

ASSESSING THE POTENTIAL THREAT OF WIDELY USED AGROCHEMICALS
TO HONEY BEE (*APIS MELLIFERA* L.) DRONES AND WORKERS

A Dissertation

by

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ABSTRACT

Honey bees (*Apis mellifera*) have been experiencing an ongoing decline in population over the last half century despite their tremendous importance to agriculture. A variety of environmental pressures have been implicated in this decline including pesticides commonly found within the hive and foraging environments. To assess the potential effect of exposure to in-hive pesticides on drone spermatozoa viability, we compared the viability of spermatozoa collected from drones reared in pesticide-free wax to that of drones reared in wax contaminated with field-relevant doses of several common agrochemicals, including chlorothalonil and chlorpyrifos, and miticides, including fluvalinate, coumaphos and amitraz. Our results suggest a significant negative effect of in-hive pesticide exposure during development on spermatozoa viability.

The greatest risk of exposure to pesticides, however, is faced by a subset of workers, foragers, that function in food collection from floral resources. The activity of honey bee foragers contributes approximately \$17 billion annually in pollination services for several major crops in the United States including almond, which is completely dependent on honey bees for pollination. To assess the effects of pesticides commonly used during almond bloom on honey bee forager survival, foragers were exposed to label dose variants of the fungicides iprodione, boscalid/pyraclostrobin formulation and azoxystrobin alone and in combination. Label dose variants of two insect growth regulators, methoxyfenozide and pyriproxyfen, as well as an acaricide, bifenthrin, were also assessed for their effects on forager survival. We utilized a wind tunnel and

atomizer set up (wind-speed: 2.9 m/s) to simulate field-relevant exposure of honey bees to these chemicals during aerial application in almond orchards. Our results indicate a significant decrease in forager survival resulting from exposure to pesticides commonly applied during almond bloom. To assess the effects of commonly used mosquito control insecticides on honey bee forager mortality, foragers were exposed using a wind tunnel-atomizer assembly simulating exposure from ultra-low volume spray truck application (wind-speed: 1.8 m/s). Significant acute forager mortality resulted from exposure to the mosquito control insecticides when applied above and below the manufacturer application rate.

DEDICATION

I would like to thank, first and foremost and above all else, God, my Lord and Savior Jesus Christ. I dedicate this dissertation to my parents, my mother Rosa Elia Fisher (nee Renteria) and my father Adrian Leon Fisher Sr. As a young woman, my mother left her home in Mexico to move to the United States, she had to leave her own educational opportunities but always remembered the importance of education and hard work in raising my sisters and I. We were blessed with her love and example of the kind of person to strive to be as we grew up, my educational and career pursuits are a reflection of the values and hard work my mother instilled in me. My father learned the value of discipline and hard work in his service to the nation as a part of the Air Force during the Vietnam War. He always strove to provide a better life for my sisters and I, greatly aiding our growth and my pursuits. I find reflections of my father in my own journey to earn a doctoral degree, the first in my family, and hope to pass on the lessons I have learned from him. I would also like to thank my younger sisters, Virginia and Ivette Fisher, for their constant support and friendship, for our ability to positively influence one another.

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CHAPTER I

INTRODUCTION

The honey bee (*Apis mellifera*) is the most significant crop pollinator in the United States, their services worth approximately \$17 billion annually (Calderone 2012). Additionally, American agriculture has undergone a tremendous increasing shift to animal pollinated crops in the last half century for which the honey bee is an essential pollinator (Aizen and Harder 2009). However, honey bee and other pollinator populations have undergone a substantial decline within the same timeframe, raising concerns about sustainability of food security (Aizen and Harder 2009). An estimated 45% reduction in the number of honey bee colonies has occurred since the 1960s (Johnson et al. 2010). although declines in bee populations were noted prior to widespread reporting of CCD (Watanabe 1994). Colony loss was highlighted most dramatically by the occurrence of colony collapse disorder (CCD), which entails the unexplained disappearance of most workers from a hive typically leaving only the queen, young workers and brood present in a hive (Oldroyd 2007). Although colony losses attributable to CCD were reported at exceptional rates in 2006 and 2007 (vanEngelsdorp et al. 2008), loss of colonies below the beekeeper-accepted level remains an ongoing issue (Seitz et al. 2016).

A multitude of factors have been implicated in declining bee populations including pressure from the loss of habitat and forage, pathogens and associated diseases, hive pests such as the ectoparasitic mite *Varroa destructor*, and pesticide exposure within the

hive and in the general environment external to the hive (Naug 2009, Guzmán-Novoa et al. 2009, Johnson et al. 2010, Williams et al. 2010, Le Conte et al. 2010, Becher et al. 2013, Smith et al. 2013). Pesticides, particularly beekeeper applied miticides, are a ubiquitous component of the in-hive environment found at high rates in food resources and wax (Mullin et al. 2010). Additionally, widespread use of pesticides in various crop systems ensures frequent exposure to honey bees in major foraging resources (Johnson et al. 2010). Much of the recent research on the effects of pesticides on honey bee health have focused on neonicotinoid insecticides, the most widely used insecticides worldwide (Goulson et al. 2013). A number of studies have uncovered detrimental effects to workers from exposure to the neonicotinoids imidacloprid, clothianidin and thiamethoxam (Colin et al. 2004, Aliouane et al. 2009, Alaux et al. 2010, Schneider et al. 2012, Di Prisco et al. 2013, Ciereszko et al. 2017).

However, a lack of detrimental effects of neonicotinoid exposure has also been observed (Chauzat et al. 2009, Cresswell et al. 2011) and neonicotinoids were detected at low rates in hive components compared to other pesticides (Mullin et al. 2010). In addition, there exists concern over the field-relevant applicability of the neonicotinoid doses and exposure methods employed in many studies (Blacquièrre et al. 2012). Despite the widespread application of other chemicals with high potential for bee exposure, such as fungicides, in key cropping systems (Fisher et al. 2017) several widely used pesticides remain understudied. To elucidate field-relevant risks of exposure incurred by honey bees we simulated field exposure scenarios to assess realistic doses of pesticides commonly encountered inside the hive and in the foraging environment.

Our evaluation of the sub-lethal effects of in-hive pesticides on drone reproductive quality was conducted by contaminating beeswax-coated frames with pesticides applied at rates detected by a previous pesticide residue analysis of multiple wax samples from commercial beekeeping operations across the United States (Mullin et al. 2010). To assess the effects of field realistic concentrations of fungicides, insect growth regulators and an acaricide commonly used in almond orchards, a crop that receives the majority of managed hives for pollination services during bloom (Sumner and Boriss 2006, Carman 2011), we utilized a wind tunnel-atomizer set up (wind speed = 2.9 m/s) to simulate forager exposure to these chemicals from aerial applications. Finally, we examined the adverse effects of mosquito abatement insecticides on honey bee health, which are increasingly being used in urban environments where honey bees might be present (Farajollahi et al. 2012, Faraji et al. 2016). Exposure was accomplished through the use of a wind tunnel-atomizer set up (wind speed = 1.8 m/s) designed to simulate insecticide exposure from terrestrial applications by an ultra-low volume spray truck.

CHAPTER II
IN-HIVE PESTICIDES NEGATIVELY AFFECT HONEY BEE
(HYMENOPTERA: APIDAE) DRONE SPERMATOZOA VIABILITY

2.1. Introduction

Among species of eusocial Hymenoptera (ants, wasps, and bees), males live sheltered lives inside their hives, where they are raised by sister workers until they are ready to mate (Stürup et al. 2013). A substantial amount of colony resources is invested in the care and nurturing of males, as they provide no contributions to colony maintenance apart from reproduction (Holldobler and Bartz 1985). Male rearing by workers appears regularly among eusocial insects (Boomsma et al. 2005), especially in swarm-founding species such as honey bees in the genus *Apis*, which exhibit an extreme male-biased sex ratio among reproductives (Winston 1987; Baer 2005). In the honey bee, *Apis mellifera*, colonies are composed of one queen and a few thousand seasonal males (drones) that are reared only during the reproductive season, and only when colony resources are plentiful (Winston 1987). Honey bee queens mate with an average of 12 to 15 drones (Tarpy et al. 2004), collecting 4 to 7 million spermatozoa for up to five years in a sperm-storing organ known as the spermatheca (Harbo 1986; Wilde 1994; Cobey 2007). During the reproductive season, thousands of drones congregate in areas where they locate and attempt to mate with virgin queens (Winston 1987). As competition between individuals for a chance to mate is intense, those drones with higher sexual competitiveness are likely to be of higher reproductive fitness compared to other drones (Baer 2005). For

example, drone weight and size have been found to significantly influence mating success and paternal representation following mating (Couvillon et al. 2010). Despite the importance of drones as genetic reservoirs of a colony's traits, few studies have looked at the effects of environmental conditions on drone reproductive quality.

Among the environmental factors that could potentially negatively impact drone spermatozoa viability is exposure to pesticides within the hive. Mullin et al. (2010) found dozens of pesticides in wax samples collected from over 250 commercial beekeeping operations in the United States, some at alarmingly high concentrations. An especially troubling group of chemicals are that used to combat the ectoparasitic mite, *Varroa destructor*, a pest of honey bees that, when found in large numbers and if left untreated, causes colonies to collapse and die (Boecking and Genersch 2008). Over the last few decades, continuous treatment of varroa with the miticides fluvalinate and coumaphos has led to resistant mite populations (Elzen et al. 1998; Elzen and Westervelt 2002) and prolonged contamination of wax inside hives (Mullin et al. 2010). Few studies have explored the effects of exposure to pesticides on drone reproductive quality. The beekeeper-applied miticides fluvalinate (a pyrethroid) and amitraz (a formamidine), active ingredient of many varroa control products currently in use, have been found to lower body weight and frequency of mating flights in drones (Rinderer et al. 1999; Shoukry et al. 2013). In addition, the organophosphate coumaphos has been found to significantly lower drone spermatozoa count and viability (Burley 2007). A similar negative impact on spermatozoa counts was observed in drones exposed to fluvalinate and amitraz (Shoukry et al. 2013). Interestingly, Johnson et al. (2013) reported no

impact of in-hive miticides on drone spermatozoa viability. However in that study, miticide treatments were applied topically on adult drones, thus leaving a knowledge gap on the potential effects of miticide exposure during drone development. New adult drones emerge from their cells with all the spermatozoa they will ever possess (Baer 2005; Al-Lawati et al. 2009). In fact, they undergo only minor anatomical changes after emergence (Czekonska et al. 2013), an interesting aspect of male biology found in other Hymenoptera (Holldobler and Bartz 1985). Therefore, environmental conditions influencing drones during development may affect fitness and selection throughout adulthood. Furthermore, the beeswax environment within hives is contaminated with several miticides at once (Mullin et al. 2010), so future studies on the effects of chemical contamination of the wax on drone reproductive quality should build on the aforementioned findings and focus on field-relevant combinations of these chemicals, not simply focus on one product at a time.

Several agrochemicals have also been found to negatively impact drone reproductive health. In particular, oral exposure to the neonicotinoid insecticides thiamethoxam and clothianidin significantly lower spermatozoa viability in adult drones (Straub et al. 2016). Similar effects on spermatozoa viability and mitochondrial activity have been observed from imidacloprid exposure, although variation in the intensity of the results between colonies was noted (Ciereszko et al. 2017). These findings suggest a general negative impact on spermatozoa number and viability caused by contamination of the beeswax environment with a number of common miticides and agrochemicals.

In this study, we used the five most ubiquitous agrochemicals found in wax samples by Mullin et al. (2010) and used the reported concentrations of each pesticide to impregnate the wax used for drone comb construction with different combinations of these chemicals. We reared drones in these conditions and upon reaching sexual maturity, we measured drone spermatozoa viability. We make recommendations regarding the use of these pesticides near or within honey bee colonies based on our findings.

2.2. Materials and Methods

This study was conducted during two different reproductive seasons at the at the Janice and John G. Thomas Honey Bee Facility research apiary of the Texas A&M University RELLIS Campus in Bryan, TX. The first experiment was conducted from May to October 2014, and involved the use of beeswax-coated drone frames that were either pesticide-free, or contaminated with a combination of the miticides fluvalinate and coumaphos, or with a combination of the pesticides chlorothalonil and chlorpyrifos. The second experiment was conducted from July to September 2016, and involved the use of beeswax-coated frames that were either pesticide-free, or contaminated with the miticide amitraz. The experimental procedures for each experiment are outlined below.

2.2.1. Experimental Procedures in 2014

2.2.1.1. Drone Frame Preparation

Approximately 20 lbs of pesticide-free, cosmetic grade bees wax was melted down in a large water bath. The beeswax was purchased from Koster Keunen Inc. (Watertown, CT,

USA). Once the beeswax was melted, plastic drone frames (Brushy Mountain Bee Farm, Moravian Falls, NC, USA) were submerged one at a time into the wax bath twice until fully coated. Each coated frame was then allocated to one of three treatment groups or a control group (Table A-2). Frames allocated to treatment one were each coated with 10 mL of miticide solution (i.e., 20.4 mg of fluvalinate and 9.2 mg of coumaphos / 100 mL of acetone) applied to both sides of the frame using a 750 mL all-purpose sprayer. Frames allocated to treatment two were each coated with 10 mL of agrochemical solution (i.e., 5.4 mg of chlorothalonil and 0.09 mg of chlorpyrifos / 100 mL of acetone) applied to both sides of the frame using another 750mL sprayer. The control group consisted of no pesticides and represented an uncontaminated rearing environment. Each frame allocated to the control group was sprayed with 10 mL of acetone on both sides using a separate 750 mL sprayer. The pesticide concentrations used for the treatment groups were derived from the high concentrations of each pesticide detected in wax by Mullin et al. (2010) (Table A-1). All pesticides used were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

2.2.1.2 Drone Rearing and Capture

Pairs of experimental frames were placed in different host hives. Drone brood reared on experimental frames remained in their respective host hives throughout larval development. Following pupation and the capping of drone cells by workers, the frames were removed from their host hives and placed in five-frame nucleus colony boxes (“nucs”) allocated to each experimental group approximately one day prior to the

anticipated emergence of adult drones. A food frame and a small group of young workers was included in each nuc box to tend to new drones as they emerged. The nucs were placed in an incubator set at 34°C with $\approx 75\%$ relative humidity. Emergence of adult drones was monitored daily, and emerged drones were gathered and marked by applying a spot of acrylic paint onto the mesonotum (Figure A-1a). A different paint color was used for each treatment and for each day of drone emergence to keep track of the drones' age and beeswax rearing environment. Marked drones were then returned to their host hive where they underwent sexual maturation. All labeled drones were captured approximately 18 days later. Drone capture occurred over four periods between May and October 2014 and included the placement of queen excluders at the hive entrance to block drones from re-entering the hive, as well as opening host hives to search for any appropriately-aged painted drones housed therein.

2.2.1.3. Semen Collection and Sample Preparation

Captured drones underwent forced ejaculation through the application of pressure on the thorax and abdomen, which triggers the eversion of the endophallus (Cobey 2007).

Semen samples were collected using 10 mL syringes and were then transferred individually to labeled Eppendorf tubes containing 100 μL of saline solution (0.24 g HEPES, 0.88 g NaCl and 1 g BSA diluted in 100 mL of dH_2O) to extend spermatozoa longevity for analysis by flow cytometry (Rzymiski et al. 2012, Tofilski et al. 2012).

Test tubes were labeled corresponding to each individual semen sample. Each tube received 40 μL of Phosphate Buffered Solution (PBS), 10 μL of semen solution, 3 μL of

sybr-14 (Life Technologies™, Carlsbad, CA, USA) and 3 µL of propidium iodide (PI) (Life Technologies™, Carlsbad, CA, USA). Sybr-14 and PI are dyes that differentially stain viable spermatozoa green and non-viable spermatozoa red, respectively (Collins et al. 1999). The prepared tubes were then gently mixed and placed in a closed cabinet for approximately 15 min to allow for the interaction between spermatozoa, sybr-14 and PI. A second set of labeled test tubes received 400 µL of PBS and 10 µL of the stained semen solution from the first set of test tubes and was gently mixed.

2.2.1.4. Spermatozoa Viability Analysis

Spermatozoa viability was analyzed using a Becton Dickson FACScan Flow Cytometer System and the BD CellQuest™ software at by the Equine Theriogenology Service Lab of the Texas A&M University College of Veterinary Medicine and Biomedical Sciences in College Station, TX. Each test tube from the second set was inserted, one at a time, into the Becton Dickson FACScan Flow Cytometer for approximately 30 s. The BD CellQuest™ software was run simultaneously to the processing of samples in each tube by the flow cytometer, which calculated and recorded the proportion of viable to non-viable spermatozoa in each sample. Output from the BD CellQuest™ software typically depicted the viable and non-viable spermatozoa subpopulations. Occasionally, a third subpopulation comprised of live spermatozoa that was viable but damaged, emerged in analyzed samples. When encountered, this third subpopulation of “transitional” spermatozoa was pooled with the viable subpopulation in accordance with previous studies (Love et al. 2003).

2.2.2. Experimental Procedures in 2016

2.2.2.1. Drone Frame Preparation

Drone frame preparation and rearing was generally similar to the methods described in previous sections (2.1, 2.2) with a few changes. Plastic frames were allocated to either the amitraz treatment group or an untreated control group. Frames in the control group were sprayed with 10 mL of acetone on both sides using a 750 mL sprayer, while amitraz designated frames were sprayed with 10 mL of amitraz solution (i.e., 4.3 mg of amitraz / 100 mL of acetone) applied to both sides of the frame using a separate 750 mL sprayer (Table A-3). The amount of amitraz used was the highest concentration detected in beeswax by Mullin et al. (2010) (Table A-1).

2.2.2.2. Drone Rearing and Capture

Control and amitraz-treated frame pairs were placed in the same hives, and drone brood was reared on the experimental frames by the colony's workers. The frames were removed just prior to adult emergence and placed in nucs allocated for each experimental group. A frame with food resources and a group of young workers were included in each nuc to tend to emerged drones. The nucs were then placed in an incubator set at 34°C with $\approx 75\%$ relative humidity, and emergence of adult drones was monitored daily. Emerged drones were then marked with a spot of paint on the mesonotum. Marked drones were collected into drone cages consisting of a wooden frame and sliding queen excluder side panels and were approximately 16.51 cm x 15.24

cm x 7.62 cm (Figure A-1b). Once loaded with drones, the cages were placed back in the original host hives. The use of drone cages was implemented in 2016 to increase drone recapture rate, which was low when drones were allowed to roam freely in 2014. Additionally, caged drones were retrieved at ten and 18 days post emergence, representing a sexually immature and a sexually mature subset of drones, respectively. This additional measure was also implemented to further increase the sample size for both experimental groups. Drones were successfully retrieved from cages between July and September 2016.

2.2.2.3 Semen Collection and Sample Preparation

Semen was collected from mature drones 18 days post emergence through forced eversion of the endophallus. Semen collection from immature drones, collected ten days post emergence, included the removal of the reproductive tract of live drones through dissection. The seminal vesicles were then removed from the rest of the reproductive tract and crushed in an Eppendorf tube containing 100 μ L of saline solution (0.24g HEPES, 0.88g NaCl and 1g BSA diluted in 100mL of diH₂O), allowing for the escape of spermatozoa and seminal fluid into solution. Each semen sample was then combined with 3 μ L of sybr-14 and 3 μ L of PI, which differentially stained viable and non-viable spermatozoa, respectively (see section 2.1.3. for details).

2.2.2.4. Spermatozoa Viability Analysis

Spermatozoa viability of drones reared in either pesticide-free or amitraz-contaminated beeswax was analyzed using a Nexcelom Cellometer[®] Vision CBA Image Cytometer (Nexcelom Biosciences LLC., Lawrence, MA) at the Heep Center in the Department of Entomology at Texas A&M University, College Station, TX. Prepared semen samples were incubated in a closed cabinet for 8 min, and then a 20 μ L aliquot of each sample was loaded into separate cell counting chambers of cartridges for the Nexcelom Cellometer[®] Image Cytometer. The cell counter automatically quantified the proportion of viable to non-viable spermatozoa in each sample.

2.2.3. Statistical Analysis

Differences in the spermatozoa viability between treated and control groups in 2014 and 2016 were assessed using Student's *t*-tests. All tests were carried out using the JMP[®] v12 statistical software (SAS Institute Inc., Cary, NC). The level of statistical significance was set for all tests at $\alpha = 0.05$. All descriptive statistics are reported as the mean \pm the standard error of the mean (S.E.M.).

2.3. Results

2.3.1. Effects of Miticides and Agrochemicals on Spermatozoa Viability

Significant effects of agrochemicals and miticides in the drone-rearing environment were found on spermatozoa viability in 2014. Specifically, drones exposed to the miticide treatment group, which included a combination of fluvalinate and coumaphos

("F+C"), had an average spermatozoa viability of $34.7\% \pm 4.2\%$ ($n=15$), which was less than half, and significantly lower than the viability of $73.6\% \pm 5.5\%$ ($n=6$) in the control group ($t=5.12$, $P<0.0001$; Figure A-2). Similarly, drones exposed to the agrochemical treatment group, which included chlorothalonil and chlorpyrifos ("C+C"), had an average spermatozoa viability of $41.3\% \pm 10\%$ ($n=3$), which was significantly lower than drones in the control group ($t=3.39$, $P=0.009$; Figure A-2). Thus, the most commonly encountered contaminants in beeswax (Mullin et al. 2010) clearly affect drone reproductive health at field-relevant concentrations.

2.3.2. Effect of Amitraz on Spermatozoa Viability

Amitraz also caused a significant drop in spermatozoa viability for drones reared in 2016, although not as pronounced as the treatment groups in 2014. Even though drones reared in pesticide-free or amitraz-laden beeswax were collected at two different post-emergence ages (i.e., ten and 18 days post emergence), no significant difference was found between drones in the two age cohorts for either the control group ($t=0.06$, $P=0.48$) or the amitraz-treated group ($t=0.23$, $P=0.83$). Therefore, the data were pooled from both age cohorts and we performed an overall comparison of all drones in each treatment group. The average viability of spermatozoa in the control drone group was $99.2\% \pm 0.2\%$ ($n=31$), while that for drones in the amitraz group was $80.1\% \pm 1.0\%$ ($n=50$). Statistically, rearing drones in the amitraz-laden beeswax detrimentally impacted spermatozoa viability compared to the control group ($t=19.8$, $P<0.0001$; Figure A-3). As another frequent contaminant in beeswax that is currently widely used in varroa mite

control (Mullin et al. 2010), amitraz clearly affects drone reproductive health by negatively impacting spermatozoa viability during development.

2.4. Discussion

This is the first study that assesses the combined effects of miticides and agrochemicals impregnating the wax-rearing environment on drone spermatozoa viability. The miticides fluvalinate, coumaphos and amitraz, as well as the pesticides chlorothalonil and chlorpyrifos, severely impair drone reproductive quality, as they significantly decrease spermatozoa viability. Our results complement previous studies of the sub-lethal effects of common miticides and agrochemicals on drone reproductive health (Rinderer et al. 1999, Burley 2007, Shoukry et al. 2013, Straub et al. 2016, Cierezsko et al. 2017). When combined at field-relevant concentrations, these chemicals negatively impact drones during development, which is the key period when spermatogenesis occurs (Baer 2005, Al-Lawati et al. 2009, Johnson et al. 2013). However, our sample size in 2014 was suboptimal because of our low success rate in drone recapture, which would need to be improved before we make strong inferences about our results. In addition, even though we used drone source colonies that were headed by sister queens in 2014, we did not use the same colonies in 2016, and thus, between-colony genetic variation, which naturally occurs between honey bee breeds (Rhodes et al. 2011), could have been a factor in the results we obtained. Also, the occurrence of a transitional subpopulation in some samples analyzed using flow cytometry in 2014 may have presented a confounding variable. Even though transitional subpopulations are often

interpreted as damaged but living spermatozoa, they may have been other cell types or non-cellular material inadvertently included in semen samples. The cell counter used in 2016 allowed for specific calibration to the size and dimensions of drone spermatozoa, and thus other cell types were likely excluded with greater reliability than with the flow cytometry technique utilized in 2014.

Pesticide residue analyses were not conducted to verify miticide and agrochemical concentrations in the wax combs used to rear drones. However, experimental frames were sprayed with pesticides after an initial coat of wax was applied, and bees subsequently drew out comb on the pesticide-treated foundation. Thus, drone larvae were likely exposed to pesticide concentrations lower than those initially applied to the experimental frames. Pesticide diffusion rates in beeswax have not been assessed, although Wu et al. (2011) noted substantial accumulation of pesticide residues, including fluvalinate and coumaphos, in wax previously free of contamination within three months. In addition to apparent rapid diffusion, fluvalinate and coumaphos undergo slower degradation in beeswax with an approximate half-life of five years (Bogdanov 2004). The half-life of fluvalinate in general environmental settings is approximately three days (Fantke et al. 2014), while that of coumaphos is approximately ninety-nine days (Malek et al. 1997).

Drone reproductive health might be compromised through a combination of factors including pesticide exposure, temperature, nutrition, etc. Honey bee drone spermatozoa were recently shown to undergo a high degree of environmental pressure, being highly

sensitive to fluctuations in temperature and threats to the immune system (Stürup et al. 2013), although the study was conducted mainly in laboratory conditions. Nutrition may also be an important factor affecting drone fertility (Czekońska et al. 2015), as access to a reduced pollen supply was recently shown to result in drones with reduced semen volume and ejaculatory success (Czekońska et al. 2015). However, pollen deprivation was not found to affect either overall spermatozoa counts (Czekońska et al. 2015) or spermatozoa viability (Stürup et al. 2013). Clearly, the environmental factors that influence honey bee male fertility in field conditions remain poorly understood.

An interesting aspect of drone biology is the process of sexual maturation, which describes the migration of spermatozoa generated during development through the reproductive tract (Rhodes 2008, Rousseau et al. 2015). Spermatozoa migrate from the testes to the seminal vesicles where they receive nourishment and support from the mucus glands, which are accessory reproductive organs (Rhodes 2008, Rousseau et al. 2015). This process directly precedes the completion of maturation where semen is transferred to the drone's intermittent organ, or endophallus. Various estimates have been proposed for the time needed to complete sexual maturation in drones, from lower estimates of 6 to 8 days (Bishop 1920, Mackensen & Roberts 1948) to higher estimates of two weeks or more (Moritz 1989, van Niem Nguyen 1995, Rhodes 2002). In 2014 semen samples were successfully obtained through forced eversion of the endophallus 18 days post emergence, which is a longer post-emergence time than has been previously reported. We were highly unsuccessful when we tried to obtain semen samples from drones younger than 18 days post emergence. Our discovery of this later

time for maturation completion came at the cost of prematurely and unsuccessfully sampling younger drones via forced eversion of the endophallus for all treatment groups. In particular, we lost dozens of samples that could have yielded spermatozoa viability results because sampling occurred about 10-14 days post emergence, which is the timeline at the upper end of what has been reported before (Moritz 1989, van Niem Nguyen 1995, Rhodes 2002).

The ubiquitous presence of in-hive pesticide residues (Mullin et al. 2010) and their negative effects on drone reproductive health may indirectly affect the reproductive quality of queens that mate with sub-standard drones. Queen longevity is a function of her brood production rate, with reductions in productivity triggering supersedure, the replacement of the old queen with a new queen (Rangel et al. 2013, Sandrock et al. 2014, Rangel et al. 2015, 2016). Since drones are the sources for half of the genetic material required for the production of worker brood, a queen that mates with compromised drones may undergo faltering brood production sooner than otherwise expected. Of particular note, the long-held view that queens retained only viable spermatozoa in their spermathecae (Ruttner & Koeniger 1971) has been refuted by recent discoveries of the retention of non-viable spermatozoa in the spermatheca (Collins 2000, Bienkowska et al. 2011). The apparent negative impact of common in-hive pesticides on drone fertility may confer a larger proportion of non-viable spermatozoa coming from drones that successfully mate with a queen, thus contributing a reduction in the queen's supply of usable spermatozoa. Baer (2005) suggested that ejaculate size directly affects successful spermatozoa storage in queens following mating, while

successful fertilization depends on individual spermatozoa quality. In this regard, beekeepers seem to be inadvertently selecting against drone fitness through the use of in-hive miticide treatments. Annual comb replacement, including the exclusion of comb over five years old, is a recommended practice for beekeepers (Berry 2008). This practice, however, is often viewed by beekeepers as costly to them and taxing for the bees, since they have to draw out new comb (Berry 2008). In light of our findings, it may be advisable for beekeepers to consider the long-term benefits of regular comb replacement to reduce the influence of these detrimental chemicals on drone fertility and to reduce in-hive miticide accumulation, which will likely lead to improved colony health.

CHAPTER III

THE SYNERGISTIC EFFECTS OF ALMOND PROTECTION FUNGICIDES ON HONEY BEE (HYMENOPTERA: APIDAE) FORAGER SURVIVAL*

3.1. Introduction

Honey bees (*Apis mellifera* L.) contribute about \$17 billion annually to the United States economy, primarily through pollination of major agricultural crops (Calderone 2012, Zhu et al. 2015). Among the main crops pollinated by honey bees is almond (*Prunus dulcis*), which relies almost entirely on honey bee pollination for nut set (Klein et al. 2012). The almond industry in California produces about 80% of the almonds consumed worldwide (Klein et al. 2012), employing ~60% of all managed honey bee hives in the country to provide pollination services during the crop's bloom in mid- to late-winter (Sumner and Boriss 2006). In protecting almond orchards from various pests and pathogens, heavy chemical treatments are employed during bloom (Bosch and Blas 1994). However, despite their ubiquitous use, the effects on honey bee health of the various pesticides used repeatedly in almond orchards are not well understood.

In particular, little is known about the effects on honey bee health of fungicides used in almond orchards during bloom, although a few studies have shown that some fungicides affect colony health at various stages of bee development. For example, Mussen et al. (2004) and Mussen (2013) reported negative impacts on brood survival in vitro and in

*Reprinted with permission from “The Synergistic Effects of Almond Protection Fungicides on Honey Bee (Hymenoptera: Apidae) Forager Survival” by Adrian Fisher II, Chet Coleman, Clint Hoffmann, Brad Fritz and Juliana Rangel, 2017. *Journal of Economic Entomology*, 110(3), 802-808, Copyright 2017 by Oxford University Press.

the field when the brood was fed pollen that was artificially contaminated with fungicides (Mussen et al. 2004, Mussen 2013). Similarly, Kubik et al. (1999) recorded high levels of the fungicides vinclozolin and iprodione in stored pollen and honeycollected from colonies used for pollination in cherry orchards (Kubik et al. 1999). Furthermore, Vandamme and Belzunces (1998) observed negative sublethal effects of combinations of the fungicides prochloraz and difenoconazole, and the insecticide deltamethrin, on thermoregulation in adult workers (Vandamme and Belzunces 1998). Combinations of fungicides, acaricides, and insecticides have also been shown to cause synergistic detrimental effects on adult worker and queen mortality (Pilling and Jepson 1993, Johnson and Purcell 2013). Moreover, an examination of the combined application of some insecticides, a fungicide (tetraconazole), and a herbicide in crop systems such as cotton, rice, and corn, revealed significant negative synergistic impacts of these chemicals on adult worker survival in vitro (Zhu et al. 2015).

A recent review of studies on the toxicity to honey bees of fungicides and other pesticides found in pollen, wax, and honey, noted the persistence of fungicide residues in most samples, including noticeable detection levels of the fungicides boscalid, captan, and myclobutanil in pollen and adult bees, often at higher frequencies than other pesticide classes (Johnson et al. 2010). In a different study, the fungicide chlorothalonil was found at high frequency and concentration in stored pollen from commercial apiaries from across the United States (Mullin et al. 2010). Chlorothalonil contamination is associated with entombing behavior, whereby workers cap contaminated pollen cells with propolis, presumably to protect the colony from further exposure to the chemical (vanEngelsdorp et al. 2009). However, the precise health effects of high levels of

chlorothalonil in pollen are not well understood. Honey bee foragers, which begin their food-seeking tasks 21d post-emergence (Huang and Robinson 1996, Abou-Shaara 2014), comprise the age group that is most susceptible to direct exposure to agrochemicals when visiting flowers for pollen and nectar collection (Pettis et al. 2013). Foragers are thus an attractive age cohort for studying the effects of field-relevant concentrations of fungicide tank mixes used in agricultural crops on colony health. In this study, foragers were exposed to three fungicides widely applied during the almond bloom in California to assess the potential synergistic effects of these agrochemicals in simulated tank mixes on forager mortality. In light of these findings, a more careful consideration of fungicide application in almond orchards or any agricultural crop during bloom should be considered, because it might negatively affect honey bee colony health in ways that are poorly understood.

3.2. Materials and Methods

3.2.1. Fungicides Used

The fungicides selected for our study were iprodione (Iprodione 2SE Select™ :23.8% iprodione; Prime Source, LLC, Evansville, IN), BP (Pristine®: 25.2% boscalid, 12.8% pyraclostrobin; BASF Corporation, Research Triangle Park, NC), and azoxystrobin (Quadris®: 22.9% azoxystrobin; Syngenta Crop Protection, Inc. Greensboro, NC), which were among the top 50 pesticides most widely used during the almond bloom in California in 2012 (Pesticide Action Network Pesticides Database 2012; Table B-1). All fungicides were purchased from a commercial source (Amazon Inc.) and applied

individually or in combination with other fungicides at either the manufacturer's recommended label dose, or at different concentrations.

3.2.2. Experimental Treatment Groups

To assess the effects on forager mortality of each selected fungicide, alone and in various combinations with other fungicides, seven experimental groups were formulated including a fungicide-free control group. The fungicide treatment groups included iprodione at differing concentrations derived from the recommended label dose (Table B-2). Other treatment groups included in the study were combinations of iprodione and BP or iprodione and azoxystrobin at the recommended label dose. The control group consisted only of the solvent, distilled water, which was used to dissolve all fungicides used in the treatment groups. Three separate trials were conducted between September and November 2015 (Table B-2), and increased rainfall and corresponding decreases in available forage were observed over this period. A fourth experimental trial was conducted in December 2015 by applying each fungicide at twice the recommended label dose rate, reflecting a potential worst-case scenario in the application of these chemicals to honey bee foragers (Table B-3).

3.2.3. Forager Capture

Honey bee foragers were collected from a designated hive located at the Janice and John G. Thomas Honey Bee Facility of Texas A&M University's Riverside Campus in Bryan, TX. Honey bee adults covering frames that contained little to no brood but contained

ample food resources were selected for the exposure experiments. Such frames were targeted because they likely had a higher number of older adult bees on them, including foragers, which take on food collection rather than brood maintenance, the task of younger workers (Winston 1987). Foragers were gently brushed off the frames into bioassay cages composed of a circular cardboard frame, holding rings, and mesh side panels. The bioassay cage frames had a diameter of ~15.2 cm. To enclose the cages, a single sheet of mesh fabric was stretched over either side of the cage, then a thin cardboard holding ring with a slightly larger diameter than the cage frame was forced around the frame securing the mesh in a taut position. Approximately 40–50 foragers were loaded into each bioassay cage, and a total of six bioassay cages were allocated to each experimental treatment group (Figure B-1a). The bioassay cages were disposed of after every use.

3.2.4. Fungicide Exposure

Bioassay cages loaded with 40–50 foragers were divided into experimental groups that were either exposed to fungicides at various concentrations, or exposed to fungicide-free water, the diluent used in every treatment (Tables B-2 and B-3). Contact exposure was conducted utilizing a wind tunnel atomizer setup at the USDA Agricultural Research Service Aerial Application Technology Laboratory at the Riverside Campus of Texas A&M University, located in Bryan, TX. Large fans at one end of the wind tunnel setup propelled air at a speed of 2.9 m/s down the wind tunnel chamber, simulating the wind speed of pesticides dispensed from agricultural aircraft. Labeled bioassay cages were

loaded, one at a time, onto a holding fork near the end of the wind tunnel chamber opposing the large fans (Figure B-1b). The fungicides were diluted in water and sprayed at concentrations corresponding to the label dose or preselected label dose variants. Approximately 10 ml of each fungicide solution was loaded into the twin fluid atomizer located at the end of the wind tunnel chamber corresponding to the large fans. A 10ml syringe was used to transfer fungicide solution through a plastic tube attached directly to the atomizer. A compressed air tank was connected to the atomizer and activated along with the wind tunnel fans propelling fungicide solution through the atomizer and down the wind tunnel chamber. Each application lasted for ~5 s to ensure the complete expulsion of fungicide solution from the atomizer and propulsion down the chamber to the bioassay cage on the opposing end. Following exposure, bioassay cages were removed from the holding fork and the atomizer was cleansed with acetone between the application of each experimental treatment. This process was repeated for all bioassay cages allocated to each treatment group. The control group bioassay cages were loaded into the wind tunnel but were spared fungicide exposure; instead, they were sprayed with water propagated through the atomizer.

3.2.5. Monitoring Forager Survival

Following the application of fungicide treatments, foragers in each bioassay cage were transferred to a labeled plastic containment unit (~1 quart in volume), containing strips of wax foundation attached to the side and bottom of the unit (Figure B-1c). A wide brimmed funnel was placed over a containment unit, and then one of the holding rings

on a bioassay cage was removed to facilitate the transfer of foragers. The bioassay cage was secured over the funnel and one of the mesh side panels was removed, allowing foragers in the bioassay cage to migrate into the containment unit. The containment unit was gently shaken and a lid was swiftly placed over it to secure the foragers within. This process was repeated until all foragers in each bioassay cage were transferred to corresponding containment units. A pair of 1.5-ml Eppendorf tubes was inserted into premade holes in the lid of each unit. The Eppendorf tubes served as feeders and water dispensers. Feeder tubes were loaded with ~1 ml of sugar syrup composed of a 50:50 mixture of water and sucrose, while water dispensing tubes were loaded with ~1 ml of water. The containment units were kept in an incubator set at 34.5°C and 75% relative humidity. The units were checked every 24 h for 10 consecutive days, noting the number of dead workers to determine the total number of workers that died every day within the 10-d period. A forager was considered dead if it exhibited a complete lack of movement, which was often accompanied by the forager lying on its side with its proboscis permanently extruded.

3.2.6. Statistical Analysis

To compare the average forager mortality over a 10d period between the untreated control group and each individual fungicide treatment, Student's t-tests (JMP 12.0, SAS Inc., Cary, NC) were performed. To compare the survival rate between control and treatment groups, Kaplan–Meier survival analysis was performed (JMP 12.0, SAS Inc.,

Cary, NC). For all tests, the level of statistical significance was set at $\alpha=0.05$. All descriptive statistics are reported as the mean \pm standard error of the mean (S.E.M.).

3.3. Results

Following exposure to iprodione alone at various concentrations, and iprodione in combination with other fungicides (Table B-2), foragers tended to experience significantly high mortality that increased in severity over the course of the first three trials (Figure B-2). For instance, during the September 2015 trial, no statistically significant difference was observed when the treatment groups were compared with the untreated control (Figure B-2a). But in the October 2015 trial, this difference was statistically significant ($t=1.06$, $P=0.04$), with foragers exposed to iprodione at twice the label dose having an average 10d-mortality of $24\% \pm 11\%$ compared with $8.4\% \pm 2.3\%$ for the untreated control group (Figure B-2b). Differences in worker mortality were more striking during the November 2015 trial (Figure B-2c), with a significantly higher average mortality observed in the iprodione treatment at the label dose ($32.5\% \pm 13.1\%$; $t=1.99$, $P=0.04$), the iprodione at twice the label dose ($64.5\% \pm 17.9\%$; $t=3.23$, $P=0.01$), the combinations of iprodione and azoxystrobin at the label dose ($40.1\% \pm 15.3\%$; $t=2.21$, $P=0.04$), and the combinations of iprodione and BP at the label dose ($43.9\% \pm 17.8\%$), compared with the untreated control group ($5.5\% \pm 3.1\%$; $t=2.11$, $P=0.04$).

Average forager mortality data were also analyzed over the course of 10d within each trial using Kaplan–Meier survival analysis. In addition to the assessment of the general effect of the collective treatment groups on forager mortality for all treatment groups, the

survival rate of foragers exposed to each individual treatment was compared with the untreated control group. The treatment groups at twice the label dose of iprodione, iprodione+azoxystrobin, and iprodione+BP significantly decreased forager survival in all three trials of the first experiment (Table B-4). Similarly to our average mortality results, fungicide-treated foragers experienced a significant and progressive decrease in their survival rate, which occurred earlier in time over the course of the first three trials, compared with the untreated control group (Figure B-3). In the first trial, which was done in September 2015 (Figure B-3a), a significant decrease in forager mortality was observed as a general effect of fungicide exposure ($\chi^2=25.04$, $P<0.001$). Iprodione at twice the label dose, the combination of iprodione and azoxystrobin at the label dose, and iprodione and BP at the label dose significantly decreased forager survival compared with the untreated control group. In the second trial, which was done in October 2015 (Figure B-3b), the overall effect of fungicide exposure was more pronounced ($\chi^2=30.30$, $P<0.0001$). The treatment groups iprodione at the label dose and iprodione at half the label dose significantly decreased forager survival. Also, as with the September 2015 trial, the treatments of iprodione at twice the label dose, iprodione in combination with azoxystrobin, and iprodione in combination with BP all demonstrated a significant decrease in forager survival compared with the untreated control group. Interestingly, forager survival rate was most highly impacted in the November 2015 trial (Figure B-3c), whereby all treatment groups experienced a significant decline in survival relative to the control group ($\chi^2=328.7$, $P<0.0001$). The treatment groups included iprodione at a quarter of the label dose, at half the label dose, at the label dose, and at twice the label

dose, iprodione combined with azoxystrobin, and iprodione combined with BP (Table B-4).

In a second experiment (with only one trial) conducted in December 2015, foragers were exposed to individual applications of iprodione, BP, and azoxystrobin, each at twice the label dose rate (Table B-5). During this trial, foragers experienced significantly higher average mortality when exposed to iprodione at twice the label dose rate compared with the untreated control group ($t=2.22$, $P=0.04$; Figure B-4). Mortality data using Kaplan–Meier survival analysis revealed an overall significant effect of all three of the fungicide treatment groups in significantly decreasing forager survival rate over the 10-d experimental period ($\chi^2=31.5$, $P<0.0001$; Figure B-5). When the survival rate of each individual fungicide treatment group was compared with the control group, each pairwise treatment comparison revealed a significant decrease in forager survival rate (Table B-5).

3.4. Discussion

The most frequently used fungicides during almond bloom in California during the 2012 season revealed a significant negative effect of iprodione, alone and in combination with azoxystrobin or BP, on honey bee forager survival. Overall, significant drops in forager survival (as measured by Kaplan–Meier survival analyses) when foragers were exposed to the various fungicides treatment groups compared with untreated control groups. These effects were consistent in three separate trials conducted in Fall 2015, with the overall effect of the fungicide treatments progressively intensifying in trials conducted

later in the year, thus suggesting a severe seasonal effect of fungicide exposure on forager mortality.

The causes of decreased forager survival due to fungicide exposure are likely due to disruption of key physiological processes within exposed foragers. However, the mode of action of fungicides on honey bee physiology has been poorly examined. It is possible that exposed workers may have inadvertently increased the concentration of fungicides that end up inside their bodies through allo- and self-grooming (Scheiner et al. 2013), thus licking fungicides on their cuticle while confined in the containment units after being treated with the fungicides. This idea remains to be tested, however. In addition, the transition to winter physiology in honey bees appears to coincide with reduced immunological processes (Steinmann et al. 2015), specifically in the reduction of the expression of genes associated with microbial resistance. This process of reducing immune strength apparently coincided with our continuous application of fungicides, perhaps clarifying why the same treatment groups had an enhanced negative effect on forager survival over time.

Interestingly, when the fungicide prochloraz was topically applied along with pyrethroid insecticides to workers, it enhanced the toxicity of both compounds by inhibiting the activity of detoxifying cytochrome P450 monooxygenases (Pilling et al. 1995). In a more recent study, topical application of prochloraz was observed to interact with the acaricides tau-fluvalinate, coumaphos, and fenpyroximate, increasing their toxicity to treated workers (Johnson et al. 2013). Four other fungicides, including chlorothalonil,

boscalid, pyraclostrobin, and a combination of boscalid and pyraclostrobin, similarly increased the toxicity of tau-fluvalinate (Johnson et al. 2013) via topical applications to workers. In that study, inhibition of detoxifying P450 monooxygenases was proposed as the mechanism by which the fungicides enhanced the toxicity of the acaricides used (Johnson et al. 2013). In fungal targets, boscalid and pyraclostrobin function as a succinate dehydrogenase inhibitor and a quinone outside inhibitor, respectively (Fernández-Ortuño et al. 2012). Thus BP disrupts mitochondrial metabolism and ATP synthesis within cells. Azoxystrobin functions in a similar manner in that it disrupts the electron transport chain, thereby inhibiting ATP synthesis (Bartlett et al. 2002). Azoxystrobin also induces oxidative stress through electron liberation from the process of respiration (Kim et al. 2007). Finally, iprodione, though its mode of action is not fully understood, appears to inhibit glutathione synthesis (Dierickx 2004), which is essential for detoxification processes in the cell's mitochondria (Ribas et al. 2014).

The previously demonstrated lethal effects of field-relevant doses of iprodione on honey bee brood, although applied in vitro (Mussen et al. 2004), suggest the potential for contaminated foragers to inadvertently cause rapid population declines in exposed colonies. Intensive application of iprodione in almond orchards may lead to its prevalence in food stores, particularly pollen, as has been noted in other agricultural systems such as cherry orchards (Kubik et al. 1999). The resilience in wax of fungicide residues with respect to other pesticide classes (Johnson et al. 2010) may make them a greater threat to colony health, especially when considering the general lack of attention to this class of agrochemicals. Interestingly, boscalid, one of the active ingredients of

Pristine[®], was among the most highly detected fungicides found in wax by a review of studies examining in-hive pesticide toxicity (Johnson et al. 2010). This suggests that along with iprodione, BP may also impact colony health through persistent contamination of pollen reserves. Mussen et al. (2004) may provide an accurate basis of comparison for the sustenance of brood on contaminated pollen in field conditions, an indication of further colony effects beyond short-term forager mortality.

Given their effects on fungal targets and insight on the effects of a few fungicides on honey bees (Pilling et al. 1995, Johnson et al. 2013), fungicides may overall exert similar effects to those caused by acaricides and other chemicals in inhibiting detoxifying components and processes. However, despite reports of the persistence of fungicides in a hive environment (Kubik et al. 1999, Johnson et al. 2010), the focus to honey bee health has been on the effect of fungicides in augmenting the toxicity of other pesticides classes (Pilling and Jepson 1993, Pilling et al. 1995, Vandamme and Belzunces 1998, Johnson and Purcell 2013, Johnson et al. 2013), therefore leaving a distinct vacancy in our understanding of the precise effects of fungicides on their own on honey bee health. By examining fungicides as individual applications and in combination with other fungicides, we will create a stronger basis for understanding the potential threat that fungicides alone may pose to honey bee health.

In conclusion, despite the importance of honey bee foragers in supplying food resources to their colony, the precise mechanisms of action of fungicides used during floral bloom have not been characterized in honey bees. When considering the use of fungicides during the almond bloom, cautious fungicide application in almond orchards is

recommended to prevent unplanned forager exposure to these chemicals. Perhaps avoidance of such applications during bloom or applying fungicides during times of low honey bee forager activity, such as late evenings, would help mitigate the direct and potential secondary effects of fungicides to honey bee colony health.

CHAPTER IV

THE EFFECTS OF THE INSECT GROWTH REGULATORS METHOXYFENOZIDE
AND PYRIPROXYFEN AND THE ACARICIDE BIFENAZATE ON HONEY BEE
(HYMENOPTERA: APIDAE) FORAGER SURVIVAL

4.1. Introduction

In the United States, honey bees (*Apis mellifera*) make the largest contribution to pollination services for agricultural crops, which amounts to approximately \$17 billion annually (Calderone 2012). Almond (*Prunus dulcis*) is among the major crop recipients of honey bee pollination services, being completely dependent on honey bees for nut set (Klein et al. 2012). The California almond industry produces approximately 80 percent of the world almond supply (Klein et al. 2012), a feat accomplished through the use of about 60 percent of all managed honey bee colonies in the country, which are transported to almond orchards during the crop's bloom in mid to late winter (Sumner and Boriss 2006). To avoid problems associated with various pests and pathogens, almonds are frequently defended with repeated chemical treatments during the bloom period (Bosch and Blas 1994). Given the particularly widespread use of insecticides in agricultural fields, several insecticides have been the focus of much concern and investigation with respect to their impacts on bee health (Goulson 2013).

Neonicotinoid insecticides are the most widely utilized insecticides around the world (Goulson 2013). Numerous studies have uncovered sublethal and lethal effects on honey

bee health from exposure to neonicotinoids. For example, foraging activity and navigation have been shown to be reduced by sublethal doses of imidacloprid (Colin et al. 2004), clothianidin (Schneider et al. 2012) and thiamethoxam (Henry et al. 2012). Furthermore, topical exposure to thiamethoxam reduces worker olfactory memory and learning performance, and oral exposure reduces responsiveness to high sucrose concentrations (Aliouane et al. 2009).

Imidacloprid also induces a synergistic effect with respect to infection with the microsporidian parasite *Nosema ceranae*, as the presence of both increases stress and even death through the inhibition of glucose oxidase (Alaux et al. 2010). Similarly, clothianidin exposure inhibits transcription factors involved in immune response, facilitating viral pathogen replication (Di Prisco et al. 2013). Despite the many studies on the impact of neonicotinoids on honey bee health, not all results have been consistent. For instance, no effect of field-relevant dosages of imidacloprid was observed on locomotion, feeding or mortality (Cresswell et al. 2011). Also, despite the widespread presence of imidacloprid residues in pollen and honey stores, contamination was not correlated with colony mortality (Chauzat et al. 2009). Furthermore, the presence of imidacloprid and other neonicotinoids in food stores and other hive products seems to vary regionally, as another study examining in-hive pesticide residues found neonicotinoids at very low rates (Mullin et al. 2010).

Unlike neonicotinoids, the effects of other pesticides such as insect growth regulators (IGRs) and acaricides to honey bee health have been generally overlooked. The IGR

diflubenzuron significantly increased mortality in early honey bee instar larvae and pupae while exposure to adults resulted in physical abnormalities (Chandel and Gupta 1992). In a subsequent study, diflubenzuron exposure reduced worker weight and suppressed hypopharyngeal gland development (Gupta and Chandel 1995). Exposure to another IGR, pyriproxyfen, has been observed to negatively impact workers by inhibiting vitellogenin synthesis in the hemolymph (Pinto et al. 2000), as well as by conferring morphological abnormalities such as misshapen wings and increased rejection by nest mates (Fourrier et al. 2015). Furthermore, although targeted toward other arthropod taxa, several acaricides are found frequently in hive products (Mullin et al. 2010) and thus may constitute a significant threat to honey bee health. Several acaricides including thymol, coumaphos and formic acid negatively affect honey bee immune responses, including the expression of the c-Jun amino-terminal kinase pathway involved in detoxification (Boncristiani et al. 2012). Furthermore, coumaphos induces immunosuppression in workers by decreasing lysozyme expression (Garrido et al. 2016).

Honey bee foragers are the subset of the worker force tasked with food collection, a duty that typically begins 21 days post emergence (Huang and Robinson 1996, Abou-Shaara 2014). Foragers are thus the age group most susceptible to direct exposure to pesticides outside the hive (Pettis et al. 2013), making them particularly important in studies of the health effects of pesticides used in agricultural crops. In this study, we exposed foragers to two IGRs and an acaricide widely applied during the almond bloom in California and assessed their potential effects on forager mortality. Based on our results, increased caution in the application of these pesticides in almond orchards or any agricultural crop

during bloom should be encouraged in the almond industry's Best Management Practices efforts, as they negatively affect honey bee foragers and thus, potentially impact overall colony health.

4.2. Materials and Methods

4.2.1. Pesticides Used

The pesticides selected for the study were the IGR methoxyfenozide (Intrepid[®]: 22.6% methoxyfenozide; Dow AgroSciences LLC, Indianapolis, IN), the IGR pyriproxyfen (Nyguard[®]: 10% pyriproxyfen; McLoughlin Gormley King Company, Minneapolis, MN), and the acaricide bifentazate (Floramite[®]: 22.6% bifentazate; MacDermid Agricultural Solutions, Waterbury, CT). These pesticides were chosen because they were widely applied during the almond bloom in California in 2014 (Summary of Pesticide Use Report Data; Table C-1). All pesticides were applied individually at the manufacturer's recommended label dose or at different concentrations below and above the label dose rate (see below) following the experimental procedures outlined by Fisher et al. (2017). The label dose variants utilized ranged from half the label dose to three times the label dose rate. Dose variants above the label dose (2x, 3x) represented scenarios where foragers could have been exposed repeatedly with the label dose (1x) application either in different fields or multiple times while performing the pollination services.

4.2.2. Experimental Treatment Groups

To assess the effects of the three pesticides on honey bee forager survival, five experimental groups were formulated including a pesticide-free control group for each pesticide tested. The first set of trials included treatment with methoxyfenozide at differing concentrations derived from the recommended label dose (Table C-2).

Methoxyfenozide was tested in three separate trials conducted between March and May 2016. A second experimental trial set was conducted using pyriproxyfen at concentrations derived from the label dose (Table C-2) compared to an untreated control group. Pyriproxyfen treatment groups were used in three separate trials between January and March 2017. Finally, a third set used the acaricide bifenthrin at differing concentrations derived from the recommended label dose compared to a control group (Table C-2) in three separate trials conducted between July and October 2016. For all trials the control group consisted only of the solvent, distilled water, which was used to dissolve the pesticides used in the treatment groups.

4.2.3. Forager Capture

Honey bee foragers were collected from a designated hive at the Janice and John G. Thomas Honey Bee Facility of Texas A&M University's RELIS Campus in Bryan, TX. Forager capture was conducted by collecting bees from frames containing mostly food resources and no brood, since these frames are typically covered by older adults, including foragers, which engage in tasks including food collection and unloading, rather than brood maintenance, which is associated with younger workers (Winston 1987).

Foragers were gently brushed off frames into bioassay cages composed of a circular cardboard frame of 15.2 cm in diameter, a single sheet of mesh fabric that was stretched over either side of the cage, and another thin cardboard holding ring slightly larger in diameter than the cage frame to secure the mesh in a taut position. Approximately 30 to 40 foragers were loaded into each bioassay cage and six bioassay cages were allocated to each experimental treatment group (Figure C-1a). Each bioassay cage was used only once then discarded to avoid cross contamination between trials.

4.2.4. Pesticide Exposure

The bioassay cages loaded with foragers were divided into experimental groups that were either exposed to pesticides at various concentrations, or to pesticide-free water (Table C-2). Foragers were exposed to the pesticides using a wind tunnel atomizer setup at the USDA-Agricultural Research Service Aerial Application Technology Laboratory in Bryan, TX. The wind tunnel-atomizer setup was designed and assessed to accurately simulate the droplet size of field applications for specific chemicals (Fritz and Hoffmann 2016). Large fans at one end of the wind tunnel setup propelled air at a speed of 2.9 m/s down the wind tunnel chamber simulating the wind speed of pesticides dispensed from an agricultural spraying aircraft. The bioassay cages were loaded, one at a time, onto a holding fork near the end of the wind tunnel chamber opposing the large fans (Figure C-1b). Each pesticide was diluted in water separately and sprayed at concentrations corresponding to the label dose or other label dose variants. Approximately 10 mL of each pesticide solution was loaded into the twin fluid atomizer using a 10 mL syringe

located at the end of the wind tunnel chamber where the large fans were located. A compressed air tank was connected to the atomizer and activated along with the wind tunnel fans propelling pesticide solution through the atomizer and down the wind tunnel chamber. Each application lasted approximately 5 s to ensure the complete expulsion of the pesticide solution from the atomizer and propulsion down the chamber to the bioassay cage on the opposing end. Following exposure, the bioassay cages were removed from the holding fork and the atomizer was cleansed with distilled water between applications of each experimental treatment. This process was repeated for all bioassay cages allocated to each treatment group. For the control group, bioassay cages were loaded into the wind tunnel and were sprayed with water, but were not exposed to pesticides.

4.2.5. Monitoring Forager Survival

Following the application of each pesticide treatment, foragers in each bioassay cage were transferred to a labeled plastic containment unit (~1 quart in volume) containing strips of wax foundation attached to the side and bottom of the unit (Figure C-1c). To load bees, a wide brimmed funnel was placed over a containment unit and one of the holding rings on a bioassay cage was removed to facilitate transfer. The bioassay cage was secured over the funnel and one of the mesh side panels was removed allowing foragers in the bioassay cage to migrate into the containment unit, which was then gently shaken and topped with a lid to prevent foragers from escaping. This process was repeated until all foragers in each bioassay cage were transferred to the corresponding

containment units. Two 1.5 mL Eppendorf tubes were inserted into pre-made holes in the lid of each containment unit to serve as a feeder and a water dispenser. Feeder tubes were loaded with approximately 1 mL of a 1.5M sucrose solution, and water dispensing tubes were loaded with approximately 1 mL of water. The containment units were kept in an incubator set at 34.5 °C and ~75% relative humidity. The units were checked every 24 h for ten consecutive days and the number of dead foragers at each 24 h interval was recorded. A forager was considered dead if it exhibited a complete lack of movement, which often entailed the forager lying on its side with its proboscis permanently extruded.

4.2.6. Statistical Analysis

Due to unequal variances in the data, non-parametric Wilcoxon tests were performed to compare average forager mortality over a 10-day period between the untreated groups and each pesticide treatment group. A MANOVA test was performed to assess interaction effects between trial and treatment for each pesticide. Kaplan-Meier survival analysis was performed to compare the survival rate between the untreated control group and the pesticide treatment groups in each set of trials. All statistical tests were performed with the software JMP® 12.0 (SAS Inc., Cary, NC). The level of statistical significance was set at $\alpha = 0.05$ for all tests. All descriptive statistics are reported as mean \pm standard error of the mean (S.E.M.).

4.3. Results

Three separate trials were conducted for each pesticide tested. The data for all three trials were pooled together to assess average forager mortality and survival rate. However, we obtained an interaction effect of trial and treatment for methoxyfenozide ($F=9.69$, $P<0.0001$) and bifenazate ($F=2.82$, $P=0.009$). Some potential underlying reasons for this variation are proposed in the discussion. A significant increase in average forager mortality was found as a general effect of methoxyfenozide exposure ($\chi^2=13.44$, $P=0.009$). Foragers were specifically affected by methoxyfenozide exposure at three concentration variants of the label dose (Table C-2; Figure C-2a). Namely, methoxyfenozide at the label dose significantly increased average forager mortality compared to the control group ($Z=2.86$, $P=0.004$). Methoxyfenozide at twice the label dose ($Z=3.36$, $P=0.0008$) and three times ($Z=2.61$, $P=0.009$) the label dose also caused a significant increase in forager mortality compared to the control group.

A significant increase in average forager mortality was likewise observed as a general effect of exposure to pyriproxyfen ($\chi^2=15.13$, $P=0.004$) with two particular label dose variants significantly impacting average mortality (Figure C-2b). Specifically, pyriproxyfen at twice ($Z=3.39$, $P=0.0007$) and three times the label dose ($Z=3.08$, $P=0.002$) significantly affected forager mortality compared to the control group.

Lastly, foragers exposed to bifenazate at concentration variants of the label dose (Table C-2) also experienced a significant increase in average mortality ($\chi^2=18.27$, $P=0.001$) across all treatment groups (Figure C-2c). Bifenazate at the label dose significantly

increased forager mortality compared to the control group ($Z=3.33$, $P=0.0009$), which was also the case for all label dose variants tested including half ($Z=2.94$, $P=0.003$), twice ($Z=3.22$, $P=0.001$) and three times the label dose for bifentazate ($Z=3.50$, $P=0.0005$).

The Kaplan-Meier survival analysis was used to assess forager survival rate over a ten-day period in which foragers were monitored following exposure to the pesticide treatments. Similarly to average mortality, foragers exposed to the concentration variants of each pesticide experienced a significantly lower survival rate compared to the untreated control groups (Figure C-3). For methoxyfenozide-exposed foragers (Figure C-3a), a significant decrease in forager survival was observed as a general effect of pesticide exposure ($\chi^2 = 429.50$, $P<0.0001$). Each label dose variant induced a similar effect when compared individually to the control group (Table C-3) including half ($\chi^2 = 148.31$, $P<0.0001$), one time ($\chi^2 = 343.98$, $P<0.0001$), twice ($\chi^2 = 329.84$, $P<0.0001$) and three times the label dose of methoxyfenozide ($\chi^2 = 398.48$, $P<0.0001$). A similar outcome resulted from pyriproxyfen exposure, inducing a significant decrease in survival rate compared to the control group due to pesticide exposure (Figure C-3b). Significant decreases in forager survival were observed in the comparison between each individual concentration variant and the control (Table C-3) including half ($\chi^2 = 31.62$, $P<0.0001$), one time ($\chi^2 = 93.03$, $P<0.0001$), twice ($\chi^2 = 173.30$, $P<0.0001$) and three times the label dose of pyriproxyfen ($\chi^2 = 180.04$, $P<0.0001$). Finally, bifentazate had a similarly negative effect of pesticide exposure on forager mortality ($\chi^2 = 273.13$, $P<0.0001$) and in the individual comparison of each treatment to the control (Table C-3).

This included exposure to bifenazate at half ($\chi^2 = 69.54, P < 0.0001$), one time ($\chi^2 = 174.17, P < 0.0001$), twice ($\chi^2 = 192.28, P < 0.0001$) and three times the label dose ($\chi^2 = 246.41, P < 0.0001$).

4.4. Discussion

Our examination of three pesticides used widely during the 2014 almond bloom in California revealed significant negative effects to honey bee forager survival from exposure to the IGRs methoxyfenozide and pyriproxyfen, and the acaricide bifenazate. Kaplan–Meier survival analyses revealed a significant decrease in forager survival over time when foragers were exposed to various pesticide concentration treatment groups compared to untreated control groups. Both insect growth regulators and the acaricide caused significant negative impacts to average forager mortality after 10 days. The observed effects varied slightly between the three pesticides used. Exposure to methoxyfenozide caused significantly lower forager survival at all label dose variants (except for half the label dose) compared to untreated controls. Conversely, only the dose variants of pyriproxyfen above the label dose affected average forager mortality, while all label dose variants of bifenazate contributed a significant negative effect of forager mortality. Additionally, an interaction effect between trial and treatment was observed for methoxyfenozide and bifenazate. The intensity of the impact of exposure increased progressively across trials for both of these pesticides, suggesting a potential seasonal effect on forager survival that should be considered when exposing honey bees to these chemicals.

The trials for methoxyfenozide were conducted from March to May, while those for bifentazate were conducted from July to October. Thus, the trials encompassed a period of time that spanned from one season to another. The progressively pronounced negative effects of bifentazate exposure on forager survival over time may be accounted for the fact that honey bee physiology changes between seasons, particularly in the transition to winter physiology, which entails reduced expression of genes involved in pathogenic resistance (Steinmann et al. 2015).

Aside from pyrethroids, honey bees are generally considered to be highly susceptible to insecticides (Johnson et al. 2006). While cytochrome P450 enzymatic activity has been implicated in the detoxification of some insecticides, particularly neonicotinoids such as thiacloprid and acetamiprid (Iwasa et al. 2004), immunological pathways such as oxidative stress and antimicrobial peptide production appear to be the main targets of many insecticides including neonicotinoids and IGRs (James and Xu 2012). Insecticide activity on honey bees may extend beyond the individual to potentially reduce overall colony immunity, particularly in the inhibition of glucose oxidase pathways, which is important for antimicrobial defenses (James and Xu 2012). For instance, the frequently studied neonicotinoid imidacloprid has been observed to induce with *Nosema ceranae*, albeit synergistically, a reduction in glucose oxidase activity (Alaux et al. 2010).

Even though pesticides are often applied at levels below acute mortality in honey bees, our examination of the commonly used methoxyfenozide, pyriproxyfen and bifentazate suggests that honey bee forager survival is impacted over time and may be significantly

reduced even at levels below the manufacturer's recommended application dose rates. Fisher et al. (2017) recently conducted a similar study looking at the effects of fungicides commonly used in California for the protection of almond orchards (Fisher et al. 2017). The effects of dose variants of iprodione ranging from one fourth to twice the label dose rate as well as iprodione in combination with BP or azoxystrobin at the label dose, were tested on honey bee forager mortality over a 10-day period. Iprodione at all concentration variants and combinations negatively affected forager survival, particularly during trials conducted in fall and winter (Fisher et al. 2017). As observed in our examination of methoxyfenozide and bifenazate, the effect of iprodione and iprodione combinations were increasingly pronounced over time, verifying a potential seasonal effect on foragers' susceptibility to pesticide exposure. As more studies are published on the sub-lethal effects of pesticides on honey bee health (Colin et al. 2004, Henry et al. 2012, Schneider et al. 2012, Fourrier et al. 2015, Garrido et al. 2016), a more careful consideration of the importance of integrated pollinator and pest management needs to be addressed. For instance, measures reducing pesticide exposure to honey bees during almond bloom are being more thoroughly described by the Almond Board of California's Best Management Practices (<http://www.almonds.com/pollination#BeeBMPs>), including recommendations for pesticide application during alternative hours coinciding with low honey bee forager activity (i.e., between dusk and dawn) to aid in reducing the impact of pesticides on honey bee health. Avoidance strategies may present the best option for defending crops

while minimizing pollinator loss, as the lack of immediate or long-term mortality may not indicate a lack of health risk for bees.

CHAPTER V
THE ACUTE EFFECTS OF MOSQUITO ABATEMENT INSECTICIDES ON
HONEY BEE (*APIS MELLIFERA*) FORAGER MORTALITY

5.1. Introduction

Honey bees (*Apis mellifera*) are the most important beneficial insects in agriculture, providing an estimated \$17 billion annually to the United States economy, particularly through crop pollination (Calderone, 2012). Throughout the year, over half of the estimated 2.6 million colonies of managed honey bees are transported across the country for crop pollination and honey production (vanEngelsdorp and Meixner, 2010; Seitz et al., 2016). Consequently, colonies are constantly exposed to a wide array of pesticides when workers forage for nectar and pollen, as well as when they are treated by beekeepers for pests and pathogens (Mullin et al., 2010). Exposure to pesticides has been proposed as a mitigating factor in the decline of honey bee populations in the United States (Finley et al., 1996; Faucon et al., 2002; Mullin et al., 2010; Krupke et al., 2012; Johnson, 2015; Doublet et al., 2015; Seitz et al., 2016). Many pesticides commonly used for the control of insect pests that cause damage to agricultural crops are toxic to honey bees (Halm et al., 2006; Decourtye et al., 2004; Desneux et al., 2007; Henry et al., 2012; Charreton et al., 2015; Dively et al., 2015; Pisa et al., 2015).

Honey bee health is also at risk from exposure to pesticides used to control insect vectors of humans and animal pathogens, particularly mosquitoes (Caron, 1979; Pankiw and Jay,

1992). As a preventative measure against vector-borne disease outbreaks, insecticides sprayed for mosquito control are widely administered in the United States. Some of the most common mosquito abatement insecticides include the broad-use insecticide fipronil (El Hassani et al., 2005; Tingle et al., 2003), the organophosphates fenthion, dichlorvos and malathion (Atkins et al., 1981; Womeldorf et al., 1974), the carbamate bendiocarb (Atkins et al., 1981), and the organic pyrethrins (Atkins et al., 1981), among others. Many of these insecticides are also used to control other household pests including ants, beetles, fleas, and mites.

Pesticide use is regulated nationally by the Environmental Protection Agency, which requires the testing and registration of all mosquito abatement products used in the United States. However, states and smaller governing bodies may authorize the establishment of mosquito control districts. For example, the Mosquito Abatement District Act of Illinois afforded the state of Illinois the legal ability to establish districts for mosquito abatement using the parameter of having at least three hundred residents in a given district (Illinois General Assembly, 2015). Similarly, the California Health and Safety Code permits the establishment of mosquito abatement and vector control districts within state lines. State governmental policies concerning mosquito control programs are also known in a few Atlantic states including Delaware and Connecticut.

To comply with state and federal regulations for mosquito abatement, insecticides used for this purpose must be tested for toxicity to target, as well as non-target organisms. Several methods are utilized to assess the toxicity of mosquito abatement insecticides to

honey bees, which are among the most beneficial non-target organisms potentially affected by these chemicals. A common assessment technique involves topical application of insecticides on the bee's thorax at various concentrations above and below the recommended label dose rates (Anderson and Atkins, 1968). Alternatively, honey bees may be placed in cages which are then sprayed with the pesticides of interest dissolved in a solvent at spray rates similar to those used in the field (Womeldorf et al., 1974; Anderson and Atkins, 1968; Atkins et al., 1981). The simplicity and repeatability of these cage bioassays allows for an effective comparison between experimental groups (Bonds et al., 2010). However, there are many products currently used for mosquito abatement that have not been thoroughly tested for their effects on honey bee health. Our main objective was to examine the effects of varying concentrations of two commonly-used public health insecticides used for mosquito control on honey bee forager mortality. To do so, foragers were placed in bioassay cages which were then inserted into a wind tunnel with a wind speed of 1.8 m/s. After exposing honey bees to varying concentrations of the two mosquito abatement products, acute worker mortality was assessed at 24 and 48 hours after exposure. Based on these findings we make recommendations for public health officials on the proper delivery of mosquito abatement insecticides.

5.2. Materials and Methods

5.2.1. Insecticide Selection and Forager Capture

The insecticides selected for this study were two products used in the control of mosquitoes: Insecticide (I) 1 (Duet™: 1% prallethrin, 5% sumithrin® and 5% piperonyl butoxide; Clarke Mosquito Control Products Inc., Roselle, IL), and Insecticide (I) 2 (Aqua-Pursuit™: 20.6% permethrin and 20.6% piperonyl butoxide; Precision Control Technology Inc., Baltimore, MD). Our treatment groups consisted of concentration variants ranging from the 0.2 to 1.6 times the label dose rate for I1 (Table D-1) and 0.5 to 3.3 times the label dose for I2 (Table D-2). In a previous study examining I1, I2 and other common mosquito control insecticides, Anderson (2015) evaluated the LC₁₀, LC₅₀ and LC₉₀ values for each insecticide. However, those experiments generally assessed dose variants far below or far above the application rate of the insecticides of interest (Anderson 2015). Thus, our study focused on the intermediate dose variants more closely revolving around the manufacturer's label dose for those products.

Honey bee foragers were collected from two source colonies located at the Janice and John G. Thomas Honey Bee Facility at the Texas A&M University REllIS Campus in Bryan, TX. Approximately 20 to 30 captured foragers were placed in bioassay cages consisting of a round hollow cardboard frame with a diameter of 15.2 cm and a depth of 7.6 cm covered by a mesh fabric that was tightly stretched over both ends of the frame and held in place by thin cardboard rings (Figure D-1a). Once the foragers were caged, they were exposed to the insecticides using the wind tunnel setup described below.

5.2.2. Wind Tunnel Exposure

To test honey bee worker mortality 24 h and 48 h after insecticide exposure, a bioassay cage containing 20 to 30 bees was inserted into a wind tunnel apparatus. The wind tunnel was constructed from a World Health Organization (WHO) template utilized in the evaluation of insecticide sprays (WHO, 2009). The wind tunnel consisted of a galvanized duct pipe with a diameter of 15.2 cm that was capped at the entrance. Airflow regulation was obtained with the cap, which contained ten pores with a diameter of 2 cm each (Figure D-1b). Insecticide exposure was attained through the use of an atomizer with a volume median diameter of $15 \pm 2 \mu\text{m}$. The atomizer was inserted in the center pore of the tunnel entrance allowing for a thorough droplet dispersal of the insecticide solution. The droplet size dispensed was set such that the atomizer caused the solvent (acetone) to evaporate before the insecticide permeated the bioassay cages. The atomizer was connected to a small nitrogen tank set at $\sim 70 \text{ kPa}$, which served to propel the insecticide solution out of the atomizer and down the wind tunnel shaft.

For every insecticide concentration and trial replicate, a clean bioassay cage was carefully nestled into a groove along the tunnel's length. Once the bioassay cages were placed individually inside the tunnel, the nitrogen tank was activated and 0.5 mL of insecticide solution was loaded into the nozzle of the atomizer. The atomizer took approximately 4 s to dispense 0.5 mL of insecticide solution through the nitrogen tank, and was allowed to run for 10 s to ensure complete dispersal of all loaded solution through the tunnel. Each bioassay cage was removed following exposure and was then

replaced with a fresh cage. Cages in the control groups were subjected to identical experimental conditions but were sprayed only with insecticide-free acetone.

Approximately 0.5 mL of acetone was sprayed through the atomizer between each experimental trial to rid the atomizer and tunnel of lingering residues from preceding sprays.

Following exposure, the caged foragers were transferred to capped 1-quart plastic cups and were provided with 1.5 M sucrose solution *ad libitum* through an Eppendorf tube inserted through a pre-cut hole in the lid of the cup. Once stocked with foragers, the cups were placed in an incubator maintained at 34°C and 75% RH. Forager mortality was assessed 24 h and 48 h post exposure and the number of dead foragers was recorded for each cage. Foragers were considered deceased if they exhibited a complete lack of movement, which was typically accompanied by the permanent expulsion of the proboscis. Each insecticide was assessed over the course of six trials, with each trial consisting of three replicates (i.e., three bioassay cages) per experimental group.

5.2.3. Statistical Analysis

Student's *t*-tests were performed to compare the average forager mortality between each insecticide concentration variant and the untreated control groups using the statistical software JMP 12.0 (SAS Inc., Cary, NC). For each insecticide, forager mortality between control and treatment groups was assessed over the course of the entire study period (i.e., overall average) as well as for each time point (24 h and 48 h post exposure) at which mortality was monitored independently. All descriptive statistics are reported as

the mean \pm standard error of the mean (S.E.M.). The level of statistical significance for all tests was set at $\alpha = 0.05$.

5.3. Results

5.3.1. Acute Forager Mortality Following Exposure to Insecticide 1

Exposure to varying concentrations of the insecticide Insecticide (I) 1 resulted in an overall significant increase in average forager mortality for two different concentrations (Figure D-2). Specifically, exposure to the concentration 60% above the label dose of I1 (“1.6x I1”) caused $20.1\% \pm 8.0\%$ mortality, which was significantly different from the control group ($t=2.50$, $P=0.02$). Likewise, the concentration at 80% of the label dose of I1 (“0.8x I1”) caused $15.1\% \pm 6.8\%$ mortality, which was significantly higher relative to the control groups ($t=2.21$, $P=0.04$).

When mortality was compared individually at the 24 h and 48 h post-treatment time periods, a significant increase in average forager mortality was observed only within the first 24 h (Figure D-3). Specifically, the 1.6x D dose caused $14.4\% \pm 6.7\%$ mortality ($t=2.14$, $P=0.04$), while the 0.8x D dose caused $14.9\% \pm 6.9\%$ mortality ($t=2.17$, $P=0.04$), both of which were significantly higher than the control group (Figure D-3). None of the treatments significantly impacted forager mortality relative to the control at 48 h post exposure (Figure D-4). Notably, mortality for the control groups was zero for both, the 24 h, and the 48 h time periods.

5.3.2. Acute Forager Mortality Resulting from Insecticide 2 Exposure

Foragers exposed to varying concentrations of the insecticide Insecticide (I) 2 exhibited higher mortality rates post exposure compared to the I1 treatments (Figure D-5).

Exposure to the concentrations 330% above the label dose of I2 (“3.3x I2”) caused an overall mortality of $44.2\% \pm 8.7\%$, which was significantly higher than the control group ($t=5.09$, $P<0.0001$). Likewise, exposure to the concentration 60% above the label dose (“1.6x I2”) caused $24.7\% \pm 8.2\%$ mortality, which was significantly different from the control ($t=3.00$, $P=0.008$), while the concentration at 80% of the label dose (“0.8x I2”) caused $15.2\% \pm 7.0\%$ mortality, being also significantly different from the control group ($t=2.17$, $P=0.04$).

When mortality was compared individually at the 24 h and 48 h post-treatment time periods, a significant increase in average forager mortality was observed only within the first 24 h but not 48 h post exposure. Specifically, the 3.3x AP dose caused $39.4\% \pm 8.4\%$ mortality ($t=4.69$, $P=0.0002$), while the 1.6x D dose caused $21.6\% \pm 8.1\%$ mortality ($t=3.66$, $P=0.02$), both of which was significantly higher than the control group (Figure D-6). None of the treatments significantly impacted forager mortality relative to the control 48 h post exposure (Figure D-7). Mortality for the control groups was again zero for both, the 24 h, and the 48 h time periods.

5.4. Discussion

We evaluated the effects to honey bee forager mortality caused by two insecticides commonly used in mosquito abatement programs when applied at levels above and below the label dose. Following exposure to varying concentrations of each insecticide, a significantly high forager mortality for concentrations above the label dose was observed for both products. Likewise, exposure to either I1 or I2 at 80% of the label dose rate significantly increased forager mortality. A previous study examining I1 and I2, among several mosquito control insecticides, tested concentrations inducing LC values ranging from 10% to 90% mortality (Anderson 2015). I1 was examined over the course of three trials with low concentrations ranging from 20-164 $\mu\text{g/mL}$ and high concentrations ranging from 830-9,000 $\mu\text{g/mL}$. I2 was also examined over three trials with low concentrations ranging from 114-912 $\mu\text{g/mL}$ and high concentrations ranging from 780-200,000 $\mu\text{g/mL}$ (Anderson 2015). Our study investigated intermediate concentration ranges with reduced deviation from the label dose rate. Specifically, I1 (label rate: 529.2 $\mu\text{g/mL}$) was examined in concentrations ranging from 105-847 $\mu\text{g/mL}$ (Table D-1) while I2 (label rate: 840.74 $\mu\text{g/mL}$) was examined in concentrations ranging from 420-2802 $\mu\text{g/mL}$ (Table D-2).

Even though our primary objective was to test the ability of these insecticides to induce acute honey bee mortality, they may affect honey bee health through long-term, chronic exposure to these chemicals. Sub-lethal effects on honey bee health have been found for other insecticides used to control arthropod pests of humans and animals. For example,

fipronil, which is widely used in veterinary medicine against ticks, fleas, and other insect pests, has been found to affect honey bee foragers by impairing olfactory memory and sucrose perception (El Hassani et al., 2005; Tingle et al., 2003). Furthermore, when applied directly on foraging honey bees, high concentrations of fenthion and dichlofos caused high mortality rates (Womeldorf et al., 1974). However, the effects were not as drastic when using colonies exposed in the field through aerial applicators, perhaps because the chemicals did not persist in the environment long enough to enter the hives and expose the entire colony. Atkins et al. (1981) had similar results with bendiocarb, malathion, and chlorpyrifos. They found that, while these chemicals were deadly to honey bees when applied directly on their cuticle, the mortality rate was negligible when the bees were inside the hive at the time of spraying. The authors also found that pyrethrins with a final concentration of 2.5% and applied at a rate of 2 fl. oz./min had very little effect even when directly applied to caged honey bees. Furthermore, to examine the effects of fenitrothion exposure to native pollinators, Kevan (1975) sampled native bee populations in response to noted declines in pollinator abundance observed in New Brunswick, Canada during peak pollinator activity in blueberry fields. A connection between bee decline and fenitrothion application was concluded based on application sites and concentrations relative to bee abundance. These findings imply that precautions should be taken to ensure that honey bees are not foraging or clustering outside hives when most insecticides are sprayed near apiaries.

The primary active ingredients of I1 include prallethrin and sumithrin, while the primary active ingredient of I2 is permethrin, all of which are pyrethroids. Pyrethroids act on

voltage-gated sodium channels of neuronal axons by keeping them permanently open preventing repolarization resulting in paralysis (Soderlund et al. 2002). Honey bees are generally considered to be resilient to pyrethroid exposure but highly vulnerable to other insecticides (Johnson et al. 2006). More recently there has been increasing interest in the effects of neonicotinoid insecticides and their mode of toxicity on honey bees (see review by Dively et al. 2015). For example, Iwasa et al. (2004) topically applied various neonicotinoids on adult workers, including imidacloprid, clothianidin, thiamethoxam, dinotefuran, nitenpyram, acetamiprid, and thiacloprid, to determine the toxicity of each chemical to honey bees. The toxicity of cyano-substituted neonicotinoids was observed to be much lower than that of nitro-substituted neonicotinoids (Iwasa et al. 2004). Likewise, sub-lethal treatments with the neonicotinoid thiamethoxam induced cell condensation and elimination in the mushroom bodies and midgut of exposed honey bees, which contributed to reduced worker longevity (Oliveira et al. 2014). Neonicotinoids function as agonists for the neurotransmitter acetylcholine by competitively binding to nicotinic acetylcholine receptors causing hyperstimulation of the central nervous system (Yamamoto 1999). Due to the structural composition of insect acetylcholine receptors they are particularly affected by exposure to neonicotinoids as they bind more strongly to insect receptors than those of other animal taxa (Tomizawa 2004).

Although they are frequently employed, cage bioassays can sometimes yield inconsistent results. For example, the density of mesh material used in cage structures may significantly affect the resulting outcome of exposure, whereby lower mesh densities

yield greater effects of pesticides on bee mortality (Hoffmann et al., 2008). Furthermore, cages also provide an opportunity for secondary exposure to tested chemicals resulting from direct contact with the cage surface (Bonds et al., 2010). For instance, in a study involving mosquitos, Hoffmann et al. (2008) tested the efficacy of two types of cages and a wind tunnel on insecticide delivery rate, and found that cage mesh density decreased the amount of spray, droplet size, and wind speed within the cages.

Nevertheless, our results may serve to inform mosquito abatement programs on the potential hazards of commonly used control measures to non-target organisms, such as the honey bee, and the need for avoidance strategies in the application of control insecticides. The increasing distribution and urban occurrence of mosquito vectors for the causative agents of chikungunya and dengue fever (Farajollahi et al. 2012, Faraji et al. 2016) may need additional considerations for co-occurrence of pest and beneficial insects in rural and urban settings. In conclusion, based on our results we suggest that public health officials should take strong precautions when applying mosquito abatement insecticides during the time of honey bee foraging activity when spraying is done near apiaries.

CHAPTER VI

CONCLUSION

By examining commonly used, yet understudied, pesticides we described significant negative effects on honey bee health of these chemicals frequently encountered within the hive and in foraging environments. We observed a significant negative impact of the most frequently detected miticides and agrochemicals in beeswax on drone spermatozoa viability. Forager survival rates were negatively affected following exposure to fungicides used to defend almond orchards from fungal pests during bloom. Similarly, exposure to insect growth regulators and an acaricide also used during bloom in almond orchards significantly reduced forager survival. Foragers exposed to insecticides commonly used in mosquito abatement programs experienced significant acute mortality of exposure. Our findings help augment understanding on the effects of pesticides on honey bee health with particular focus on largely neglected chemicals that are nevertheless frequently encountered in-hive (Mullin et al. 2010) and in many major crop systems (Johnson et al. 2010, Fisher et al. 2017).

In considering the importance of our findings to the bigger picture, bee hives used in almond orchards for pollination endure several other stress factors in addition to pesticide exposure. Over a million hives are transported into California from out of state for almond pollination, Simone-Finstrom et al. (2016) discovered a significant negative effect of long distance transportation on honey bee worker lifespan and a significant increase in oxidative stress (Simone-Finstrom et al. 2016). A larger negative effect on

worker survival, however, was accounted for by limited forage (Simone-Finstrom et al. 2016). Almond pollen, while providing adequate protein resources (Somerville 2001), was found to contain a high omega-6 to omega-3 fatty acid ratio (Arien et al. 2015). Bees maintained on a diet low in omega-3 fatty acids or on *Eucalyptus* pollen, a relative of almonds that contains a similar fatty acid ratio, exhibited significantly lower olfactory and tactile learning capabilities (Arien et al. 2015). Almond orchards are a monocultural crop system presenting little to no alternative floral sources for bees (Arien et al. 2015). Incidentally, honey bee colonies typically undergo rapid growth heading into spring, which coincides with their occurrence in almond orchards during bloom (Arien et al. 2015).

Another potential variable affecting colony health is seasonal change in bee physiology (Steinmann et al. 2015). Honey bee physiology during winter, when bees are present in almond orchards (Sumner and Boriss 2006), includes reduced expression of genes involved in immune responses (Steinmann et al. 2015). Inhibition of immunological capability appears to be the main effect of exposure to insecticides (James and Xu 2012). Similarly, fungicides are generally suspected of impairing honey bee immune responses including cytochrome P450 inhibition, facilitation of pathogenic infection and amplification of the toxicity of other pesticides (Pilling and Jepson 1993, Pilling et al. 1995, Pettis et al. 2013, Johnson et al. 2013). When considered along with limited foraging options (Arien et al. 2015, Simone-Finstrom et al. 2016) and substandard nutritional content (Arien et al. 2015) of almond resources, the widespread and frequent

use of pesticides (Fisher et al. 2017) may further compound stress and contribute to declining honey bee health and colony loss.

Pressure from reduced bee availability is exerted on growers as well as beekeepers, the rental price on a per hive basis for almond growers increased nearly three-fold within a decade (Carman 2011). Simultaneously, acreage increases in almond orchards drove up the number of hives required for pollination by over 84% (Carman 2011). The ubiquitous presence of in-hive pesticide residues and their apparent negative effects on male reproductives may contribute to the various stress factors impacting honey bee populations by negatively impacting queen longevity and brood production.

Additionally, urban mosquito control practices may threaten sedentary, non-commercial hives further facilitating a progressive decline in honey bee abundance and availability for pollination services.

Through our work, we suggest key changes on the part of growers and beekeepers in control practices of pests within and outside of the hive. Greater utilization of non-chemical control methods for hive pests and corresponding reductions in dependence on hard chemical treatments may improve drone reproductive competency. Avoidance strategies such as those recommended by the Almond Board of California, may reduce forager exposure to pesticide treatments in almonds and other crop systems. Given the efficacy of night spraying for mosquito control (Farajollahi et al. 2012) strict application at times of honey bee inactivity at floral resources may reduce risk of exposure to honey bees in urban settings.

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APPENDIX A

Table A-1. Summary of pesticide detections in wax samples found in commercial beekeeping operations across the United States (Adapted from Mullin et al. 2010, Table 4).

Pesticide detected	Class	Number of samples analyzed	Positive detection rate (%)	Highest detection found in wax (ppb)
Fluvalinate	Pyrethroid	259	98.1	204,000
Coumaphos	Organophosphate	259	98.1	91,900
Chlorpyrifos	Organophosphate	258	63.2	890
Chlorothalonil	Fungicide	258	49.2	53,700
DMPF (amitraz)	Formamidine	177	60.5	43,000

Table A-2. Pesticide concentrations used to assess the effects of contamination of wax with miticides and agrochemicals on drone spermatozoa viability in 2014

Experimental group	Pesticides used	Concentration (mg/100mL of acetone)
Control	None	0
F+C	Fluvalinate	20.4
	Coumaphos	9.19
C+C	Chlorothalonil	5.37
	Chlorpyrifos	0.09

Table A-3. Concentration used to assess the effects of contamination of the wax with the miticide amitraz on drone spermatozoa viability in 2016

Experimental group	Pesticides used	Concentration (mg/100mL of acetone)
Control	None	0
A	Amitraz	4.3



Figure A-1. A) Honey bee drones were collected shortly after emergence and marked on the thorax before being returned to their host hive. Paint marks of different colors for each treatment and each day allowed us to distinguish experimental drones from non-experimental drones in the hive, and to track the precise age of each drone. B) Drone holding cages were utilized to increase the recapture rate of control and amitraz-treated drones in 2016.

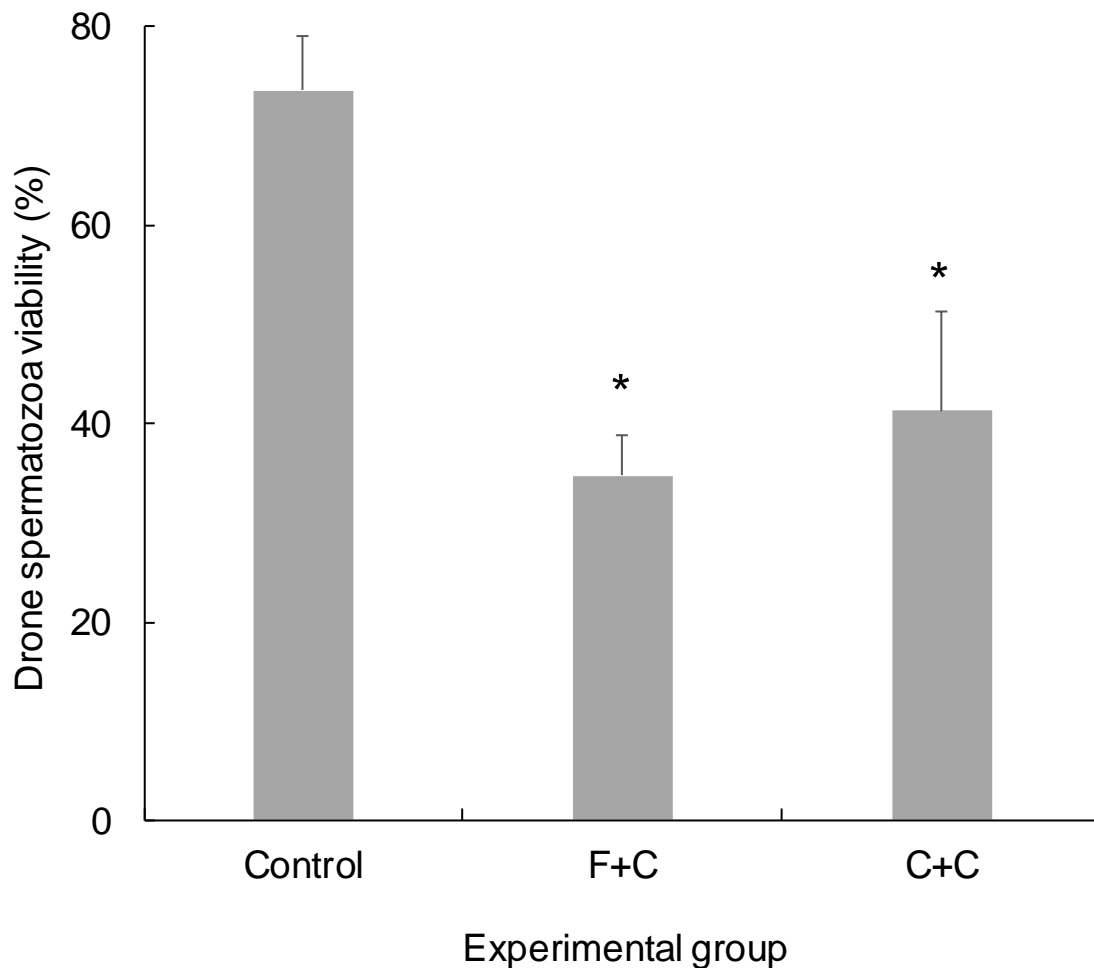


Figure A-2. Average spermatozoa viability observed in sexually mature honey bee drones reared on frames coated with pesticide-free wax (Control), wax contaminated with the miticides Fluvalinate and Coumaphos (F+C), or wax contaminated with the agrochemicals Chlorothalonil and Chlorpyrifos (C+C) in 2014. The “*” symbols represent values for which $P < 0.05$.

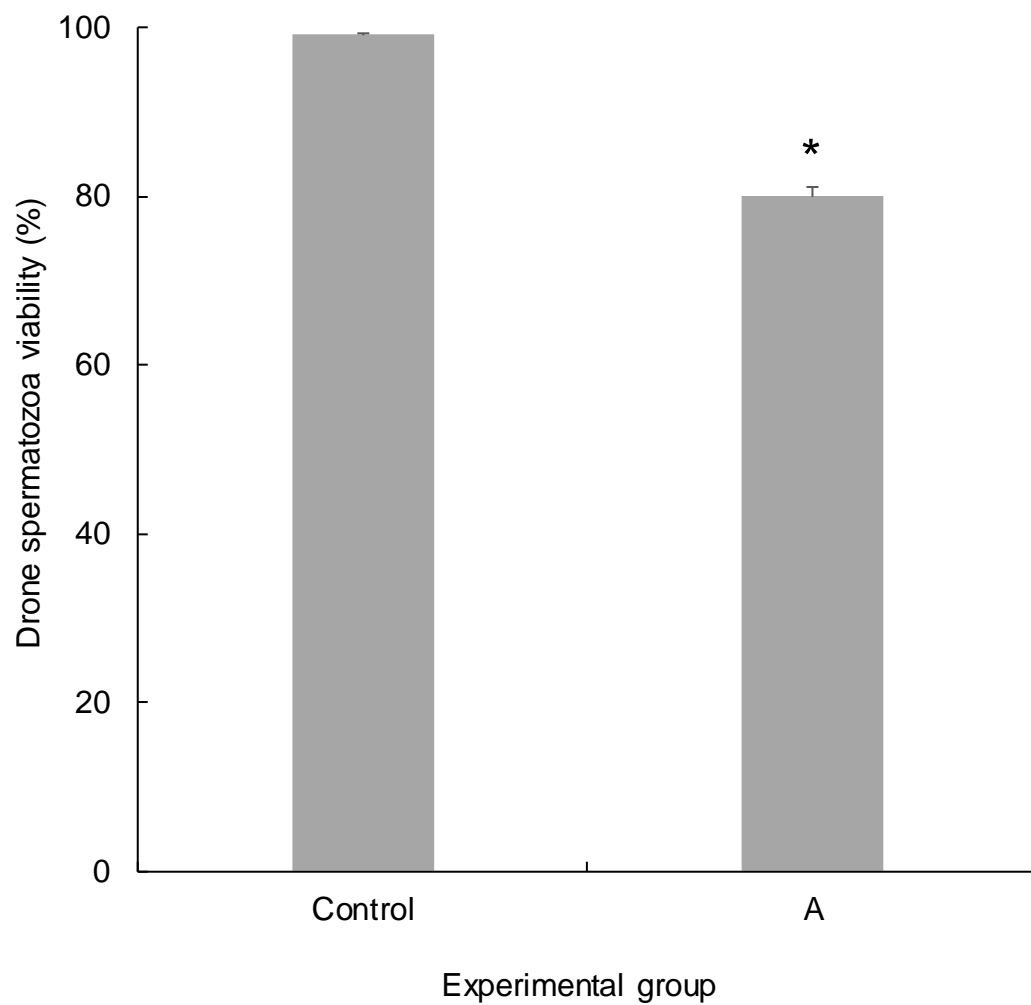


Figure A-3. Average spermatozoa viability observed in sexually mature honey bee drones reared on frames coated with pesticide-free wax (Control) or wax contaminated with the miticide Amitraz (A) in 2016. The “*” symbol represents a value of $P < 0.05$.

APPENDIX B

Table B-1. Top four fungicides used in California during the almond bloom in 2012^a

Fungicide	Chemical class	Gross number of pounds applied	Application rate (lbs/acre)	Number of acres planted	Number of acres treated	Percentage of acres treated
Azoxystrobin	Strobin	44,481	0.19	223,847	231,044	103
Boscalid	Anilide	62,143	0.21	337,528	327,122	97
Pyraclostrobin	Strobin	34,589	0.11	337,242	327,122	97
Iprodione	Dicarboximide	151,968	0.48	310,766	315,097	101

^aData obtained from the Pesticide Action Network Pesticides Database “*Pesticide Use on Almonds in 2012*”

Table B-2. Fungicide treatment groups devised to test the effects of Iprodione at various concentrations and iprodione in combination with azoxystrobin and BP to test the effects of fungicides on honey bee forager mortality

Experimental Treatments	Fungicides used (percentage of label dose rate)	Fungicide concentration (mL or g/L H ₂ O)
Control	N/A	N/A
1	1/4x Iprodione	5.5 mL
2	1/2x Iprodione	11 mL
3	1x Iprodione	22 mL
4	2x Iprodione	44 mL
5	1x Iprodione + 1x Azoxystrobin	22 mL + 125 mL
6	1x Iprodione + 1x BP	22 mL + 21.8 g

Table B-3. Fungicide treatment groups devised to test the effects of iprodione, azoxystrobin and BP, at twice the recommended label dose rate, on honey bee forager mortality

Experimental treatment	Fungicides used (label dose rate)	Fungicide concentration (mL or g/L H ₂ O)
Control	N/A	N/A
1	2x Azoxystrobin	250 mL
2	2x BP	43.6 g
3	2x Iprodione	44 mL

Table B-4. Fungicide treatment groups used in experiment 1 that resulted in a significant decrease in honey bee forager survival relative to the control group

Trial	Experimental month	Pairwise (t-test) treatment comparison	X^2 value	<i>P</i> value
1	September 2015	2x Iprodione vs. Control	11.05	<0.001
		1x Iprodione + 1x Azoxystrobin vs. Control	11.21	<0.001
		1x Iprodione + 1x BP vs. Control	6.14	0.01
2	October 2015	1/2x Iprodione vs. Control	6.26	0.01
		1x Iprodione vs. Control	4.19	0.04
		2x Iprodione vs. Control	18.11	<0.0001
		1x Iprodione + 1x Azoxystrobin vs. Control	9.15	0.003
		1x Iprodione + 1x BP vs. Control	15.9	<0.0001
3	November 2015	1/4x Iprodione vs. Control	72.5	<0.0001
		1/2x Iprodione vs. Control	43.6	<0.0001
		1x Iprodione vs. Control	65.9	<0.0001
		2x Iprodione vs. Control	266.4	<0.0001
		1x Iprodione + 1x Azoxystrobin vs. Control	126.2	<0.0001
		1x Iprodione + 1x BP vs. Control	172.3	<0.0001

Table B-5. Fungicide treatment groups used in experiment 2 that resulted in a significant decrease in honey bee forager survival relative to the control group

Pairwise treatment comparison	X^2 value	<i>P</i> value
2x Iprodione vs. Control	21.64	<0.001
2x BP vs. Control	27.31	<0.001
2x Azoxystrobin vs. Control	32.12	<0.001



Figure B-1. Experimental set up used to test the effects of fungicides on honey bee forager mortality. First, a) about 40-50 bees were loaded into clean bioassay cages. Then, b) cages were consecutively placed in a wind tunnel and exposed to either a fungicide-free control or fungicide-laden treatment in increasing concentrations as shown in Tables 2 and 3. Once treated, the caged bees were transferred into c) plastic holding units with feeders containing 50:50 sucrose solution ad libitum and placed in an incubator held at 34°C to measure worker mortality every 24 h for 10 days.

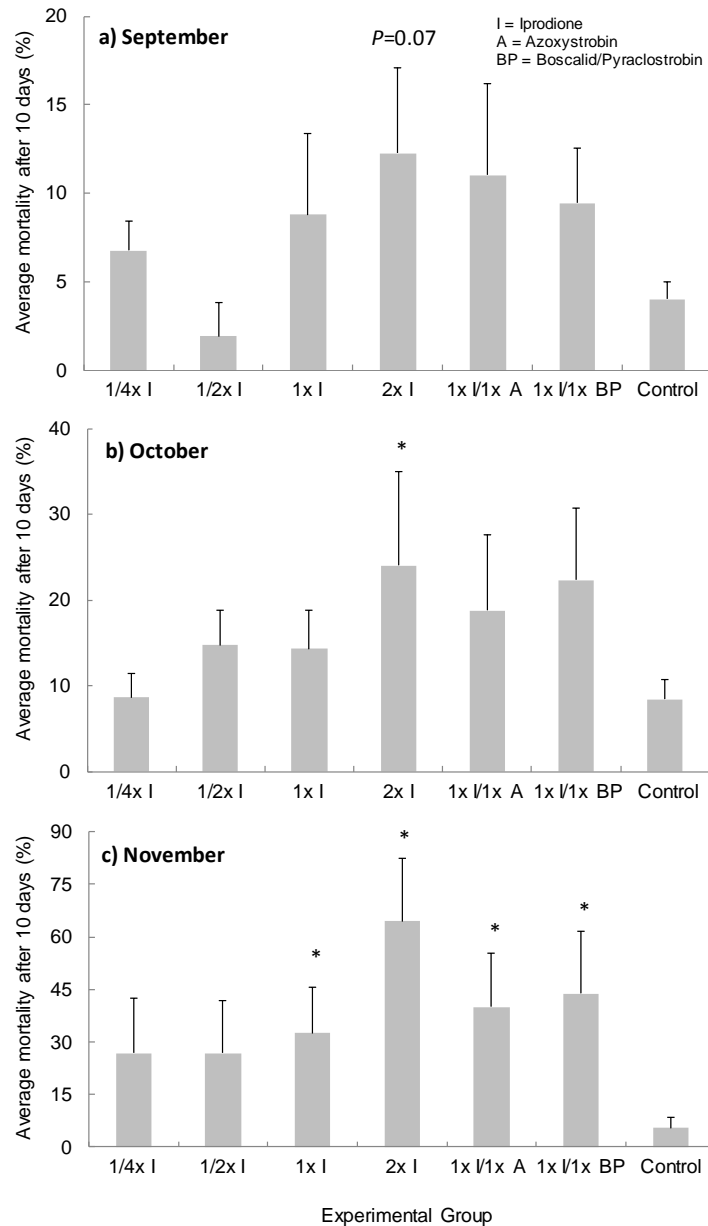


Figure B-2. Average honey bee forager mortality observed after 10 days during three trials in which bioassay cages containing 40-50 foragers were exposed in a wind tunnel to six fungicide treatment groups and an untreated control group. The trials were conducted in a) September 2015, b) October 2015, and c) November 2015. The treatments included iprodione at 1/4x, 1/2x, 1x, and 2x the label dose rate, as well as combinations of iprodione and azoxystrobin, and iprodione and BP, all at the label dose rate. “*” symbols represent values of $P < 0.05$.

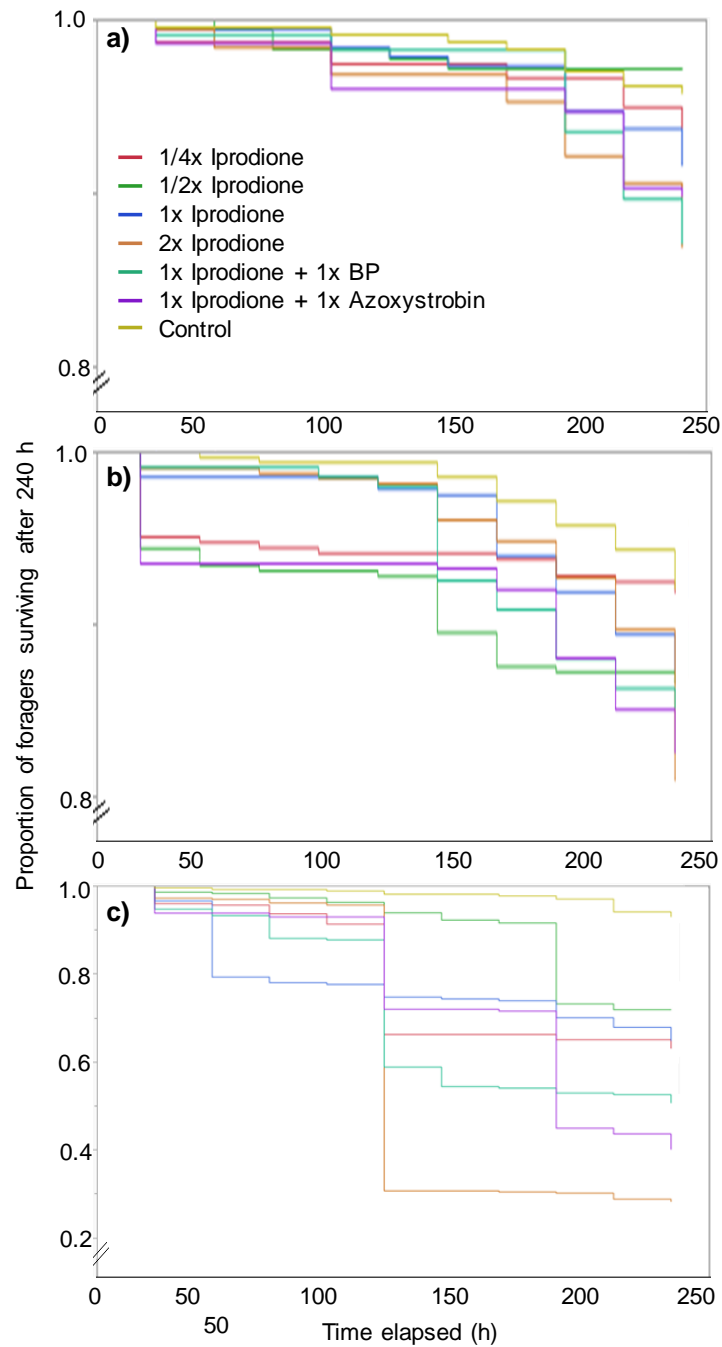


Figure B-3. Proportion of honey bee foragers contained in groups of 40-50 individuals that survived in an incubator held at a constant temperature of 34.5°C, 240 h after exposure in a wind tunnel to either one of six fungicide treatment groups or an untreated control group. Trials were conducted in a) September 2015, b) October 2015, and c) November 2015. See “Materials and Methods” for more details.

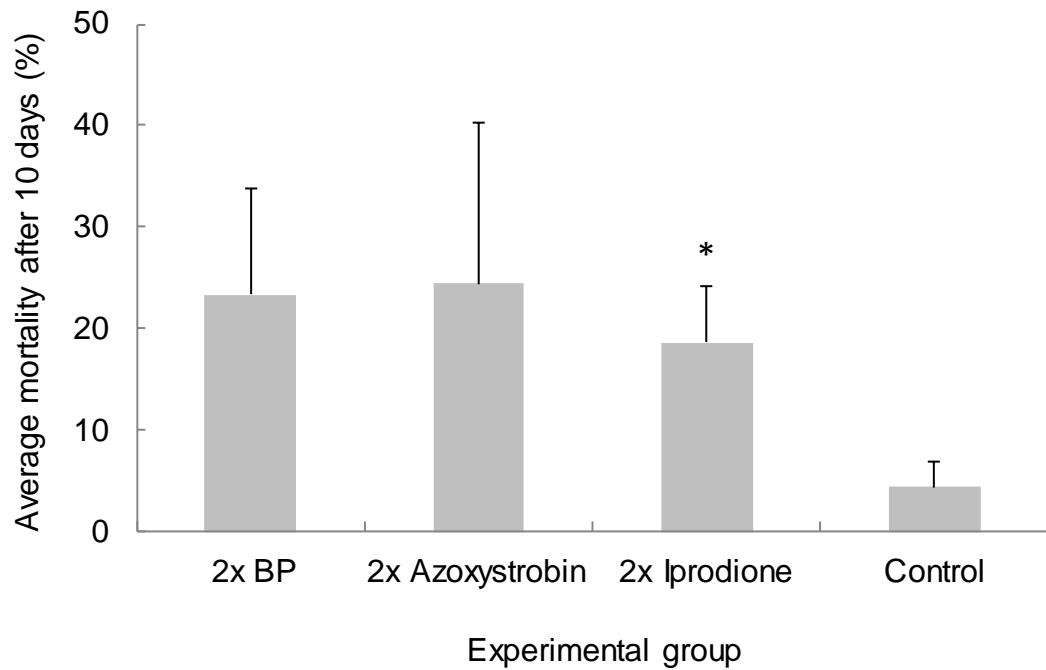


Figure B-4. Average honey bee forager mortality observed after 10 days during a trial conducted in December 2015 in which bioassay cages containing 40-50 foragers were exposed in a wind tunnel to three fungicide treatment groups and an untreated control group. The fungicide treatments included BP, azoxystrobin, and iprodione at twice the label dose rate. The “*” symbol represents a value of $P < 0.05$.

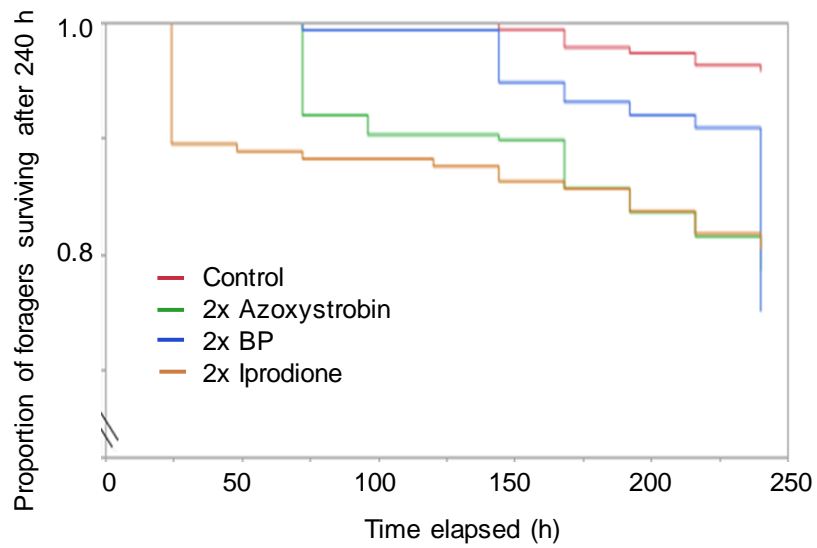


Figure B-5. Proportion of honey bee foragers contained in groups of 40-50 individuals that survived in an incubator held at a constant temperature of 34.5°C, 240 h after exposure in a wind tunnel to either azoxystrobin, BP, or iprodione at twice the label dose rate, or to an untreated control group.

APPENDIX C

Table C-1. Top insecticides used during the California almond bloom in 2014^a

Active ingredient	Main insecticide group	Mode of Action	Number of lbs applied	Number of agricultural applications	Number of acres treated
Methoxyfenozide	Insect growth regulator	Ecdysone receptor agonist	160,411.22	7,330	559,294.06
Pyriproxyfen	Insect growth regulator	Juvenile hormone mimic	4,529.84	2,285	240,012.20
Bifenazate	Acaricide	Electron transport inhibitor	48,673.22	1,071	73,446.86

^a Application and treatment data obtained from the California Department of Pesticide Regulation "Summary of Pesticide Use Report Data 2014"

Table C-2. Experimental treatment groups used to test the effects of the IGRs methoxyfenozide and

Experimental treatment group	Application dose relative to label dose rate	Methoxyfenozide concentration (mL/L H ₂ O)	Pyriproxyfen concentration (mL/L H ₂ O)	Bifenazate concentration (mL/L H ₂ O)
Control	0 (no pesticide applied)	0	0	0
1	1/2x label dose	0.37	0.53	0.32
2	1x label dose	0.75	1.06	0.64
3	2x label dose	1.5	2.12	1.28
4	3x label dose	2.25	3.18	1.92

Table C-3. Pesticide treatment groups that resulted in a significant decrease in forager survival relative to the untreated control group

Pesticide	Pairwise treatment comparison	χ^2 value	<i>P</i> -value
Methoxyfenozide (M)	1/2x M vs. Control	148.31	<0.0001
	1x M vs. Control	343.98	<0.0001
	2x M vs. Control	329.84	<0.0001
	3x M vs. Control	398.48	<0.0001
Pyriproxyfen (P)	1/2x P vs. Control	31.62	<0.0001
	1x P vs. Control	93.03	<0.0001
	2x P vs. Control	173.3	<0.0001
	3x P vs. Control	180.04	<0.0001
Bifenazate (B)	1/2x B vs. Control	69.54	<0.0001
	1x B vs. Control	174.17	<0.0001
	2x B vs. Control	192.28	<0.0001
	3x B vs. Control	246.41	<0.0001



Figure C-1. Experimental set up used to test the effects of pesticides on honey bee forager survival. First, a) about 30-40 bees were loaded into clean bioassay cages. Then, b) cages were placed in a wind tunnel and exposed to either a pesticide-free control or pesticide-laden treatment in increasing concentrations as shown in Tables 2, 3, and 4. Once treated, the caged bees were transferred into c) plastic holding units with feeders containing 50:50 sucrose solution ad libitum and placed in an incubator held at 34°C to measure worker mortality every 24 h for 10 days.

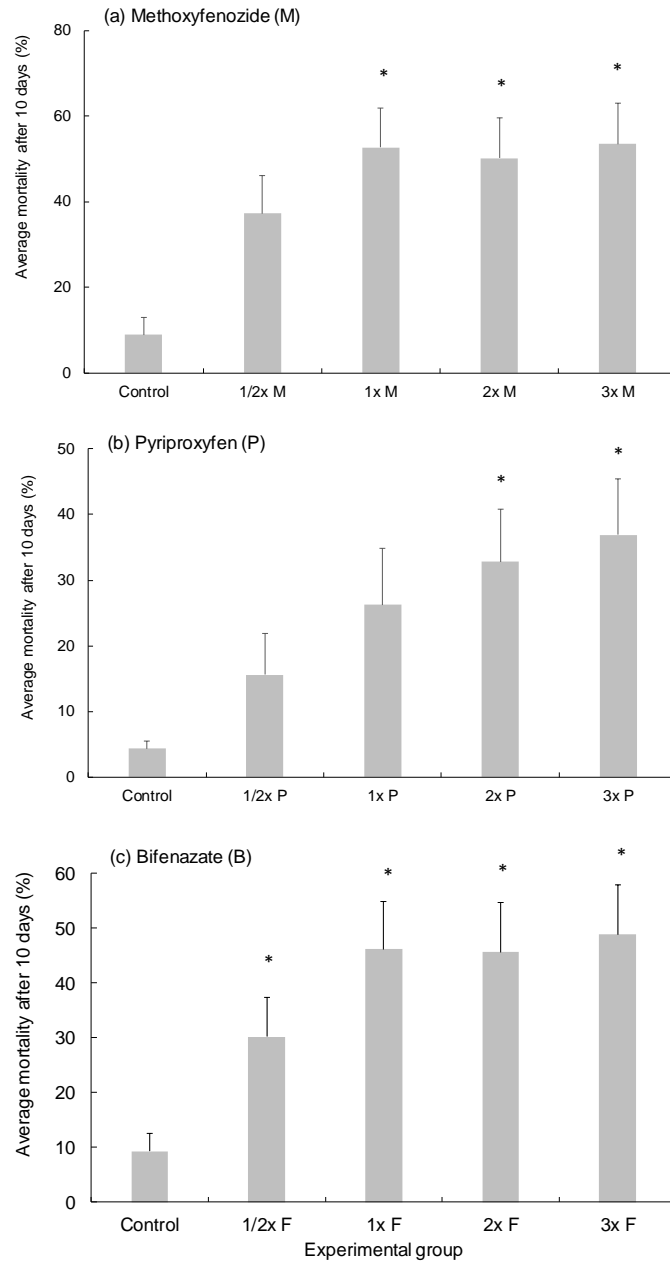


Figure C-2. Average honey bee forager mortality observed after 10 days for three pesticides in which bioassay cages containing 30-54 foragers were exposed in a wind tunnel to sets of four treatment groups and an untreated control group for each pesticide. The pesticides tested included a) methoxyfenozide, b) pyriproxyfen and c) bifenazate. The treatments included 1/2x, 1x, 2x, and 3x the label dose rate for each pesticide. “*” symbols represent values of $P < 0.05$.

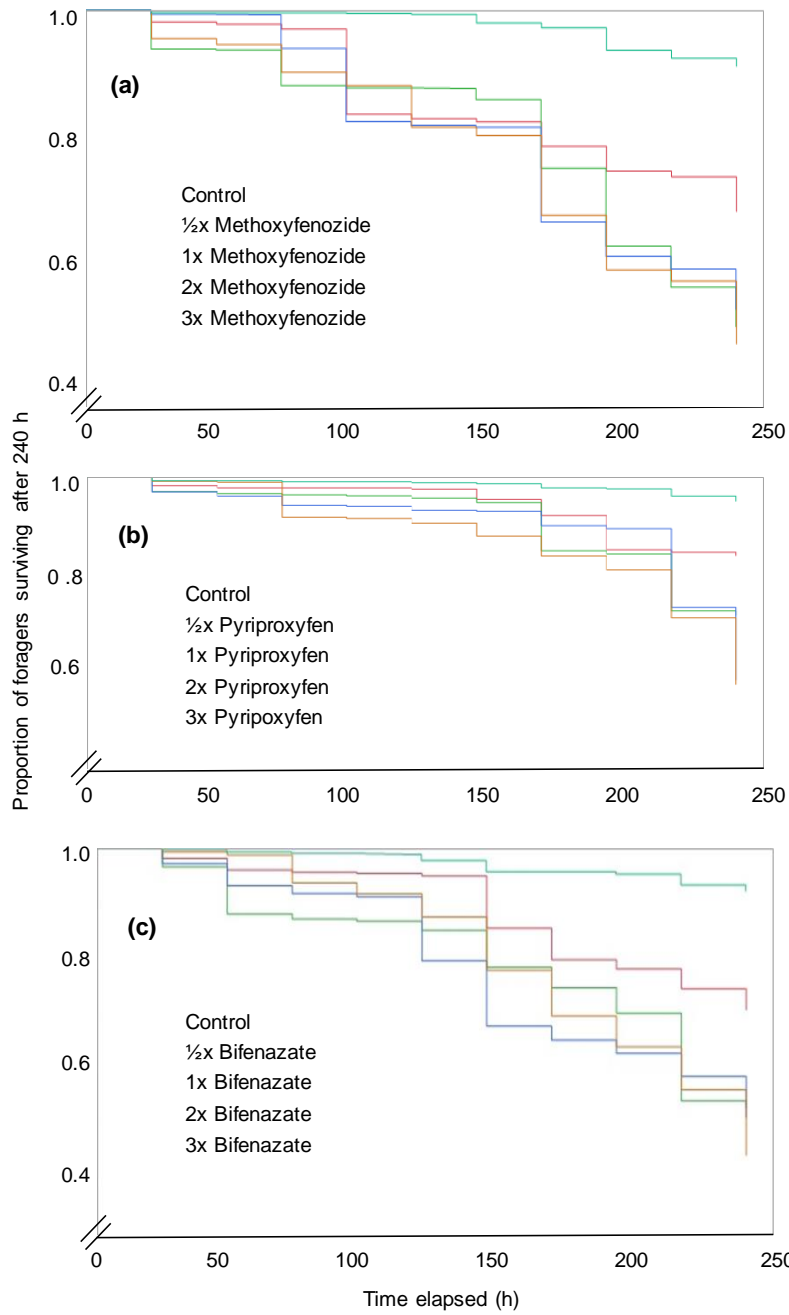


Figure C-3. Proportion of honey bee foragers contained in groups of 30-40 individuals that survived in an incubator held at a constant temperature of 34°C, 24 h after exposure in a wind tunnel to either one of four pesticide treatment groups or to an untreated control group. The pesticides tested were (a) methoxyfenozide, (b) pyriproxyfen and (c) bifentazate. See "Materials and Methods" for more details.

APPENDIX D

Table D-1. Concentration variants of I1 used to assess honey bee forager mortality following exposure to the insecticide

I1 concentration used ($\mu\text{g/mL}$)	% label dose rate
846.66	1.6x
423.33	0.8x
211.66	0.4x
105.83	0.2x
0	Control

Table D-2. Concentration variants of I2 used to assess honey bee forager mortality following exposure to the insecticide

I2 concentration used ($\mu\text{g/mL}$)	% label dose rate
2802.47	3.3x
1401.24	1.6x
700.62	0.8x
420.37	0.5x
0	Control

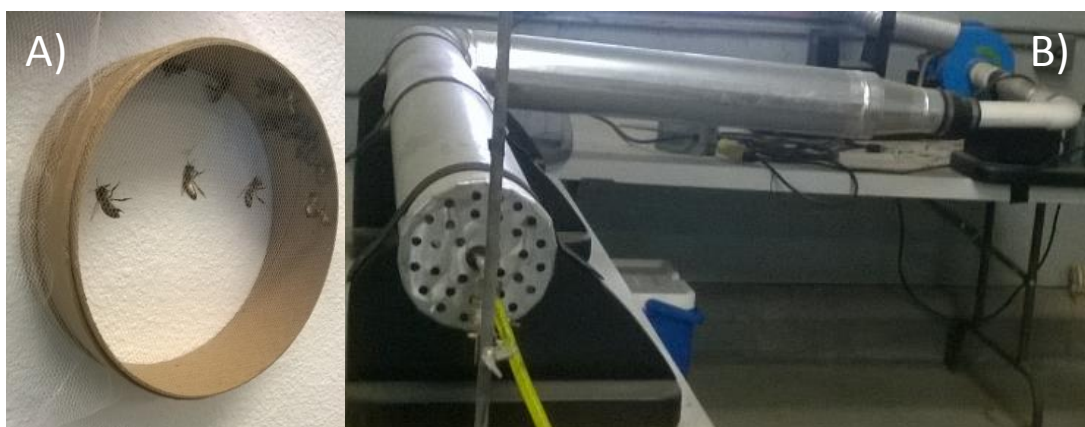


Figure D-1. A) Bioassay cages were loaded with 20-30 honey bee foragers before being inserted into B) a wind tunnel (wind speed: 1.8m/s) where foragers housed in a bioassay cage were sprayed with 0.5 mL of insecticide solution. See Materials and Methods for details.

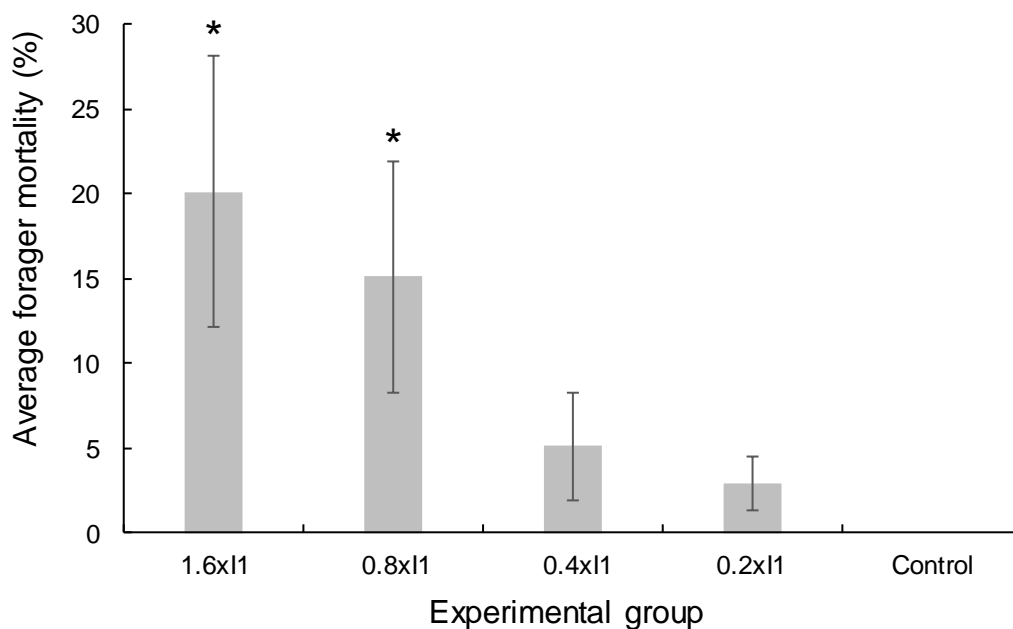


Figure D-2. Overall average honey bee forager mortality resulting from exposure to label dose variants of I1 and to an untreated control group. Data are presented as the mean \pm SEM for six trials with three replicates per experimental group. The “*” symbols represent values for which $P < 0.05$.

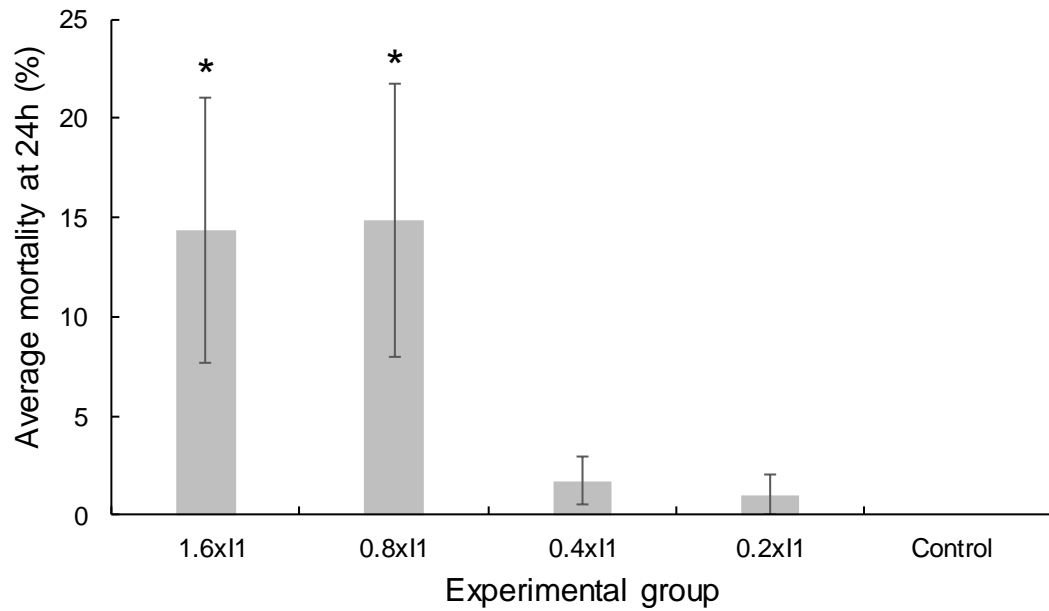


Figure D-3. Average honey bee forager 24 h after exposure to label dose variants of I1 and to an untreated control group. Data are presented as the mean \pm SEM for six trials with three replicates per experimental group. The “*” symbols represent values for which $P < 0.05$.

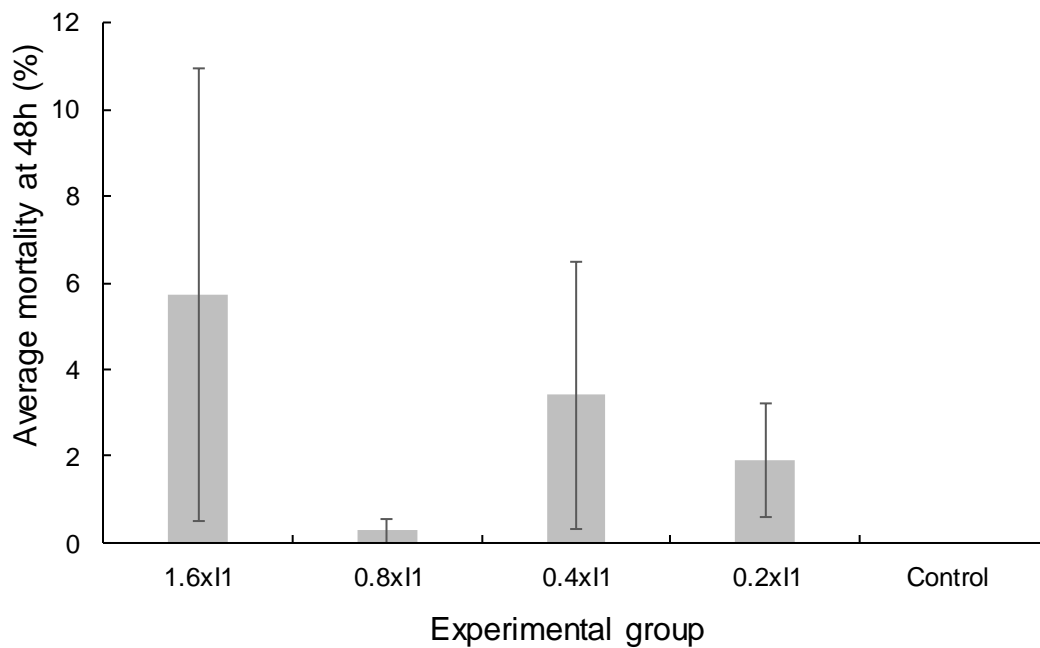


Figure D-4. Average forager 48 h after exposure to label dose variants of I1 and to an untreated control group. Data are presented as the mean \pm SEM for six trials with three replicates per experimental group.

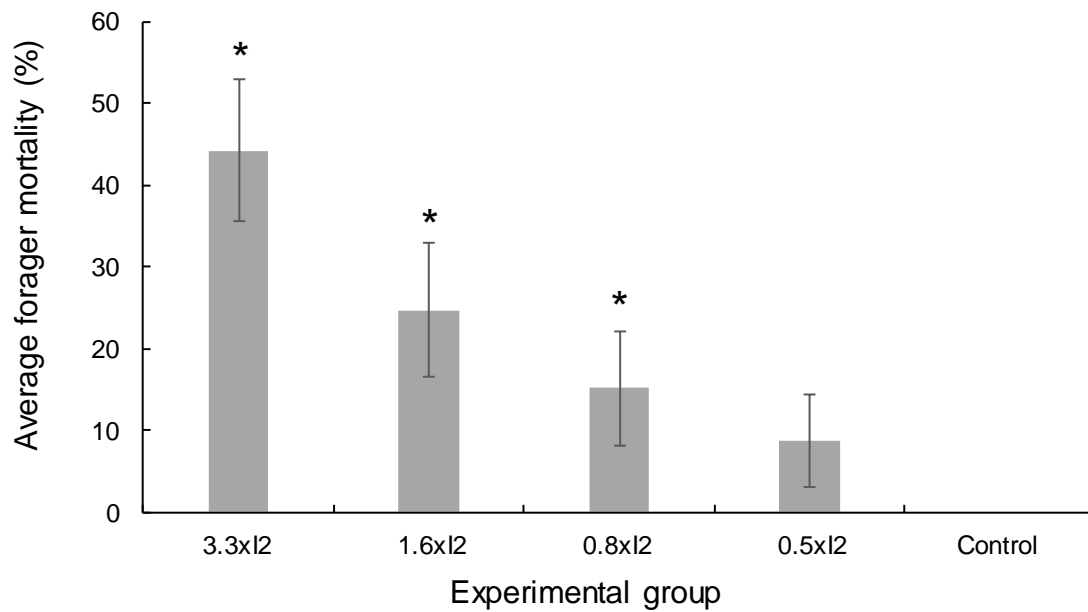


Figure D-5. Overall average honey bee forager mortality resulting from exposure to label dose variants of I2 and to an untreated control group. Data are presented as the mean \pm SEM for six trials with three replicates per experimental group. The “*” symbols represent values for which $P < 0.05$.

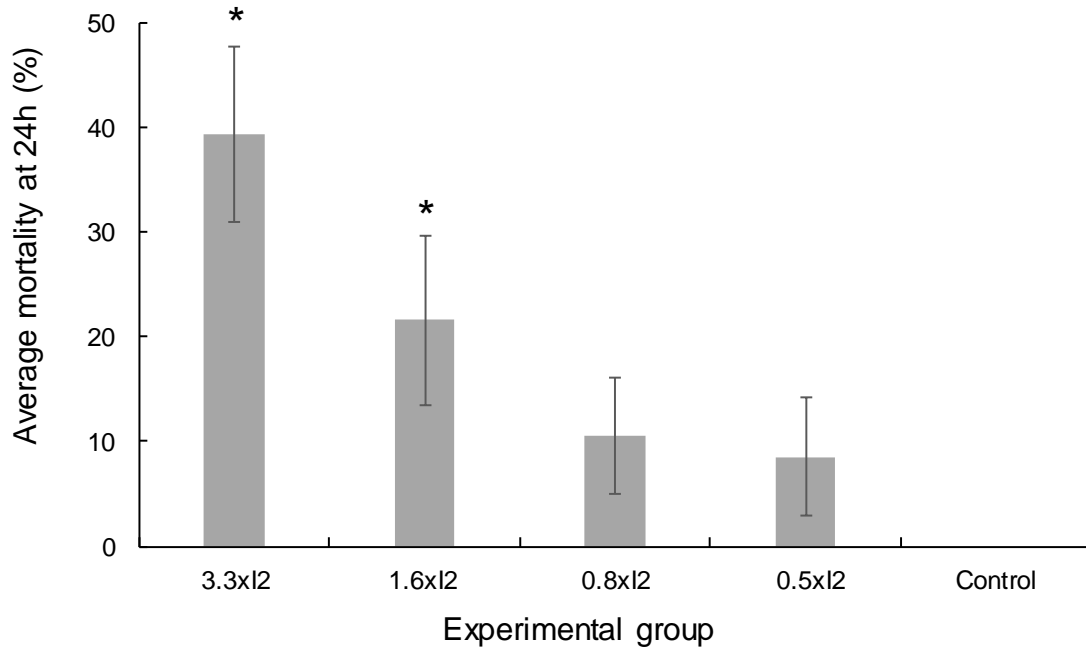


Figure D-6. Average honey bee forager 24 h after exposure to label dose variants of I2 and to an untreated control group. Data are presented as the mean \pm SEM for six trials with three replicates per experimental group. The “*” symbols represent values for which $P < 0.05$.

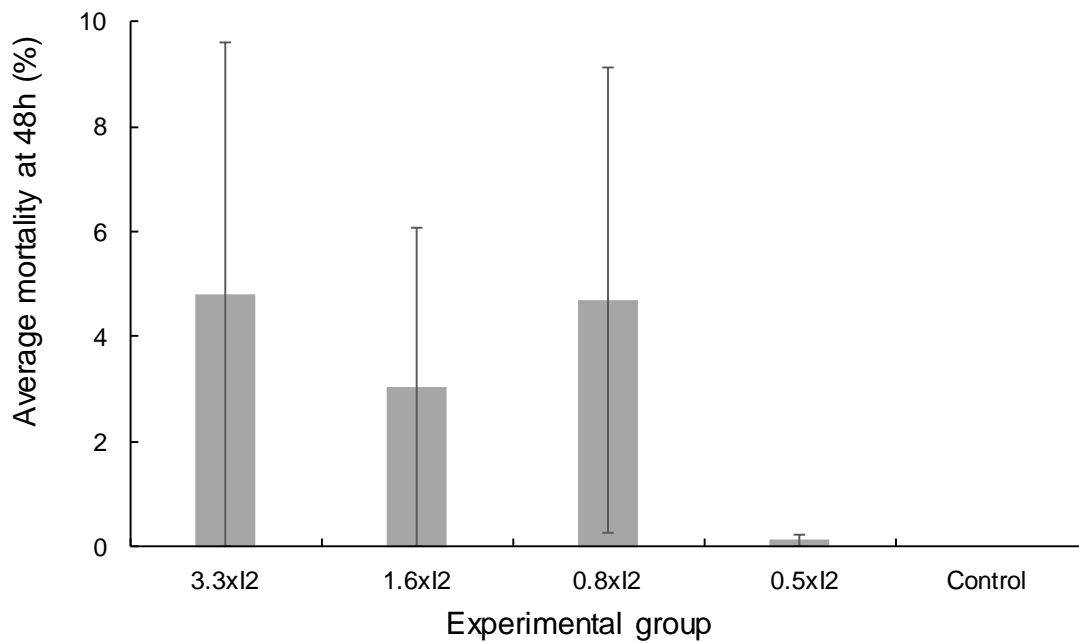


Figure D-7. Average forager 48 h after exposure to label dose variants of I2 and to an untreated control group. Data are presented as the mean \pm SEM for six trials with three replicates per experimental group.