trips) relative to the control group. Additionally, D-Phenothrin alone (Secondary Hypotheses 1) showed no significant changes in the number of hive trips (foraging or short trips), while the solvent control acetone (Secondary Hypotheses 2) did result in a significant decrease in the number of short/bathroom trips. Honeybees are vital for crop production across the globe. This study is intended to contribute to the scientific understanding of honeybee population declines and ultimately point scientific and agricultural communities toward increasing colony health and vitality and reversing the trend of increasing death rates.

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INTRODUCTION

1.1 Economic Importance and Decline of Honey Bees

The honeybee (*Apis mellifera*) is essential to agriculture worldwide, and apiculturists struggle to maintain the health and vitality of hives for crop production. It is estimated that annually, honey bees add \$15 billion in added crop value in the United States, and \$235-\$577 billion worth of annual global food production depends on pollination from honey bees, native bees, and flies alone (Bayer, 2019; Medicine, 2020). Preliminary results for the year of April 1st, 2020 – April 1st, 2021, estimated that 45.5% of managed honeybee colonies in the United States perished, the second-highest annual loss recorded to date. This loss rate is 1.8% higher than last year's loss rate of 43.7% and 6.1% higher than the average (39.4%) over the last ten years (Steinhauer et al., 2021). The decrease in colony health and vitality is not a result of a single influence. Various afflictions, including pathogens, parasites, management practices, nutrition inadequacies, and pesticide exposure, are plaguing colony survival rates. Beekeepers have experienced an increase in insecticide-related die-offs, and while it is understood that many insecticides are toxic to honeybees, the issue is complex. Researchers and the agricultural community have been actively making strides toward protecting pollinators and reducing the risks of exposure to honeybees by working together to understand insecticide, herbicide, and fungicide effects on pollinators and change application processes (Sanchez-Bayo &

Goka, 2014). However, despite the strides made in improving agricultural practices, insecticide exposure is still a critical issue for honeybees.

1.2 Pesticide Contamination of Honey Bees' Hives

On average, hives contain six different pesticides, and in recent studies, as many as 121 insecticides have been discovered in hives. Several of these insecticides, organophosphates, pyrethrins, and pyrethroids, are commonly used in misting systems for the control of adult mosquitoes from the genera *Anopheles*, *Aedes*, *Culex*, *Culiseta*, *Mansonia*, *Coquillettidia*, and *Psorophora* (Long & Krupke, 2016; Koenraadt, 2015; Mullin *et al.*, 2010). The highest levels of contamination in various areas of hives come from pyrethrins and pyrethroids. Pyrethroids are similar to natural pyrethrins produced by the flowers *Chrysanthemum cinerariaefolium* and *C. coccineum*. Pyrethrins are a broad-spectrum insecticide that and kill a wide variety of insects. Approximately 30% of the world's market for insecticides is pyrethroids, with over 1,000 pyrethroids being synthesized. Pyrethroids are registered for commercial and residential use for insect pest control and are found being used in ships, aircraft, homes, crops, vector control, and more (Bao *et al.*, 2020; Pfeil, 2014; United States Environmental Protection Agency (US EPA), 2013, 2013, 2015, 2017, 2020). Due to the diversity of use, the United States has about 23 synthetic pyrethroids approved and over 3,500 pyrethrin and pyrethroid insecticide products registered by the Environmental Protection Agency (California Department of Pesticide Regulation, 2021; US EPA, 2015, 2017) Of the pyrethroid insecticide residues

found within hives, D-Phenothrin is known for its use as mosquito adulticides; therefore, D-Phenothrin was the focus compound of this experiment.

1.3 Pyrethroids as Vector Control.

While insecticides have numerous uses in agriculture, including but not limited to crop protection and preservation of food, insecticides are also widely used in the prevention of vector-borne diseases. Pyrethroids and organophosphates are commonly used vector control for fleas, ticks, lice, and mosquitos, with mosquitos being a major vector for many diseases worldwide (Chaskopoulou et al., 2014). These management practices include source reduction, larval control, and adult mosquito control. (Fouet & Kamdem, 2019; Howard et al., 2007; US EPA, 2013, 2013). Despite the significant advances in research and treatment as of 2018, mosquito-borne diseases such as dengue fever, yellow fever, malaria, chikungunya, West Nile, and Zika fever still account for an estimated 17% of all infectious disease deaths globally (Audain et al., 2017; Chandrasegaran et al., 2020; Gao et al., 2020). Although management programs need to be designed according to regional needs, insecticides such as pyrethroids are considered a fundamental tool for controlling mosquito populations (Fouet & Kamdem, 2019; Howard et al., 2007; US EPA, 2013, 2013). Some places, such as the United States, use bacterial insecticides, synthetic pyrethroids, insect growth inhibitors, organophosphate insecticides, and mineral oils or monomolecular films to control larval mosquitos (US EPA, 2013, 2013). Another practice is the use of ultra-low volume (ULV) application of insecticides for adult mosquitos. In the United States, synthetic pyrethroids and organophosphates are

registered for application by truck-mounted and aircraft misting systems. D-Phenothrin is one pyrethroid commonly used in these misting systems in the United States and Greece (Chaskopoulou et al., 2014; Luo et al., 2016; US EPA, 2013, 2013).

1.4 Impact of D-Phenothrin on Honey Bees

A) D-Phenothrin mechanism of action in insects

In the United States, a majority of insecticides are neurotoxic that inhibit neurotransmitters or affect voltage-gated sodium channels (Hénault-Ethier, 2016). Pyrethroids are organic compounds that are axonic excitotoxins that interfere with the voltage-gated sodium channels in the axon of neurons (Figure 1) (Hénault-Ethier, 2016). As a synthetic version of pyrethrins, pyrethroids mimic the effects of the pyrethrin esters while having longer residual effects and increased stability in storage and are considered extremely toxic to fish and non-target invertebrates (Devine & Denholm, 1998; Krejci, 2020; Li et al., 2017; Long & Krupke, 2016; Reregistration Eligibility Decision (RED) D-Phenothrin, 2008; Soderlund et al., 2002). Pyrethroids are divided into two classes, Type I and Type II. Type I pyrethroids lack the α -cyano group, which enhances toxicity in Type II pyrethroids (Hénault-Ethier, 2016). D-Phenothrin is a synthetic Type I pyrethroid affecting the central and peripheral nervous system (Devine & Denholm, 1998; Soderlund et al., 2002; US EPA, 2008). In a typical neuron, voltage-gated sodium channels of the axonal membrane open, allowing sodium to pass and close after the action potential. The singular action potential propagates through the axon of the nerve and triggers muscle contraction. When exposed to pyrethroids, the sodium channels begin

to malfunction. The voltage-gated sodium channels of the axonal membrane are prevented from closure for extended periods causing repetitive nerve discharge and increased excitation (Type I) or leaving the axonal membrane depolarized permanently (Type II) (Figure 1). The repetitive firing (in Type I pyrethroids) or depolarization (in Type II pyrethroids) leads to tremors or involuntary movements, salivation, paralysis, and death of the insect (Costa, 2008; Devine & Denholm, 1998; Hénault-Ethier, 2016; Lushchak et al., 2018; Soderlund et al., 2002; Song & Narahashi, 1996; US EPA, 2013, 2013, 2015, 2017, 2020). While most literature indicates Type I and II pyrethroids are associated with specific symptoms, this has been found not to be the case, and therefore insects can exhibit any of the above symptoms when exposed to pyrethroids of any class (Hénault-Ethier, 2016).

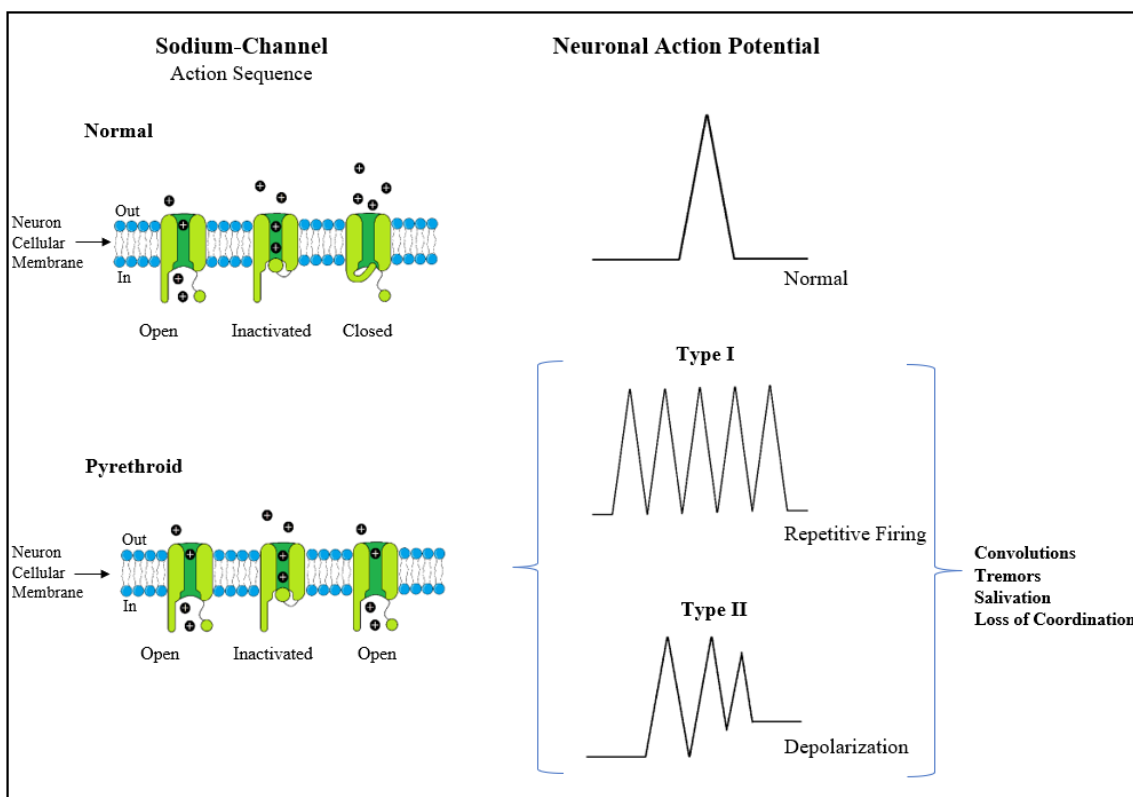


Figure 1. Mechanism of action of pyrethroids used in adult mosquito control.

Normal functioning voltage-gated sodium channels open allowing sodium ions to pass through the neuron cellular membrane and close after the action potential (top). The singular action potential propagates through the axon of the nerve creating muscle contraction. Pyrethroids bind to the voltage-gated sodium channels (bottom). After initiation of the action potential, sodium channels malfunction leaving them open resulting in repetitive firing (Type I) or depolarization (Type II). Repetitive firing and depolarization lead to convulsions, tremors, salivation, and loss of coordination.

Pyrethroids such as D-Phenothrin have enhanced effectiveness by mixing them with common synergistic compounds such as piperonyl butoxide (PBO) and MGK-264

(Hénault-Ethier, 2016). The main route for detoxification in insects is through the mixed-function oxidase system. In insects, P450 enzymes are found in virtually all tissues and perform a multitude of important tasks, one of which is the metabolism of foreign chemicals of natural or synthetic origin (Peng et al., 2017). In an insect, PBO inhibits the mixed-function oxidase (MFO) system; specifically, it inhibits natural detoxification by the enzyme Cyt P450 (Figure 2) (Hénault-Ethier, 2016). Therefore, when PBO is included in an insecticide, the levels of the insecticide within the insect remain elevated, increasing their lethality (Casida, 1970; Jones, 1998; Moores et al., 2009).

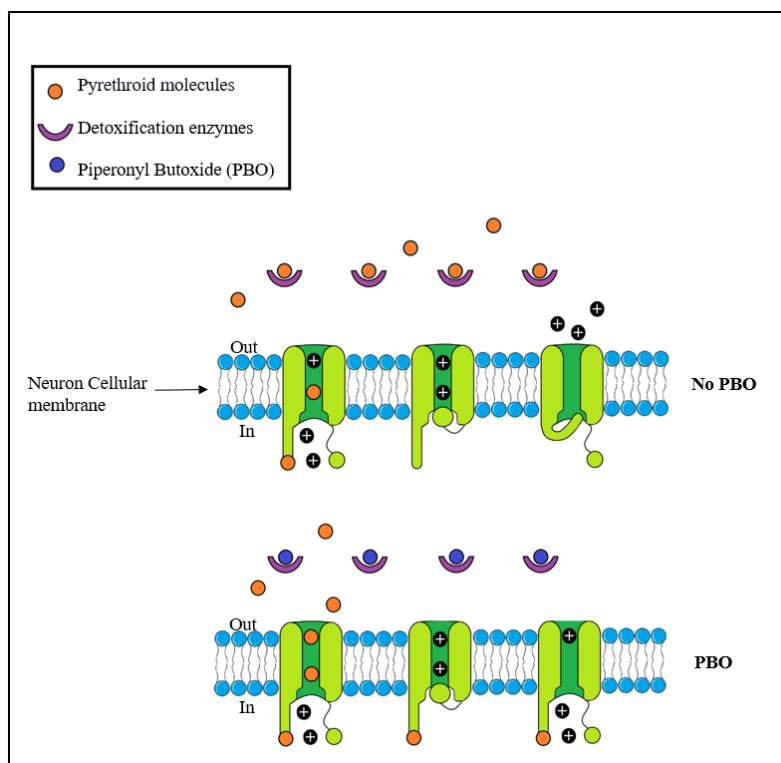


Figure 2. Pyrethroid effectiveness on voltage-gated sodium channels. Top: A normal voltage-gated sodium channel with an intact mixed-function oxidase (MFO) system. The detoxification enzymes (purple U) cause the pyrethroid molecules (orange circles) to become inactive, preventing binding to the sodium channels. Bottom: In the presence of PBO (blue circles), detoxification enzymes are blocked, and the active pyrethroid molecules are not bound and detoxified, resulting in higher concentrations of the pyrethroid molecules reaching the sodium channel binding sites.

B) Honey bees mixed-function oxidase system

The honey bee's mixed-function oxidase (MFO) system has significantly fewer P450 enzymes for detoxification than some other insects. Compared to mosquitos, honey bees have less than a quarter of those found in mosquitos. This lack of detoxification enzymes

could put honey bees at a higher risk for adverse effects from pyrethroids, especially when combined with piperonyl butoxide to inhibit the mixed-function oxidase system (Claudianos et al., 2006; Feyereisen, 2011, 2018; Hénault-Ethier, 2016; Jones, 1998; Moores et al., 2009).

C) Motor, sensory, and cognitive impacts of D-Phenothrin on honey bees

In previous pyrethroid honeybee toxicity research, Type I and II pyrethroids have been shown to have a negative effect on motor functions (Gupta & Milatovic, 2014; Oliver et al., 2015). Similar to D-Phenothrin, permethrin has been associated with alterations in feeding, communication, and maintenance at low rates of exposure. Honey bees exposed to permethrin topically at 0.001 µg demonstrated an increase in trembling dances, abdomen tucking and rotation, self-cleaning, and leg-rubbing; and spent less time giving food, antennae touching, and walking (Palmquist et al., 2012). When exposed to sublethal amounts of deltamethrin, a synthetic pyrethroid, honeybees exhibited less time grooming and impaired memory and learning, resulting in cognitive disorder (Oliver et al., 2015; Palmquist et al., 2012). Exposure to deltamethrin has been shown to cause honeybees to have less precise waggle dances, altered homing abilities and flight patterns, and reduced learned orientation toward odor stimulus (Palmquist et al., 2012; Zhang et al., 2020). While lacking information on piperonyl butoxide, these findings on pyrethroids lead to the possibility of D-Phenothrin, another pyrethroid and voltage-gated sodium channel-targeting insecticide, as having a negative impact on motor functions resulting in the inability for foragers to leave the hive to bring back essential resources.

1.5 Honey Bee Exposure to Adult Mosquito Insecticides.

A) Ultra Low Volume (ULV) application pyrethroids for mosquito control

Research has found varying degrees of safe ULV application of pyrethroids and organophosphates for adult mosquitos near honeybee colonies (Rinkevich et al., 2017). In some instances, D-Phenothrin has no significant effect on honeybees when used during periods of pollinator inactivity, low doses, and at great distances from hives (Caron, 2019; Pankiw & Jay, 1992; Pokhrel et al., 2018). One study found, using the recommendation of the World Health Organization for ULV applications of 7.5 g/ha D-Phenothrin (Pesguard S102) and 1.0 g/ha deltamethrin (Aqua K-Othrine) for mosquito management had no significant nontarget mortalities over a 2-year study with five spray trials. Nor did honeybees exhibit any effects from sublethal exposure, performing as well as control hives (Chaskopoulou et al., 2014). While these studies and others show no significant issues for honey bees exposed to ULV application of pyrethroids, these studies and others do not consider particular factors such as label recommendations for long term spraying, recommended length mortality studies for honey bees, synergistic effects from other pesticides, including other pyrethroids, nor the inclusion of synergistic compounds such as piperonyl butoxide (California Department of Pesticide Regulation, 2021; US EPA, 2013)

B) Pesticide exposure and the use of pyrethroid for mosquito abatement

Understanding and tracking the rates of pesticide exposure for honey bees is complex. Detailed data on pyrethroid use and pesticides, in general, are lacking or nonexistent for

most countries (Li et al., 2017). Currently, the complete pesticide database is from the California Department of Pesticide Regulation. However, in this author's experience, the database lacks sufficient searching tools to find specific data on specific pesticides (Li et al., 2017). Even with the amount and number of pesticides used being tracked in California, there is no set regulation to cap the overall amount and number of pesticides used in the United States. The only law that governs the application of pesticides in the United States is the requirement to follow the individual pesticide label. In 2018, California's Department of Pesticide Regulation (DPR) documented pesticide use was 20,900,664 pounds of active ingredients. Of those active pesticide ingredients, 792,549 pounds were pyrethroids, and 59949 pounds was piperonyl butoxide (PBO) (California Pesticide Information Portal: *Summary of Pesticide Use Report Data 2018, 2021*). In many countries, there are no regulations for mosquito abatement as well. In the United States, the responsibility falls on the state and county to spray for mosquitos, with spray rates being determined by local governments using only insecticide label recommendations and no set guidelines for abatement in or around apiaries. The Environmental Protection Agency (EPA) has label requirements for pyrethrin and pyrethroids stating not to use products near or on crops and weeds that are in bloom where bees are visiting. These labels statements, however, are only required for liquid pesticide products designed for outdoor agricultural use and exclude any pesticide products used for residential use and/or Ultra Low Volume (ULV) wide area mosquito control applications (US EPA, 2013, 2013, 2015, 2017, 2020). While it is possible to contact local abatement programs state and county-wide to request no spraying near hive

locations, programs are not required to skip areas, nor are they required to maintain a safe distance from hives and any deaths as a result of mosquito abatement practices are also excluded from the ability to report a pesticide-related hive death to the EPA for tracking active ingredient lethality (Tehama County Mosquito and Vector Control District, personal communication, May 03, 2021; US EPA, 2013, 2013, 2015, 2017, 2020)

1.6 Purpose of Study and Hypothesis

D-Phenothrin, a mosquito insecticide, is known to impair motor function in honeybees. In addition, the finding that honeybees naturally have weaker enzymatic defenses to break down D-Phenothrin suggests that the common practice of the adding enzyme inhibitor, PBO, to D-Phenothrin sprays, can be especially harmful to honeybees. The current study was therefore designed to test the effects of D-Phenothrin alone and in combination with PBO, on honeybee behavior. Specifically, bees were individually fed sucrose solutions containing insecticide or control compounds, then fitted with microchip trackers and freed. The trackers allowed automated measurement of the number and timing of trips to or from the home hive. I hypothesized that D-Phenothrin with the addition of PBO (Primary Hypothesis) would have the most significant magnitude of disruption to hive trips, and D-Phenothrin alone (Secondary Hypotheses 1) would have the second most significant disruption to hive trips compared to the sugar control. Additionally, I hypothesized that the solvent control acetone (Secondary Hypotheses 2) would result in no significant disruption to hive trips.

METHODS

2.1 Colony Establishment and Study Site

Hives started with 15 five-frame nucleus colonies with approximately 8,000 bees and a mated Italian queen in an apiary located in Platina, CA, USA. Sixteen new colonies were established, in March 2021, following typical beekeeping protocols and compensating for current die-off rates in the United States. Hives where the queen left or died had Italian replacement queens installed from Olivarez Honey Bees, Inc. Colonies, were fed 1:1 sucrose syrup and pollen for 90 days and transferred to 10-frame hives when required, based on growth (Bruckner *et al.*, 2019; Medrzycki *et al.*, 2013). In August 2021, colonies were transported to a new location near Corning, CA, USA.

2.2 Establishing Pesticide Dosage Concentration for Behavioral Studies

A) Dosage establishment

To assess the sublethal effects of D-Phenothrin, the development of a nonlethal dose to administer to experimental bees was necessary. Dosage establishment began with a published LD₅₀ and included piperonyl butoxide (PBO) due to its use with D-Phenothrin to enhance the effectiveness against mosquitos (Casida, 1970; Jones, 1998; Moores *et al.*, 2009). Many of the adult mosquito insecticides registered for use in the United States contain equal parts of pyrethroids and piperonyl butoxide (California Department of Pesticide Regulation, 2021). Therefore, the dosages in this experiment did as well. The United States Environmental Protection Agency Pesticide Ecotoxicity Database's oral

administration study of D-phenothrin found $0.13 \mu\text{g bee}^{-1}$ (contact LD50 studies) to be lethal and $0.094 \mu\text{g bee}^{-1}$ as a NOAEL (no-observed-adverse-effect level) (*OPP Pesticide Ecotoxicity Database*, 2018).

B) Dosage establishment trials

Initial experimental dosing began with $0.10 \mu\text{g bee}^{-1}$ of D-phenothrin and $0.10 \mu\text{g bee}^{-1}$ piperonyl butoxide in each bee. This number was used because it was lower than the LD50 and higher than the NOAEL published. Forty bees from the experimental colony were placed in California Mini Queen Cages for each dosage to be evaluated (Mann Lake Ltd. Woodland, CA, USA) (Figure 3). Each queen cage contained one bee and was kept at ideal foraging temperature, 14°C - 38°C , during dose testing (Medrzycki et al., 2013; Tautz et al., 2003). Bees were starved for up to two hours, and the dosage was micro pipetted directly into/onto the proboscis of each bee. The average nectar intake of an adult forager per foraging trip is $20\text{-}40 \mu\text{g}$. Based on these criteria, each dosage was diluted to $10 \mu\text{l}$ with 50% sucrose solution to increase likelihood of each bee taking the total dose (Feuerbacher et al., 2003; Medrzycki et al., 2013). Bees were observed at 30 minutes, 12 hours, 24 hours, and 48 hours post dosage and had access to food, 50% sucrose solution, and water during observations. (Knopper et al., 2016; Pamminger et al., 2019; Tautz et al., 2003). If the death rate hit 50% before 48 hours or was still increasing at 48 hours beyond 50%, the dose was considered lethal, the evaluation ended, and a new lower dosage was administered to a new group of bees (Medrzycki et al., 2013; US EPA, 2014, 2016, 2016, 2018). A nonlethal dosage was determined to be $0.08 \mu\text{g bee}^{-1}$ D-phenothrin and $0.08 \mu\text{g bee}^{-1}$ piperonyl butoxide in each bee. The D-phenothrin available

for this experiment came dissolved in acetone, an approved solvent at 5% concentration or lower for honey bee toxicology (California Department of Pesticide Regulation, 2021; US EPA, 2014, 2016, 2016, 2018; Medrzycki et al., 2013). Acetone was an additional control (acetone only group) in this study because it differs from petroleum distillates solvents used in available products for mosquito abatement (Table 1). In a typical mosquito adulticide product, petroleum distillates are solvents used to mix the pesticides with other ingredients (*Petroleum Distillates*, 2020).



Figure 3. Queen Cages. Queen cages with individual bees for dosage. Cages were kept together to reduce the stress of the bees and to minimize unwanted changes to normal behavior (Medrzycki et al., 2013).

Table 1. Five treatment groups: sugar control, PBO only, D-Phenothrin only, acetone control, and D-Phenothrin + PBO. Each dose was added to a 1:1 sucrose solution to ensure the bees would be willing consume the full dose.

Group	D-Phenothrin	Piperonyl Butoxide	Acetone	1 :1 Sucrose & Water
1: Sugar Control	--	--	--	10 μ l
2: PBO Only	--	0.08 μ l	--	9.92 μ l
3: D-Phenothrin Only	0.08 μ l	--	--	9.92 μ l
4:D-Phenothrin + PBO	0.08 μ l	0.08 μ l	--	9.84 μ l
5: Acetone Control	--	--	0.05 μ l	9.95 μ l

2.3 Measuring D-Phenothrin Effects on Honey Bee Foraging Behavior

A) Hive and reader setup

For the foraging assessments, the experimental hive was moved onto a 1m tall table and had the iID®BEE reader attached to the entrance. A hive reducer was added to channel bees through the reader. A landing platform was added to the hive for ease of entering and exiting the hive. The hive was allowed to acclimate for an initial 24 hours before the collection of bees began (Decourtye et al., 2011; Medrzycki et al., 2013; US EPA, 2014, 2016, 2016, 2018). RFID (radio frequency identification) tags were programed from MircoSensys with an individual identification number (UID). Tags were further programmed using the iID®BEE PENsolid mobile reader with the title of one of five experimental groups: sugar control, PBO only, D-phenothrin only, D-phenothrin plus PBO, and acetone (Table 1). The reader was then connected to the iID®BEE controller, 12V power supply, and power source. Every UID detected was stored on a 14Gb USB flash drive attached to the controller. Every 15 seconds, a timestamp and flying direction

were recorded for any identified UIDs. Each experimental group was monitored for four full foraging days of 12 hours each.

B) Tagging foragers

The age of a honey bee may affect the foraging behaviors being measured independently of the experimental pesticide treatment (Cardoso-Júnior et al., 2018; Herb et al., 2012; Johnson & Frost, 2012; Medrzycki et al., 2013; Rinkevich et al., 2015). Foragers were randomly sampled and randomly assigned into treatment and control groups, therefore distributing age effects equally among groups to control for this source of error variability.

For each of the 5 treatment groups 50 bees were collected and tagged. Foragers of an undefined age were collected going through the entrance of the hive using a Pipe Queen Catcher to be tagged (Mann Lake Ltd. Woodland, CA, USA) (Figure 4) (Johnson & Frost, 2012). The caste a female worker bee is in dictates what behaviors and duties individual bees perform. Workers that are at the entrance of the hive are thus foragers or guards. Based on this behavior, female workers leaving the hive were determined to be foragers and collected (Johnson & Frost, 2012). Collection of bees began in the morning at the start of foraging hours between 6 and 8 am and ended in the afternoon when bees began to return to the hive between 4 and 6 pm. Bees were collected in queen pipes in groups of 10-12 individuals. Individual bees were then transferred into a Queen Marking Tube modified with a 2mm nylon mesh (Mann Lake Ltd. Woodland, CA, USA) (Figure 5). In the queen tube, the bee was compressed gently to prevent movement. Using a combination of tweezers and a wax gemstone picker, tags were glued to the bees using

Krazy Glue, Max Bond Gel (Decourtye et al., 2011; Tutun et al., 2020). Bees were then transferred to queen cages for dosing. Any bees that died during the process of tagging or while in the queen cage before being dosed were discarded and replaced.



Figure 4. Pipe Queen Catcher. Sponge was removed and catcher was placed over bee at hive entrance. Once a bee was in the tube, the cork was removed, and bee was coaxed into the marking tube for tagging.

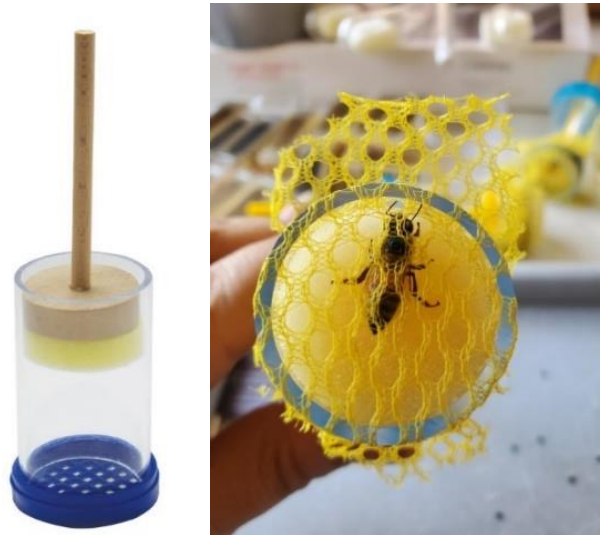


Figure 5. Modified Queen Marking Tube. Bees were placed inside a modified tube and a plunger was slowly pushed to trap the bee against a mesh top for gluing of the RFID tag.

C) Dosing foragers

Five experimental groups of 50 individual foraging bees received one of the five treatments (sugar control, PBO only, D-Phenothrin only, acetone control, and D-Phenothrin + PBO; Table 1). Each bee was confined to a queen cage at an ideal foraging temperature, above 14°C, and starved for up to two hours before the dosage was micro pipetted directly into/onto the proboscis of each bee (Figure 6; Medrzycki et al., 2013; Tautz et al., 2003). Bees were starved an additional hour and offered a standard 50% sucrose solution before being placed back at the entrance of the hive (Decourtye et al., 2011).



Figure 6. Dosing of bees. Each bee had a single dose in 50% sucrose solution micro pipetted directly into/onto the proboscis for one of the five experimental groups. Bees received $0.05 \mu\text{l}$ acetone, $0.08 \mu\text{g bee}^{-1}$ D-phenothrin, $0.08 \mu\text{g bee}^{-1}$ piperonyl butoxide, $0.08 \mu\text{g bee}^{-1}$ D-phenothrin and $0.08 \mu\text{g bee}^{-1}$ piperonyl butoxide, or $10\mu\text{l}$ 50% sucrose solution.

D) Behavioral measures

The bees were observed using RFID tags to investigate the mosquito insecticides' effects on the frequency and duration of foraging and non-foraging trips from the hive (Decourtye et al., 2011; Schneider et al., 2012). For assessing motor function, tagged experimental bees were monitored with the reader, and the dependent measure was the number of foraging and non-foraging trips from the hive. The dependent measure for assessing cognitive function was the number of bees that did not make it back to the hive over the 72-hour (three-day) foraging period (Figure 7).



Figure 7. Reader on hive entrance. The reader was screwed into place on the hive entrance with a reducer used to prevent bees from navigating around the reader. A ramp was added to aid in landing at the reader, and to create additional landing space that had been lost with the reader attachment.

2.4 Data Formatting and Exclusion

Data collected by the iID@BEE program was copied to Excel for the initial inspection. The data was converted to USA time and date reporting standards, and 9 hours were subtracted from each timestamp to convert the summertime zone from Germany (UTC, the program's default) to California (PDT). Four data exclusion criteria were then applied: 1. Reads from 6:01 pm to 5:31 am were removed from the data because bees are not expected to be foraging during those hours; 2. The first timestamp (recorded hive entrance or exit) for each bee was removed because immediately after tagging with the active reader, the experimenter placed the bees at the hive entrance to encourage them to crawl through, producing a reading; 3. Timestamps that were <15 seconds apart were

considered duplicate readings of the same trip, most likely caused by bees lingering at the hive entrance, so those were removed from the data set; 4. Bees that had over 200 timestamps, including multiple timestamps at < 15-second intervals, were considered to be acting as guard bees at the hive entrance and therefore had their data removed (Johnson & Frost, 2012). The data were then split into two categories: short trips lasting less than 3 minutes and 30 seconds, which were likely to be bathroom trips, and long trips lasting more than 3 minutes and 30 seconds, which were considered foraging trips (Thompson et al., 2016; Perry et al., 2015). Trips were only counted if there was a confirmed “departure” and an “arrival” or “unknown” following that departure. The tracker produced “unknown” readings when bees came through the reader during a time recording or at an angle; the reader was unable to confirm direction. These were considered likely returns and therefore counted.

2.5 Tests of Statistical Assumptions

The formatted/cleaned data was used to test assumptions for planned statistical tests. Exploratory analyses were performed to check for equivalent variances, normal distribution, and outliers for the two categories (short trips and long trips). Data transformations (square root and \log_{10}) were to create normal distribution and reduce the effects of outliers. Research has shown that when data has asymmetrical distribution and large sample size, a non-parametrical Kruskal-Wallis test has a higher power compared to a one-way ANOVA (Hecke, 2010). Therefore, the data were left in the original form (i.e., no transformations or outlier exclusion), and a Kruskal-Wallis test was performed. The

outliers in the data were genuinely unusual values, so they were not removed while performing the statistical analysis as they were considered to reflect significant effects of experimental manipulations potentially. Additionally, outliers do not heavily influence the Kruskal-Wallis test. The Kruskal-Wallis test was used to test the equality of the distributions by comparing the mean ranks of each distribution of foraging trips. The mean rank was used to determine whether the number of foraging trips in one group(s) was lower or higher than in the other group(s). Data for each of the five treatment groups were checked for similar shapes, and a Kruskal-Wallis test based on differences in group medians was also performed (Laerd Statistics, 2015; 2015).

RESULTS

3.1 Statistical Assumptions

A) Foraging trip assumptions

Each group (Sugar, PBO, D-Phenothrin, D-Phenothrin + PBO, and Acetone) was shown to have equivalent variances, but the data did not fit a normal distribution as required for standard parametric statistical tests. The data were skewed positively (indicating that a few bees in each group had much higher numbers of foraging trips than others) and contained many outliers (Figure 8). While the data were not normally distributed, the data for each of the five treatment groups did have similar shapes allowing for the Kruskal-Wallis test based on differences in group medians (Laerd Statistics, 2015; 2015).

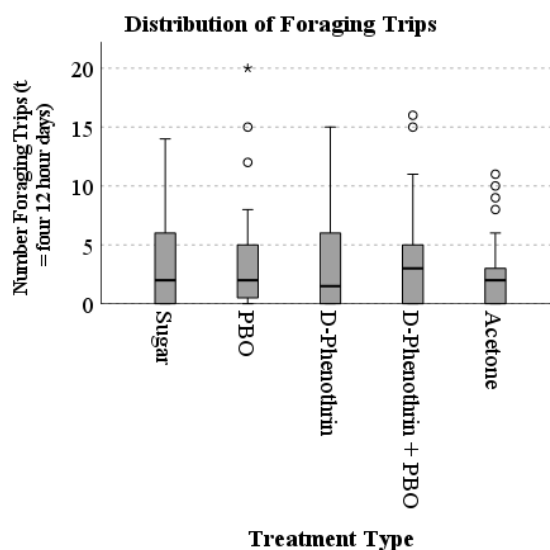
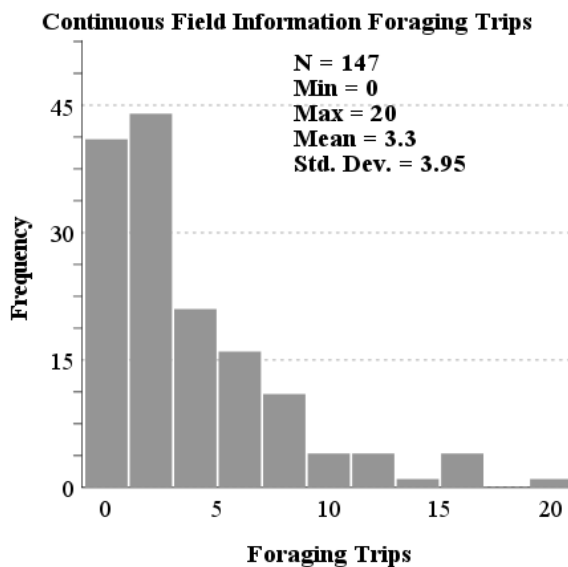
Figure 8B**Figure 8A**

Figure 8. Distribution of foraging trips. A. Error bars represent SD, circles represent outliers, and asterisks (*) are extreme outliers where data points are more than 3 box-lengths away from the edge of their box. Each Treatment group (sugar, PBO, D-Phenothrin, D-Phenothrin + PBO, and acetone) had equivalent variances and data did not fit a normal distribution. **B.** The positive skew and high number of outliers indicated that some bees in each group had a higher number of foraging trips than others.

B) Non-foraging assumptions

Similar to the foraging trip data, groups (sugar control, PBO, D-Phenothrin alone, D-Phenothrin + PBO, and acetone control) had non-equivalent variances, and the data did not fit a normal distribution as required for standard parametric statistical tests. The data was skewed positively (indicating some bees in each group had much higher trips than others) and contained many outliers (Figure 9). The short non-foraging trip data also had similar shapes among treatment groups allowing for the Kruskal-Wallis test based on differences in group medians (Laerd Statistics, 2015; 2015).

Figure 9A

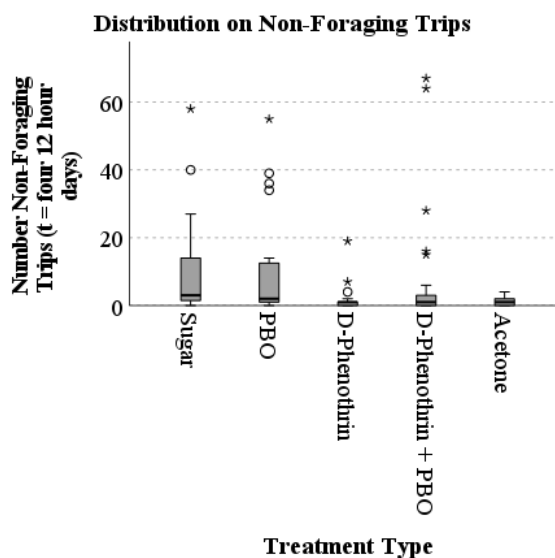


Figure 9B

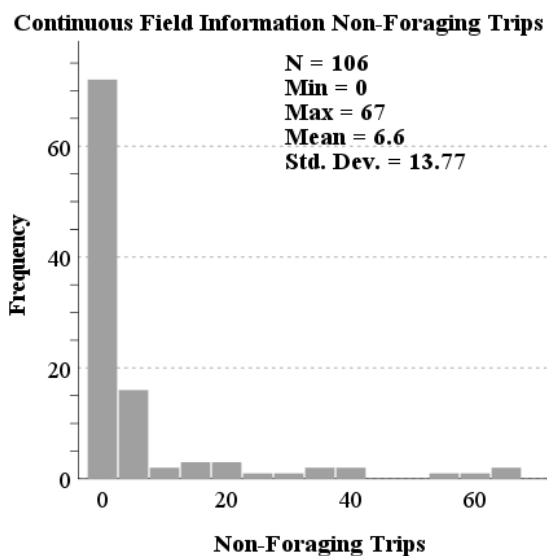


Figure 9. Distribution of non-foraging trips. A. Error bars represent SD, circles represent outliers, and asterisks (*) are extreme outliers where data points are more than 3 box-lengths away from the edge of their box. Each Treatment group (sugar, PBO, D-Phenothrin, D-Phenothrin + PBO, and acetone) had equivalent variances and data did not fit a normal distribution. **B.** The positive skew and high number of outliers indicated that some bees in each group had a higher number of non-foraging trips than others.

3.2 Foraging Trips

Distributions of foraging trips were similar for all groups, as assessed by visual inspection of a boxplot. Median (Mdn) comparison indicated D-Phenothrin + PBO had the highest number of foraging trips among all groups (Mdn = 3.00), and D-Phenothrin alone had the lowest (Mdn = 3.00; Table 2). Additionally, when considering mean ranks (M), the acetone treatment group had the lowest number of foraging trips (M = 68.94) while D-Phenothrin + PBO had the highest number of foraging trips (M = 77.77; Table 3).

Table 2. Foraging trip means and medians.

Treatment	Kurtosis	Std. Deviation	Median	Mean	Range Minimum	Range Maximum
Sugar	1.273	3.770	2.00	3.28	0	14
PBO	4.232	4.773	2.00	3.61	0	20
D-Phenothrin	1.269	4.172	1.50	3.55	0	15
D-Phenothrin + PBO	2.530	4.081	3.00	3.63	0	16
Acetone	1.755	3.007	2.00	2.56	0	11
Total	2.943	3.953	2.00	3.31	0	20

Table 3. Foraging trip mean ranks comparison.

Treatment	N	Mean Rank
Sugar	25	73.84
PBO	31	74.47
D-Phenothrin	22	75.34
D-Phenothrin + PBO	35	77.77
Acetone	34	68.94
Total	147	

The Kruskal-Wallis analysis of foraging trips (trips lasting >3 minutes and 30 seconds) revealed no statistically significant difference among groups, therefore, Hypothesis I was not supported ($\chi^2(5) = 0.805, p = 0.938$; Table 4).

Table 4. Independent-Samples Kruskal-Wallis Test summary foraging trips. A. The test statistic is adjusted for ties. **B.** Multiple comparisons (post hoc tests) were not preformed because the overall test does not show significant differences among groups.

Total N	147
Test Statistic (χ^2)	0.805 ^{A, B}
Degrees Of Freedom	4
Asymptotic Sig. (2-sided test)	0.938

3.3 Short Non-Foraging Trips

Distributions of non-foraging trips were similar for all groups, as assessed by visual inspection of a boxplot. Median (Mdn) comparison indicated Median non-foraging trips were equivalent for D-Phenothrin, D-Phenothrin + PBO, and acetone (Mdn = 1.00). Sugar control treatment group had the highest non-foraging trips (Mdn= 3.00) and PBO had the second highest (Mdn= 2.00) (Table 5). Additionally, the Mean ranks (M) indicated D-Phenothrin (M = 42.61) had the lowest number of non- foraging trips, while sugar control (M = 69.68) had the highest number of non- foraging trips (Table 6).

Table 5. non-foraging trip means and medians

Treatment	Kurtosis	Std. Deviation	Median	Mean	Range Minimum	Range Maximum
Sugar	3.564	15.916	3.00	10.74	0	58
PBO	1.492	16.643	2.00	11.26	0	55
D-Phenothrin	12.381	4.509	1.00	2.28	0	19
D-Phenothrin + PBO	6.619	18.392	1.00	8.48	0	67
Acetone	.042	1.128	1.00	1.24	0	4
Total	8.347	13.774	1.00	6.62	0	67

Table 6. Non-foraging trip mean ranks comparison.

Treatment	N	Mean Rank
Sugar	19	69.68
PBO	19	67.00
D-Phenothrin	18	42.61
D-Phenothrin + PBO	25	49.18
Acetone	25	43.10
Total	106	

The Kruskal-Wallis analysis revealed significant differences among groups in the number of non-foraging trips, $\chi^2(5) = 15.379$, $p = 0.004$ (Table 7). Pairwise comparisons were performed, between all groups, using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Adjusted p -values and unadjusted p -values are presented. Adjusted p -values revealed statistically significant differences in median non-foraging trips between sugar and acetone treatment groups ($p=0.035$; Table 8). While the Kruskal-Wallis analysis of foraging trips (trips lasting < 3 minutes and 30 seconds) revealed a statistically significant difference among groups, a pairwise comparison among all groups indicated that Hypothesis II was not supported, as no other treatment group combinations were statistically significant under the adjusted p -value (Figure 10).

Table 7. Independent-Samples Kruskal-Wallis Test summary non-foraging trips. A.

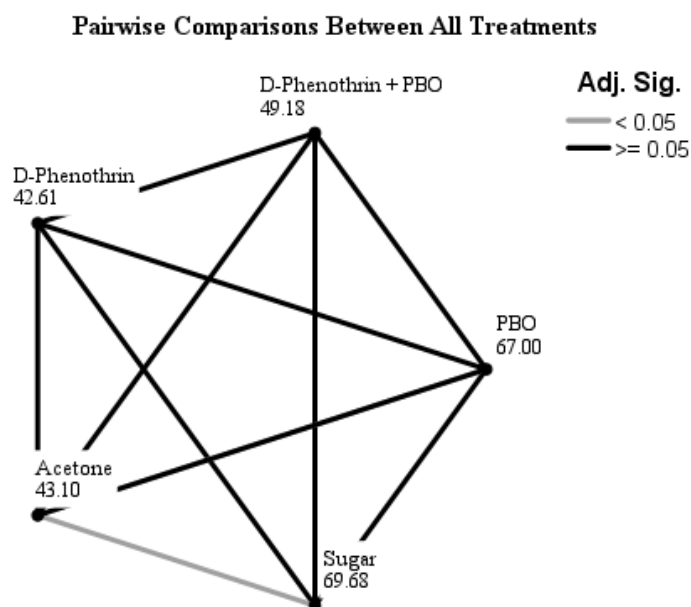
The test statistic is adjusted for ties. **B.** There were statistically significant differences and multiple comparisons (post hoc tests) were performed.

Total N	106
Test Statistic	15.379 ^{A, B}
Degrees Of Freedom	4
Asymptotic Sig. (2-sided test)	0.004

Table 8. Pairwise comparisons among all treatments examining mean ranks of non-foraging trips. Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed, and the significance level is 0.05. **A.** Significance values have been adjusted by the Bonferroni correction for multiple tests.

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig. ^A
D-Phenothrin-Acetone	-.489	9.241	-0.053	0.958	1.000
D-Phenothrin-D- Phenothrin + PBO	-6.569	9.241	-0.711	0.477	1.000
D-Phenothrin-PBO	24.389	9.833	2.480	0.013	0.131
D-Phenothrin-Sugar	27.073	9.833	2.753	0.006	0.059
Acetone-D-Phenothrin + PBO	6.080	8.456	0.719	0.472	1.000
Acetone-PBO	23.900	9.099	2.627	0.009	0.086
Acetone-Sugar	26.584	9.099	2.922	0.003	0.035*
D-Phenothrin + PBO-PBO	17.820	9.099	1.959	0.050	0.502
D-Phenothrin + PBO- Sugar	20.504	9.099	2.254	0.024	0.242
PBO-Sugar	2.684	9.699	0.277	0.782	1.000

Figure 10. Pairwise Comparison Plot showing comparisons among all treatments on the number of non-foraging trips. Each node shows the sample average rank of treatment. Black lines between treatment groups represent no significance based on adjusted significant values. Gray lines represent significance between treatment groups.



DISCUSSION

4.1 Main Purpose and Results

A) Main purpose

To date, studies have determined that the synthetic (chemical) insecticide classes organochlorines, carbamates, organophosphates, pyrethroids, and neonicotinoids are detrimental to honey bee health and behavior and are a significant driver of managed honey bee colony losses (Appendix A; Connelly, 2012; Dolezal, 2022; Johansen et al., 2013; Kiljanek et al., 2016; Kumar et al., 2020; Oliver et al., 2015; Ostiguy et al., 2019; Traynor et al., 2021; Williams et al., 2015; Wu et al. 2011). Common insecticides used in agriculture crops and public health are indeed highly toxic to honey bees. Even so, risk assessment studies determining effects on non-target pollinators in the field are limited and a highly debated topic. Additionally, there is less understanding of the effects of sub-lethal amounts or long-term exposure to pesticides due to fewer studies using oral exposure or field studies; many studies have relied on a contact LD⁵⁰ use laboratory settings with low ecological validity to determine risks (Caron, 2019; Connelly, 2012; Dolezal, 2022; Johansen et al., 2013; Kiljanek et al., 2016; Kumar et al., 2020; Long & Krupke, 2016; Oliver et al., 2015; Ostiguy et al., 2019; Pankiw & Jay, 1992; Pokhrel et al., 2018; Traynor et al., 2021; Williams et al., 2015; Wu et al. 2011). This study was designed to assess the impacts on honeybee colony health of one insecticide (D-Phenothrin) that honey bees are exposed to through mosquito control practices,

specifically misting systems to control adult mosquitoes within the foraging ranges of apiaries.

B) Interpretations of results and theories

Results indicated that the use of realistic doses of oral D-Phenothrin and an ecologically valid measurement showed no effect. When exposed to D-Phenothrin, there were no significant changes in honey bee foraging behaviors. D-Phenothrin alone, or D-Phenothrin combined with the synergistic compounds piperonyl butoxide (PBO), did not influence honey bees' number of foraging trips. Non-foraging trip numbers were also unaffected by D-Phenothrin and D-Phenothrin with PBO. However, acetone, the solvent control, did result in bees having fewer short, non-foraging trips outside the hive.

Although acetone was shown to be significant in this study, ecologically, the significance is not relevant in the context of this study. Although used as a solvent for D-Phenothrin in laboratory settings, acetone is not used as the solvent for mosquito adulticide products.

Acetone was tested as a control in this study because the available D-Phenothrin came dissolved in acetone and not a petroleum distillate used in mosquito adulticides. While it is potentially important to consider the results of acetone in other contexts, it does not have clear implications for assessing the risks of D-Phenothrin to honey bees (See Methodological limitations and recommendations; *Petroleum Distillates*, 2020; SPEX CertiPrep, 2020).

While conservative comparisons indicated no statistical significance for non-foraging trips, biologically speaking, there was an indication that the foragers had a change in behavior surrounding non-foraging short trips. The mean ranks, medians,

and unadjusted p-values indicated that foragers exposed to P-Phenothrin alone or D-Phenothrin in combination with PBO had fewer non-foraging short trips compared to the sugar control. The complexities of honey bee behavior and the limited research on foraging behavior make it difficult to determine the correlation between D-Phenothrin and a reduction in short non-foraging trips without additional research. Two potential theories that need to be explored are that exposure to D-Phenothrin and D-Phenothrin with PBO (Pyrethroids) has disrupted the foragers' gut microbiota and feeding or caused a shift in division of labor and the performance of particular foraging tasks.

Honey bees are hygienic overall, and defecation is never done inside a hive unless it is the queen, which is cleaned up after, or if a worker is sick. Honey bee gut microbiotas are vital for the health of bees and the processing and storage of food resources in the hive. Pesticide exposure is known to disturb these microbiotas. Pesticide exposure can directly affect the growth of beneficial gut bacteria, disrupt gut homeostasis, and lower immune defenses to pathogens in honey bees. Additionally, insecticides are known to cause a reduction in feeding behaviors (Connelly, 2012; Dolezal, 2022; Hotchkiss et al., 2022; Johansen et al., 2013; Kiljanek et al., 2016; Kumar et al., 2020; Oliver et al., 2015; Ostiguy et al., 2019; Traynor et al., 2021; Williams et al., 2015; Wu et al., 2011). One possible reason P-Phenothrin and D-Phenothrin with PBO had fewer non-foraging short trips is that the exposed foragers had disrupted gut microbiotas and were eating less, reducing the number of times each bee defecated. Additional research on the effects of pyrethroids on feeding and defecation would need to be performed to test this hypothesis.

In a hive, foragers are classified as scout bees looking for resources and nest sites and reticent bees that become resource gatherers. Scout bees will leave the hive searching for the best resources while the reticent bees wait to be given information on the resource location (Abou-Shaara, 2014). Scout bees exhibit distinct patterns of gene activity in the brain compared to non-scout foragers and are risk-takers who are more likely to search for new resources than go to established resource locations (Liang et al., 2012). Hives also have the ability to follow a push-pull model where caste changes are triggered by environmental stressors and primer pheromones that require a hive to necessitate colony-level and individual shifts in castes (Johnson & Frost, 2012). Research has indicated that insecticide exposure in foragers results in transcriptional alterations of endocrine-related genes of the brain and changes in worker bee response to primer pheromones (Favaro et al., 2022; Fent et al., 2020). Fewer non-foraging trips may indicate a shift to risk-taking behavior. The bees exposed to D-Phenothrin might have become scouts, meaning less time was spent in the hive waiting for information on resources, decreasing the number of short trips outside the hive for defecation. Research indicating changes in honey bee behavior, endocrine-related gene expression in the brain, and responses to primer pheromones when exposed to insecticides indicate a need for a study to determine changes in foraging behavior beyond the number of trips made.

4.2 Methodological Reasoning

Assessing risks to honeybees is a very complex matter. A combination of factors rather than a single cause can be blamed for the observed loss of pollinators. Current losses can

be attributed to pathogens, parasites, management practices, nutrition inadequacies, genetic constraints, socio-economic factors, and pesticide exposure/environmental toxins (Medrzycki et al., 2013; Steinhauer et al., 2021). While it can be confirmed that pesticides are harmful to honey bee health, the number of studies exploring exposure in the field is limited. In the United States, to recognize the risks of pesticide exposure for honey bees, studies must go through 3 tiers. Tier 1 screens are conducted under laboratory conditions, tier 2 are semi-field studies with confinement to a tunnel, and tier 3, are full-field studies (US EPA, 2013, 2013, 2015, 2017, 2020). Due to a majority of studies being laboratory conditions and the stress confinement to a tunnel can cause on honey bees, a full field experiment design was chosen to analyze changes in bee behavior exposed to mosquito insecticide and was more representative of actual use conditions and likely exposure scenarios (US EPA, 2013, 2013, 2015, 2017, 2020). Multiple factors contribute to potential adverse effects on honey bees from an ecological standpoint, and simulating real-world occurrences can improve our understanding of these factors and maximize the ecological validity of the findings even when there is an inability to have control over extraneous variables (Schick et al., 2017; See Methodological limitations and recommendations; Appendix B).

4.3 Methodological Limitations and Recommendations

Field studies for assessing pesticide exposure are challenging, and every factor cannot be quantified. RFID technology, however, could lead to better ways to assess exposure and non-lethal effects of pesticides. The use of RFID technology is relatively new for honey bee research, but is a powerful tool for entomologists and scientists to monitor insect behavior. As an initial attempt at a field experiment using RFID tracking to explore the effects of mosquito adulticide, the knowledge gained is valuable in generating more ecologically valid experiments. If this study were to be repeated, the study would benefit from additional hives, larger treatment group sizes, and multiple trials throughout the entire foraging season. Below explores the methodological limitations of this study and their remediations.

A) Hive deaths

Of the 16 colonies initially established, five colonies perished, including the D-Phenothrin exposed hive, with a death rate of 30.25%. While there were no significant changes in honey bee foraging behaviors when exposed to the mosquito adulticide D-Phenothrin, the D-Phenothrin exposed hive did experience decline and eventual death. From September to January, the hive experienced a steady decline. In January, the hive size was approximately 20 bees, and no queen, meaning the hive was dead. Behaviorally, the bees were facing the corners of the hive and did not react to disturbances. Two colonies perished within the first month of installation. After transportation of the Nucleus colonies, both became queenless, and new queens were unable to be established

in either hive, resulting in hives declining. An additional two hives died shortly after transportation to a new location in Corning, CA, USA. One hive showed the classic signs of acute pesticide poisoning, with an excessive number of dead bees inside and in front of the hive (Connelly, 2012; Johansen et al., 2013; Kumar et al., 2020). The last hive did have signs of Colony Collapse Disorder (CCD). There were baby bees left behind, food in the hive, and the workers were gone. The queen, however, was also no longer present. With the definition and symptoms of CCD a highly debated topic, it cannot be confirmed that the hive's death was CCD (vanEngelsdorp et al., 2009; vanEngelsdorp et al., 2017; US EPA, 2013). Based on the number of hives that died during the experiment, it is unknown if the decline of the D-Phenothrin exposed hive resulted from the experiment or the many abiotic and biotic factors that are currently influencing the death trends of managed colonies (Medrzycki et al., 2013; Steinhauer et al., 2021).

B) Unknown variability in bee ages and caste

Honey bees are known for their complex division of labor (DOL) within a hive. Cohort-level DOL patterns, where bees of the same age transition between castes together and abruptly, are generally accepted (Johnson & Frost, 2012). Research, however, has indicated Individual-level patterns of DOL are also present in hives. Bees can transition between castes gradually, with bees being in multiple castes at once, skipping castes, and having the ability to revert from forager to nurse bee (Cardoso-Júnior et al., 2018; Herb et al., 2012; Johnson & Frost, 2012). The age of honey bees has been shown to affect their susceptibility to various pesticides (Medrzycki et al., 2013). A recent study revealed that as honeybees age, their sensitivity to Naled, an Organophosphate similar to Malathion,

increases significantly while their sensitivity to D-Phenothrin decreases significantly (Rinkevich et al., 2015). The foragers captured as adults could have been at any age, making them more or less susceptible to the effects of the D-Phenothrin or transitioning between castes. A follow-up experiment should have honeybees sampled in the age demographic of forager 21-45 days old (Vance et al., 2009). For each experimental analysis, bees should be of the same age, and experimental trials need to be done for youngest (21-28 days old), middle-aged (28-35 days old), and oldest (35-45 days old) foragers to account for age-based pesticide sensitivity (EPA, 2014, 2016, 2016; Vance et al., 2009). Additionally, trials for younger foragers (21-28 days old) should also account for changes in caste (Appendix B).

C) Capture and tagging stress

During the capture and tagging of the bee, the bees were not anesthetized but were manually immobilized. Capture and manual immobilization could have resulted in the death of tagged bees due to increased stress and energy expenditure during the process (de Souza et al., 2018). Research, however, has shown that anesthetizing honey bees using cold temperatures or CO₂-induced anesthesia results in changes in the behavior and physiology of honey bees. The longer the duration of cold temperatures, the greater the influences on worker longevity and learning and foraging behavior (Tutun et al., 2020). CO₂ has been shown to cause workers to age faster, start forage earlier, and shorten their life span (Tutun et al., 2020). Manual immobilization is required to forgo anesthetizing influences on foraging behavior; thus, an improved method to reduce capture stress and manual immobilization would be using queen pheromones on capture and tagging

equipment. Queen pheromones are known to regulate the behavior of worker bees. Not only do these pheromones promote a calming influence on workers, but the queen mandibular pheromone (QMP) has been found to block aversive learning in young worker bees (Beggs & Mercer, 2009; Maisonnasse et al., 2010; Slessor et al., 2005). Introducing queen pheromones during capture could reduce tagged workers' stress and possible mortality (Appendix B).

D) Acetone as insecticide solvent

As stated above, acetone was statistically significant; however, ecologically, the significance was not relevant for this study. Due to D-Phenothrin and D-Phenothrin + PBO showing no significance for changing foraging behavior, acetone's significance statistically did not correlate to an ecologically significant measure in this study. However, acetone as a solvent for other toxicology research or during a repeat of the current study could be significant. Acetone is an approved solvent for honey bee toxicology risk assessment research by the United States Environmental Protection Agency and, in general, is used in most toxicology studies. Acetone at a 5% concentration or lower is considered safe to use (California Department of Pesticide Regulation, 2017; EPA 2014, 2016, 2016; Medrzycki et al., 2013; SPEX CertiPrep, 2020). With the concentration of acetone used during this study showing a significant change in foraging and non-foraging trips, more research on using this solvent for honey bee toxicology should be considered. A superior methodology would be to use petroleum distillates as the solvents for pesticide toxicology studies in honey bees. Petroleum distillates are solvents used to mix pesticides. By law, in the United States, pesticide

products must be labeled if the product contains more than 10% petroleum distillates (*Petroleum Distillates*, 2020). Future honey bee toxicology studies should use solvents from the target pesticides product to better determine the effects on non-target pollinators in the field (Appendix B).

E) Damage during tagging, hygienic behavior, and post tagging release

The actual tagging and release of bees was also a potential confound. Honey bees are known for their hygienic behavior and often groom themselves and each other. This behavior, coupled with how experienced one is at placing the tags on the bees, can reduce the retention of the RFID tags. Additionally, the quality of the glue, moisture levels, and temperature can all affect the retention of the tags (de Souza et al., 2018).

Damage during tagging was also another potential confound. Tags placed over the eyes, pressing on the thorax too hard, or glue on the wings can damage a bee, killing it or making it unable to fly. Some bees have been shown to be sensitive to the smell of some glues as well (de Souza et al., 2018). Bees should be captured, tagged, and held for 24 hours to monitor for damage, retention of the tag, and death to reduce the possible effects of the above. During release tagged bees, bees should have been placed inside the hive and not made to crawl through the reader. As shown above, the process was stressful on the bees, and the potential to expend too much energy could have meant bees were not physically able to make it into the hive and died of starvation due to too much time between feedings (Medrzycki et al., 2013). After tagging, bees should be placed inside the hive to increase their chances of survival (Appendix B).

F) Tag placement

Where the RFID tags were placed on the thorax could have changed the number of foraging trips an individual could perform, making it a potential confound. If the initial tagging did not kill the bee or prevent it from its ability to fly, the tag might have generated a hindrance for the bee. The foraging behavior of honey bees is very complex, and factors within the hive and externally can impact the behavior of foragers (Abou-Shaara, 2014). Typically honey bees will begin foraging in the early morning and finish in the evening. Forager bees can forage up to 5 miles and are able to remember at what time of day specific food resources that give the most significant returns are available (Abou-Shaara, 2014). Resource availability, weather conditions, energy expenditure, forager age, and forager category (reticent bees or scout) can all influence the number of foraging trips a honey bee will make in a day (Rodney & Purdy, 2020). Recently researchers have been quantifying the amount of time and number of foraging trips bees make, though there are still gaps in our understanding. When foraging naturally, research has shown bees will make 1 to 13.5 trips per day, with a maximum of 24 trips. In addition, foraging locations with unnaturally high resources available increase the number of foraging trips a bee can make dramatically. Foragers also spend, on average, 0.96 to 3.06 hours outside the hive flying per day (Rodney & Purdy, 2020).

A honey bee can carry about 70 mg of nectar and 10 mg of pollen, and the RFID tags were about 3 mg making the extra weight low and less likely to influence foraging trips (Decourtye et al., 2011). While the weight of the tag itself may not have affected the number of trips a forager could make, misplacement of the tags could have altered the

trip durations and the number of trips an individual bee could perform. Covering the eyes or damaging the wing structure could impair individual foragers' navigation abilities and flight abilities (de Souza et al., 2018).

When proving methods, the effects of tag placement on the number of foraging flights are more challenging to track without considering the impacts of D-Phenothrin or other pyrethroids. Research has shown that honey bees exposed to neonicotinoids in a single acute dose have an initial increase in flight duration, and long term or multiple doses cause a decrease in flight duration and a reduction in the number of foraging trips (Schneider et al., 2012; Tosi et al., 2017). Research in the field or lab on the effects of pyrethroids on the foraging behavior of honey bees is limited, making it difficult to determine if the impact would be similar to neonicotinoids; however, based on how different pesticide classes are seen to have similar behavior effects on honey bees it is import to consider when improving methodology (Appendix A; Connelly, 2012; Dolezal, 2022; Johansen et al., 2013; Kiljanek et al., 2016; Kumar et al., 2020; Oliver et al., 2015; Ostiguy et al., 2019; Traynor et al., 2021; Williams et al., 2015; Wu et al. 2011). An improved methodology should focus on a more extended data collection period and compare the number of foraging trips at an individual level. Analysis of delayed reactions to the pyrethroid and analysis of individually tagged bees to determine if they fall into typical foraging behavior (number of average trips and hours spent foraging) after being chipped must be included in a repeat of the study. The purpose of doing a field experiment was to maximize ecological validity, so it was an unfortunate by necessary

compromise to alter the bees themselves through chipping in a way that may have lowered ecological validity.

G) One reader

In this experiment, there was only one reader placed at the entrance of the hive. Not all trips to and from the hive could be counted as a foraging trip with one reader. Trips were only able to count if there was a confirmed “departure” and an “arrival” or “unknown” following that departure. The reader produced “unknown” readings when bees came through. An additional reader on a feeder would increase the number of confirmed trips by showing a timestamp for both readers. This way, when an unknown or a duplicate direction appears, if there is a following timestamp at the feeder, then confirmation of a foraging trip is available (Appendix B; Decourtye et al., 2011; de Souza et al., 2018; Schneider et al., 2012).

H) Wildfires and rolling blackouts

In California, wildfires are a significant risk. Drought conditions and heatwaves have increased this risk. Before tagging of bees could begin in the experimental location of Platina, CA, USA, went under emergency evacuation due to the McFarland Fire.

Experimental hives were transported to a new location during the emergency to allow the fire crews access to the fire and prevent the hives' death. The moving of hives, while common, can cause undue stress on a hive and behavior changes to honey bees. One study found a decrease in decreased ribosomal and protein-folding activity and an increase in methylation. Following recovery from transportation, bees showed an increased production of antibiotic peptides and a decrease in transcripts associated with

immune activity and defense response (Melicher et al., 2019). Additionally, Fluctuations in temperatures and vibration can result in the death of brood and adult bees, including the queen (Appendix B; Hristov et al., 2020; Melicher et al., 2019).

To reduce the risk of wildfires, California has implemented rolling blackouts during times of high wind advisory. The power source to run the RFID equipment changed with hives at the new location. Although still being powered by a battery pack, the capacity to run was approximately four days, and there was no ability to charge the pack as needed. The lack of power reduced the duration of the study and prevented the following of the EPA's Pesticide Risk Assessment Process for Bees under Tier II (semi-field studies) or Tier III studies (full-field studies) (EPA, 2013, 2013, 2015, 2017, 2020). A shortened study time also prevented time delay analysis of results, and treatment groups were not all tagged during the same week. It is still unknown if D-Phenothrin and PBO affect foraging behavior long-term due to the shortened duration of the study (Costa, 2008; Devine & Denholm, 1998; Hénault-Ethier, 2016; Kiljanek et al., 2016; Lushchak et al., 2018; Soderlund et al., 2002; Song & Narahashi, 1996). Improvements can be made to the study by using a combination of a battery pack and solar panels to prevent loss of power during the duration of the studies (Appendix B).

4.4 Conclusions

This study gave further insight into how RFID technology can be used to assess pesticide exposure risks for honey bees. While this experiment dealt with classic issues related to the control over extraneous variables and requires a significant number of resources, the

following experiment allows for the results of mosquito adulticide and future insecticides to be interpreted on colony-wide effect. The use of RFID technology in field study generates environmentally realistic results and improves risk assessments within managed colonies. Furthermore, this study paves the way for a more efficient methodology for assessing using RFID technology and opens the door to research using RFID technology to understand the complexities of honey bee behavior beyond toxicology. Foraging behavior and division of labor of honey bees are complex, and factors within the colony and the environment impact this behavior. Many studies, including this one, have investigated these factors, and the combination of video tracking and RFID technology could ameliorate breeding and management practices (Abou-Shaara, 2014; de Souza et al., 2018; Johnson & Frost, 2012).

The impacts of mosquito insecticides on pollinators are a concern to mosquito control professionals, beekeepers, and others. Whereas beekeepers and agricultural companies have made strides towards protecting pollinators, more research on mosquito adulticides' lethal and sub-lethal effects is still required. The result of this study is one step closer to informing the agricultural community, which relies on the health and vitality of honeybees for crop production, of any detrimental alterations to behavioral ecology, specifically foraging. This study should be repeated with the methodology recommendations above to determine if the results of D-Phenothrin having no effects on foraging and non-foraging trips are conclusive. The information can then be used to improve current best management practices for agriculture and mosquito abatement

programs, such as the timing, location, and concentration of insecticide spraying to minimize the potential for unintended harm to honeybees.

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APPENDICES

Appendix A. Synthetic Insecticide Classes and Negatively Associated Changes in Behavior

Insecticide Class	Pesticide Class Effects on Behavior
Most insecticides	<ul style="list-style-type: none"> • Stupefaction, paralysis, and tremors, salivation, convulsions, and loss of coordination. • Increased defensiveness • Navigation and Forager disorientation: less precise waggle dances, altered homing abilities, flight patterns and reduced foraging efficiency. • Immobile, lethargic bees unable to leave flowers, unable to right themselves, unable to fly. • Lack of foraging bees on a normally attractive blooming crop. • Excessive grooming • Excessive numbers of dead and dying honey bees in front of the hives.
Carbamates, Organophosphates, and Neonicotinoids	<ul style="list-style-type: none"> • Significant impairments to the memory and olfactory learning capabilities and taste sensitivity. • Reduction in flight distance and duration & increase in velocity. • Poor queen development • Reduction in reproductive fitness of queens and drones/increase sterile queens and drones.
Carbamates	<ul style="list-style-type: none"> • Bees slow down and behave as though they have been chilled. • Dead newly emerged workers and dead brood. • Abnormal/poor laying patterns from queen. • Queenless hive
Organophosphates	<ul style="list-style-type: none"> • Fighting or confusion at the hive entrance. • Queenless hive
Organophosphate & Pyrethroids	<ul style="list-style-type: none"> • Poor brood development, increase in brood loss and/or cannibalism. • Regurgitation of honey stomach contents and Proboscis Extension.
Pyrethroids & Organochlorines	<ul style="list-style-type: none"> • Significant impairments to the memory and olfactory learning capabilities and reduced learned orientation toward odor stimulus. • Changes in feeding & reduction of giving food. • Changes in communication: less time antennae touching and in hive communication.

Appendix B. Methodological Modifications for Future Studies to Reduce Effects of Potential Confounds

Potential Confound	Description	How Results May Have Been Affected	Recommended Changes for Future Research
Unknown Variability in Bee Ages & Caste	Bees were randomly sampled and randomly assigned into treatment and control groups.	<ol style="list-style-type: none"> 1. Did not account for age-based pesticide sensitivity. 2. Did not account for bees switching castes away from forager. 	Marking of newly hatched bees to have age demographics and account for caste switching in young foragers (21-28 days old).
Capture & Tagging Stress	Bees were not anesthetized but were manually immobilized.	<ol style="list-style-type: none"> 1. Increased stress and energy expenditure 2. Stress induced death of tagged bees. 	Use of queen pheromones on capture and tagging equipment to reduce stress.
Acetone as Insecticide Solvent	Solvent used in target adulticide was acetone.	Acetone is not the solvent used in insecticide products and could create a false change in honey bee behavior significance.	Use of solvents (petroleum distillates) present in pesticide products for honey bee toxicology to reduce the chances of false significance.
Poor Tagging Methods & Hygienic Behavior	Tagging of bees using Crazy Glue, Max Bond Gel.	<ol style="list-style-type: none"> 1. Sensitivity to glue or damage to thorax and eyes resulting in death 2. Inability to navigate or fly 3. Reduction in movement and the number of trips a bee could preform 	<ol style="list-style-type: none"> 1. Hold bees for 24 hours after tagging to monitor for damage, tag retention, and death. 2. Longer data collection period 3. Analysis of delayed reactions to the pyrethroid. 4. Analysis of number of average trips and hours spent foraging per individual tagged bee
Post Tagging Release	Tagged bees were left at the front of the hive to crawl through the reader.	<ol style="list-style-type: none"> 1. Stressed bees expended too much energy and did not crawl into the hive resulting in starvation and death. 	Release bees into the hive to increase chances of survival.
Only One Reader	One reader was placed on the experimental hive.	<ol style="list-style-type: none"> 2. Inability to confirm all trips to and from the hive were foraging trips. 	Second reader on feeder to confirm more unknow trips out of the hive are in fact foraging trips.
Fires	McFarland Fire resulted in the transportation of experimental hive to another location.	<ol style="list-style-type: none"> 1. Stress and changes in behavior of the bees. 2. Death of brood, adult bees, and queens. 	-----
Rolling blackouts	RFID equipment was powered by a battery pack for the duration of the study.	<ol style="list-style-type: none"> 1. Prevented following all of the EPA's Pesticide Risk Assessment process for Bees under Tier II (semi-field studies) or Tier III studies (full-field studies). 2. Prevented time delay analysis of results and treatment groups were not all tagged during the same week 	A combination of a battery pack and solar panels to power RFID equipment.