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Title: Effects of Pesticide Exposure on Honey Bee Health: Impacts of Pesticide Interactions and Exposure Through Multiple Routes

Abstract approved:

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Honey bees (*Apis mellifera*) are responsible for approximately $17 billion in crop production per year in the United States, and are arguably the most important pollinators in the nation. The future of crop pollination and production is threatened by widespread national honey bee colony losses, which have averaged approximately 30% per year over the past decade. Many factors contribute to colony mortality, but the particular impacts of pesticides are still poorly understood. Here, we investigated the impacts of pesticides under conditions that have not been examined in previous research. Our research focused on the effects of an interaction between the neonicotinoid imidacloprid and the fungicide chlorothalonil and effects of exposure through multiple routes.

To understand the potential impacts of pesticide interactions, we exposed whole colonies to imidacloprid, chlorothalonil, or combination of both chemicals through a pollen diet for one month. We found that many of our response variables were unaffected by our treatments, and that outliers influenced the outcome of several analyses. Brood area and prophenoloxidase activity were significantly affected by different treatments when outliers were excluded, although these differences were no longer significant after the multiple comparisons confidence interval adjustment. Similarly, the number of non-pollen foragers returning to the colonies was affected by the interaction between imidacloprid and time, chlorothalonil and time, and both chemicals and time, when outliers were removed. The interactions indicated that seven weeks after the end of the exposure period, both imidacloprid and chlorothalonil reduced the number of non-pollen foragers returning to the colonies. Imidacloprid and chlorothalonil also reduced the number of total foragers returning to the colonies overall. Our results indicate that colonies may be affected by pesticide exposure long after the

exposure period, and that bees exposed to pesticides early in life may be detrimentally affected by that exposure at later stages.

To determine whether pesticide exposure through multiple routes has a greater effect on bees than single-route exposure, we conducted a laboratory experiment in which we exposed bees to imidacloprid through pollen diet, sugar syrup, or both routes. We found that exposure through sugar syrup increased the midgut proteolytic enzyme activity overall, as well as glucose oxidase activity after four weeks of exposure. Exposure through sugar syrup, as well as exposure through both routes, increased glucose oxidase activity when outliers were included and excluded from the analysis, respectively. Mortality differed significantly between bees exposed to imidacloprid through sugar syrup and those exposed through both matrices, but none of the treatments were significantly different from the control group. We also found that bees in different treatment groups consumed different amounts of sugar syrup and pollen. Our results indicate the importance of conducting laboratory experiments that better reflect field-realistic pesticide exposure by both incorporating effects over a longer period of exposure, and exposure through multiple routes.

In summary, our results provide new knowledge and insights on how pesticides impact long-term colony health. Future research must thoroughly examine statistical procedures, outliers, and statistical power, and must also determine interactions between pesticides and pathogens under different conditions, such as different types of pesticide application, honey bee subspecies, nutritional conditions, season, etc. Discerning the variability in results when these conditions vary will provide a fuller understanding of the true impacts of pesticides on colony health.

Effects of Pesticide Exposure on Honey Bee Health: Impacts of Pesticide Interactions and Exposure Through Multiple Routes

by

Stephanie Parreira

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Stephanie Parreira, Author

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Chapter 1: Introduction

1.1. Insect Pollination and Pollinator Declines

Insect pollinators are crucial to both biodiversity and food production [1]. Globally, 35% of crops depend on animal-mediated pollination, particularly by insects [2]. Across the globe, insect pollinators are declining in both diversity [3-5] and abundance [4-6]. Currently, 40% of pollinators worldwide are expected to go extinct over the next several decades [7], which is a major threat to both the ecosystem and human health. The total elimination of insect pollinators would lead to vast shortages of fruits, nuts, and vegetables [8], an additional 71 million malnourished people in low-income countries [9], and 1.42 million deaths per year from non-communicable and malnutrition-related diseases [9]. Although modern agricultural systems are not currently facing pollination shortages [10-11], they are becoming increasingly pollinator-dependent [10, 12], which is reducing the stability of agricultural yields [13] and hence increasing rates of malnutrition and micronutrient deficiency in the human population [14].

1.1.1 Honey Bee (*Apis mellifera*) Pollination

The European honey bee (*Apis mellifera*) is currently the most widely used bee for pollination of agricultural crops that need pollination. Honey bee pollination increases yields [2], crop marketability [15-17], and fruit set in both food crops and native plants [15, 17-18]. According to Klein et al. [2], 12 out of the 84 pollinator-dependent crops examined relied solely on honey bee pollination, and only nine crops did not at all depend on honey bee pollination. Without sufficient honey bee pollination, yield may also decline as much as 90% in some crops [19]. Though native bees are sometimes capable of providing adequate pollination without honey bees [20-24], and increase the efficiency of honey bee pollination [25-26], it is also true that many wild bee species are in decline and some are headed towards extinction [27-28], and are often more sensitive to various environmental stressors than honey bees [2, 4, 29-33]

Honey bees are also more efficient pollinators than native bees in some cases [2, 34] and must be present in order to maximize yields even when native pollinators are abundant [17]. While researchers often argue that pollination by native bees is a solution to honey bee colony losses [22], a healthy honey bee population is critical to sustain and maximize crop pollination and production.

1.1.2. Honey Bee Colony Losses: 2006-Present

The honey bee colony loss rates have been unsustainably high in Europe and North America for nearly a decade, averaging 30% or higher per year [35-43]. In the 2013-2014 season alone, 66% of all American beekeepers surveyed by the Bee Informed Partnership reported colony losses that were higher than they deemed acceptable [43]. Although the overall number of colonies in the world has increased since 1961 [44], the number of managed honey bee colonies in the United States has declined from nearly 6 million in 1947 to 2.5 million in 2008 [11]. Simultaneously, the demand for pollination has grown far faster than the number of colonies can support [44]. Prolonged high rates of colony loss can potentially threaten the sustainability of food production in these regions if not addressed in a timely manner.

1.2. Factors Contributing to Colony Losses

Many stressors are believed to be contributing to honey bee colony mortality, including the *Varroa destructor* mite [45-46], a gut parasite called *Nosema* [47-50], viruses [51-53], poor nutrition [54] and pesticides [55-58]. Researchers generally agree that there is no single cause of colony losses and decline, but rather, various stressors interact with each other in complex ways to weaken and ultimately kill honey bee colonies [59-61]. Despite the fact that there is widespread consensus that interactions between above mentioned factors are responsible for the significant colony losses, very few studies have actually quantified interactions between two or more stressors, especially under field conditions).

1.2.1. The Role of Pesticides

Pesticides may play a key role in both honey bee colony losses and the decline of native pollinators [62-66]. Though a relatively low percentage of U.S beekeepers perceive pesticides to be a key cause of their colony losses [39, 41, 43, 67], pesticides remain one of the top nine self-reported causes of colony mortality [67-69]. Commercial beekeepers, who provide the vast majority of honey bee pollination, cite pesticides as the cause of their colony losses more often than backyard or sideline beekeepers [42, 68]. Furthermore, beekeepers who report pesticides as the main cause of their losses more often also lose more colonies, on average, than those who report losses due to other factors [41-42].

Honey bees may be more sensitive to pesticide exposure because they have fewer detoxification genes than other insects [70]. While researchers are gaining a better understanding of how pesticides affect bee colonies, the overall impacts of pesticide exposure in the field are still not well understood [65, 71-72].

1.2.2. Interactions Between Pesticides, Pests, and Pathogens

Because pesticides are likely not the sole cause of honey bee colony losses, it is more likely that they act on honey bee colonies in subtle, indirect ways. The suggestion that exposure to pesticides increases honey bee susceptibility to pathogens is gaining traction in the scientific community. Because failing colonies have higher loads of viruses [37, 51-52, 60, 73-75], *Varroa* mites [60, 76], and *Nosema* [37, 60, 77], determining the mechanisms by which pesticide exposure makes bees more susceptible to these pathogens may be the key to understanding the contribution of pesticides to colony losses. In several studies, exposure of honey bee colonies to various types of pesticides has been linked to higher levels of pathogens [78-82], but the mechanisms by which pesticide exposure drives higher pathogen loads is still relatively unknown [72, 83]. Understanding the links between pesticide exposure,

immune physiology, pathogen loads, and mortality may be critical for understanding how pesticide exposure weakens or kills colonies.

1.2.2.1. Individual Immunity

Individual immunity in insects may include barrier defense by the insect cuticle [84-86], antimicrobial peptide activity [87-92], phagocytosis and encapsulation by hemocytes [84], and melanization by the phenoloxidase cascade [84, 90]. The phenoloxidase cascade is a well-known immune response to wounding [90, 93], bacteria [94-95], fungal pathogens [95], and potentially viruses [96], although the antiviral response in honey bees and other insects is still poorly understood [84]. A previous study found that parasitization by the *Varroa destructor* mite led to higher phenoloxidase activity in both worker and drone prepupae, indicating that the phenoloxidase cascade is also an immune response to ectoparasites [97]. Response to wounding is also a key adaptive process in mite resistance [98]. The activation of the phenoloxidase cascade is also closely associated with other cellular immune defenses, such as phagocytosis [99]. The phenoloxidase cascade may be a particularly important immune response as bees age [100-104]. Phenoloxidase activity tends to reach a plateau in adult worker bees at 8 days of age, and remains about the same throughout the rest of their lifetime [101].

Prophenoloxidase, the precursor to phenoloxidase, indicates the potential phenoloxidase activity of an individual insect. When a pathogen is recognized by the peptidoglycan recognition protein, prophenoloxidase is activated into phenoloxidase by serine proteases [84, 95, 99]. During melanization, prophenoloxidase is activated into phenoloxidase, which converts phenols into quinones [84, 95, 99]. Quinones subsequently polymerize into melanin, which forms a sealing capsule around the pathogen [84, 95]—a process otherwise known as encapsulation.

1.2.2.2. Social Immunity

Social immunity is a form of immunity that depends upon colony cooperation and social biology [105]. Social immune pathways may be a critical mechanism by which honey bees respond to pathogens (Richard et al.), since honey bees only have roughly one third of the genes encoding for individual immunity when compared to other insects such as *Drosophila* and *Anopheles* [89]. Social immunity in honey bees includes behaviors such as grooming, undertaking, behavioral fever, and sealing the colony with propolis [86, 89, 106-107], but may also be achieved through physiological mechanisms, such as the secretion of the antimicrobial peptide *defensin 1* [88, 108] and the enzyme glucose oxidase into brood food and honey [109-112]. Glucose oxidase catalyzes the reaction that converts glucose into gluconic acid and hydrogen peroxide [113-114]. The amount of glucose oxidase in honey is positively correlated with both the amount of hydrogen peroxide content and antimicrobial activity in honey [115]. Glucose oxidase activity and hydrogen peroxide inhibit microbial pathogens [113, 116-118], including American foulbrood (*Paenibacillus larvae*) [119] and *Nosema ceranae* [120]. Further, nurse bees infected with the gut pathogen *Nosema ceranae* prefer to consume honey with higher antimicrobial activity [120]. Glucose oxidase activity therefore may be a critical form of disease resistance in honey bee colonies, and adverse impacts of pesticide exposure on this form of social immunity may mean long-term susceptibility to pathogens and eventually colony loss.

1.2.2.3. Nutritional Physiology

The nutritional state of the colony and individual bees has a direct impact on the ability to cope with pathogens [121-122]. Several physiological parameters measure the quality of nutrition absorbed by the colony: hypopharyngeal gland protein content, midgut proteolytic enzyme activity, and abdominal lipid stores.

Nurse bee hypopharyngeal gland protein content is a good indicator of the quality of larval diet [123] [124-125]. Higher protein content in the larval diet is positively associated with the survival of adult bees [122, 126] and entire colonies [127]. The protein content of brood food secreted by the hypopharyngeal glands has long-term implications on the survival, longevity, and expression of antioxidant genes in worker bees [128]. Colonies also have more viruses when they are deprived of a quality protein and pollen diet [121], though whether the same applies to the protein content of the brood food has not yet been investigated.

Midgut proteolytic enzyme activity is a measurement of protein digestion [129]. Proteolytic enzyme activity tends to be highest when worker bees are nursing brood [129-130]. The proteolytic activity of the nurse bee midgut is also associated with worker bee longevity and survival [122, 126, 131-132], and positively associated with hypopharyngeal gland protein content [122, 126, 129].

Higher midgut proteolytic enzyme activity and hypopharyngeal gland protein content are directly correlated with higher survival of both individual bees and entire colonies [126-127, 131], including in the event of an immune challenge from *Nosema ceranae* [122]. Demonstrated negative impacts of pesticide exposure on these parameters would provide a clear link between pesticide exposure, nutritional stress, and colony losses.

Abdominal lipid stores are an indicator of energy reserves [133], and result in higher bee survival under nutritional stress [134]. Abdominal lipid stores are also highest at the nursing stage [135], and drop during the transition from nursing to foraging [136-137]. Bees that lose their abdominal lipid stores early engage in precocious foraging [138], which reduces the lifespan of worker bees [139-140] and is a significant predictor of colony failure [141-143]. The abdominal lipid stores (also known as the fat body) also produce vitellogenin [144], which increases worker resistance to oxidative stress [145], that is caused by exposure to some pesticides [146].

Vitellogenin also regulates immune function and longevity [147], as well as the transition from nursing to foraging [148-149].

The fat body also plays a role in individual immunity. The fat body is the site of antimicrobial peptide expression [108, 150-151]. These peptides serve as an immune defense against bacterial pathogens for both individuals and whole colonies [87-92, 94]. Therefore, understanding how pesticide exposure affects the lipid stores of worker bees is also necessary to gain a comprehensive understanding of the involvement of pesticide exposure in colony pathogen infection and mortality.

1.2.3. Neonicotinoids

Neonicotinoids are a controversial group of insecticides that have come under public scrutiny for their potential role in honey bee colony losses [152-153]. These chemicals are neurotoxins with structures similar to nicotine [154-155], and are agonists of the nicotinic acetylcholine receptors (nAChRs) in insects. Contact or oral exposure to lethal concentrations of these insecticides therefore results in unlimited action potentials in the insect neurons, paralysis, and eventually the death of the insect [155-156]. Unlike previous classes of insecticides (e.g. carbamates and organophosphates), neonicotinoids have targeted specificity towards insect receptors and are relatively non-toxic to vertebrates [154-155, 157-158]. Their lower risk to vertebrates and humans has encouraged the proliferation of their use worldwide [159]. Neonicotinoids now account for 1/3 of the global insecticide market [160], and are registered for multiple uses in 120 different countries [161] to control both sucking and chewing pests [159] [162-164]. However, because of the public perception that neonicotinoids are responsible for honey bee colony losses, the European Union issued a two-year moratorium on neonicotinoid use [165], and in the United States, beekeepers and activists have filed a lawsuit against the Environmental Protection Agency for failure to properly investigate these chemicals prior to registration [153].

1.2.3.1. Systemic Properties, Persistence and Mobility of Neonicotinoids

Neonicotinoids are water soluble and systemic [161], allowing them to move through the tissues of treated plants and translocate into pollen and nectar [166-173]. While many researchers argue that very low concentrations of neonicotinoids are present in nectar and pollen of plants [82, 173-174], and that neonicotinoids are not detected frequently in corbicular pollen (pollen collected directly from the legs of foraging bees) [80, 175] and bee bread [57] (a form of stored pollen that also includes honey [176-177]), many studies have found that neonicotinoids are frequently present in pollen [172, 178-180], and their hazard quotients (a common measure of risk, defined by the concentration in pollen divided by the LD_{50} of the chemical) tend to be higher than those of other agrochemicals [179]. Even though most neonicotinoid usage is in the form of seed treatment [160], these chemicals are also used in foliar applications [161, 164, 166], soil and irrigation applications [166, 171, 181-183], and basal trunk injections [170, 182, 184-186]. Concentrations of neonicotinoids in pollen and nectar when neonicotinoids are applied through soil and trunk injections are demonstrably higher than residues that result from seed treatment [166, 171-173, 181, 187].

Neonicotinoids may also pose a continuous threat to honey bees and other pollinators because of their persistence and mobility into untreated environments. Neonicotinoids may persist in treated plants, soil, and water, with half-lives as long as 1000 days [181, 188]. High concentrations of neonicotinoids may remain in the tissue of treated trees for longer than 4 years after a single application [186], and further accumulate in plant tissues after multiple applications [174, 189]. The water-soluble and persistent characteristics of neonicotinoids also make them highly mobile in the environment [190-191], and for this reason they are frequently detected in waterways [188, 192-195], soil [175, 188] and wildflowers that serve as alternative forage for bees [175, 196-198]. Botias et al. [198] found that 97% of the neonicotinoids bought by honey bee foragers to their colonies were from untreated wildflowers, and not from treated crops. While researchers argue that access to untreated alternative forage dilutes the potential effects of neonicotinoid exposure [199], such an assumption is less and less

likely to be true as more neonicotinoids are routinely applied to crops, forests [170, 182-184, 200] and residential gardens [201-202]. Despite the potential risks of these properties, neonicotinoid residues in the environment, pollen, nectar, and hive matrices are not regularly monitored [194, 203].

1.2.3.2. Potential Role of Neonicotinoids in Colony Declines

Neonicotinoid use in the United States increased rapidly prior to the onset of widespread and unsustainable colony losses [204]. While researchers have consistently dismissed the role of neonicotinoids as major players in honey bee colony losses [203, 205-208], there are still many gaps in our knowledge of how these chemicals affect honey bee colonies.

Although neonicotinoids have been studied for years, the current research has a limited scope [203, 209]. The vast majority of neonicotinoid research has been conducted with respect to seed treatments, particularly in corn, canola and sunflower [81, 173, 210-215]. Studies have virtually ignored potential impacts in other crops and other types of neonicotinoid applications, when concentrations of these chemicals in pollen and nectar are higher. The current available research on the impacts of neonicotinoids has also been criticized for its failure to capture the complex ways by which neonicotinoids may impact honey bee colonies, such as through cumulative exposure or by interactions with other pesticides [216].

1.2.3.3. Imidacloprid

Imidacloprid is one of the most widely used pesticides in the world, second only to glyphosate in global pesticide use [160]. It currently constitutes 41.5% of the neonicotinoid market and is registered for use in 140 different crops [161]. Imidacloprid is also widely used on various bee-pollinated crops, including pome fruits, stone fruits [217], cucurbits [171], citrus [163, 218] [189], and berries [219-220].

The role of imidacloprid in honey bee colony losses is highly contested. Its use in the seed treatment of canola is directly linked to higher colony losses in England and Wales [215], but scientists claim that field experiments in the United States do not directly link this chemical to colony mortality [82, 173]. Mullin et al. [57] found that imidacloprid was only present in 2% of bee bread samples in North American colonies, casting doubt on whether it poses a serious risk to honey bee colonies, but in another study it was the 4th most frequently detected pesticide in dead honey bees in citrus and stone fruit orchards, at concentrations as high as 223 ppb [58]. It is also detected far more frequently in corbicular pollen samples [179-180, 198] and at concentrations that are more hazardous to bees than concentrations of all other detected agrochemicals, except phosmet [179]. Workers consume imidacloprid-contaminated food more quickly than uncontaminated food [221], which may explain discrepancies between detected residues in corbicular pollen, dead bees, and bee bread. Current sampling methods may not be able to capture realistic residues of imidacloprid in bee bread due to relatively quicker consumption of the contaminated bee bread by bees in a colony.

Sublethal, field-realistic exposure to this chemical has been linked to higher infections of *Nosema* [79], higher *Varroa* mite infestations of honey bee colonies [82], altered ability to carry foraging tasks [222-224], decreased immunity [225], and altered gene expression [226-228], but not colony mortality [82, 187, 229]. While imidacloprid is the most well-studied of all the neonicotinoids [214], making up 78% of the neonicotinoid research on honey bees [214] and 66% of the neonicotinoid toxicity studies of aquatic invertebrates [230], much is still unknown about how it might interact with other pesticides and increase colony susceptibility to pathogens.

1.2.4. Fungicides

Though neonicotinoids currently receive the most attention in research on honey bee toxicology [214], the impacts of fungicides are also becoming a greater concern.

Fungicides generally have low acute lethal toxicity to honey bees [231-234] and have thus far been considered as safe chemicals. For this reason, they are applied to crops during the bloom while bees are foraging [232], and are exposed to bees through both contact and oral routes. Fungicides make up the majority of the pesticide load in pollen [57, 80] and are detected more frequently in pollen than systemic insecticides [80, 235-236]. Though there are currently not enough data on record to determine whether fungicides play a critical role in colony losses [205], high fungicide loads have been linked to lower isolates of beneficial fungi in pollen [237], increased honey bee susceptibility to *Nosema* [80], and higher risk of colony disorders such as queen loss [236]. Some fungicides are also highly toxic to honey bee larvae [238], although this is not always the case [232], and it is unknown whether such findings are relevant to field conditions.

1.2.4.1. Chlorothalonil

Chlorothalonil is a substituted benzene [234, 239] fungicide that acts on glutathione [240-241], and is highly toxic to both aquatic and terrestrial invertebrates [242-244]. Chlorothalonil is relatively non-toxic to adult honey bees in comparison with insecticides [234], but is also more toxic to bees compared to other fungicides [234]. Chlorothalonil is more toxic to honey bee larvae when compared to adult worker bees [245]. It is currently unknown how much chlorothalonil is present in brood food under field-realistic conditions, but its presence in pollen is widespread. Chlorothalonil is the most frequently detected pesticide in bee bread, and has been detected in at least 47% of bee bread samples [57]. It also comprises the majority of fungicide loads in both corbicular pollen [80] and bee bread [57]. High levels of chlorothalonil in beeswax is a potential predictor of Colony Collapse Disorder (CCD) [59], but the impact of chlorothalonil residues in pollen is not entirely clear [57]. In one study, chlorothalonil was detected in samples of trapped pollen and dead bee samples, but not in returning foragers [235]. Bees fed on pollen containing high chlorothalonil residues in the laboratory were more susceptible to *Nosema* [80].

However, pollen type in that laboratory experiment appeared to be a confounding factor and may have influenced the findings.

High levels of chlorothalonil have been detected in entombed pollen, which is defined as pollen sealed into comb with wax and propolis [246]. Brood reared in the laboratory on entombed pollen had lower survival than brood reared on normal pollen [246], but it is still unclear whether that was due to chlorothalonil or due to other factors associated with entombed pollen. The single field experiment that determined the effects of chlorothalonil on entire colonies found that bees reared in colonies exposed to low levels of chlorothalonil in sugar syrup had reduced wing length and body weight [247], but how these traits are implicated in overall colony fitness is currently not known.

1.3. Research Gaps and Impetus for Thesis Research

Although the effects of pesticides (particularly neonicotinoids) on honey bees have been heavily investigated in recent years, many knowledge gaps still exist in the current scientific literature. Below, we discuss several research gaps addressed in Chapters 2 and 3.

1.3.1 Field-Realistic Exposure to Pesticides

While a variety of lethal and sublethal effects of pesticides on bees have been detected in the laboratory, and occasionally in the field, such effects typically occur under conditions that do not constitute field-realistic exposure [207, 248]. Researchers frequently emphasize the need for research investigating the impacts of field-realistic exposure to bees [72, 216, 229, 248-249]. However, despite the call for further research on the impacts of field-realistic pesticide exposure, many components of field-realistic exposure have been overlooked. Below, I address ways in which field-realistic pesticide exposure has been underestimated or misrepresented. Understanding to what degree field-realistic exposure differs for different types of

worker bees, different cropping systems, and different types of pesticide application will be critical to understanding the true impacts of pesticides on honey bee health.

1.3.2. Multiple Routes of Pesticide Exposure

Previous research has also ignored the exposure of an individual bee to the same chemical via different avenues over the same period of time [197, 250]. Foraging bees may be exposed to pesticides through pollen [57, 171-173, 175, 178-179, 198, 235], nectar and honey [172-173, [251-253], wax [55, 57, 254-255] and water [195, 256] that are collected and stored in the colony, as well as via neonicotinoid-contaminated dust [250, 257-258]. However, both laboratory and field experiments have thus far only examined effects through one exposure route at a time, usually via pollen diet [79, 82, 259] or sugar syrup [221, 223, 277, 260-263]. Because worker bees tend to consume different amounts of pollen and sugar syrup based on their age and behavioral task [264-267], and as consumption of different resources also coincides with various changes in physiology [135, 137-138], worker bees may be differentially affected by pesticide exposure through different routes at different stages of life.

1.3.3. Effects of Pesticides on Whole Honey Bee Colonies

Whether the sublethal effects of pesticide exposure in the field are connected to colony survival in the long term is still uncertain [268]. Most field experiments in the United States and elsewhere have not found a direct causative link between sublethal levels of pesticide exposure and colony mortality [207, 213, 229, 269]. However, the current design and structure of field experiments is not robust, with high variability, low replication and short duration [152, 229]. Treatments often consist of placing honey bee colonies in treated fields versus untreated fields [81, 210, 213], which are sometimes not separated adequately in terms of distance to keep bees of different treatments from foraging in the other treatment fields [152, 210, 213]. The pesticide being investigated is also often detected in control colonies [82, 210, 213]. Though

these issues exist in the current literature, researchers who detect these aberrations have thus far not thoroughly discussed them when making inferences regarding effects of the pesticides being investigated. Experiments that involve direct feeding of pesticides to whole honey bee colonies are more likely to yield more meaningful, causative results. However, field experiments in which colonies are exposed to pesticides through direct feeding are few [79, 82, 259, 262], and have thus far not quantified interactions between pesticides (see Pesticide Interactions, page 17). The single colony-level experiment that quantified an interaction between two pesticides was only ten days long [270], and therefore failed to capture any effects that may take place during pollination of crops that may bloom as long as one month, such as citrus [252].

1.3.4. Long Term Effects of Sublethal Pesticide Exposure

As it is generally assumed that field-realistic pesticide exposure does not directly kill honey bee colonies[210, 248], it has become more important for researchers to determine whether (and how) chronic, sublethal effects lead to colony failure in the long term. A population growth model that considers sublethal stress suggests that chronic sublethal exposure to neonicotinoids can cause bumble bee colony failure [271], but whether or not this is true for honey bee colonies is yet to be confirmed. In laboratory experiments, sublethal pesticide exposure has been shown to have delayed effects [272] and cumulative effects with chronic exposure [260, 273], which would not otherwise be captured in short-term experiments. Rondeau et al. [272] determined that delayed impacts of imidacloprid exposure did not take effect until 30 days later, when concentrations as low as 4 ug/L killed 50% of the adult worker population. Further field research is needed in order to determine whether these effects also exist when whole honey bee colonies are exposed to these chemicals under field-realistic conditions.

1.3.5. Effects of Pesticides on Nurse Bees

While there is extensive literature on the effects of imidacloprid on honey bees, most of the literature is specific to effects on foragers [223, 274-277]. However, focusing only on forager bees leaves out the potential impacts of pesticide exposure on other worker bees performing in hive tasks that are critical for colony growth and survival. The impacts of pesticide exposure on nurse bees may be particularly important for understanding how pesticides weaken colonies. Nurse bees play a critical role in the colony, by both raising brood and tending to the queen [278-279]. Worker bees become foragers after 2-3 weeks [280], although this may vary depending on whether they forage precociously. Because nurse bees consume both nectar and bee bread [176, 281-282], a form of stored in-hive pollen that includes both pollen and honey [176-177], they are estimated to consume higher amounts of pesticides than forager bees [267]. Rortais et al. [267] estimated that nurse bees in particular consume approximately four times more imidacloprid over a 10-day period than forager bees. The nurse bees might also be specifically impacted by pesticide exposure through more than one route due to their consumption of both resources (pollen and nectar) simultaneously.

1.3.6. Variation in Field-Realistic Pesticide Concentrations

Many of the lethal and sublethal effects of pesticides on honey bees are detected when concentrations are much higher than those typically considered to be field-realistic [274, 283] [284-287]. However, the concentrations currently deemed to be "field realistic" currently only include those under a very narrow scope of conditions. Neonicotinoid concentrations in pollen and nectar have been widely documented, but mostly for seed-treated crops [214], when concentrations are generally lower than 10 ppb [173-174]. However, seed treatment accounts for only 60% of neonicotinoid use [161], and concentrations in pollen and nectar under conditions other than seed treatment are scarcely mentioned in the current literature. This may be particularly important to consider, since various types of soil application may result in average

concentrations as high as 80 ppb in cucurbit pollen, and foliar applications may result in pollen concentrations greater than 100 ppb [171]. Soil treatment of ornamental plants with label rates of imidacloprid also produces concentrations in flowers that are 793 to 1,368 times higher than those detected in seed-treated crops [168]. Furthermore, researchers have thus far failed to recognize that these chemicals persist in the tissues of plants after soil or trunk treatment at high concentrations [186, 200], which may pose a potential long-term risk to pollinators and other beneficial insects. There is currently very little data on the persistence of neonicotinoid concentrations in the tissues of orchard trees pollinated by honey bees [162, 252]. One study found that imidacloprid concentrations in the nectar of citrus flowers may be as high as 40 ppb after recommended soil treatment with imidacloprid, and up to 55 ppb at twice the recommended field application rate [252]. The degree to which residues of neonicotinoids and other pesticides in pollen and nectar vary demonstrates the need for both crop-specific and application-specific research on field-realistic pesticide exposure.

During the processing of in-hive nectar into honey, both sugars and systemic pesticides become more concentrated in stored nectar and honey [252, 288], which may increase pesticide concentrations in bee bread. Byrne et al. [252] found that mean concentrations of imidacloprid in stored nectar were approximately three times higher than the mean concentrations detected in nectar collected from citrus flowers. The concentration of pesticides in ripened nectar likely cause pesticides in bee bread to be higher as well. In cherry orchards, fungicide concentrations may be as much as three times higher in stored pollen than those in corbicular pollen [288]. Imidacloprid concentrations in bee bread in a study by Mullin et al. [57] were also far higher than residues previously detected in crops, with a mean concentration of 39 ppb and a maximum concentration of 206 ppb. The increased concentrations of pesticides within the hive pose a particular risk for nurse bees, but despite this risk, the current literature has barely investigated these patterns and their implications on long-term colony health. One previous colony-level experiment determined that imidacloprid concentrations of 20 ppb and 100 ppb in pollen diet led to reduced overwintering

survival of honey bee colonies, but the authors disregarded the implications of these effects due to current perceptions of what is considered field-realistic [82]. It is necessary that researchers truly understand what constitutes field-realistic concentrations, so that the impacts of various pesticides are not either underestimated or overestimated.

1.3.7. Pesticide Interactions

Most research to date only examines the impacts of bee exposure to a single pesticide, with two-way pesticide interactions determined at best. The significance of such studies is somewhat questionable, considering that during pollination and under most field conditions, bees are rarely exposed to a limited number of pesticides and are more often exposed to multiple chemicals at once [57-58, 80, 178, 235]. Despite this knowledge, the vast majority of experiments have investigated the effects of a single chemical.

There is seemingly endless potential for interactions between pesticides in honey bee colonies, as up to 121 different active ingredients were detected in hive matrices such as bees, pollen and wax [57]. However, despite the consistent call for research on pesticide interactions [202, 214, 269], very few studies have sought to explore them [71]. Those studies that have attempted to explore interactions thus far have focused on interactions between pyrethroid insecticides and ergosterol biosynthesis inhibitor (EBI) fungicides [289-294], interactions between fungicides and miticides [245, 295], and interactions between miticides [296]. Interactions between neonicotinoid insecticides and other chemicals have been particularly neglected [56, 71, 202, 214, 249, 297].

1.4. Conclusions

Many factors contribute to honey bee colony mortality and losses, including various pathogens and pesticides. Both beekeepers and the public have scrutinized pesticides,

particularly neonicotinoids, for their potential impacts on honey bees, but most research has failed to detect a significant causal link between neonicotinoid exposure and colony mortality. Because neonicotinoids and other pesticides typically occur at low, sublethal levels in the field, it has become more important to investigate the long-term impacts of sublethal pesticide exposure. Here, we describe two experiments that begin to fill some of the knowledge gaps that impede our ability to fully understand the impacts of sublethal pesticide exposure.

Although honey bees are commonly exposed to more than one pesticide at once, very few studies have determined how pesticides interact to weaken or kill honey bee colonies. In Chapter 2, we determine the impacts of an interaction between a neonicotinoid (imidacloprid) and a fungicide (chlorothalonil) and how it impacts number of colony health indicators, including colony size, food stores, foraging activity, and nurse bee physiology. This study is the first to investigate a neonicotinoid-fungicide interaction under open-field conditions, the first to investigate interactions between imidacloprid and chlorothalonil, and the most comprehensive study to date that determines the long-term impacts of sublethal pesticide interactions on colony health, foraging, and disease susceptibility.

Honey bees are also typically exposed to pesticides through multiple routes throughout their lifetime: through pollen, nectar, water, and dust. Despite researchers' understanding that bees are exposed to pesticides through different matrices at different life stages, no previous research has investigated whether exposure through multiple routes exacerbates the effects of sublethal pesticide exposure. In Chapter 3, we explore how the neonicotinoid imidacloprid affects worker mortality and physiology when it is present in pollen, sugar syrup, or both matrices. This study provides new insight into the effects of pesticide exposure when experiments accurately mimic pollen- and nectar-feeding behaviors in the field, and therefore more closely represent field-realistic exposure.

Finally, the majority of pesticide research has been narrowly focused on foraging workers. Research has largely neglected effects on young bees, which are exposed to higher sublethal concentrations of pesticides inside the colony. Researchers must understand the impacts of pesticides on younger workers (nurse bees) in order to fully understand the implications of sublethal pesticide exposure in honey bee colonies. Both experiments described here investigate the effects of pesticide exposure affects the immune and nutritional physiology of nurse bees.

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Chapter 2

Effects of A Neonicotinoid, A Fungicide, and Their Interaction on Honey Bee Colony Health

2.1. Abstract

Beekeepers in the United States have experienced unsustainably high rates of colony loss for nearly a decade. While various factors contribute to these losses, pesticides have received particular scrutiny from beekeepers and the public. Most research to date has been unable to identify pesticide exposure as a major cause, but also has largely focused on the effects of a single chemical. Because bees are typically exposed to more than one agrochemical at any given time, researchers repeatedly emphasize the need to investigate interactions between pesticides, particularly under field-realistic conditions. Despite the call for research on pesticide interactions, such studies have rarely been conducted. In this experiment, we tested the impacts of imidacloprid (a neonicotinoid insecticide), chlorothalonil (a fungicide), and their interaction on various parameters pertaining to foraging, colony health, and worker physiology. This experiment is the first to quantify the effects of agrochemical interactions under field-realistic exposure conditions over several months, as well as the first to investigate interactions between these specific agrochemicals.

We determined that most of our physiological parameters (midgut proteolytic enzyme activity, hypopharyngeal gland protein content, glucose oxidase activity and abdominal lipid stores), as well as total adult bee population and brood area, were not significantly affected by our agrochemical treatments. *Varroa* infestation and *Nosema* infection levels were also not significantly different between our treatments (although our analyses excluded all 0 values). Colony survival was high in all treatments, and therefore was unlikely to have been affected by these pesticides. The only variables differentially affected by our treatments were prophenoloxidase activity, the number of non-pollen foragers returning to the colonies, and the total number of foragers returning to the colonies. Our analyses were only able to detect these differences when outliers were excluded. These results shed light on the need for more thorough

exploration and discussion of the statistical procedures used to quantify the impacts of pesticides.

The most notable effect was the interactive effect on the number of returning non-pollen foragers between imidacloprid, chlorothalonil. Seven weeks after the end of the exposure period, the combination of both chemicals reduced the number of returning non-pollen foragers by approximately 50% compared to the control group. This reduction was significantly greater than the reduction caused by exposure to either chemical alone. From these results, it is apparent that either nurse bees or bee larvae were impacted by pesticides in pollen in a way that impacted their foraging abilities at later stages. Because the number of foragers returning to the colony have critical implications for food storage, nurse bee behavioral maturation, and colony survival, the ways in which pesticides and their metabolites in pollen and brood food impact the later foraging abilities of worker bees deserve particular attention. We conclude that further long-term experiments are needed in order to fully understand the impacts of pesticide exposure on entire colonies. We further emphasize the need for research that documents interactions between different pesticides, as well as interactions between pesticides and pathogens, bee genetics, and nutrition.

2.2 Introduction

In the United States, the honey bee (*Apis mellifera*) is the predominant pollinator of several food crops. Each year, $17 billion worth of crops rely directly and indirectly on honey bee pollination [1]. Although the importance of honey bees versus native pollinators has been debated in recent years [2-6], honey bee pollination remains a top priority in American agriculture [7]. Pollination by honey bees increases seed set, fruit size, and crop yields, even in some native plants [8-10]. In some cases, pollination by non-*Apis* bees is inadequate to maximize production, even when these bees are abundant [9]. While native bees contribute to pollination in agricultural systems, healthy honey bee populations are indispensable in order to sustain efficient agricultural production [6].

As the need for pollination services worldwide has risen substantially over the past four decades [11], honey bee populations have been unable to keep up with increasing demand [12]. This is particularly true in the United States, where the number of managed colonies declined by 50% from 1961 to 2006 [13] and rates of colony loss have been unsustainably high over the past decade [14-19]. This decline has not yet led to pollination shortages [1, 20], but increasing acreage of bee-pollinated crops [12] may increase the likelihood of pollination shortages and reduce the stability of agriculture in the future [21]. Furthermore, high rates of colony loss and the increasing cost of maintaining honey bee colonies [22] further reduce the stability of honey bee pollination services. In order to reduce colony mortality and maintain honey bee populations that are sufficient to meet current pollination demands, it is critical to understand the factors driving these losses.

Although many different causes of colony losses and colony collapse have been proposed [16, 23-29], it is general scientific consensus that there is no single cause [30]. Rather, the colony losses are a result of multiple factors interacting in complex ways leading to colony mortality [30-35]. Studies that quantify interactions between factors involved in colony losses are lacking and direly needed, especially in the field, and at the colony level [36]. However, despite the high demand for research on interactions, honey bee research continues to utilize a single-factor approach, especially in studies that seek to determine the impacts of pesticide exposure [37].

The roles that various pesticides play in colony losses are still somewhat unclear [38]. Pesticides remain one of the top nine self-reported causes of colony loss [14, 15, 39], and commercial beekeepers tend to cite pesticides as the cause of their colony losses more often than backyard or sideline beekeepers [15]. Additionally, beekeepers who cite pesticides as the main cause of their losses experience higher losses, on average, than those who report losses due to other factors [17]. Despite beekeepers' suspicions that pesticides are heavily involved in colony losses, scientists frequently conclude that pesticide exposure is not directly responsible for high colony mortality [27, 40-

43]. A potential reason for these discrepancies may be that current research methods for determining the impacts of pesticides do not capture the complexity that exists in the field [44]. The failure of bee researchers to account for this complexity and to take seriously beekeepers' understanding of their colonies may potentially lead to pesticide management decisions that are detrimental to pollination services [45]. Because commercial beekeepers provide the vast majority of pollination services in the nation [22], the long-term impacts of various pesticides, and the many ways they may increase the likelihood of colony failure, deserve further scientific attention.

In order to understand the complex ways in which pesticide exposure may impact honey bee colonies under field conditions, researchers must conduct studies that measure long-term impacts of sublethal pesticide exposure. Because field-realistic pesticide exposure does not typically directly kill honey bee colonies [46-47], it has become more important for researchers to determine how chronic, sublethal effects lead to colony failure in the long term[48]. These effects are still underexplored under field conditions. While some published data document the effects of chronic pesticide exposure (primarily neonicotinoid exposure [43, 49-50]) to entire colonies, there are still very few studies that track those effects over longer periods of time [43, 47, 50-52]. The majority of these experiments are too short in duration to determine long-term effects [53], and the single semi-field study that examined an agrochemical interaction was only ten days long [54]. In the field, crops may bloom for as long as one month [55], and long-term monitoring is critical to capture the potential delayed effects [56-57] of pesticide exposure.

Accounting for complexity in honey bee toxicological research also requires the investigation of pesticide interactions. There is seemingly endless potential for interactions between pesticides in honey bee colonies, as up to 121 different active ingredients were detected in hive matrices such as bees, pollen and wax [58]. However, despite the call for research on pesticide interactions [36, 40, 59], very few studies have sought to explore them [37]. The studies that have investigated interactions between chemicals have focused on interactions between pyrethroids and

ergosterol biosynthesis inhibitor (EBI) fungicides [60-65], interactions between fungicides and miticides [66-67], and interactions between miticides [68]. Interactions between neonicotinoid insecticides and other chemicals have been particularly neglected [36-37, 59, 69-71], especially under field conditions. In our experiment, we investigate an interaction between a neonicotinoid insecticide, imidacloprid, and chlorothalanil, a broad-spectrum fungicide.

At this point, neonicotinoids are arguably the most controversial group of pesticides—they have been linked to declines in various organisms, including birds [72], aquatic wildlife [73-74], and native bees [75-78] but they are not directly linked to honey bee colony declines in the United States [27, 41]. The potential risk that neonicotinoids pose to honey bees and other pollinators has led to a full ban on the use of neonicotinoids in the European Union [79] and a lawsuit against the United States Environmental Protection Agency [80]. The systemic properties [81-87], environmental persistence [82, 88-89], widespread use in bee-pollinated crops [55, 85, 90-93], and frequently detected environmental residues [94-97] are some of the major reasons they remain a public and scientific concern for honey bee colonies. Some neonicotinoids have also been documented to adversely impact honey bee foraging ability [98-99], learning and memory [100-102], motor function [103], and susceptibility to *Varroa* mites and various pathogens [43, 50, 57, 104-106] , which implicate the role of these chemicals in colony losses. However, there still exists a great need for field research that investigates the role these chemicals play in colony losses in the United States.

Though neonicotinoids currently receive the most attention in research on honey bee toxicology [36], the impacts of fungicides are also becoming a greater concern. Fungicides were previously thought to be safe to bees because of their low acute toxicity [107-111]. However, they are often applied to crops during the bloom while bees are foraging [109], and therefore contaminate pollen resources even when they are not systemic. Fungicides currently comprise the greatest proportion of pesticides in pollen [58, 112] and are detected more frequently in pollen than are systemic

insecticides [112-113]. Though there are currently not enough data on record to determine whether fungicides play a critical role in colony losses [27], high fungicide loads have been linked to lower isolates of beneficial fungi in pollen [114] and increased honey bee susceptibility to *Nosema* [112]. Some fungicides are also highly toxic to honey bee larvae [115], although this is not always the case [109]. Though many interactions between fungicides and other pesticides have not been explored, certain fungicide-insecticide combinations have been found to reduce queen emergence and increase virus titers of honey bee colonies [116], and certain fungicides increase the lethal toxicity of both miticides and pyrethroids insecticides [60, 62-66]. Fungicides may also demonstrably decrease the repellency of pyrethroids [65], causing bees to consume lethal doses of insecticides. However, whether fungicides adversely impact colonies specifically when in combination with neonicotinoids is still largely unknown.

Because neonicotinoids and fungicides frequently co-occur in pollen samples [58, 112-113] [117], there is high potential for these chemicals to interact with each other in honey bee colonies. Data on these interactions remain scant—while a few interactions have been explored in the laboratory [69-70, 118], only one study has documented neonicotinoid-fungicide interactions under semi-field conditions [54]. Current data on neonicotinoid-fungicide interactions focus primarily on lethal impacts on individuals rather than sub-lethal impacts that have implications for colony health. Under laboratory conditions, ergosterol biosynthesis inhibitor (EBI) fungicides and demethylation inhibitor (DMI) fungicides increase the lethal toxicity of neonicotinoids, though these results are highly variable between experiments [69-70, 118]. Fungicides may increase the toxicity of acetamiprid and thiacloprid, the two neonicotinoids by up to 1141-fold [69, 118], although the degree of this synergy is not true in all cases [70]. Thus far, no open-field experiments have explicitly quantified a neonicotinoid-fungicide interaction.

Though there are several types of neonicotinoid insecticides, imidacloprid may be of particular concern to colony health. Imidacloprid is used on various bee-pollinated

crops, including pome fruits, stone fruits [119], cucurbits [85], citrus [120-122], and berries [123-124]. The other widely used neonicotinoids thiamethoxam and clothianidin [90] are more often used to protect corn [125], which is wind-pollinated [126]. Imidacloprid use as a seed-treatment in canola is directly linked to higher colony losses in England and Wales [52], but field experiments in the United States have yet to show similar patterns [43, 81]. Sublethal, field-realistic exposure to this chemical has been linked to higher infections of *Nosema* [49], higher *Varroa* mite infestations of honey bee colonies [43], altered ability to carry out foraging tasks [99, 127-128], decreased immunity [129], and altered gene expression [130-132]. Concentrations declared to be the most field-relevant, however, do not affect colony mortality in most analyses [43, 133-134]. While imidacloprid is the most well-studied of all the neonicotinoids [36], field experiments on the impacts of this chemical are still few, and there are currently no data on how it interacts with other agrochemicals in the field. In the laboratory, the toxicity of imidacloprid has been found to only increase slightly with the addition of demethylation inhibiting (DMI) and ergosterol biosynthesis inhibiting (EBI) fungicides in the laboratory [69, 118], but field-realistic doses of these fungicides have not been shown to increase its toxicity [70]. Otherwise, interactions between imidacloprid and other fungicides, such as chlorothalonil, are not known.

Chlorothalonil is neither an EBI or DMI fungicide—it is a substituted benzene [111, 135] fungicide that acts on glutathione [136, 137], and is highly toxic to various aquatic and terrestrial invertebrates [138-140]. The toxicity of chlorothalonil to adult bees is relatively low compared to insecticides [111], but is much higher than that of other fungicides [111]. Chlorothalonil is also more toxic to honey bee larvae than it is to adult worker bees [67]. Furthermore, this chemical may be a particular concern for honey bee colonies, as it is the most frequently detected pesticide in bee bread [58] and comprises the majority of fungicide loads in both corbicular pollen [112] and bee bread [58]. High levels of chlorothalonil in beeswax is a potential predictor of Colony Collapse Disorder (CCD) [34], but the impact of chlorothalonil residues in pollen has not yet been thoroughly investigated [58].

Researchers are just beginning to unravel the sublethal and interactive impacts of chlorothalonil on honey bees. Under laboratory conditions, chlorothalonil has been found to decrease the repellency of a pyrethroid insecticide [65] and increase *Nosema* infections in adult worker bees [112]. In the field, chlorothalonil has also been linked to pollen entombing, a behavior in which bees permanently seal pollen into comb to prevent consumption [141], reduced wing length [142], and reduced body weight [142]. However, whether these effects translate into overall colony fitness is questionable, and how chlorothalonil interacts with neonicotinoid insecticides is still unknown.

The presence of both imidacloprid and chlorothalonil in pollen diet has potential for long-term negative impacts on honey bee colonies. Stored pollen in a colony is consumed by nurse bees and processed into brood food for larvae [143-144]. Because the quality of pollen and brood diet are directly linked to adult longevity [145-146], immune physiology [147], and pesticide sensitivity at later stages [148], the presence of pesticides in pollen and bee bread may have long-term consequences for honey bee colony health.

Both imidacloprid and chlorothalonil have been detected in the same corbicular pollen samples on multiple occasions [112-113], but chlorothalonil is detected in bee bread far more frequently than imidacloprid [58]. Though only 2% of bee bread samples contain detectable levels of imidacloprid [58], its presence in corbicular pollen is well-documented [112-113, 149-151], and at more hazardous levels than other chemicals [150]. Given that pesticide residues tend to be higher in bee bread than in corbicular pollen, and do not decrease over time [152], and given that bees prefer food that contains neonicotinoids to uncontaminated food [153], imidacloprid-contaminated bee bread is likely to be consumed by nurse bees more quickly than researchers have thus far sampled bee bread. We argue that the impacts of field-realistic concentrations of both imidacloprid and chlorothalonil in bee bread must be critically examined in order to understand the true impacts of both chemicals.

In this experiment, we asked the following questions: 1) Do imidacloprid and chlorothalonil have adverse effects on honey bee colonies when present in the pollen diet at field realistic concentrations? 2) Do chlorothalonil and imidacloprid act synergistically on colony health? and 3) Do the effects of these chemicals depend on time after exposure? We use colony size, as well as a number of foraging and physiological parameters, to answer these questions.

All physiological responses in this study indicate different measures of honey bee colony fitness. The responses we investigated are as follows: 1) prophenoloxidase activity, which is a measure of potential phenoloxidase activity [154] in the presence of an immune challenge such as wounding [155-156], bacterial pathogens [157], or potential parasitoids like the zombie fly [158]; 2) glucose oxidase activity, which indicates the antimicrobial potential [159] of brood food secreted by nurse bees [160-161] and honey produced by foragers [162-164]; 3) midgut proteolytic enzyme activity, which is a measure of how well nurse bees digest protein [144, 165-166], and is directly related to brood food production [167]; 4) hypopharyngeal gland protein content, which indicates the quality of secreted brood food [168]; and 5) abdominal lipid stores, which mediate the transition from nursing tasks to foraging [169-171], produce antimicrobial peptides [172-173], and are associated with longevity [174-175]. Additionally, to capture the effects of these chemicals on overall colony health (as it is typically monitored by beekeepers), we tracked colony size (adult bee population and brood area), honey stores, and foraging activity throughout the experiment.

To our knowledge, this is the first open-field study that attempts to quantify both the single and combined effects of a widely used neonicotinoid insecticide and a ubiquitously used fungicide on honey bee colonies, and the most comprehensive study examining sublethal and field-realistic pesticide exposure effects at the colony level.

2.3 Methods

2.3.1. Experimental Design & Setup

All experimental colonies were established at an Oregon State University apiary (Corvallis, OR, USA) in May 2014. Each colony was established using 1.4 kg of bees consisting of approximately 9000 bees and a queen (*Apis mellifera ligustica*). The bees for establishing experimental colonies were obtained from a local beekeeper and all the queens were purchased from a queen producer (Jackie Park-Burris Queens in Palo Verde, California). All the queens used in this study were sister queens to minimize variability that could result from varying genetics. A set of two 10-frame hive bodies housed each colony—with 16 frames of drawn comb, three frames of plastic foundation, and a 1-gallon feeder.

The experiment consisted of the following four treatments: control, imidacloprid, chlorothalonil and a combination of imidacloprid and chlorothalonil. A full-factorial (2 x 2 x 9), completely blocked experimental design was used for this study and each treatment was replicated nine times. Nine blocks of four colonies (36 colonies total) were used in the experiment. Blocks were based on baseline colony size (see *Colony Evaluations*), such that total adult bee and brood populations were approximately equal among all colonies in a block. After baseline colony evaluations were conducted, colonies within a block were equalized by removing frames of bees or exchanging brood between colonies. Pollen stores were also equalized between all colonies. After equalization, baseline colony size data was adjusted according to the estimated changes in brood and bees.

Prior to equalizing, each of the colonies in the block was randomly assigned to one of four treatments: control, imidacloprid, chlorothalonil, or both imidacloprid and chlorothalonil.

All baseline samples and colony data were collected at the end of June (June 16-17 for colony evaluations, June 18-19 for physiological parameters, June 23-24 for pathogen loads, June 30 for forager counts). Treatments were applied from June 30 to July 28.

2.3.2. Pollen Patty Diet and Pesticide Mixing

Colonies were exposed to imidacloprid and/or chlorothalonil via a pollen patty diet. Bee collected pollen from Yukon, Canada (The Pollen Man®) was finely ground and used for making the pollen patties. The pollen was analyzed for pesticide content by the USDA-AMS-NSL laboratory in Gastonia, NC. Only trace levels of few pesticides were detected in the pollen with no detectable levels of imidacloprid or chlorothalonil (See **Appendix X1**).

Pollen patties were made fresh every week for 4 weeks. Imidacloprid and chlorothalonil (pure active ingredients, Sigma Aldrich®, MO, USA) were infused to pollen through acetone solutions. The target concentrations of the two chemicals in the pollen patties were chosen based on the mean concentrations of these chemicals in stored pollen, as reported by Mullin et al. [58]: 39 ppb imidacloprid and 3014.8 ppb chlorothalonil. These concentrations are results from the only extensive documentation of pesticide concentrations in stored pollen in North America. The concentrations used here represent field-realistic concentrations in bee bread, and provide a balance between the lowest and worst-case scenario concentrations that may occur in the field. The protocol used for mixing chemicals into the pollen can be found in **Appendix X1**.

From each pollen patty in a given treatment group, we removed a 2-g sample of pollen patty. All patty samples in each treatment group were combined and sent to the USDA National Science Laboratory in Gastonia, NC to confirm concentrations. Afterward, we recorded the mass of each pollen patty (See **Appendix X1** for further details).

2.3.3. Pollen Patty Feeding and Consumption

Pollen patties were removed and replaced weekly for four weeks (June 30, July 7, July 14, July 21). During this time, pollen traps were placed on all colonies during the exposure period to prevent the consumption of incoming pollen and induce feeding on the pollen patty diet. Patties were placed between hive bodies for each colony using a 4.5-cm spacer.

Pollen consumption for each week was calculated as the change in pollen mass, accounting for the weight of the pie tin. Samples of the remaining pollen patties were also sent to the USDA analytical laboratory to determine whether the concentrations changed or remained stable over the course of each week.

2.3.4 Colony Evaluations

At approximately monthly intervals, colony evaluations were conducted to determine adult bee population and honey stores: June 16-17, July 23-25, August 27-29, and October 1-3. The percentage of the area covered by bees, brood, and honey were visually estimated for each frame of each colony. Estimates for each were totaled for all frames in a colony, and recorded as total frames of bees, brood, and honey. Evaluations were conducted for all colonies within the same block at the same time of day in order to evaluate colonies in all treatments equally throughout the day and minimize the differences between treatments due to foraging activity.

2.3.5. Bee Sampling

For all physiological assays, nurse bees were sampled from all experimental colonies at the same time when colony evaluations were conducted. These bees were individually collected from combs of open brood, and were identified as bees exhibiting brood-rearing behavior, such as checking brood cells or feeding brood. We used bees from this sample to quantify prophenoloxidase activity, glucose oxidase

activity, midgut enzyme activity, hypopharyngeal gland protein content and abdominal lipid stores.

Samples of approximately 200-250 bees were taken from a frame of open brood for each colony at these same intervals. These samples were stored in 75% ethyl alcohol and used to quantify parasite (*Varroa* and *Nosema*) loads.

2.3.6. Forager Counts

Weekly, beginning June 30 (before treatments were applied) and ending September 22, we counted the number of bees returning to each hive over a 3-minute period. Forager counts took place between 11 am and 12 pm on sunny days that did not follow any other disturbance of colonies (e.g. during feeding or colony evaluations). Pollen foragers and non-pollen foragers were counted separately, and were only counted as they entered the colony.

2.3.7. Varroa/Nosema Parasitism Records

Varroa and *Nosema* infestation of each colony at each month was determined using the sample of bees stored in alcohol. *Varroa* mite loads were quantified using the protocol described in Lee et al. [176]. The abdomens of one hundred bees from this sample were used to quantify *Nosema* infection intensity, using the methods of Cantwell [177] and Jack et al. [178]. (See **Appendix X1** for further details.)

2.3.8. Hypopharyngeal Gland Protein Content

We quantified hypopharyngeal gland protein content using a standard BCA assay (Pierce Biotech BCA Assay Kit, Thermo Scientific, IL, USA). Hypopharyngeal glands (HPG) were dissected from 10 nurse bees per colony. All glands from the bees of one colony were pooled together in 125 μL of phosphate-buffered saline (PBS; 10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4, Sigma

Aldrich), and stored at -80˚C until time of analysis. The protocol was adapted from Jack et al. [178], and modified for protein quantification in the glands of 10 heads instead of 1. The details for the assay can be found in **Appendix X1**.

2.3.9. Hemolymph Extraction

We extracted hemolymph from 10-15 live nurse bees per experimental colony per sampling period (June 18-19 for baseline, July 30-31 and September 9-10 for post-treatment). Bees were anesthetized on dry ice for several minutes, until most bees were no longer moving. We then pinned each bee through the thorax and made an incision on the second or third abdominal tergite with a small pair of scissors. A micropipette was used to draw approximately 2-5 µL of hemolymph from the incision. Hemolymph from all the bees of a colony was pooled together on ice and 25 ul of the pooled sample was added to 0.5 ml of ice-cold sodium cacodylate buffer (NaCac, 1 mM sodium cacodylate, 5 mM calcium chloride, pH 6.5), and stored at -80°C. Samples were then thawed in a 5°C ice water bath, centrifuged at 4 °C to pellet cellular debris, and aliquoted. Aliquots were stored at -20 °C until time of prophenoloxidase analysis.

2.3.10. Prophenoloxidase Activity (ProPO)

Prophenoloxidase activity (ProPO) was measured as maximum linear rate (Max V) of the substrate (L-Dopa) conversion per milligram of hemolymph protein. Prophenoloxidase activity was determined using a protocol adapted from Laughton and Siva-Jothy [179], Laughton and Siva-Jothy [180], Wilson-Rich et al. [181], and Zufelato et al. [182]. (See **Appendix X1** for details.)

We quantified the amount of hemolymph protein per sample with a standard BCA assay similar to that used to quantify hypopharyngeal gland protein content. Hemolymph BCA assays differed from hypopharyngeal gland BCA assays only in

that the BSA standards were diluted in 1:1 NaCac:PBS, and samples were plated as ½ dilutions (in PBS).

2.3.11. Glucose Oxidase Activity

We measured glucose oxidase (GOX) activity in pooled samples of 10 whole heads, using a colorimetric kinetic assay. These heads were immediately frozen in dry ice and then stored at -80°C until time of homogenization and GOX analysis.

Each sample was homogenized in 500 µl of sodium phosphate lysis buffer (80 mM sodium phosphate monobasic/sodium phosphate dibasic pH 7.4, 20 mM sodium chloride, 1% Triton X-100, 1X Halt™ EDTA-free protease inhibitor (Thermo Scientific, IL, USA)), with one 3-mm tungsten carbide bead in a Qiagen® TissueLyser II at 30 oscillations/second for 2 rounds of 1.5 minutes. The resulting homogenates were centrifuged at 14,000 rpm for 15 minutes, 4 °C. Supernatants were then pipetted into clean tubes and centrifuged again for 4 minutes at 14,000 rpm, 4 °C to further pellet any remaining cellular debris. From those supernatants we made 1/5th dilutions in lysis buffer.

The assay was performed on a BioTek® Synergy 2 plate reader, with a hydrogen peroxide standard curve (nmoles H_2O_2 produced/microplate well). Ten microliters of each standard or sample dilution were combined with 180 µl of sample reaction mix (100 µl distilled, autoclaved water, 50 µl 0.5 M potassium phosphate pH 7, 20 µl 0.5 M D-glucose, and 2.5 U horseradish peroxidase (10 µl)) in each of two replicate wells in a chilled Greiner Bio-One® non-binding 96-well plate. For sample blanks, 10 µl of each diluted sample were combined with 180 µl of a similar mixture—differing only in that glucose was replaced with an additional 20 µl of water. After shaking the plate for 2 minutes at 37 °C, 20 µl of o-dianisidine dihydrochloride (0.95 mg/ml, Sigma-Aldrich®, MO, USA) were added to each well. Absorbance at 430 nm, 37 °C, was measured every 34 seconds for 1.5 hours, with continuous shaking. We then calculated the units of glucose oxidase activity present in each sample (See **Appendix**

X1). One unit of GOX activity is denoted as the amount of the enzyme required to generate 1.0 µmole of H_2O_2 per minute at 37 °C.

The final glucose oxidase values were calculated as milliunits per mg of protein in each sample. We quantified the protein concentration of the samples using a standard BCA assay, as previously described. Samples were analyzed as $1/20^{th}$ dilutions (in PBS).

2.3.12. Abdominal Lipid Stores

A pooled sample of 10 nurse bees per colony were used to quantify abdominal lipid stores using the protocol of Wilson-Rich et al. [181]. (Details can be found in **Appendix X1.**)

2.3.13. Midgut Proteolytic Enzyme Activity

Midgut proteolytic enzyme activity (MEA) was determined on pooled samples of 10 nurse bees per colony. Abdomens were dissected from the bees and added to tissue lyser tubes containing 300 µL of chilled 0.1 M Tris buffer (pH 7.9) and a tungsten carbide bead, and homogenized using the Tissue Lyser II® (2 rounds of 30 oscillations/second, 1.5 minutes each). Each sample was disrupted in 300 ul of chilled 0.1 M Tris buffer (pH 7.9) with one 3–mm tungsten carbide bead at 30 oscillations/second for two round of 1.5 minutes in a Qiagen Tissue Lyser II.

After homogenization, samples were centrifuged at 13,300 rpm and 4°C for 7 minutes. Following centrifugation, 5 µL of the supernatant was added to each of three 1.5-mL centrifuge tubes containing 25 ul of chilled 0.1 M Tris buffer. 60 µL of 2% azocasein (diluted in 0.1 M Tris buffer, pH 7.9) were then added to all tubes. After vortexing gently, all tubes were incubated for three hours. For a given sample, one tube was incubated in ice in order to serve as the sample blank, while the remaining

two tubes—duplicate sample reaction tubes—were incubated at 37°C. We then placed all tubes on ice and added 300 μL of 10% trichloroacetic acid (TCA). Tubes were then vortexed and centrifuged at 13,300 rpm for 5 minutes at room temperature.

Midgut proteolytic enzyme activity was measured by recording absorbance at 440 nm with a Beckman® DU spectrophotometer as in Sagili et al. [167] and Jack et al. [178].

2.3.14. Colony Maintenance During the Study Period

Colonies were treated for *Varroa* mites from September 4, 2014 to October 15, 2014 using Apivar® strips. Each colony was fed 1 gallon of 50% (v/v) sugar syrup on August 1 and August 22, 2014. Colonies were again fed with 66% (v/v) sugar syrup and half of a Global Patties® protein supplement on September 24, 2014 and once again with 66% sugar syrup on October 15, 2014. Colonies were also fed 500 g of hard sugar candy on February 11, February 20, and March 13, 2015.

2.3.15. Overwintering Survival

Overwintering survival was recorded on March 13, 2015. On this day, colonies were recorded as "alive" (live bee cluster) or "dead" (no live bees in the colony).

2.3.16. Statistical Analyses

All response variables were analyzed with full-factorial repeated-measures linear mixed models using the "lme" function in R (package "nlme"). (See **Appendix X1** for description of explanatory variables, response variables, included random effects, and selected correlation structures.) We determined main and interactive effects using Type III F-tests (anova.lme function, with "type" set to "marginal"). We report significance for all analyses at both the 0.05 and 0.10 alpha level. While it is a common practice to declare small p-values above 0.05 to be "insignificant," it is important to note that the alpha level used to determine significance is arbitrary [44,

183-187]. Failing to acknowledge the usefulness of the p-value can lead to inappropriate conclusions and poor management decisions. Furthermore, researchers have recently argued that p-values below 0.10 and above 0.05 should be taken seriously in scientific research [187].

Where there were statistically significant differences, we performed multiple comparisons using a Tukey HSD adjustment using the packages "lsmeans" and "multcomp." For variables that were log-transformed, we back-transformed means and confidence intervals and report differences as a ratio of medians. The results for data that were not log-transformed are reported on means. The list of variables for which we used these remedies is included in **Appendix X1**.

For much of the data, residuals plots indicated right skew or unequal variances. We remedied this by running the models on log-transformed data, by allowing for unequal variances between time periods, or both. Many of the data also contained outliers (see **Appendix X1**), here defined as a data point with a residual less than -2 or greater than 2. We paid close attention to the results of each analysis with and without these data points—in cases where the assumptions of the model appeared to be met when outliers were both included and excluded, we ran the model under both conditions. For analyses in which results differed when outliers were included and excluded, we reported results for both analyses. In some cases, the assumptions were only met when outliers were included or when outliers were excluded. This is further described for each response variable in **Appendix X1**.

Thus far, the presence or influence of outliers in data from field experiments has not received adequate attention. Outliers are well-known to increase the likelihood of Type II errors (the statistical error in which researchers conclude that an independent variable has no effect on a response variable, when in fact it does have an effect) and reduce statistical power [188]. Previous papers have called for better examination and reporting of statistical outliers [189] and higher statistical power [134]. Although statisticians typically only recommend removing outliers if they are due to errors in

data entry, they may also be removed in the case that the data are not well standardized or are due to sampling error [190]. We argue that although we standardized our experiment by keeping all colonies in the same apiary and blocking by colony size, there may be other factors in our experiment that are outside of the control of the experimental design, as is the case with any open-field experiment with honey bee colonies. For example, foragers in the same apiary may forage up to 9.5 km away from the colony [191], which makes it nearly impossible for all colonies in an apiary to forage on the exact same resources. This is one example in which uncontrollable variation in the field may contribute to wide variation in the response variables. Although bees forage close to their colonies when adequate forage is nearby [192], our experiment took place after the major blooms of the season were over, which would have made it more likely that foraging bees would fly longer distances from the colonies to find food and increased the potential for uncontrolled variation between colonies.

For the *Varroa* and *Nosema* analyses, we only included colonies that had detectable levels of the given parasite in the model (0 values were excluded from the analysis). This is also further explained in the Appendix.

2.4. Results

2.4.1 Pesticide Concentrations

The results from the USDA analytical laboratory confirmed that imidacloprid concentrations used in this study were consistently near the intended concentration, averaging 35 ppb. Our chlorothalonil concentrations were, however, only 3.6% of the intended concentration, averaging 108 ppb. Potential reasons for these discrepancies are outlined in the Discussion section.

Imidacloprid concentrations also remained stable in our pollen patties throughout each week, as whereas there were no longer detectable levels of chlorothalonil in pollen patties by the end of the week.

2.4.2. Pollen Patty Consumption

Total pollen patty consumption for all four weeks of treatment did not depend on imidacloprid exposure ($F_{1,22} = 0.776$, $p = 0.388$), chlorothalonil exposure ($F_{1,22} = 0.0003$, $p = 0.986$), or the combination of both chemicals ($F_{1,22} = 0.085$, $p = 0.774$).

2.4.3. Adult Bee Population

Adult bee population was not significantly different between levels of imidacloprid ($F_{1, 24} = 0.090$, $p = 0.767$), chlorothalonil ($F_{1, 24} = 1.806$, $p = 0.193$), or the combination of both chemicals ($F_{1, 24} = 0.295$, $p = 0.592$). Adult bee population was significantly affected by time ($F_{3, 96} = 99.351$, $p < 0.0001$). Neither the effect of imidacloprid ($F_{3, 96} = 0.517$, $p = 0.672$), chlorothalonil ($F_{3, 96} = 0.368$, $p = 0.777$) nor was there an interaction between both chemicals and time ($F_{3, 96} = 0.337$, $p = 0.798$).

2.4.4. Percent Change in Adult Bee Population

The percent change in adult bee population was not significantly affected by imidacloprid exposure ($F_{1, 24} = 0.564$, $p = 0.460$), chlorothalonil exposure ($F_{1, 24} = 0.572$, $p = 0.457$), or the combination of both chemicals ($F_{1, 24} = 0.313$, $p = 0.581$). There were no significant interactions between any of the chemicals and time (See **Appendix X2**).

2.4.5. Brood Area

In the model containing all data points, brood area was not affected by imidacloprid ($F_{1,24} = 0.281$, $p = 0.601$) or chlorothalonil ($F_{1,24} = 0.0008$, $p = 0.9775$). The

interaction between chlorothalonil and imidacloprid was nearly significant at the 0.10 alpha level ($F_{1,24}$ = 2.886, p = 0.102). Brood area was also significantly different between months for all groups ($F_{3,96}$ = 269.021, p < 0.0001). There were no significant interactions between any of the treatments and time (See **Appendix X2**.)

In the model in which the two outliers were removed, brood area was still unaffected by imidacloprid ($F_{1,24}$ = 0.813, p = 0.376) and chlorothalonil ($F_{1,24}$ = 0.158, p = 0.694) alone. The interaction between imidacloprid and chlorothalonil became significant at the 0.10 alpha level ($F_{1,24}$ = 3.173, p = 0.088). The effect of time remained significant ($F_{3,94}$ = 303.490, p < 0.0001), and all interactions between the chemicals and time remained insignificant (See **Appendix X2**).

The interaction between these chemicals appeared to be one in which the colonies treated with both chemicals had more total brood than colonies treated with either chemical alone, but neither group was significantly different from the control. However, the interaction between the two chemicals became insignificant between groups after the Tukey adjustment (**Figure 2.1**).

2.4.6. Percent change in Brood Area

Percent change in brood area was not significantly affected by imidacloprid exposure ($F_{1, 24}$ = 0.030, p = 0.863) or chlorothalonil exposure ($F_{1, 24}$ = 0.128, p = 0.723). The interaction between both chemicals also did not significantly affect change in brood area ($F_{1, 24}$ = 1.012, p = 0.325). Neither the effect of imidacloprid ($F_{2, 64}$ = 0.1091, p = 0.897) nor the effect of chlorothalonil ($F_{2, 64}$ = 1.272, p = 0.287) had a significant interaction with time. There was also no significant interaction between imidacloprid, chlorothalonil, and time ($F_{2, 64}$ = 0.384, p = 0.683).

2.4.7. Honey Stores

There was no effect of imidacloprid ($F_{1, 24} = 0.001$, $p = 0.974$), chlorothalonil ($F_{1, 24} = 0.027$, $p = 0.870$), or their interaction ($F_{1, 24} = 0.828$, $p = 0.373$) on honey stores in the model that included outliers. Neither imidacloprid, chlorothalonil, or their combination of the two interacted with time (See **Appendix X2**). Honey stores did differ between months ($F_{3, 95} = 81.728$, $p < 0.0001$), which we generally expected and did not further explore.

When the model was run again on the data without outliers, there was no main effect of chlorothalonil ($F_{1, 24} = 0.112$, $p = 0.741$), but the interaction between time and chlorothalonil nearly became significant at the 0.10 alpha level ($F_{3, 89} = 2.090$, $p = 0.107$). In this potential interaction, it appeared that colonies exposed to chlorothalonil had slightly higher honey stores than colonies not exposed to chlorothalonil at month 1 and month 3, but slightly lower honey stores than colonies not exposed to chlorothalonil at months 0 and 2 (**Figure 2.2**). The greatest raw difference between these groups was 0.4 frames of honey, at months 1 and 2. There was no effect of imidacloprid ($F_{1, 24} = 0.012$, $p = 0.915$), its interaction with time ($F_{3, 89} = 1.121$, $p = 0.345$), or its interaction with chlorothalonil ($F_{1, 24} = 0.823$, $p = 0.373$). Further, there was also no interaction between both chemicals and time ($F_{3, 89} = 0.057$, $p = 0.982$).

2.4.8. Pollen Foragers

The median number of pollen foragers in the colonies was not affected by imidacloprid ($F_{1, 24} = 1.288$, $p = 0.268$) or chlorothalonil ($F_{1, 24} = 0.334$, $p = 0.569$). There was no interaction between the chemicals ($F_{12, 371} < 0.0001$, $p = 0.993$).

The median number of pollen foragers in the colonies was different between different weeks ($F_{12, 371} = 14.866$, $p < 0.0001$). However, neither the effect of imidacloprid ($F_{12, 371} = 0.991$, $p = 0.457$) nor chlorothalonil ($F_{12, 371} = 0.967$, $p = 0.480$) interacted with

time. There was also no interaction between both chemicals and time ($F_{12, 371} = 0.918$, $p = 0.529$).

2.4.9. Non-Pollen Foragers

Results were drastically different between the model containing outliers and the model excluding them. The model containing outliers detected no significant effect of imidacloprid ($F_{1, 24} = 1.944$, $p = 0.176$), chlorothalonil ($F_{1, 24} = 1.866$, $p = 0.185$), or their interaction ($F_{1, 24} = 0.940$, $p = 0.342$). There were no interactions between the chemicals and time (See **Appendix X2**). Time was the only factor that had a significant effect on non-pollen foragers in this model ($F_{12, 384} = 40.799$, $p < 0.0001$).

In the model that excluded outliers from the previous model, the effect of both imidacloprid ($F_{1, 24} = 0.580$, $p = 0.454$) and chlorothalonil ($F_{1,24} = 1.680$, $p = 0.207$) remained insignificant, as did their interaction ($F_{1,24} = 0.285$, $p = 0.598$). The effect of time remained significant ($F_{12, 364} = 55.226$, $p < 0.0001$). However, when outliers were removed, the interaction between imidacloprid and time became significant ($F_{12, 364} = 2.058$, $p = 0.019$). The interaction between chlorothalonil and time was significant at the 0.10 alpha level ($F_{12, 364} = 1.577$, $p = 0.0664$). There was also an interaction between both chemicals and time ($F_{12, 364} = 2.274$, $p = 0.009$). Below, all comparisons between treatments reported are those from the model excluding outliers.

The interaction between imidacloprid exposure and time indicated that colonies exposed to imidacloprid had different numbers of returning non-pollen foragers than colonies not exposed to imidacloprid prior to treatment (at week 0), and 7 weeks after the conclusion of the exposure period (week 11). Prior to treatment, the median number of returning non-pollen foragers in colonies assigned to imidacloprid exposure was nearly 1.5 times greater than colonies not assigned to imidacloprid exposure (**Figure 2.3**). At Week 1, these differences no longer existed, indicating that the number of returning non-pollen foragers increased significantly more in colonies

that were not exposed to imidacloprid than colonies that were during the first week of exposure. Further, seven weeks after the exposure period ended, the median number of returning non-pollen foragers for colonies not exposed to imidacloprid was 1.4 higher than that of colonies exposed to imidacloprid.

In the interaction between chlorothalonil and time, chlorothalonil exposure led to a significantly different number of returning non-pollen foragers at the conclusion of the exposure period (week 4) and again seven weeks after the conclusion of the exposure period (week 11). After four weeks of exposure, the median number of returning non-pollen foragers for colonies not exposed to chlorothalonil is estimated to be 1.3 times that of colonies treated with chlorothalonil (**Figure 2.4**). Seven weeks later, the median number of returning non-pollen foragers was also estimated to be 1.4 times higher in colonies not exposed to chlorothalonil than that of colonies that were exposed to chlorothalonil.

The interaction between imidacloprid, chlorothalonil, and time indicated that the median number of non-pollen foragers differed between treatment groups at week 0 (before the exposure period), week 10, and week 11 (**Figure 2.5**). Prior to treatment, the median number of returning non-pollen foragers in colonies assigned to chlorothalonil exposure was nearly half (54.1%) that of colonies assigned to exposure to both chemicals. At this same time point, control colonies and imidacloprid-only colonies were not significantly different from each other, nor were they different from chlorothalonil-only colonies and colonies assigned to exposure of both chemicals. These differences disappeared after a week of exposure, and were not different between treatments until week 10 and week 11.

At week 10, the median number of returning non-pollen foragers in colonies exposed solely to chlorothalonil was 1.6 times greater than that of colonies exposed to both chemicals. The chlorothalonil-only group was also not different from the control or imidacloprid-only group, and the colonies exposed to both chemicals were not different from the control or imidacloprid-only group. At week 11, there was a clear

synergistic interaction between chemicals in which colonies treated with both chemicals had significantly fewer returning non-pollen foragers than colonies in the control group, while colonies exposed to either chemical alone were different from neither the control nor colonies exposed to both chemicals. At that week, the median number of non-pollen foragers returning to the colonies in the control group was over double that of colonies exposed to both chemicals.

2.4.10. Total Foragers

In the model that contains all data points, there was no significant effect of imidacloprid ($F_{1, 24} = 0.960$, $p = 0.337$) nor chlorothalonil ($F_{1, 24} = 0.719$, $p = 0.405$) on the total number of foragers returning to the colonies. There was also no additional effect of the combination of both chemicals on total returning foragers ($F_{1, 24} = 0.047$, $p = 0.830$).

The number of total foragers returning to the colony differed significantly between weeks ($F_{12, 384} = 27.541$, $p < 0.0001$). However, the interactions between the chemicals and time were not significant (See **Appendix X2.**)

When outliers were excluded, the total number of returning foragers still differed significantly between weeks ($F_{12, 365} = 31.825$, $p < 0.0001$). The effect of imidacloprid ($F_{12, 365} = 3.368$, $p = 0.079$) and chlorothalonil ($F_{12, 365} = 2.987$, $p = 0.097$) became significant at the 0.10 alpha level. There was still no interaction between the two chemicals ($F_{1, 24} = 0.604$, $p = 0.445$), nor were there any interactions between the chemicals and time (**See Appendix X2**).

The effect of imidacloprid was such that overall, colonies not exposed to imidacloprid had an overall greater number of returning foragers than colonies exposed to imidacloprid (**Figure 2.6**). The same was true for the effect of chlorothalonil (**Figure 2.7**). We estimate that there were approximately 8.6 more foragers returning every 3 minutes to colonies not exposed to imidacloprid than those colonies exposed to

imidacloprid, and 8.1 more foragers returning every 3 minutes to colonies not exposed to chlorothalonil than for those exposed to chlorothalonil. Furthermore, based on pairwise comparisons, these differences did not exist between these groups prior to treatment.

2.4.11. *Varroa* Mite Infestation

When the outlier was included in the model, there was no effect of imidacloprid ($F_{1,24}$ = 1.093, p = 0.353), chlorothalonil ($F_{1,24}$ = 0.394, p = 0.536), or interaction between the chemicals ($F_{1,24}$ = 0.354, p = 0.558). At the 0.10 alpha level, *Varroa* mite infestations varied with time ($F_{3, 44}$ = 2.324, p = 0.088), but there were no interactions between the chemicals and time (See **Appendix X2**).

When the outlier was excluded from the model, all effects remained insignificant (see **Appendix X2**) except the effect of time ($F_{3, 43}$ = 2.878, p = 0.047).

2.4.12. *Nosema* Infection

We did not detect any significant effects of imidacloprid ($F_{1,21}$ = 2.287, p = 0.145), chlorothalonil ($F_{1,21}$ = 0.389, p = 0.540), or their interaction ($F_{1,21}$ = 0.400, p = 0.534) on the *Nosema* spore counts of colonies infected with *Nosema* throughout the experiment. The effect of time on *Nosema* infections was nearly significant at the 0.10 alpha level ($F_{3,31}$ =2.213, p = 0.106). However, there were no interactions between any of the treatments and time (see **Appendix X2**).

2.4.13. Hypopharyngeal Gland Protein Content

Hypopharyngeal gland protein content did not differ significantly between levels of imidacloprid ($F_{1, 24}$ = 1.050, p = 0.316) or chlorothalonil ($F_{1, 24}$ = 2.066, p = 0.164). It was also unaffected by the combination of both chemicals ($F_{1, 24}$ = 0.071, p = 0.792). Furthermore, there were no interactions between either of the chemicals, their

combination, and time (see **Appendix X2**). Furthermore, hypopharyngeal gland protein content was not significantly different between months ($F_{2, 64} = 0.372$, $p = 0.691$)

2.4.14. Prophenoloxidase Activity

The model containing outliers showed that prophenoloxidase activity was not significantly affected by imidacloprid ($F_{1,24} = 1.879$, $p = 0.183$) chlorothalonil ($F_{1,24} = 0.017$, $p = 0.897$), or their interaction ($F_{1,24} = 0.666$, $p = 0.423$).

Prophenoloxidase activity was significantly different between different months ($F_{2,64} = 20.076$, $p < .0001$). The interaction between chlorothalonil and time was significant at the 0.10 alpha level ($F_{2, 64} = 2.400$, $p = 0.099$), but the differences between groups were insignificant after the Tukey p-value adjustment. The interaction between imidacloprid and time was insignificant ($F_{2, 64} = 0.578$, $p = 0.564$), as was the interaction between the two chemicals and time ($F_{2, 64} = 0.092$, $p = 0.912$).

When the two outliers were removed, the interaction between chlorothalonil and time became significant ($F_{2,61} = 6.401$, $p = 0.003$). All other main effects and interactions remained insignificant (see **Appendix X2**), except for the main effect of time, which remained significant ($F_{2,61} = 34.963$, $p < 0.0001$).

The interaction between chlorothalonil and time was such that colonies exposed to chlorothalonil and unexposed to chlorothalonil were different from each other prior to treatment (month 0) and at the end of the exposure period (month 1). Prior to treatment, the median prophenoloxidase activity was estimated to be 1.3 times greater in colonies not exposed to chlorothalonil than in colonies that were exposed to chlorothalonil. At the end of the exposure period, the median prophenoloxidase activity in colonies exposed to chlorothalonil was estimated to be 1.4 times greater than that of colonies not exposed to chlorothalonil. The median prophenoloxidase activity in colonies exposed to chlorothalonil therefore decreased by a lesser degree

over the course of the exposure period than it did in colonies exposed to chlorothalonil (**Figure 2.8**).

2.4.15. Glucose Oxidase Activity

Neither imidacloprid ($F_{1, 24} = 2.333$, $p = 0.140$) nor chlorothalonil ($F_{1, 24} = 0.004$, $p = 0.952$) had a significant effect on glucose oxidase activity. There was no interaction between chemicals, nor did chemicals interact with time (see **Appendix X2**). The effect of time was also not significant ($F_{1, 24} = 1.901$, $p = 0.158$).

2.4.16. Abdominal Lipid Stores

The results differed slightly when the influential outlier was included in the analysis, versus when it was excluded. In the model that included the outlier, neither imidacloprid ($F_{1, 24} = 0.930$, $p = 0.345$) nor chlorothalonil ($F_{1, 24} = 0.654$, $p = 0.427$) had a significant effect on abdominal lipid stores. The interaction between the two chemicals also had no effect on abdominal lipid stores ($F_{1, 24} = 0.022$, $p = 0.883$), and there were no interactions between any of the chemicals and time (see **Appendix X2**). Abdominal lipid stores were, however, significantly different between months ($F_{2, 63} = 5.916$, $p = 0.004$).

In the model that excluded the outlier, neither imidacloprid ($F_{1, 24} = 0.690$, $p = 0.414$), chlorothalonil ($F_{1, 24} = 0.597$, $p = 0.447$), nor the combination of both chemicals ($F_{2, 62} = 0.086$, $p = 0.772$) had an effect on abdominal lipid stores. The effect of time remained significant ($F_{2, 62} = 5.977$, $p = 0.004$). The interaction between imidacloprid exposure and time also remained statistically insignificant, but came closer to significance at the 0.10 alpha level ($F_{2, 62} = 2.241$, $p = 0.115$). There was also no significant interaction between both chemicals and time ($F_{2, 62} = 1.875$, $p = 0.162$).

2.4.17. Midgut Proteolytic Enzyme Activity

Neither imidacloprid ($F_{1, 24} = 2.232$, $p = 0.148$) nor chlorothalonil ($F_{1, 24} = 0.077$, $p = 0.784$) had an effect on midgut proteolytic enzyme activity. There was also no significant interaction between imidacloprid and chlorothalonil ($F_{1, 24} = 0.205$, $p = 0.655$). Neither the effects of the chemicals on midgut enzyme activity nor their combination interacted with time (see **Appendix X2**). Midgut enzyme activity was significantly different between months ($F_{2, 64} = 34.514$, $p < 0.0001$), but this effect was not further explored due to the nature of the research questions.

2.4.18. Overwintering Survival

Overwintering survival was high in all treatments—only one colony in three of the treatment groups (control, imidacloprid and both chemicals) did not successfully overwinter.

2.5 Discussion

In this experiment, we examined how field realistic concentrations of imidacloprid and chlorothalonil in pollen affect various parameters of colony health. To our knowledge, this is the first open-field study that quantifies long-term effects of an interaction between a fungicide and a neonicotinoid at the colony level. This is also one of few studies to investigate causal relationships between chlorothalonil and declining colony health [34, 142], as opposed to correlative or observational relationships [58, 112, 141]. While our chlorothalonil concentrations turned out to be lower than intended, the concentrations quantified in the pesticide analyses are within the range that has been detected in bee bread and pollen in previous field studies [58, 112, 193], and therefore may still be characterized as field-realistic.

Chlorothalonil concentrations in this experiment remained consistently near 100 ppb, which is approximately 3% of our intended level. There are several potential

explanations for this result. The difficulties of working with chorothalonil are well-documented in analytical chemistry [194]. The QuEChERS method was used to quantify the chlorothalonil concentrations of our experimental pollen patties. Recovery rates from this method are typically below 70% [195]. Much of the chlorothalonil originally present in our pollen was also potentially lost during the analytical process, meaning that the concentration in the pollen patties fed directly to our colonies was likely higher than indicated by the analytical test. However, it is still likely that chlorothalonil was lost during the mixing and experimental process. Chlorothalonil is known to degrade quickly in acetone [196] and photodegrade in the presence of the organic matter [197-198], such as that of the pollen. Many previous experiments with chlorothalonil and honey bees have used acetone as a solvent [67, 130, 199-200], but have not investigated the stability of chlorothalonil in the solvent prior to experimentation. Given that our concentrations were far lower than intended, we suggest that target concentrations of cholorothalonil used in previous studies should be reassessed, if feasible. We also recommend that methods for achieving intended concentrations of chlorothalonil be confirmed prior to experimentation in future studies.

Chlorothalonil is also easily degraded by microbes [201], many of which are present in pollen [202-205] and may be inhibited by repeated fungicide applications [206-208]. Furthermore, whereas high fungicide loads reduce the microbial communities in bee bread and pollen [114], our experimental pollen contained no fungicides prior to pollen manipulation. Our pollen likely contained a wide array of microbes that quickly degraded the chemical and were not inhibited by our single application of chlorothalonil, and may have become even more active with the addition of sugar syrup [209-210]. This would explain why the pollen patties contained no detectable levels of chlorothalonil at the end of each week. Further research is needed to determine the best methods of achieving target concentrations of this compound.

In our experiment, honey bee colony size and brood area were not significantly affected by exposure to imidacloprid, cholorothalonil or the combination of both

chemicals. These results are similar to several other field studies, especially with respect to imidacloprid [51, 54, 81, 211]. We did not observe any periods in which there were no brood in the colonies, which differs from the patterns previously observed at concentrations of 20 ppb and 100 ppb [43]. Considering that our experimental concentration was within this range, it is possible that the concentration of imidacloprid was not the sole reason for breaks in the brood cycle in the previous experiment. For example, the subspecies used in that study was not disclosed. This may be potentially because of diet differences (see discussion of *Varroa* and *Nosema* results), but may also be due to differences in the experimental subspecies.

It is possible that the subspecies used in our experiment, *Apis mellifera ligustica*, may not be affected by these pesticides in the same way that other honey bee subspecies may be. Subspecies of honey bee may differ widely in longevity [212], metabolism [213], antioxidative physiology [213], and the quantity and quality of secreted brood food [213-214]. Differences in royal jelly secretions may influence the outcome of pesticide exposure in scientific studies by influencing detoxification abilities [215] and response to oxidative stress [145, 216-218]. Low levels of the glutathione-S-transferase (GST) detoxification enzymes have been detected in the brood food of multiple subspecies [214], but it is currently unclear whether they secrete significantly different amounts of this enzyme.

The potential for subspecies to influence the outcome of a pesticide experiment has been documented in some recent studies. Sandrock et al. [219] found that long-term changes in colony size and brood area in response to neonicotinoid exposure differed between *A.m. carnica* and *A.m. mellifera*. Effects on *A.m. ligustica* were not examined in that study. Another previous study also demonstrated that pesticide sensitivity varies between both honey bee subspecies, though this varies between pesticides [220]. Otherwise, studies determining how different honey bee subspecies respond to pesticide exposure are rare. Further research should be conducted to determine whether specific subspecies are more robust to long-term pesticide exposure than are others.

The results of our physiological assays support the findings of some previous studies, while contradicting others—even when field realistic concentrations of chlorothalonil were used. Similar to a previous experiment, we found that chlorothalonil exposure does not affect the lipid stores of nurse bees in experimental colonies [142]. The findings for hypopharyngeal gland-related analyses, however, may contradict previous findings that chlorothalonil reduces total protein content and increases glucose oxidase activity of nurse bees [142]. Previous data on the effects of chlorothalonil on prophenoloxidase activity are also mixed. Our results differ from the findings in which chlorothalonil did not affect prophenoloxidase activity in nurse bees or foragers bees in the field [142], but are in agreement with a laboratory experiment in which chlorothalonil increased transcription of prophenoloxidase-activating enzymes [130]. The contradiction of the field experiment may be due to our higher experimental concentration (~100 ppb instead of 10 ppb [142], and exposure through pollen diet instead of sugar syrup. Because nurse bees consume more pollen than they do sugar syrup [221], pesticides in pollen may pose a greater risk for nurse bees than do pesticides in nectar. Furthermore, because chlorothalonil concentrations are well-documented for pollen but virtually unknown in nectar, we can be more certain that studies pertaining to exposure through pollen are field-realistic. However, we recognize the need to quantify chlorothalonil concentrations in nectar and honey in order to gain a clearer understanding of field-realistic exposure through multiple exposure routes. Future research should also focus on determining the impacts of chlorothalonil when concentrations are at the higher end of the field-realistic spectrum.

We found that imidacloprid had no significant effect on activity of the immune enzymes, glucose oxidase, prophenoloxidase, and hypopharyngeal gland protein content. In a laboratory experiment, Alaux et al. [222] demonstrated a similar lack of effect on glucose oxidase activity, but previous findings on effects of imidacloprid on prophenoloxidase activity are mixed [222] [129]. Very few studies have examined the protein content of hypopharyngeal glands as it relates to imidacloprid exposure, but

some research has investigated how the effects of imidacloprid exposure might manifest in hypopharyngeal gland size—with mixed results [223-225]. It may be possible that smaller hypopharyngeal gland size does not necessarily translate into lower hypopharyngeal gland protein content in honey bees, but this is unlikely based on previous research [226-227]. We also may not have detected effects on hypopharyngeal gland protein content because our data is from an open-field experiment, not a laboratory experiment. The social component of honey bee biology that is incorporated into open-field experiments—including the presence of brood and a queen, which both alter worker bee behavior and physiology [226, 228-233]—may buffer the effects of pesticide exposure in a way that has not been discovered. Previous experiments explored effects at much lower concentrations than used in this experiment [222-225], but all were conducted in the laboratory, thereby leaving out critical elements of sociality that may mitigate such effects. Furthermore, we exposed our colonies to contaminated pollen instead of contaminated sugar syrup. Because worker bees tend to consume different amounts of pollen and sugar syrup based on their age [144, 221, 234-235], and because the times at which they consume different resources also coincides with changes in physiology [169, 236], the potential for pesticides to affect bees differently through different exposure routes should not be overlooked in experiments and risk assessments. Multiple exposure routes are frequently ignored in the current literature. With the exception of one experiment [41], laboratory experiments have largely determined the impacts of imidacloprid exposure through sugar syrup only [222-224]. Lastly, none of these experiments focused solely on *A.m. ligustica*, and only one included this subspecies (mixed with *A.m. mellifera*) [222]. Because hypopharyngeal gland size and number of secreted brood food proteins differ widely between specific honey bee strains and subspecies [213-214], it is important to note that the impacts of pesticide exposure on hypopharyngeal gland protein content may also differ between these subspecies.

Though the impact of imidacloprid exposure on abdominal lipid stores of nurse bees is not thoroughly explored in previous research, a previous experiment found that different genes involved in lipid metabolism were significantly upregulated and

downregulated in larvae from colonies that were exposed to an even lower concentration of imidacloprid in sugar syrup [237]. Low concentrations of imidacloprid also alter levels of antimicrobial peptides [129], which are produced in the abdominal lipid stores (the fat body). However, how these changes translate into actual changes in lipid stores is not yet clear. Additionally, another previous experiment determined that two metabolites of imidacloprid, which are more likely to impact brood in this experiment (see the discussion of effects on foraging), caused precocious foraging in bees as young as 8 days old [238]. Because precocious foraging is associated with reduced longevity [175, 239], and because precocious foraging occurs in conjunction with the early loss of abdominal lipids [169], further work is needed to confirm whether these effects take place at field-realistic concentrations.

Our findings contribute to the currently narrow body of knowledge pertaining to the effects these two chemicals on the honey bee midgut. One prior study determined that high concentrations of both chlorothalonil and imidacloprid increased honey bee midgut cell death [240], though their combined effects were not explored. This may be pertinent to midgut proteolytic enzyme activity, especially if cell death is increased in the digestive cells, which secrete proteolytic enzymes [241-242]. It has been previously suggested that midgut cell death in honey bees and digestive enzyme secretions are linked [243]. Degenerating and dying digestive cells in honey bees exhibit deteriorating microvilli, which are extensions of the cells that secrete digestive enzymes [166, 244]. Peritrophic membrane thickness and midgut wall thickness, which may also be impacted by cell death in the midgut, are also directly associated with proteolytic enzyme activity [166, 245]. Exposure to the pesticides boric acid and fipronil have been found to induce such changes [246], but the experiment that tested the effects of chlorothalonil and imidacloprid did not examine impacts on digestive cells [240]. The experimental concentrations in Gregorc & Ellis [240] were also as much as four times higher than those considered to be field realistic concentrations. Future research should continue to explore how various field-realistic concentrations

affect midgut physiology and proteolytic enzyme activity in order to better understand the range of concentrations that can affect digestive abilities.

We did not detect any significant effects of these two pesticides on *Varroa* mite infestation or *Nosema* infections. These results differ from those of previous studies that documented higher *Varroa* mite infestation in colonies exposed to imidacloprid in their pollen diets [43], and higher *Nosema* infections in bees that were previously exposed to imidacloprid [49] and chlorothalonil [112]. It is possible that we did not detect differences in *Nosema* infections because previous patterns that associated these chemicals with higher *Nosema* infections were detected in the laboratory. Laboratory research that examines the impacts of pesticides on *Nosema* infection typically involve feeding specific amounts of *Nosema* spores to bees, but it is unknown whether feeding bees known amounts of spores reflects *Nosema* transmission under field-realistic conditions. It is also important to note that our analyses were only conducted for colonies with detectable levels of these pathogens, and ignored colonies without detectable levels. Further analysis should be conducted to determine whether exposure to these pesticides not only increase *Nosema* and *Varroa* mite infestations, but also determine whether they increase the prevalence (presence or absence) of these pathogens among whole colonies. We should also not discount the role of our experimental pollen in potentially buffering the effects of these chemicals on pathogen levels. While our experiment utilized pure pollen patties to apply pesticide treatments, previous experiments applied treatments through protein supplement. The potential of these diet differences to influence the outcome of pesticide exposure is discussed further on page 88.

Research on possible interactions between these chemicals and the pathogens investigated in this experiment is direly needed. We did not explore these interactions in this experiment in order to conserve degrees of freedom and maintain the highest possible level of statistical power. However, it is worth mentioning that in a number of other laboratory and field experiments, *Varroa* or *Nosema* have been found to impact a number of related physiological and foraging parameters either alone [247-

253] or in synergy with pesticides such as imidacloprid [222, 254]. Thorough assessment of pesticide-pathogen interactions in the field is necessary for researchers to understand the complex mechanisms responsible for colony losses, but these assessments are severely lacking in current investigations [255-256]. Higher replication and complex statistical analyses will be required to quantify these interactions in a way that has not yet been achieved in previous experiments. We suggest that researchers and statisticians collaborate to develop these analyses and document the interactive effects of pesticides and pathogens in the field.

The effects of both imidacloprid and chlorothalonil on the number of non-pollen foragers were apparent only when outliers were excluded from the data in this experiment. Both of these chemicals affected numbers of non-pollen foragers at different weeks, and both chemicals also consistently reduced the number of returning non-pollen foragers seven weeks after the last exposure period. With outliers excluded, imidacloprid and chlorothalonil also reduced the total number of foragers returning overall, likely because of their effects on non-pollen foraging. However, these chemicals did not affect the number of returning pollen foragers.

The effects we detected in non-pollen foraging, seven weeks after the end of the exposure period suggests that larvae fed with chemical contaminated pollen were affected as adults. This is consistent with previous findings that imidacloprid significantly impacts the olfactory behavior of adults exposed to imidacloprid as larvae [127], and significantly alters the expression of genes related to flight performance, metabolism and longevity in exposed larvae [237]. Imidacloprid itself has only been detected in brood food when imidacloprid is at much higher concentrations—100 ppb—in the pollen diet of the nurse bees [43]. Based on the rapid pace at which imidacloprid is metabolized in adult honey bees and the presence of its metabolites in the honey bee head soon after oral exposure [257], it is more likely that metabolites, rather than the active ingredient, are causing these effects. Dively et al. [43] did not detect any imidacloprid metabolites in brood food after exposing colonies to protein diet spiked with imidacloprid, but noted this may be due

to the high level of detection for those metabolites. Although previous work has estimated how much imidacloprid different workers consume within a specified period of time [221], there are currently no such estimations for the amount of metabolites consumed by worker larvae, and no data on the presence of chlorothalonil metabolites in brood food.

It is possible that the cumulative exposure to these pesticides via pollen diet impacted our colonies at both the larval and nurse bee stages, which may have been exacerbated by exposure to multiple generations of bees. Larvae consume pollen for 5 days, and nurse bees consume pollen for a minimum of 8 days [221], but experiments have thus far only determined how exposure affects larval [67, 105, 258] and adult bee mortality [36, 105] separately, but has thus far neglected cumulative impacts of consumption of these pesticides at more than one of these stages. This might lead us to underestimate the effects pesticide exposure on a single generation of bees. However, whether bees in this experiment were affected by pesticide exposure differently at different stages is unknown. Further research should seek to determine if this is the case.

It is important to note that while adult bees fed with contaminated pollen were affected as non-pollen foragers, this was not necessarily true for all generations of adults throughout the experiment. Based on the 21 days it takes for worker bees to develop from egg to adult [259] [260], and the 2 to 3 weeks after adult emergence to begin foraging activities [259], the effects of pesticides in pollen could have taken place any time after week 5 to week 7, when the first generation fed on treated pollen likely began to forage. Based on this same timeline, workers that were larvae in week 4 began foraging during week 10. When outliers were excluded from the model, significant interactions between chemicals did not exist until week 10-11, which indicates that pesticide exposure may not have affected all generations equally. While the life cycle of the worker bee, as well as the effects we observed particularly seven weeks after the end of the exposure period, suggest that the final generation of larvae exposed to the chemicals was the only generation to be affected by the exposure to

these chemicals, whether that is true is not yet certain. Depending on whether exposure to these chemicals also impacted developmental time, the onset of foraging, and longevity, which would all play a role in which generations were foraging at that time [175, 228, 261-262], multiple generations may have been affected at that time. Because we did not measure the exact dates of emergence, the time of foraging onset, or the longevity each generation, we cannot conclude whether or not this was the case. Impacts on precocious foraging and adult longevity may be a critical mechanism by which agrochemical exposure reduces colony mortality in the long term, since both of these are directly linked to colony mortality [175, 263-264]. However, there is a dearth of information on these effects, and hence we urge that future research should explore these mechanisms.

Until we are able to make conclusions about generation-specific impacts, we suggest that pesticide exposure reaches a critical limit after a certain duration of exposure that leads to reduced foraging success and potentially the long-term weakening of colonies. Previous research has established some time-cumulate effects of pesticides on adult worker bees [57, 265-266], but there are currently no such data for entire colonies. It is possible that pesticide exposure may only negatively impact honey bee colonies, especially in terms of successful nectar foraging, only after several generations of nurse bees or young larvae are exposed (versus only a single generation). The duration of pesticide exposure, and in turn, the number of generations of exposed workers, may be a critical component of the proposed overflow of the pesticide "risk cup" [267] for honey bees. We suggest that further research should be conducted to determine the critical duration of pesticide exposure that makes colonies susceptible to different individual pesticides and their combinations, as well as the specific number of generations that may be exposed to these chemicals without the colony being ultimately adversely impacted.

While our results pertaining to the effects of imidacloprid on foraging contribute to existing knowledge, the findings regarding negative effects of chlorothalonil on foraging are novel. Because chlorothalonil is relatively non-toxic to bees, it is

possible that its effects on foraging result indirectly from its effects on pathogens such as *Nosema*. Previous findings have linked both chlorothalonil and imidacloprid exposure to higher *Nosema* infection [49, 112], which also impairs foraging abilities and worker survival [253, 268-270]. We did not detect such patterns in this experiment, but this may be due to the sampling methods we used to quantify *Nosema* infection. We quantified *Nosema* in bees that were collected from comb area with open brood, and were therefore more likely to be nurse bees. Because forager bees typically have higher *Nosema* loads than nurse bees [271-272], our findings may have been different if we had specifically sampled forager bees from our experimental colonies. Further research should be conducted to determine whether these chemicals affect *Nosema* loads specifically in forager bees, therefore better capturing interactions that impact foraging success.

The discrepancies between our results for non-pollen foragers and pollen foragers may be due to differences in further pesticide exposure during foraging activities. Nectar foragers are estimated to consume much greater quantities of nectar and systemic insecticides than do pollen foragers [221]. It is possible that bees exposed to these chemicals at the larval or nurse stages may be more sensitive to pesticide exposure during foraging activity. Field-realistic levels of neonicotinoids in nectar have already been demonstrated to impair foraging abilities even when bees are not exposed to neonicotinoids at earlier stages [98-100, 128, 273-274]. Given our findings, these effects may be further exacerbated by pesticide exposure at earlier stages. Other larval conditions, such as rearing temperature, have also been found to affect susceptibility to pesticides at the adult stage [275], and it is not unreasonable to speculate that other larval stressors may do the same. However, whether or not larval or nurse bee pesticide exposure affects pesticide susceptibility at later stages has not yet been explored.

Our total and non-pollen forager findings have potential implications for the amount of honey collected by the colony, although that was not the case in this experiment. Although the number of returning non-pollen foragers were significantly lower in the

treatment group exposed to both chemicals than in the control group at week 11, honey stores did not show the exact same differences. Our results are similar to Dively et al. [43], in which imidacloprid exposure from May to August led to 12% lower rates of returning foragers in August and September, but did not lead to significantly different honey stores. This may be because the effects of these chemicals appeared at a time after which the majority of the honey flow had passed. If our exposure period had been earlier in the season, and the reduction in non-pollen foragers took place during the honey flow, it is possible that honey stores would have also differed between the treatments. It is necessary to conduct similar research to determine whether agrochemical exposure during the spring (the season during which the most pollination takes place) leads to significant differences in honey stores. Such work is critical for researchers to establish the link between pesticide exposure, poor overwintering abilities, and starvation, which beekeepers frequently rate as top culprits of colony loss [14-15, 19, 39]. Bee sensitivity to pesticides also differs between seasons [276], which further stresses the need for spring experiments.

It is important to note that the effects we detected when outliers were removed from the forager analysis were during the weeks from which the most outliers were removed. It may be argued that the reason we detected significant effects during these weeks was because removing the outliers led to abnormally small variances, and incorrectly determined significant effects [277]. However, the findings from our analysis without outliers also substantiate previous findings that in the fall, fewer foragers returned to colonies exposed to imidacloprid through pollen diet during the summer time [43]. Furthermore, the confidence intervals for these effects demonstrated distinct differences among groups, which demonstrates the magnitude of these effects better than p-values alone [183-187]. For example, considering that the confidence interval for the group exposed to both chemicals remained outside of the confidence interval for the control group during week 11, we have reason to believe that these effects are real. Failure to address the presence of abnormally large outliers may mask the true long-term effects of pesticide exposure, which likely leads to misleading conclusions and ineffective management recommendations.

As demonstrated in our analyses, outliers may play a critical role in our ability to detect detrimental effects of pesticides. Extreme outliers substantially increase the probability of Type II errors and reduce the power of statistical analyses [188, 278], but whether or not outliers were present in previous field data is not known [43, 51, 77, 81, 211]. If outliers and other deviations from statistical assumptions are ignored, the statistics generated from such analyses will lead to the erroneous conclusions [189]. We emphasize the need to be clear about the presence and handling of outliers in this type of data, especially since most field experiments already have low statistical power [279].

Statistical power also may have played a critical role in our ability to detect the effects of these field realistic concentrations of chemicals on honey bee colony health. Cresswell [279] found that only 50% of field experiments had high enough statistical power to detect adverse effects of pesticides. While our experiment utilized higher replication than most other field experiments [47, 51, 54, 81, 211, 219, 280] and minimized variation present in other experiments by keeping all colonies in the same apiary and using sister queens to minimize genetic variability [43, 47, 51], our replication is still lower than is typically recommended to detect effects [187, 189]. Statistical power may not have been high enough to detect effects on one or more of these response variables, especially if such effects led to slight differences. Our statistical power may have prevented us from detecting significant effects and interactions for responses where p-values were considerably low, but not low enough to be considered statistically significant at either the 0.10 or 0.05 alpha level. We suggest that researchers conduct further studies that maximize replication, minimize variation and background noise. It is possible that an experiment with higher replication would have more precisely estimated the distribution of non-pollen foragers, reduced the presence of outliers in the data, and more accurately estimated differences between groups. Future studies should seek to identify an optimal number of replicates to detect these effects. However, it is also important to note that experiments with higher replication are near impossible to do if it is critical to take all

measurements and samples on the same day, or within the same time frame. Some of the parameters we estimated, such as colony size and food stores, took up to 2 days to survey all 36 experimental colonies.

While we did not detect significant effects on many of the response variables we explored, we also recognize that these results apply to a relatively narrow range of conditions. Our experimental colonies faced no migration stress, were fed pure wildflower pollen with few residues of other pesticides, and were closely monitored and managed to prevent starvation and damaging levels of *Varroa* mites. The pollen diet supplied to our colonies in this experiment may have played a particularly important role in many of our findings. Diet quality influences many of our response variables, including glucose oxidase and prophenoloxidase activity [147, 281], abdominal lipid stores [147], hypopharyngeal gland size and protein content [282-283], colony development and survival [283], and midgut enzyme activity [282-283]. It has also been found to increase adult longevity [145, 284], increase tolerance to *Nosema* and *Varroa* [281, 285], and reduce worker bee susceptibility to pesticides [286]. Though we did not measure the specific protein content, amino acid profile, or diversity of the pollen used for our experimental patties, we do not dismiss the possibility that this pollen may have played a role in these results. Previous colony-level experiments applied pesticide treatments through pollen supplement [43, 49], which may vary significantly in terms of protein content [287] and may influence physiology or resistance to pesticides. It is possible that the pollen diet supplied to our colonies may have masked the potential impact of pesticides infused into it. We also acknowledge that our results may have differed if more pesticide residues were present in our experimental pollen. For these reasons, we do not conclude that our results are the all-encompassing answer to whether imidacloprid and chlorothalonil affect our parameters of interest. Rather, they demonstrate that these chemicals have limited effects on colony health when other conditions are optimal for colony health and survival.

In conclusion, this experiment provides a better foundation for the design and analysis of future field experiments investigating effects of pesticides. Further work is needed on interactions between chemicals, especially since so many different chemicals have been detected in hive matrices [58, 112, 193]. We emphasize the need for further research that 1) quantifies interactions between pesticides and pathogens through complex high-level statistical analyses, 2) maximizes statistical power and addresses the role of outliers in related statistical analyses, and 3) quantifies the effects of chemicals over a longer time span in the life of honey bee colonies.

2.6. Conclusions

To our knowledge, this is the first experiment to quantify interactions between a neonicotinoid insecticide and a fungicide on entire colonies, and the first experiment to track the impacts of an agrochemical interaction over the course of several months.

Our results provide a more comprehensive understanding of both pesticide interactions and the various ways that sublethal pesticide exposure may influence colony health and survival. The effects we have detected in this experiment, especially those that pertain to foraging activity and prophenoloxidase activity, support the need for pesticide experiments that reflect long-term pesticide exposure and delayed effects. Our results stress the need for the following: 1) further experiments that quantify interactions between agrochemicals, pathogens, nutrition and potentially genetics, 2) studies that quantify residues of pesticides and their metabolites in both bee bread and brood food during the pollination of different crops, and 3) thorough explanations of statistical analyses, especially with regard to statistical power and handling outliers. While researchers have emphasized a particular need for research on pesticide and pesticide-pathogen interactions, the current research continues to be limited by short study durations, low replication and statistical power, single-chemical treatments, and a potential underestimation of pesticide exposure in the field.

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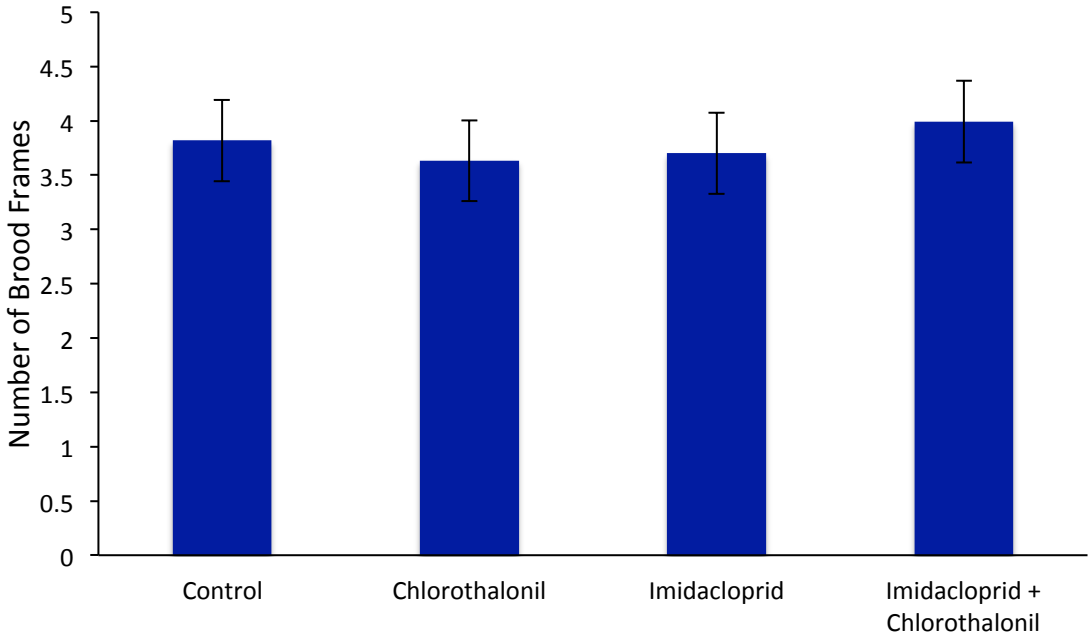

Figure 2.1 Mean brood area (+90% Tukey-adjusted confidence intervals) by treatment overall, averaged across the entire experiment, with outliers excluded.

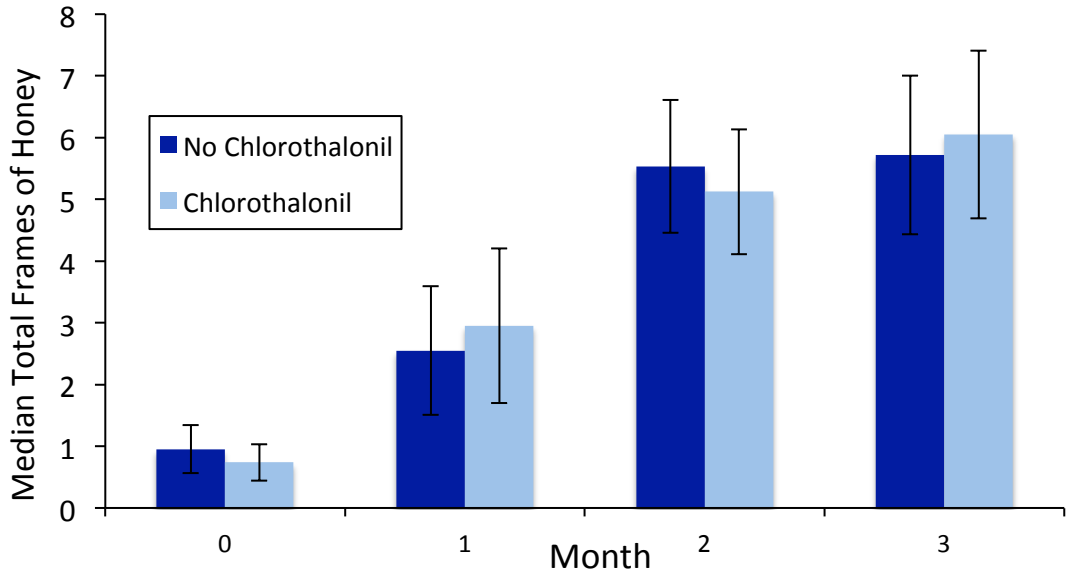

Figure 2.2 Median total frames of honey (+ 90% Tukey-adjusted confidence intervals) for colonies exposed to chlorothalonil and not exposed to chlorothalonil each month (4 months total), outliers excluded.

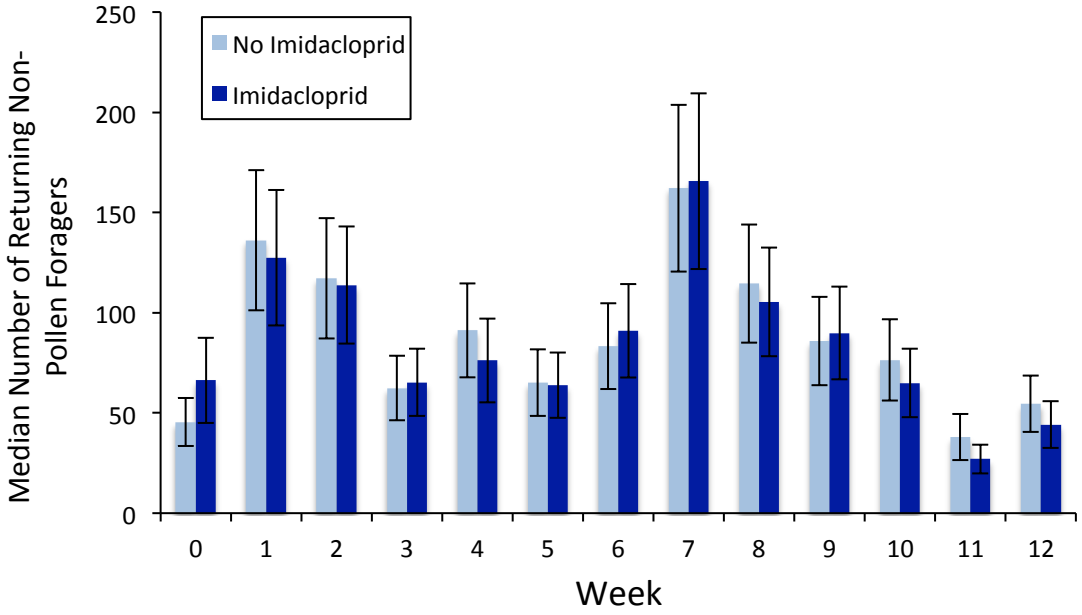

Figure 2.3 Median number of returning non-pollen foragers (+ 95% Tukey-adjusted confidence intervals) for colonies exposed to imidacloprid and not exposed to imidacloprid each week (13 weeks total), with outliers excluded.

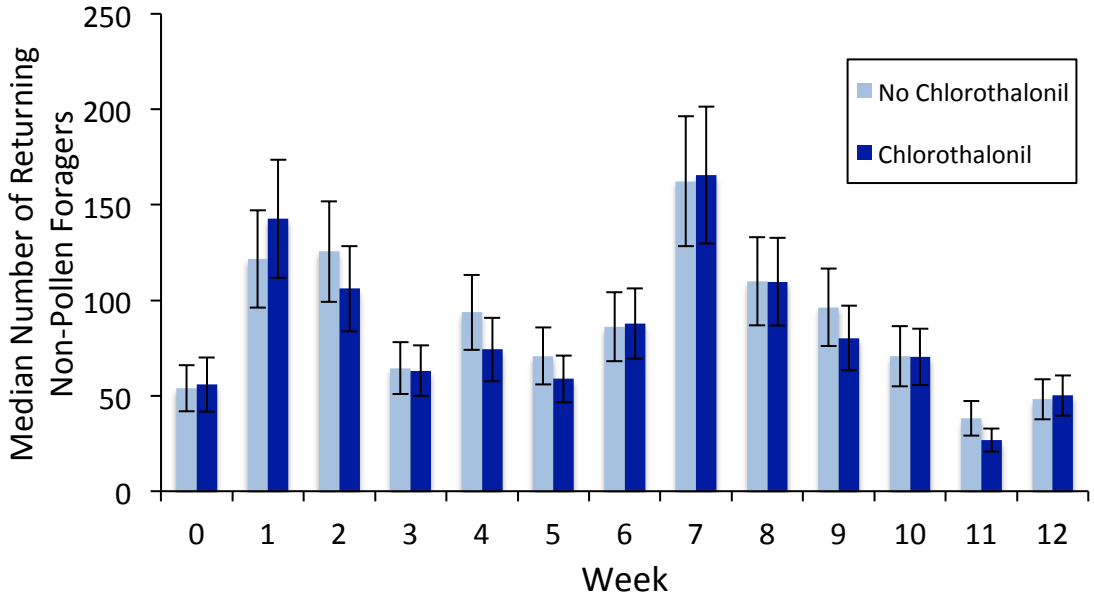

Figure 2.4 Median number (+ 90% Tukey-adjusted confidence intervals) of returning non-pollen foragers for colonies exposed and not exposed to chlorothalonil each week (13 weeks total), with outliers excluded.

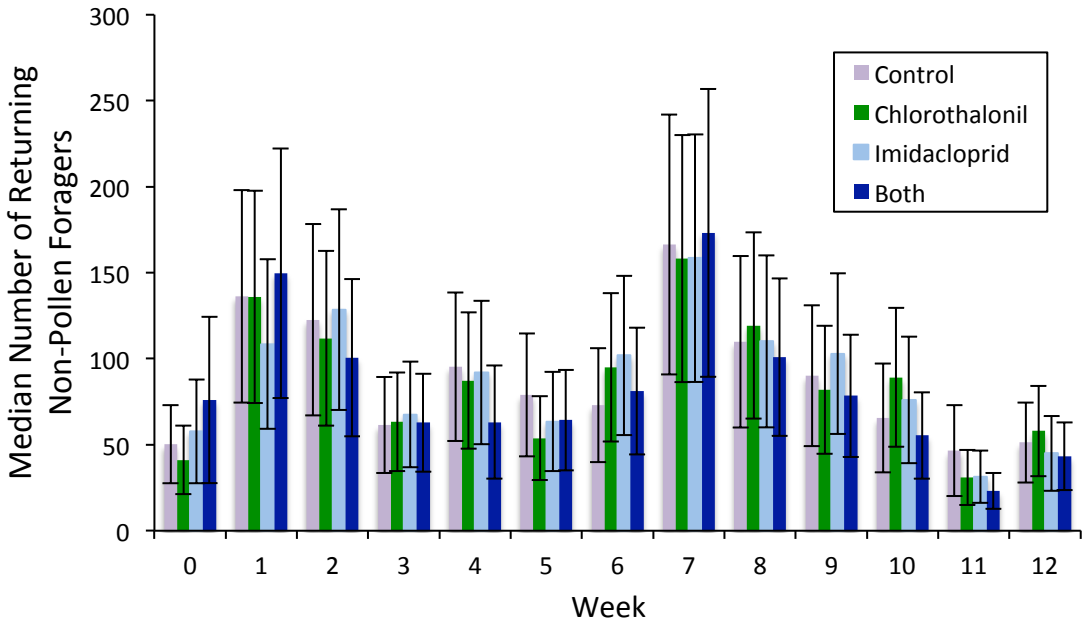

Figure 2.5 Median number of non-pollen foragers (+ 95% Tukey-adjusted confidence intervals) returning to colonies in all treatments each week (13 weeks total), with outliers excluded.

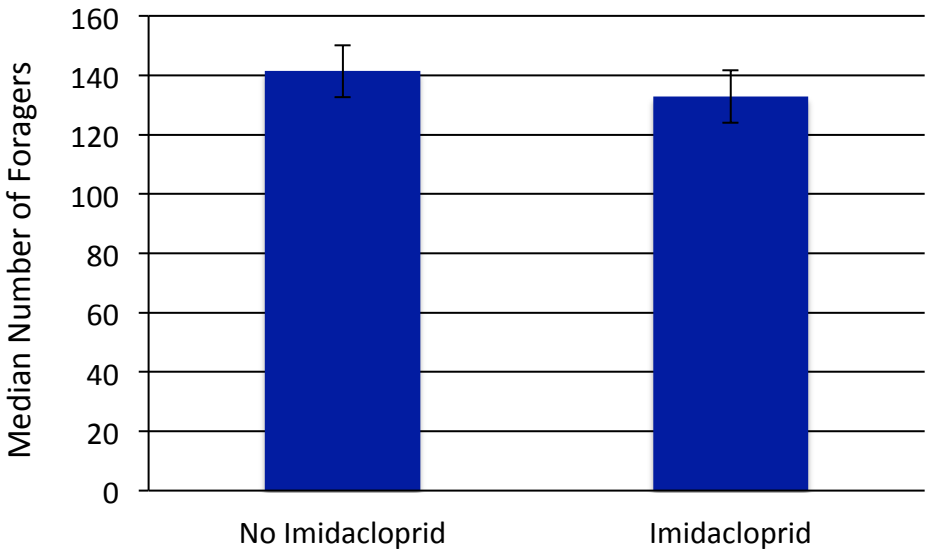

Figure 2.6 Median (+ 90% Tukey-adjusted confidence interval) number of total foragers returning to colonies exposed and not exposed to imidacloprid, averaged across all weeks.

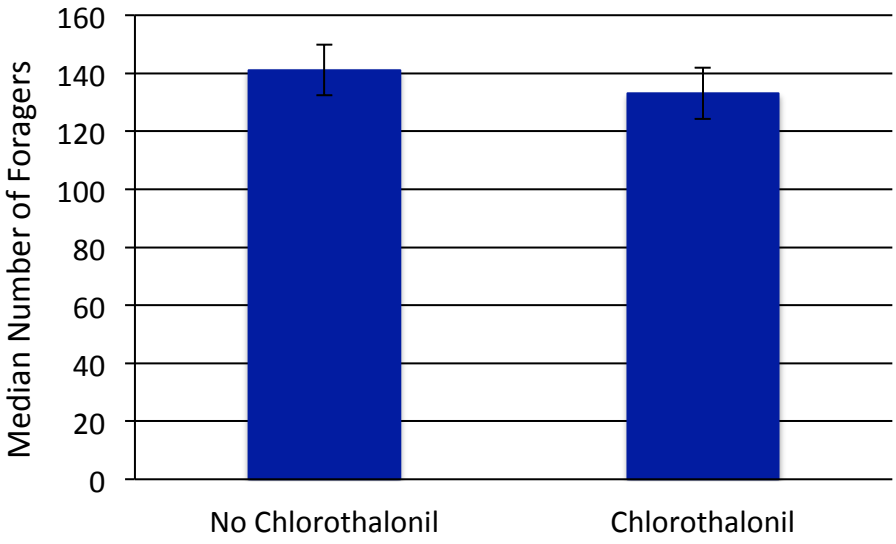

Figure 2.7 Median number (+ 90% Tukey-adjusted confidence interval) of total foragers returning to colonies exposed to chlorothalonil and not exposed to chlorothalonil, averaged across all weeks.

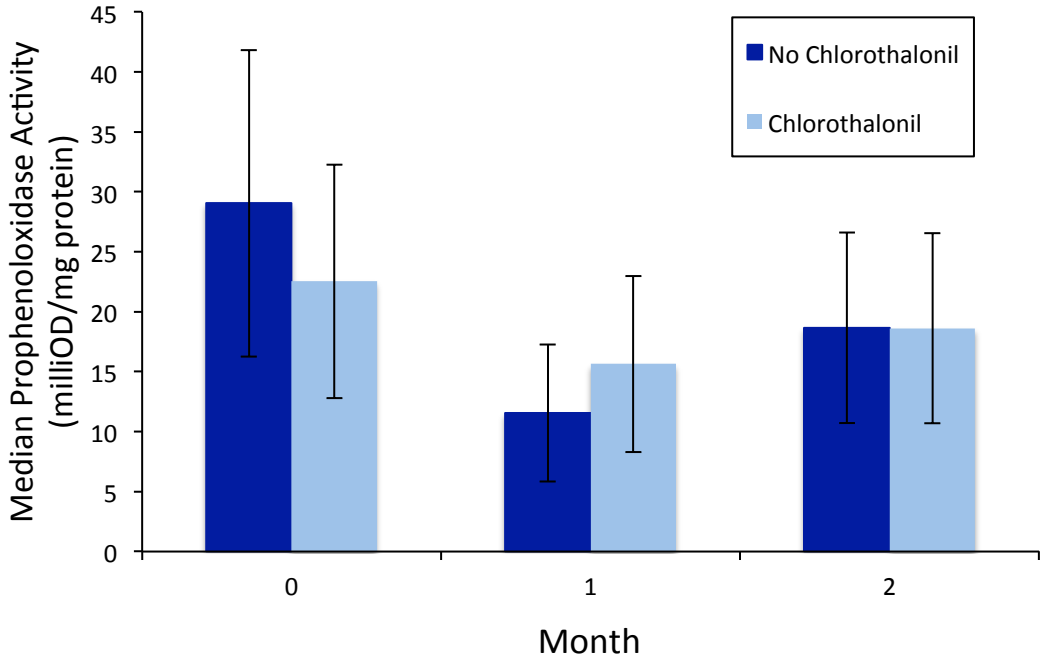

Figure 2.8. Median prophenoloxidase activity (+ 90% Tukey-adjusted confidence intervals) of nurse bees in colonies exposed to chlorothalonil and not exposed to chlorothalonil, with an outlier excluded.

Chapter 3: Impacts of a Neonicotinoid (Imidacloprid) on Honey Bee Mortality and Physiology: Effects of Multiple Exposure Routes

3.1. Abstract

Pesticides, particularly neonicotinoids, are currently under intense public scrutiny for their potential involvement in honey bee colony losses. While most previous research has not directly linked colony losses to neonicotinoid exposure, the ways in which neonicotinoid exposure affects colony health and survival are still poorly understood. Many of these studies have failed to capture the complexity of neonicotinoid exposure in the field, as they have overlooked the effects of neonicotinoid exposure through multiple routes. In this experiment, we examined whether exposure to the neonicotinoid imidacloprid at concentrations previously detected in stored pollen and nectar had (1) detrimental effects on worker physiology and mortality, (2) whether these effects were significantly different when bees were exposed through both routes as opposed to a single route and (3) whether these effects differed over time. The physiological responses we tested included glucose oxidase activity, prophenoloxidase activity, abdominal lipid stores, hypopharyngeal gland protein content, and midgut proteolytic enzyme activity. We also tracked the consumption of pollen and sugar syrup throughout the experiment.

Glucose oxidase activity and midgut proteolytic enzyme activity were differentially affected by the treatments. Midgut proteolytic enzyme activity was significantly higher in bees exposed to imidacloprid through sugar syrup when compared to bees not exposed to imidacloprid through sugar syrup. When outliers were included in the model, glucose oxidase activity was higher in bees exposed to imidacloprid through sugar syrup than it was for bees in cages not exposed to imidacloprid through sugar syrup, most notably after 4 weeks of exposure. Without outliers, there was an interaction between exposure through both routes and time, in which bees exposed to imidacloprid through both routes had significantly higher glucose oxidase activity than those exposed through sugar syrup alone.

Our mortality analysis indicated that a significantly fewer bees survived in the treatment group exposed to imidacloprid via both routes when compared to the group exposed only through sugar syrup, and fewer bees survived on Day 23 in the treatment group exposed to imidacloprid through pollen diet than those that were not exposed through pollen diet. Effect sizes, however, were very small, and may not be biologically significant to colony survival.

Bees exposed to imidacloprid through pollen diet consumed more pollen and sugar syrup, on average, than bees that were not exposed to the chemical through pollen diet. Bees exposed to imidacloprid through sugar syrup also consumed significantly less sugar syrup than bees exposed to imidacloprid through both routes, a pattern that was strongest on Day 29 of the experiment. Differences in food consumption likely played a significant role in our findings, and may have long-term consequences for resource depletion and overwintering starvation in whole colonies.

Our results indicate the need for further laboratory (and field) research that examines the impacts of neonicotinoids and other pesticides when bees are exposed to these chemicals through more than one route. Our results also further highlight the need for studies longer than two weeks in duration, as well as the need to better understand the influence of outliers in pesticide research, and the need to develop better statistical methods for analyzing survival of bees grouped into cages.

3.2. Introduction

Honey bee pollination contributes to $17 billion in crop production in the United States each year [1]. Dependence on bee pollination has grown 50% in developed countries and 62% in developing countries from 1961 to 2006 [2], but beekeepers have experienced unsustainably high rates of colony loss in the last decade [3-9]. Though colony losses have not yet led to pollination shortages overall [10], pollination and food production will be threatened in the long term if they continue [11-13]. Researchers generally agree that there is no single cause or solution to this

problem [14-17]. Multiple factors—such as parasites, viruses, poor nutrition, and pesticides—interact to weaken and kill honey bee colonies over an extended period of time [18-26]. It is critical that researchers determine how these factors contribute to colony loss in the long term in order to maintain the stability of honey bee pollination services and agricultural yields.

Pesticides, particularly those of the neonicotinoid class, have come under scrutiny for their potential involvement in colony losses and collapse [27-30]. Neonicotinoid use in the United States increased rapidly before colony losses became a widespread problem [31], but scientists frequently dismiss claims that neonicotinoids play a critical role in colony declines [16, 29, 32-33]. These chemicals have been demonstrated to adversely impact honey bee foraging abilities [27, 34], learning and memory [35-37], motor function [38], and susceptibility to *Varroa* mites and various other pathogens [33, 39-43]. They have also been linked to declines in various other wildlife, including aquatic invertebrates [44-45], birds [46], and native bees [47-50]. Some researchers have argued that neonicotinoids have not been identified as a cause of colony losses because experiments are poorly designed or do not account for the many complex ways by which bees are exposed to neonicotinoids [28, 51].

Research pertaining to the impact of neonicotinoids on honey bee colony health has largely ignored the potential effects of exposure through multiple routes [52-53]. Bees may be exposed to pesticides through pollen [23, [54-61], nectar and honey [56, 60, 62-64], wax [23, 65-67] and water [68-69] that is brought back to the colony, as well as via neonicotinoid-contaminated dust [53, 70-71]. However, laboratory and field experiments have thus far only examined effects through one exposure route at a time, usually via pollen diet [30, 33, 72] or sugar syrup [35, 73-78]. Because worker bees tend to consume different amounts of pollen and sugar syrup based on their age [79-82], and because the times at which they consume different resources correspond with changes in worker physiology [83-85], worker bees may be differentially affected by pesticide exposure through different routes at different stages.

While there is extensive literature on the effects of neonicotinoids on honey bee workers, most of the literature is specific to effects on foragers [27, 74, 86-88]. Focusing only on forager bees excludes potential impacts on other bees that are vital for colony growth and development, such as nurse bees. Nurse bees play a critical role in the colony, by both raising brood and tending to the queen [89-90]. These bees also serve as the next generation of foragers, as most adult workers become foragers after 2-3 weeks of work inside the nest [91]. It is therefore necessary to fully understand the impacts of pesticide exposure on younger bees.

Because nurse bees consume both nectar and bee bread [92-94], a form of stored, in-hive pollen that includes both pollen and honey [94-95], they may be particularly impacted by exposure through multiple routes. Rortais et al. [82] estimated that between consumption of nectar and stored pollen, nurse bees in particular consume approximately 4 times more imidacloprid (one of the most widely used neonicotinoids [96]) over a 10-day period than do forager bees. Thus, nurse bees may face a different threat from multiple routes of neonicotinoid exposure that has not been adequately addressed in the current literature.

The neonicotinoid concentrations to which bees (particularly nurse bees) are exposed in the field have also been discussed with a very narrow focus. Many researchers argue that very low concentrations are present in the nectar and pollen of plants [33, 56, 97]. However, neonicotinoids tend to be more concentrated in nectar and pollen stored inside bee colonies than in the nectar and pollen of flowers [23, 63]. Furthermore, most of the data on neonicotinoid residues in nectar and pollen only pertain to seed treatment—particularly in corn, canola and sunflower [32, 41, 56, 98-102]. While seed treatment is the most common use of neonicotinoids [96], these chemicals are also used in foliar applications [103-105], soil and irrigation applications [55, 105-108], and basal trunk injections [107, 109-112], which lead to demonstrably higher concentrations in the pollen and nectar of crops [55-56, 60, 105-106, 113]. In this experiment, we investigated the effects of imidacloprid

concentrations previously detected in citrus trees treated with a soil application of imidacloprid [63].

The persistence and mobility of neonicotinoids may also influence the concentrations to which bees are exposed in the field. The half-lives of neonicotinoids may be as long as 1000 days [106, 114], and high concentrations may remain in the tissue of treated trees for longer than 4 years after a single application [112]. Neonicotinoids have also been demonstrated to accumulate in plant tissues when the chemicals are applied more than once [97, 115]. Additionally, the water-soluble characteristics of neonicotinoids make them highly mobile in the environment [116] [117], allowing them to move into previously untreated areas. Researchers argue that access to untreated, alternate forage dilutes the potential effects of neonicotinoid exposure [118]; but this hypothesis is unlikely to be true as neonicotinoids are detected in untreated fields and wildflowers that provide alternative forage to bees [52, 58-59, 119]. Botias et al. [58] found that 97% of the neonicotinoids that honey bee foragers brought to their colonies were from untreated wildflowers, and not from the treated crops. As more neonicotinoids are routinely applied to crops, forests [107-110, 120], and residential gardens [121-122], their environmental residues pose a persistent threat to honey bees and other pollinators. Because these chemicals are becoming more widespread in the environment, it is critical to examine their effects based on the assumption that no uncontaminated forage is available for bees.

While there are many different types of neonicotinoids, imidacloprid is one of the most widely used pesticides in the world, second only to glyphosate in global pesticide sales [96]. It currently comprises 41.5% of the neonicotinoid market and is registered for 140 different crop uses [104]. Imidacloprid is widely used on various bee-pollinated crops, including pome fruits, stone fruits [123], cucurbits [55], berries [124-125], and citrus [115, 126-127].

Whether or not imidacloprid plays a significant role in honey bee colony losses is highly contested. Its use in the seed treatment of canola is directly linked to higher

colony losses in England and Wales [102], but scientists claim that field experiments in the United States do not directly link this chemical to colony mortality [33, 56]. Sublethal, field-realistic exposure to imidacloprid has been demonstrated to increase susceptibility to *Nosema* [72], increase *Varroa* mite infestations in honey bee colonies [33], alter ability to carry out foraging tasks [34, 74, 128], decrease immunity [129], alter gene expression [77, 130-131], and promote viral replication [39] in honey bees. Imidacloprid is the most well-studied of all the neonicotinoids [101], making up 78% of the neonicotinoid research on honey bees [101]; but much is still unknown about how it increases colony susceptibility to pathogens [43, 132]. It may be possible that imidacloprid drives colony losses by impacting immunity, or otherwise affecting bee physiology in a way that increases susceptibility to pathogens. Here, we examine the effects of imidacloprid exposure on multiple physiological responses that may influence susceptibility to pathogens in worker bees.

The degree to which bees are exposed to imidacloprid in the field is still not well understood. Mullin et al. [23] found that imidacloprid was only present in 2% of the bee bread samples in North American colonies, casting doubt on whether it poses a serious risk to honey bee colonies. In another study, however, it was the 4th most frequently detected pesticide in dead honey bees collected in citrus and stone fruit orchards, and was detected at concentrations as high as 223 ppb [133], which is over twice the chronic LC_{50} for imidacloprid [134]. It is also detected far more frequently in corbicular pollen samples than in bee bread [54, 58, 135], and at concentrations that are more hazardous to bees than those of all other chemicals, except the organophosphate phosmet [54]. Imidacloprid concentrations are also higher in stored pollen and nectar than they are in flower nectar and corbicular pollen. Mullin et al. [23] also determined that imidacloprid concentrations in bee bread averaged 39 ppb, a concentration higher than those previously demonstrated to induce sublethal effects on individual honey bees [74] and entire colonies [33], and Byrne et al. [63] determined that concentrations in stored nectar average 30 ppb when bees are restricted to foraging only on citrus crops that are soil-treated with imidacloprid, but may also be as high as 55 ppb.

In this laboratory experiment, we used neonicotinoid concentrations that reflect the conditions under which bees forage in citrus orchards treated with soil applications of imidacloprid, without access to uncontaminated forage. We exposed bees to imidacloprid through pollen diet, sugar syrup, or both resources. Our research questions were: 1) is bee mortality and physiology adversely affected by concentrations of imidacloprid in pollen and sugar syrup previously detected in bee bread and stored nectar? And 2) does imidacloprid exposure through both routes further exacerbate these effects? The physiological responses examined in this experiment were directly related to colony fitness, especially in terms of nutritional status and immunocompetence: 1) prophenoloxidase activity, a measure of potential phenoloxidase activity [136] that may be used to resist an immune challenge such as wounding [137-138] or bacterial pathogens [139]; 2) glucose oxidase activity, which indicates the antimicrobial potential[140] of brood food secreted by nurse bees [141-142]; 3) midgut proteolytic enzyme activity, a measure of ability of nurse bees digest protein in pollen [79, 143-144]; 4) hypopharyngeal gland protein content, an indicator of the quality of secreted brood food [145]; and 5) abdominal lipid stores, which mediate the transition from nursing tasks to foraging [84, 146-147] and produce antimicrobial peptides [148-149]. This is the first known experiment to quantify the effects of pesticide exposure through different routes on worker bee mortality and physiology.

3.3. Methods

3.3.1. Experimental Design

This experiment was carried out using a 2-factorial completely randomized, blocked design with 8 replications (1 replicate = 1 cage). Cages were housed in a Percival I-36VL biological incubator throughout this experiment. Each row of 4 cages (1 cage from each treatment) within the incubator was considered a block, and cages for each treatment were randomly assigned to a place in each block. The factorial design

consisted of four treatments: (1) control (no imidacloprid in either sugar syrup or pollen diet) (2) imidacloprid in pollen diet only (3) imidacloprid in sugar syrup only and (4) imidacloprid in both pollen diet and sugar syrup.

3.3.2. Experimental Setup

We used newly emerged adult worker bees collected from frames of capped brood from thirteen colonies at an Oregon State University apiary (Corvallis, OR, USA). On May 12, queens (all of which were *Apis mellifera ligustica* sisters) were confined to a single empty frame in their respective colonies for 48 hours using modified queen excluders that encased the entire frame. Twenty days later (June 1) these frames, then containing capped brood, were collected from the colonies and incubated in the laboratory at 55% relative humidity and 33ºC for 24 hours.

On the following day (June 2), newly emerged workers from all frames were combined into a large plastic tub and mixed thoroughly by hand to minimize any potential genetic variability. Then 350 bees were placed in each of the 32 cylindrical, wire cages (3069 cm^3). Cages were supplied with 100 mL of sugar syrup, 100 mL of deionized water, and a pollen patty, and returned to the incubator under simulated hive conditions (33 ºC, 55% RH, complete darkness, one strip of artificial brood pheromone (Superboost®), and one stick of artificial queen pheromone (Pseudoqueen®). The experiment began on June 2, 2015 and ended on June 29, 2015.

3.3.3. Sugar Syrup Treatment and Fluid Consumption

The intended concentration of imidacloprid in sugar syrup was 30 ppb, the mean concentration of imidacloprid from a flight tunnel study in Byrne et al. [63], the only study to have quantified concentrations of imidacloprid in in-hive nectar during pollination of an imidacloprid-treated orchard crop. Samples of sugar syrup used in this experiment were sent to the USDA analytical laboratory to confirm concentrations.

We used a 50% (v/v) sugar syrup for the syrup diet. Imidacloprid was added to experimental sugar syrup using an acetone stock solution; control and non-treated sugar syrup received the same volume of acetone without imidacloprid. Sugar syrup preparation and treatment details can be found in **Appendix Y1**.

Consumption of sugar syrup and water was recorded as the change in volume of both fluids over each 2-day period. Syrup and water supplies were discarded and replaced with an additional 100 mL after each consumption recording. Syrup and water consumption were normalized to consumption per bee, using the number of bees alive in each cage at the start of each 2-day period.

3.3.4. Pollen Treatment and Consumption

The target concentration of imidacloprid in pollen patties was 39 ppb, the mean concentration in Mullin et al. [23]. This is currently the only documentation of imidacloprid in bee bread throughout North America.

Pollen patties were made from bee-collected pollen (The Bee Man®, Yukon, Canada) which was finely ground as described in Chapter 2. Pollen patties were made fresh every week for 4 weeks. Each patty was made using 25 g of ground pollen and 13 mL of 33% (v/v) sugar syrup. We infused imidacloprid into the experimental pollen patties through an acetone solution, and treated pollen patties that were not assigned to imidacloprid treatment with plain acetone. Each pollen patty was mixed individually using a specified set of mixing utensils as in Chapter 1. Pollen was left to dry overnight before we mixed it with sugar syrup the following day. Further details of pollen patty preparation can be found in **Appendix Y1**. Samples of pollen patties were also sent to the USDA analytical lab to confirm concentrations.

All pollen patties were placed in 150 x 15 mm petri dishes. In order to obtain the weight of each pollen patty, each petri dish was weighed before and after adding a patty to it.

Pollen patties were removed from the cages each week, and pollen consumption was calculated as the change in mass of the pollen patty. A fresh pollen patty was supplied to each cage weekly. Pollen patty consumption was normalized as pollen consumption per bee, using the number of bees alive in each cage at the start of the week.

3.3.5. Mortality

Mortality (# of dead bees at cage bottom) was recorded every other day throughout the experiment, beginning on day 3. Bees that were presumably dying (twitching or moving slowly) but were not yet dead were not counted as dead. For a given cage, we calculated the proportion of bees that died over each 2-day interval based on the number of live bees in the cage at the beginning of that 2-day period. Additional details are provided in **Appendix Y1**.

3.3.6. Sampling of Bees for Physiological Assays

We sampled bees from all cages after 2 weeks of treatment, and again after 4 weeks of treatment. At each sampling period, 35 bees were removed for physiological analyses: 15 for hemolymph extraction (phenoloxidase and prophenoloxidase activity), 10 for midgut proteolytic enzyme activity and hypopharyngeal gland protein content, and 10 for the analysis of abdominal lipid stores and glucose oxidase activity.

Bees were sampled from cages using a Craftsman® insect vacuum. We stuffed a generous amount of cheesecloth into the vacuum canister to prevent the bees from being damaged during the sampling process. Only bees that appeared strong and

healthy (not twitching or moving very slowly) were sampled. All bees not used for hemolymph sampling were sacrificed for other physiological assays. We placed stored these bees at -80ºC until time of analysis.

3.3.7. Hemolymph Extraction

We extracted hemolymph from 10-15 live nurse bees per cage. Bees were anesthetized on dry ice for several minutes, until most bees were no longer moving. We then pinned each bee through the thorax and made an incision on the second or third abdominal tergite with a small pair of scissors. A micropipette was used to draw approximately 1-3 µL of hemolymph from each bee. Hemolymph from all the bees of a cage was pooled together in a microcentrifuge tube containing 2.5 µL of chilled sodium cacodylate (NaCac, 1 mM sodium cacodylate, 5 mM calcium chloride, pH 6.5). Then 12.5 ul of that mixture was added to 250 ul of chilled sodium cacodylate and stored at -80°C. Samples were then thawed in a 5°C ice water bath, centrifuged at 15,000 x g, 4 °C for 15 minutes to pellet cellular debris, and aliquoted. Aliquots were stored at -20 °C until time of prophenoloxidase analysis.

3.3.8. Midgut Proteolytic Enzyme Assay

Midgut proteolytic enzyme activity (MEA) was determined on pooled samples of 10 nurse bee abdomens per colony, using the protocol described in **Chapter 2** and **Appendix X1.** Total MEA was expressed as mean optical density at 440 nanometers (OD_{440}).

3.3.9. Hypopharyngeal Gland Protein Content

We quantified hypopharyngeal gland protein content using a standard BCA assay (Pierce Biotech BCA Assay Kit, Thermo Scientific, IL, USA). We pooled complete pairs of hypopharyngeal glands (HPG) from 10 bees per cage in 100 µL of

phosphate-buffered saline (PBS; 10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4, Sigma Aldrich) and followed the protocol described in **Chapter 2**. However, here we plated a ½ dilution of the homogenate supernatant, rather than a 1/10th dilution. Two samples with protein concentrations that exceeded that of the highest standard were reanalyzed with a 1/5 dilution. As in **Chapter 1**, hypopharyngeal gland was expressed in terms of mg HPG protein per bee.

3.3.10. Abdominal Lipid Stores

Abdominal lipid stores were analyzed using a protocol modified from Wilson-Rich et al. [85], as in **Chapter 2**. The protocol in this experiment was different in that we allowed abdomens to dry for four days instead of three, and rinsed abdomens in ether twice after the first ether bath. Additional details are provided in **Appendix Y1**.

3.3.11. Glucose Oxidase Assay

We determined glucose oxidase activity using the protocol described in **Chapter 2** and **Appendix X1**.

3.3.12. Prophenoloxidase Activity

Prophenoloxidase activity (ProPO) was measured as maximum linear rate (Max V) of the substrate conversion per milligram of hemolymph protein, as in **Chapter 2**, with a few notable changes. A large volume of L-Dopa was made, aliquoted and frozen at -20 C before beginning the assay; then one aliquot was thawed and vortexed for 5 minutes before being used for each assay plate. Additionally, 20 µl of hemolymph were mixed with 155 µl of dilute PBS (6.7 mM sodium phosphate, 1.8 mM potassium chloride, 91.3 mM sodium chloride, pH 7.4) rather than 135 ul water and 20 ul PBS. Lastly, the plate reader shook each plate for 3 minutes before beginning absorbance reads as well as continuously while absorbance reads were ongoing.

Prophenoloxidase activity was normalized by protein content of the hemolymph, also as in **Chapter 2**.

3.3.13. Statistical Analysis

All variables were analyzed using repeated-measures linear mixed models, with the categorical explanatory variables Pollen (No Imidacloprid or Imidacloprid), Syrup (No Imidacloprid or Imidacloprid), time (an indicator variable for each time period), and all interactions between these variables. The variable of time was different for different responses: for mortality, water consumption and sugar consumption, time was denoted by "day" (indicator variable of 1 or 0 for each day—14 days total); for pollen consumption and all physiological variables, time was denoted by "week" (indicator variable of 1 or 0 for each week—4 weeks total). All data was analyzed in the R statistical analysis program, version 3.1.2. Each model included the random effect of "cage" nested in "row" (the blocking factor). Different variables required special attention to distributional issues, handling of outliers, and correlation structures. These are further discussed in **Appendix Y1**.

Survival was analyzed using with two linear mixed models ("lme" function in package "nlme"): one to analyze the proportion of bees surviving over each two-day period, and one to analyze the proportion surviving through the end of the experiment overall (excluding bees that were sampled at week 2). Because the model for the proportion of bees surviving over the entire experiment was not a repeated-measures model, it only included the random effect of the blocking factor "row." We did not do a traditional survival analysis (generalized linear mixed model) to analyze mortality because this type of analysis incorrectly assumes that all bees die independently of each other, which has been acknowledged as a statistical problem in laboratory research with honey bees [150]. (See the Discussion for further explanation.)

3.4. Results

3.4.1. Pesticide Concentrations

The concentrations of imidacloprid in pollen patty and sugar syrup diet averaged 1.7 and 3.5 ppb, respectively, which were 10% or less than the intended concentration. These discrepancies unfortunately were a result of human error, as the calculations we used to convert parts per billion to milligrams per gram were off by one decimal point.

Although the imidacloprid concentrations in our pollen and nectar were far below our intended concentrations, the concentrations determined by the USDA analytical laboratory are still within the range of field realistic concentrations. The concentrations we ultimately detected are similar to those in the pollen and nectar of seed-treated crops [60, 97]. The results from the physiological and mortality analyses may therefore apply to seed-treated crops.

3.4.2. Midgut Proteolytic Enzyme Activity

The effect of exposure through sugar syrup on midgut proteolytic enzyme activity was significant at the 0.10 alpha level ($F_{1, 21} = 4.082$, $p = 0.053$), but there was no effect of exposure through pollen diet ($F_{1, 21} = 0.150$, $p = 0.702$). There was no interaction between exposure through sugar syrup and time ($F_{1, 27} = 0.964$, $p = 0.335$). The interaction between pollen exposure and time, though not significant, was nearly significant at the 0.10 alpha level ($F_{1, 27} = 2.808$, $p = 0.105$). There was no interaction between both exposure routes and time ($F_{1, 27} = 0.086$, $p = 0.771$), nor was there a main effect of time ($F_{1, 27} = 0.018$, $p = 0.897$).

The effect of sugar syrup exposure was such that bees exposed to imidacloprid through sugar syrup had higher midgut proteolytic enzyme activity than those not exposed through sugar syrup (See **Figure 3.1**). The mean midgut proteolytic enzyme

activity (MEA) in bees exposed to imidacloprid through sugar syrup is estimated to be 0.05 OD_{440} more than for those not exposed through sugar syrup.

The potential interaction between pollen exposure and time, though not significant, was such that bees not exposed to imidacloprid through pollen diet had slightly higher midgut proteolytic enzyme activity than bees not exposed through pollen diet in week 2, but slightly lower than bees not exposed through pollen in week 4 (See **Figure 3.2**).

3.4.3. Hypopharyngeal Gland Protein Content

Hypopharyngeal gland protein content was not affected by imidacloprid consumption through pollen diet ($F_{1, 21} = 1.684$, $p = 0.208$) or through sugar syrup ($F_{1, 21} = 0.134$, $p = 0.718$). There was no interaction between imidacloprid exposure through pollen and imidacloprid exposure through sugar syrup ($F_{1, 21} = 2.500$, $p = 0.129$), and no interactions between any combination of the exposure routes and time (See **Appendix Y2**). There was a main effect of time overall ($F_{1, 27} = 14.001$, $p = 0.001$).

3.4.4. Abdominal lipid stores

Abdominal lipid stores were not affected by imidacloprid consumption through pollen diet ($F_{1, 21} = 0.014$, $p = 0.907$) or sugar syrup ($F_{1, 21} = 0.642$, $p = 0.432$). There was no interaction between exposure through pollen diet and exposure through sugar syrup ($F_{1, 21} = 2.075$, $p = 0.165$). Additionally, there were no interactions between any combination of these variables and time (See **Appendix Y2**). The only effect that was significant was the effect of time ($F_{1, 27} = 38.952$, $p < 0.0001$).

3.4.5. Glucose Oxidase Activity

In the model including the outlier, glucose oxidase activity was not affected by exposure through pollen diet ($F_{1, 21} = 0.705$, $p = 0.411$), nor there was an interaction

between pollen exposure and time ($F_{1, 28} = 1.398$, $p = 0.247$). Further, there was no interaction between exposure through pollen diet and exposure through sugar syrup ($F_{1, 21} = 0.763$, $p = 0.392$). Time had a main effect on glucose oxidase activity ($F_{1, 28} = 20.573$, $p = 0.0001$), but there was no interaction between pollen exposure, syrup exposure, and time ($F_{1, 28} = 2.456$, $p = 0.128$).

There was both a main effect of syrup exposure ($F_{1, 21} = 5.073$, $p = 0.035$), as well as an interaction between syrup exposure and time that was significant at the 0.10 alpha level ($F_{1, 28} = 3.991$, $p = 0.056$). The interaction between syrup exposure and time showed that differences between bees exposed through syrup and those that were not were apparent during week 4, but not during week 2 (see **Figure 3.3**). At week 4, median glucose oxidase activity in bees exposed through sugar syrup was 168% of the glucose oxidase activity of bees not exposed to imidacloprid through sugar syrup. Further, the main effect of syrup exposure on glucose oxidase activity showed that across both weeks, the median glucose oxidase activity for cages exposed to imidacloprid through sugar syrup was 132% that of bees not exposed through sugar syrup (see **Figure 3.4**).

In the model that excluded the outlier, the main effect of imidacloprid through pollen diet remained insignificant ($F_{1, 21} = 0.187$, $p = 0.670$). However, the main effect of pollen exposure over time became significant at the 0.10 alpha level ($F_{1, 27} = 3.139$, $p = 0.088$). The effect of imidacloprid in sugar syrup over time became significant at the 0.05 alpha level ($F_{1, 27} = 7.079$, $p = 0.013$), as did the interaction between pollen exposure, syrup exposure, and time ($F_{1, 27} = 4.801$, $p = 0.037$). The main effect of sugar syrup ($F_{1, 21} = 8.632$, $p = 0.008$) and the main effect of time ($F_{1, 27} = 19.896$, $p = 0.0001$) also remained significant.

In the interaction between both exposure routes and time, differences between treatment groups were only apparent in week 4. During this week, the median glucose oxidase activity in bees exposed to imidacloprid through both pollen and sugar syrup was 2.8 times higher than that of bees exposed only through pollen diet, and 2.2 times

higher than that of the control group. The control group, the treatment group exposed through pollen only, and the treatment group exposed through syrup only were not different from each other (See **Figure 3.5**). The treatment group exposed through both routes was also not significantly different from the treatment group exposed through syrup only.

The interaction between pollen exposure and time was such that the group exposed to imidacloprid through pollen diet and the group not exposed to imidacloprid through pollen diet were significantly different from each other during week 2, but not during week 4 (See **Figure 3.6**). During week 2, the median glucose oxidase activity in cages exposed to imidacloprid through pollen diet was 1.3 times higher than that of cages not exposed through pollen diet.

3.4.6. Prophenoloxidase Activity

With outliers included in the analysis, prophenoloxidase activity was not affected by imidacloprid exposure through pollen ($F_{1, 21} = 0.464$, $p = 0.503$) nor sugar syrup ($F_{1, 21} = 0.900$, $p = 0.354$). There was no interaction between exposure routes ($F_{1, 21} = 0.009$, $p = 0.925$). There were no interactions between the exposure routes and time (See **Appendix Y2**). There was also no main effect of time ($F_{1, 28} = 0.024$, $p = 0.879$).

3.4.7. Pollen Consumption Per Bee

In the analysis of pollen consumption, there was no interaction between exposure through pollen diet and time ($F_{3, 83} = 0.110$, $p < 0.956$), but the main effect of pollen exposure was significant at the 0.10 alpha level ($F_{1, 28} = 3.660$, $p = 0.066$). There was no interaction between syrup exposure and time ($F_{3, 83} = 0.480$, $p = 0.695$), nor was there a main effect of sugar syrup exposure ($F_{1, 28} = 0.090$, $p = 0.772$). There was also no interaction between both exposure routes and time ($F_{3, 83} = 0.580$, $p = 0.630$), and nor there was a significant effect between both exposure routes overall ($F_{3, 83} = 0.030$,

p = 0.860). Time also had a main effect on pollen consumption ($F_{3, 83}$ = 512.38, p <0.0001).

The effect of pollen exposure was such that bees in cages exposed to imidacloprid through pollen consumed more pollen than bees in cages not exposed through pollen (See **Figure 3.7**). The difference, however, was slight—bees in cages exposed to imidacloprid through pollen diet only consumed 3% more pollen than bees in cages not exposed through pollen diet.

3.4.8. Sugar Syrup Consumption Per Bee

When outliers were included in the model, neither imidacloprid exposure through pollen ($F_{1, 21}$ = 2.240, p = 0.149) nor exposure through sugar syrup ($F_{1, 21}$ = 0.102, p = 0.753) had an effect on sugar syrup consumption per bee. There was also no interaction between exposure through pollen and exposure through sugar syrup ($F_{1, 21}$ = 2.117, p = 0.160). There was a significant main effect of time ($F_{13, 363}$ = 53.471, p <0.0001), but there were no interactions between the exposure routes and time (See **Appendix Y2**).

When outliers were excluded from the model, there was a significant interaction between pollen exposure and time ($F_{13, 347}$ = 2.990, p = 0.0004), and an additional main effect of pollen exposure overall ($F_{1, 21}$ = 4.908, p = 0.038). The main effect of imidacloprid in sugar syrup remained insignificant ($F_{1, 21}$ = 0.627, p = 0.437), and still showed no significant interaction with time ($F_{13, 347}$ = 0.706, p = 0.757). The interaction between both exposure routes and time was significant at the 0.10 alpha level ($F_{13, 347}$ = 1.693, p = 0.061), as was the interaction between both exposure routes overall ($F_{1, 21}$ = 4.026, p = 0.058). The main effect of time also remained significant ($F_{13, 347}$ = 85.189, p < 0.0001).

In the interaction between both exposure routes and time, significant differences between treatment groups were only significant on Day 29 (See **Figure 3.8**). On this

day, bees exposed to imidacloprid only through sugar syrup consumed significantly less syrup than bees exposed to imidacloprid in both pollen diet and sugar syrup. There were no other differences between treatment groups on this day. While this pattern also appeared to exist on other days, the effect on those days was not significant after the Tukey adjustment (See **Figure 3.8**). In the overall interaction between exposure routes, bees exposed to imidacloprid through sugar syrup consumed less sugar syrup overall than bees exposed to imidacloprid through both routes (See **Figure 3.9**), and there were no other differences between treatment groups.

The differences in sugar syrup consumption between cages that were exposed to imidacloprid through pollen diet and those that were not were also only apparent on Day 29 (see **Figure 3.10**). On this day, bees in cages that were exposed to imidacloprid through pollen diet consumed approximately 140% more sugar syrup than bees not exposed to imidacloprid through pollen diet. The main effect of exposure through pollen diet also indicated that overall, for all days combined, bees exposed to imidacloprid through pollen diet consumed 107% more sugar syrup than those that were not exposed through pollen diet (See **Figure 3.11**).

3.4.9. Water Consumption Per Bee

In the model that included outliers, imidacloprid exposure through pollen diet did not affect water consumption overall ($F_{1, 21} = 0.870$, $p = 0.362$), and there was no interaction between pollen exposure and time ($F_{13, 362} = 0.905$, $p = 0.548$). There was no main effect of exposure through sugar syrup ($F_{1, 21} = 0.034$, $p = 0.856$), but the interaction between sugar syrup and time was significant at the 0.10 alpha level ($F_{13, 362} = 1.569$, $p = 0.092$). Differences between bees in cages exposed to imidacloprid through sugar syrup and those that were not took place at days 13, 15, and 21, although these differences were no longer significant after the Tukey adjustment (See **Figure 3.12**). There was no additional effect of imidacloprid exposure through both pollen diet and sugar syrup ($F_{13, 362} = 0.226$, $p = 0.640$), nor did the combination of

both exposure routes interact with time ($F_{13, 362} = 1.106$, $p = 0.435$). There was, however, a significant main effect of time ($F_{13, 362} = 31.534$, $p < 0.0001$).

When outliers were removed, the main effect of imidacloprid exposure through pollen diet was not significant ($F_{1, 21} = 0.686$, $p = 0.417$), as did its interaction with time ($F_{13, 362} = 1.062$, $p = 0.391$). The main effect of exposure through sugar syrup was not significant ($F_{1, 21} = 0.090$, $p = 0.767$), but its interaction with time became significant at the 0.05 alpha level ($F_{13, 362} = 1.769$, $p = 0.046$). However, this difference was no longer apparent after the Tukey adjustment (See **Figure 3.13**). There was no additional effect of exposure through both pollen and sugar syrup ($F_{13, 362} = 0.527$, $p = 0.476$), nor did the two exposure routes interact with time ($F_{13, 362} = 1.240$, $p = 0.249$). The main effect of time remained significant ($F_{13, 356} = 34.906$, $p < 0.0001$).

3.4.10. Mortality

In the analysis of mortality (proportion of bees surviving) over time, there was no significant effect of exposure through pollen diet on survival ($F_{1, 21} = 2.38$, $p = 0.138$), nor was there a main effect of exposure through sugar syrup ($F_{1, 21} = 0.16$, $p = 0.696$). However, there was a significant interaction between exposure routes ($F_{1, 21} = 8.89$, $p = 0.007$). There was no interaction between syrup exposure and time ($F_{13, 344} = 0.870$, $p = 0.590$), nor was there an interaction between both exposure routes and time ($F_{13, 344} = 1.37$, $p = 0.169$). There was, however, a significant interaction between pollen exposure and time ($F_{13, 344} = 2.44$, $p = 0.004$), as well as a main effect of time ($F_{13, 344} = 58.65$, $p < 0.0001$).

The interaction between exposure routes showed that after the Tukey adjustment, the treatment group exposed to imidacloprid through both routes had significantly lower survival than the group exposed only through sugar syrup (See **Figure 3.14**). However, the effect size was small enough that it may not be biologically significant—the mean proportion surviving between these groups differed by less

than 2 percentage points. Both the control group and the group exposed through pollen diet only were not significantly different from either of the other two groups.

In the interaction between pollen exposure and time, mortality was only different between bees exposed to imidacloprid through pollen and those that were not on Day 23 of the experiment. On this day, fewer bees exposed through pollen survived than those that were not. The difference was slight—the mean proportion of bees surviving in cages not exposed through pollen was only 4.4 percentage points higher than that of cages exposed through pollen diet (See **Figure 3.15**). On all other days, mortality was not significantly different between these groups.

In the analysis of the proportion surviving at the end of the experiment, there was no effect of pollen exposure ($F_{1,18} = 0.242$, $p = 0.629$) or syrup exposure ($F_{1,18} = 0.068$, $p = 0.798$). There was also no significant interaction between exposure routes ($F_{1,18} = 0.652$, $p = 0.430$).

3.5. Discussion

In this experiment, we investigated the physiological effects of imidacloprid exposure to bees via pollen, carbohydrate diet, and both (pollen and carbohydrate). To our knowledge, this experiment is the first laboratory experiment to analyze the interactive effects of pesticide exposure through multiple routes, and their effects on honey bee physiology and mortality. We also tracked consumption of pollen diet, sugar syrup, and water over the course of the experiment.

In this experiment, we did not detect any effect of imidacloprid exposure through either or both routes on the hypopharyngeal gland protein content of worker bees. This result differs drastically from previous laboratory studies that have shown that imidacloprid exposure reduces the size of the hypopharyngeal glands acini [151-153], which also translates to reduced hypopharyngeal gland protein content [154]. Further, our experiment also differs drastically from these studies in a number of ways. In this

experiment, we mimicked the social conditions of the honey bee colony as closely as possible by including brood pheromone and queen pheromone in each cage, which was not done in any of the previous studies in which imidacloprid was shown to have deleterious effects on the hypopharyngeal glands. The combination of both brood and queen pheromones increase the protein content of the hypopharyngeal glands significantly [155], and therefore may influence how the hypopharyngeal glands respond to imidacloprid exposure. Because previous experiments excluded these aspects of honey bee sociality, they may have misrepresented the effects that would take place under queen-right, brood-rearing conditions. However, findings of these studies may appropriately demonstrate how workers are affected by imidacloprid when brood is absent from the colony, such as during the winter [156-157], or when the queen is poorly mated [158-159]. The effects of neonicotinoid exposure under different conditions of queen quality have thus far not been thoroughly explored, although neonicotinoid exposure has recently been linked to queen supersedure [30] and increased broodless periods [33].

Our results also indicate that imidacloprid exposure through either a single route or both routes did not affect the abdominal lipid stores of worker bees throughout the experiment. As lipid stores were not different between treatments at both week 2 and week 4, our results suggest that low concentrations of imidacloprid in both pollen and nectar are unlikely to induce precocious foraging, which is precluded by the early loss of abdominal lipids [83-84]. However, these results may differ if bees are exposed during the larval stages instead of the adult stage. Derecka et al. [160] reported that bees exposed to low levels of imidacloprid during the larval stage demonstrated various alterations in genes related to lipid metabolism, carbohydrate metabolism, and precocious foraging. We also note that investigating interactions between imidacloprid exposure and honey bee pathogens may be necessary to fully understand these patterns. A previous field experiment determined that the combination of sublethal imidacloprid exposure and high *Varroa* mite loads significantly reduced the flight distance of foragers [76], which indicates that imidacloprid and *Varroa* interact to increase energetic stress. However, only *Varroa* alone drastically reduced the body

mass and abdominal mass of workers [76]. Imidacloprid may therefore alter foraging behavior at later stages without inducing changes in abdominal lipid stores, especially when *Varroa* mite levels are high. It is necessary to examine the interactions between neonicotinoid exposure and various pests and pathogens before concluding that these chemicals play no role in lipid loss or energy metabolism.

We also did not detect any effects of imidacloprid on prophenoloxidase activity in this experiment. Our findings support previous laboratory findings where imidacloprid exposure had no impact on prophenoloxidase activity when bees were exposed through sugar syrup [161]. From these results, we may conclude that imidacloprid did not significantly reduce the ability of worker bees to cope with wounding [137-138] and other pathogens [139, 162]. However, we also recognize that the prophenoloxidase cascade may not be particularly important to honey bee immunity to many pathogens. Several studies demonstrate that the prophenoloxidase cascade remains unchanged after injection with viruses such as Acute Bee Paralysis Virus [138, 163]. Although the honey bee immune response to viruses is not well understood, other immune pathways may be more significant to virus resistance than the prophenoloxidase cascade [164]. We suggest that in addition to the immune responses explored in this experiment, future research must include the potential impacts of imidacloprid and other neonicotinoids on immune mechanisms such as antimicrobial peptide activity [138, 164-167].

The results of the glucose oxidase analysis differed drastically when outliers were included and excluded, but we detected effects of imidacloprid exposure in both analyses. The results of this analysis again demonstrate the need for researchers to pay attention to outliers in the data [168], and also warrant further investigation in future research.

The presence of imidacloprid in sugar syrup had a clear effect on glucose oxidase activity, both when outliers were included in the analysis and when outliers were excluded. Glucose oxidase activity was not different between bees exposed and not

exposed through sugar syrup after two weeks of exposure, but was higher in bees exposed through sugar syrup than in bees that were not exposed through syrup after four weeks of exposure. These results differ drastically from previous findings that imidacloprid exposure through sugar syrup does not affect glucose oxidase activity, except when such exposure coincides with *Nosema* infection [161]. We believe that we found such differences in our experiment because our experiment took place for a period of one month, which is much longer than the exposure period of 10 days in the previous experiment [161]. The differences we detected at week 4 demonstrate that exposure to imidacloprid through sugar syrup for a longer period of time does induce changes in glucose oxidase activity. These results demonstrate the need for experiments that capture long-term effects, in order to capture the effects when the bloom period and exposure period are as long as one month [63].

The differences in glucose oxidase induced by imidacloprid were somewhat unexpected, as imidacloprid exposure resulted in higher glucose oxidase activity than the control group. In this experiment, we detected that glucose oxidase activity in bees not exposed to imidacloprid through syrup declined significantly from week 2 to week 4 of the exposure period, whereas glucose oxidase activity in bees exposed through syrup did not significantly change during that time. The decrease in glucose oxidase activity in bees not exposed through sugar syrup was surprising, since glucose oxidase activity typically increases with worker age [141, 169]. It is possible that glucose oxidase activity did not increase in this experiment as honey production cannot take place under laboratory conditions. Our results may therefore demonstrate how physiological parameters differ when bees are held under laboratory conditions versus field conditions, and the need to conduct field experiments to confirm laboratory results.

In the analysis of glucose oxidase activity that excluded outliers, we also detected an interaction between both exposure routes and time. In this interaction, we found that glucose oxidase activity was not different between bees exposed to imidacloprid through sugar syrup only and those exposed through both routes, nor were there

differences between control bees, bees exposed through pollen only, and bees exposed through syrup only. Bees exposed through both pollen and sugar syrup, however, exhibited significantly higher glucose oxidase activity than both control bees and bees exposed only through pollen during week 4. This result demonstrates the need for experiments that adequately reflect exposure through multiple routes, in addition to the need for longer-term chronic exposure experiments.

The implications of the differences between glucose oxidase activity in this experiment deserve further attention. Higher production of glucose oxidase activity in bees exposed through both matrices may indicate a higher potential to cope with pathogens such as *Nosema ceranae* [170] and *Paenibacillus larvae* [140]. However, these effects must be further investigated under field conditions, especially when bees are exposed to more than one stressor. These results may have differed drastically if imidacloprid exposure had been combined with other stressors, such as *Nosema* [161] or other pesticides. Future research should seek to confirm whether imidacloprid exposure through different routes increases glucose oxidase activity under field conditions, and whether interactions with other agrochemicals or pathogens suppress glucose oxidase activity or enhance its activity. While we investigated the impacts of imidacloprid in pollen diet on glucose oxidase activity in our previous field experiment, we only did so for exposure through a pollen diet. In our field experiment, bees that were exposed to chemicals through the pollen diet were not supplied contaminated sugar syrup diet at their later stages.

In our analysis of midgut proteolytic enzyme activity, we found that midgut proteolytic enzyme activity was higher in bees exposed to imidacloprid through sugar syrup than it was in bees that were not exposed through sugar syrup. We also detected an interaction between exposure through pollen diet and time, although this interaction was no longer significant after the Tukey adjustment. This result may shed some light on the effects of imidacloprid exposure on pollen digestion. Imidacloprid may have induced higher midgut enzyme activity by influencing cell death in digestive cells, which produce proteolytic enzymes [171-172]. Cell death in digestive

cells is correlated with changes in midgut structure [173] and deteriorating microvilli [174], the extensions of the digestive cells that secrete proteolytic enzymes [171]. These changes would result in changes in the amount of proteolytic enzymes in the honey bee gut.

A previous study determined that imidacloprid also led to increased cell death in midgut cells [175], although this study did not examine impacts on digestive cells, and only determined effects using a concentration of 400 ppm. This concentration is several magnitudes of order higher than even the maximum concentrations detected in honey bee colonies in field studies, and therefore over-represents exposure under field conditions. Whether or not imidacloprid has the same effect at lower concentrations is not yet known. While exposure to 400 ppm of imidacloprid caused 61% of cells to die in a study by Gregorc & Ellis [175], it is possible that lower concentrations of imidacloprid may cause smaller increases in cell death, which would ultimately explain the relatively small differences detected in this experiment.

Our midgut proteolytic enzyme activity analysis detected an interaction between pollen exposure and time, but this interaction was not significant after the Tukey adjustment. It is somewhat surprising that the effect of pollen did not show similar patterns as exposure through sugar syrup, since bees exposed through pollen diet also consumed more pollen and sugar syrup, and therefore were exposed to even greater quantities of imidacloprid. We also expect these bees to have higher midgut proteolytic enzyme activity because increased pollen consumption generally coincides with higher proteolytic enzyme activity [79, 143]. It is possible that increased pollen consumption in these bees buffered the potential effects of imidacloprid, since pollen consumption both decreases honey bee pesticide sensitivity [176] and increases proteolytic enzyme activity [177].

Although we detected these differences in our analysis of proteolytic enzyme activity, we must also consider the possibility that the differences we detected in our analyses of midgut proteolytic enzyme activity may not necessarily be biologically significant

to worker bee survival. In Jack et al. [177], bees that exhibited a similar, though insignificant, difference in midgut enzyme activity did not show significant differences in survival.

Our mortality analysis found that fewer bees in the treatment group exposed through both pollen and syrup survived until the end of the experiment than bees exposed only through sugar syrup. This difference is likely due to increased imidacloprid intake in the group exposed through both routes. Bees exposed to imidacloprid through both routes consumed more sugar syrup, on average, than bees exposed through sugar syrup only, particularly at the end of the experiment. Furthermore, bees exposed through pollen diet consumed more pollen than bees not exposed through pollen diet. Between higher consumption of syrup containing imidacloprid and higher consumption of pollen containing imidacloprid, imidacloprid intake was much higher in the bees exposed through both routes than in bees exposed only through sugar syrup. However, survival at the end of the experiment did not differ between control bees and bees exposed to imidacloprid through both routes. The reasons for this are not known. It is possible that increased nutrient intake through both pollen and syrup in the group exposed through both routes may have buffered potential effects on mortality, which suggests that bees must consume more food to survive when exposed to realistic concentrations of imidacloprid.

In our analysis of mortality, we also detected that bees exposed to imidacloprid through pollen diet exhibited lower survival on Day 23 than bees not exposed through pollen diet. This result demonstrates the need for experiments that quantify mortality over a longer period of exposure, and that capture cumulative or delayed effects [42, 73, 150]. However, the effect size for this difference was also slight—survival was only 4% less on this day in bees exposed through pollen than bees that were not exposed through pollen, which differs from the effect sizes detected in other chronic toxicity experiments. Previous experiments have demonstrated that low concentrations of imidacloprid in sugar syrup kill 50% of adult bees 30 days after exposure [42, 150], which is not corroborated by our findings. Our findings may

differ from previous chronic exposure experiments specifically as bees in our experiment had access to a pollen diet. Pesticide sensitivity is significantly reduced [176], and longevity is increased [178-179], when worker bees are allowed to feed on pollen at the early stages of adulthood. However, in most studies that investigated the impacts of pesticides on young bees, bees were exposed to pesticides through sugar syrup without access to a pollen diet [39, 73, 78, 150, 161, 180]. Studies that investigate the impacts of pesticides on young adult workers may misrepresent the effects of these chemicals if natural pollen feeding behavior is not incorporated into these experiments. However, the results of such studies should not be completely discounted—they may still provide insights on how pesticides may impact bees under nutritional stress.

While our mortality analyses provide some insight into the effects of these pesticides, we emphasize that these results should be treated as preliminary. In the future, these data should be analyzed with a more powerful survival analysis, particularly using a generalized linear mixed model (GLMM). Traditional analyses for honey bee survival are typically conducted using generalized linear models (GLM), but these models incorrectly assume that bees are both independently exposed to the pesticide and die independently of each other [150, 181]. When bees are grouped into cages, it is more appropriate to utilize a generalized linear mixed model, which would be able to incorporate the random effects of the cages [181]. These analyses are also far more complex and accurate than linear mixed models, which were used to analyze mortality in this experiment. We did not have the statistical assistance to conduct a generalized linear mixed model, and therefore emphasize that our results should be interpreted cautiously until such an analysis is conducted.

Our consumption results are similar to previous studies where bees exposed to imidacloprid consumed more food than those that were not exposed [78, 161], except this pattern was only true for imidacloprid exposure through pollen. In previous studies, in which bees exposed to imidacloprid through sugar syrup consumed more sugar syrup, these bees were likely already under nutritional stress because they did

not have access to a pollen diet, which was probably exacerbated by exposure to imidacloprid. Our results indicate that bees exposed to imidacloprid only consumed greater quantities of sugar syrup if they were previously exposed to the chemical through pollen diet.

Higher consumption of both pollen and sugar syrup in the treatment group exposed to imidacloprid through pollen diet suggests that this type of exposure may somehow increase hunger levels. Increased food consumption in nurse bees exposed to imidacloprid in the pollen diet may therefore consume stored food more quickly than bees that are not exposed to the chemical, and rapidly deplete pollen and honey stores. These differences may contribute to colony starvation, which is one of the top self-reported causes of overwintering colony loss [7-8, 182].

Differences in diet consumption between our treatment groups, particularly for consumption of pollen, may have played a critical role in our physiological results and masked the effects of imidacloprid exposure to certain extent. Previous laboratory results demonstrating the impacts of imidacloprid on worker bees had experimental design where bees were exposed to imidacloprid through a diet of only sugar syrup, with the exception of two studies [152-153]. The presence of a pollen diet may be especially critical for understanding the effects of imidacloprid and other pesticides on honey bee health, particularly for young bees. Bees consume pollen at much higher rates than sugar syrup during the first ten days after emergence [79, 153, 178-179], and tend to consume larger quantities of sugar syrup after 10 days [153, 183]. Increased pollen consumption also increases hypopharyngeal gland protein content [184-185], midgut proteolytic enzyme activity [184-186], abdominal lipid stores [187], glucose oxidase activity [187], and prophenoloxidase activity [187]. Future experiments must account for differences in dietary needs over the entire honey bee life cycle. To expose young bees to a pesticide without accounting for these natural patterns or needs would not provide realistic insights.

The honey bee subspecies we used in this experiment may also have played a role in our findings. Subspecies of honey bee may differ widely in their longevity [188], metabolism [189], antioxidative physiology [189], and brood food quality [189-190] and quantity [190]. The cage studies cited in this discussion, particularly those that relate to hypopharyngeal gland protein content, were conducted using subspecies that were not *Apis mellifera ligustica* [151-153]. The sole study that did not determine significant effects of imidacloprid exposure on hypopharyngeal gland size was by Alaux et al. [161], where the authors used a mix of both *A.m. mellifera* and *A.m. ligustica*. The discrepancies between the studies with *A.m. ligustica* and other subspecies of honey bees suggest that different subspecies of *Apis mellifera* may respond differently to imidacloprid and other insecticides. However, further research is needed to confirm this hypothesis.

Although the concentrations of imidacloprid in this experiment were lower than our intended concentrations, these concentrations are similar to those detected in the pollen and nectar of seed-treated crops [60, 97]. Considering the very low concentrations we ultimately used in this experiment, it is surprising that we detected any differences between our treatments. Previous experiments with low concentrations did not detect any significant differences between treated and untreated bees or colonies [75, 134, 191]. The incorporation of multiple exposure routes in this experiment, as well as the longer exposure duration, is the most likely explanation we detected effects while other studies have not. Our findings demonstrate the need to conduct laboratory experiments that capture the chronic and delayed effects of imidacloprid exposure, as well as the need to conduct experiments in which bees are exposed to pesticides through more than one route. Because most cage experiments with field-realistic doses of imidacloprid range from 10-14 days or less [39, 152-153, 161], current research has largely failed to capture these effects. We note that in this experiment, the concentration of our chemical was also far lower than the intended concentration. Although we set out to determine the effects of higher concentrations of imidacloprid that have been detected in stored pollen and nectar, we are ultimately unable to extrapolate our results to such a scenario. While

our results provide some insights into how imidacloprid exposure through both of these resources may influence feeding behavior and physiology, further research must be conducted using the actual concentrations that we originally intended to use in this experiment. Future experiments should also determine the mechanisms by which imidacloprid may interact with pathogens to affect these physiological responses, particularly the glucose oxidase activity. We also recommend that researchers should explore how different honey bee subspecies differ in their response to pesticide exposure.

3.6. Conclusions

In this experiment, we investigated the impacts of field-realistic imidacloprid exposure through pollen and sugar syrup, on worker (nurse bee) physiology and mortality. Due to a miscalculation, the concentrations in our experimental pollen and sugar syrup were far lower than our intended concentrations. However, despite these low concentrations, we still detected significant effects in our analyses of glucose oxidase activity and midgut proteolytic enzyme activity. Because the concentrations ultimately detected in this experiment are close to those detected in the pollen and nectar of seed-treated crops, these results may still apply to exposure in the field.

In this experiment, glucose oxidase activity was higher in bees that were exposed to imidacloprid through sugar syrup than those not exposed through syrup. Glucose oxidase activity also declined significantly from week 2 to week 4 of the experiment in bees not exposed to imidacloprid through sugar syrup, which did not occur in the bees that were exposed through syrup. Furthermore, bees exposed to imidacloprid through sugar syrup exhibited higher midgut proteolytic enzyme activity throughout the experiment than those that were not. Mortality also differed slightly between treatments—fewer bees exposed to imidacloprid through pollen diet survived than those that were not exposed through pollen at Day 23, and fewer bees exposed through both routes survived overall than bees exposed only through sugar syrup. Differences in food consumption between different treatment groups may have played

a role in both our physiological results—bees exposed to imidacloprid through pollen diet consumed more pollen and sugar syrup, on average, than bees that were not exposed through pollen diet.

Our results indicate low levels of imidacloprid impact bee physiology and mortality after more than two weeks of exposure. We recommend that survival be re-analyzed using a generalized linear mixed model, which would more accurately compare survival curves between our treatments while still incorporating a blocking factor. Furthermore, we recommend that future research determines whether these results differ at higher concentrations realistically found stored pollen and nectar, change in response to interactions with honey bee pathogens, or differ between honey bee subspecies.

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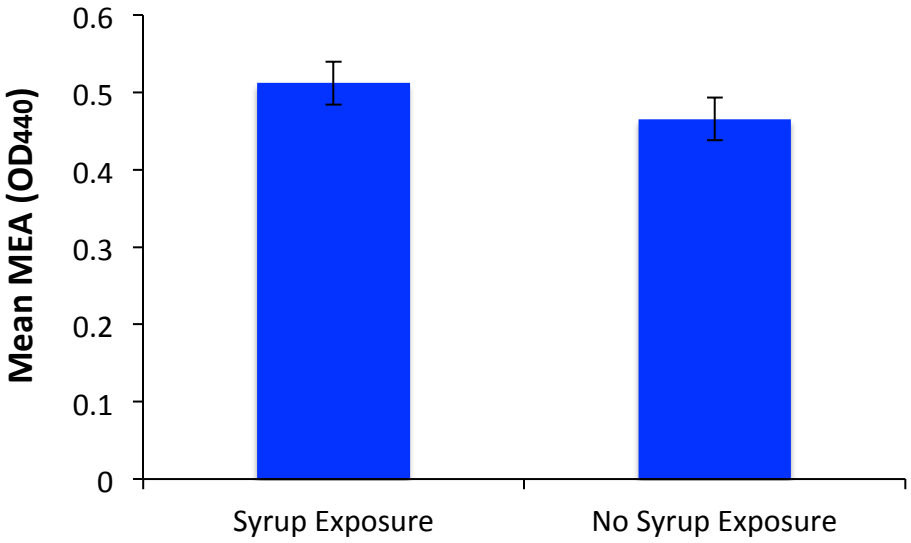

Figure 3.1 Mean midgut proteolytic enzyme activity (optical density at 440 nm, + 90% Tukey-adjusted confidence interval) of bees that were exposed to imidacloprid through sugar syrup and bees that were not exposed to through sugar syrup, averaged across the duration of the experiment.

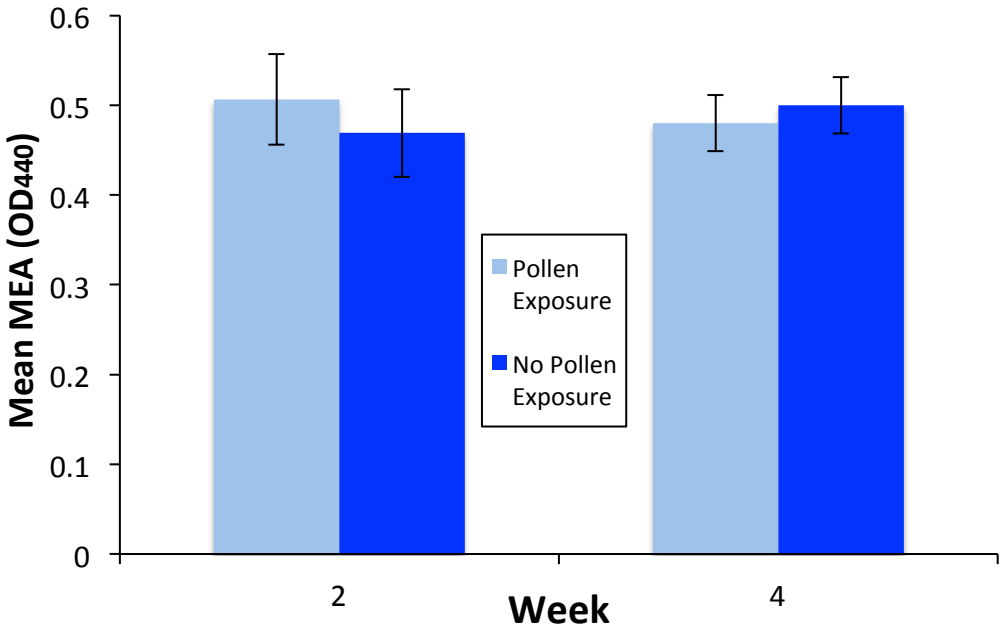

Figure 3.2 Mean midgut proteolytic enzyme activity (optical density at 440 nm, + 90% confidence intervals) of bees exposed to imidacloprid through pollen and bees not exposed through pollen diet.

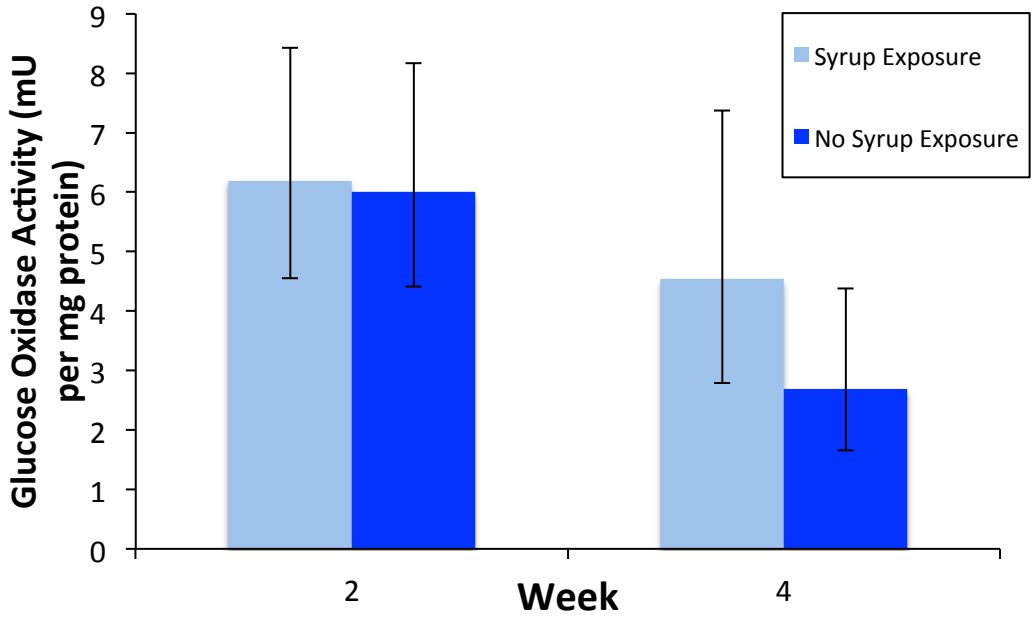

Figure 3.3 Median glucose oxidase activity (+90% Tukey-adjusted confidence intervals) of bees exposed to imidacloprid through sugar syrup and bees not exposed through sugar syrup, with outliers included.

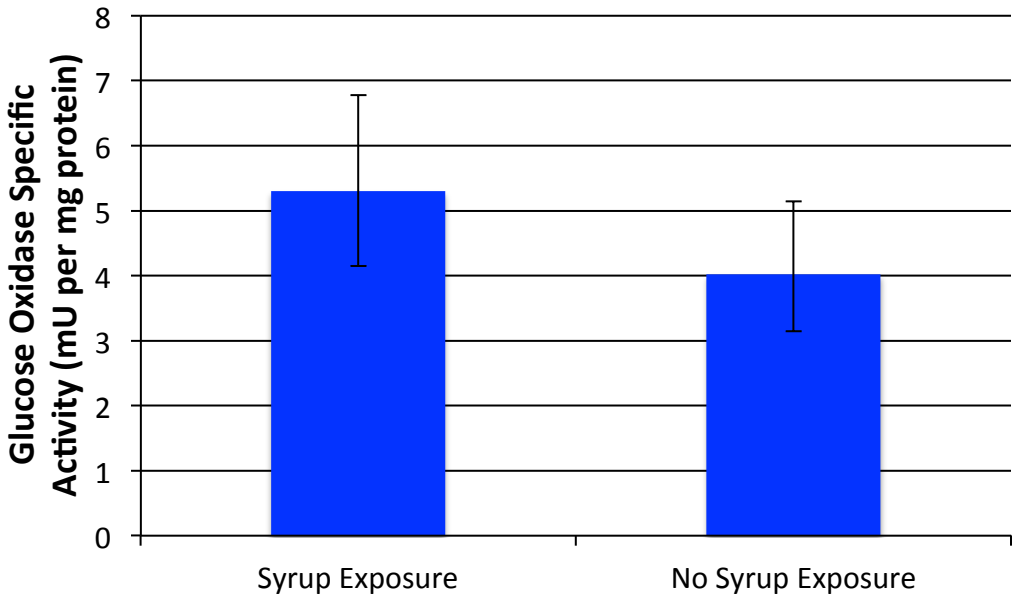

Figure 3.4 Median glucose oxidase activity (+95% Tukey-adjusted confidence interval) of bees exposed to imidacloprid through sugar syrup and those not exposed through sugar syrup, averaged across the entire duration of the experiment. Outliers are included.

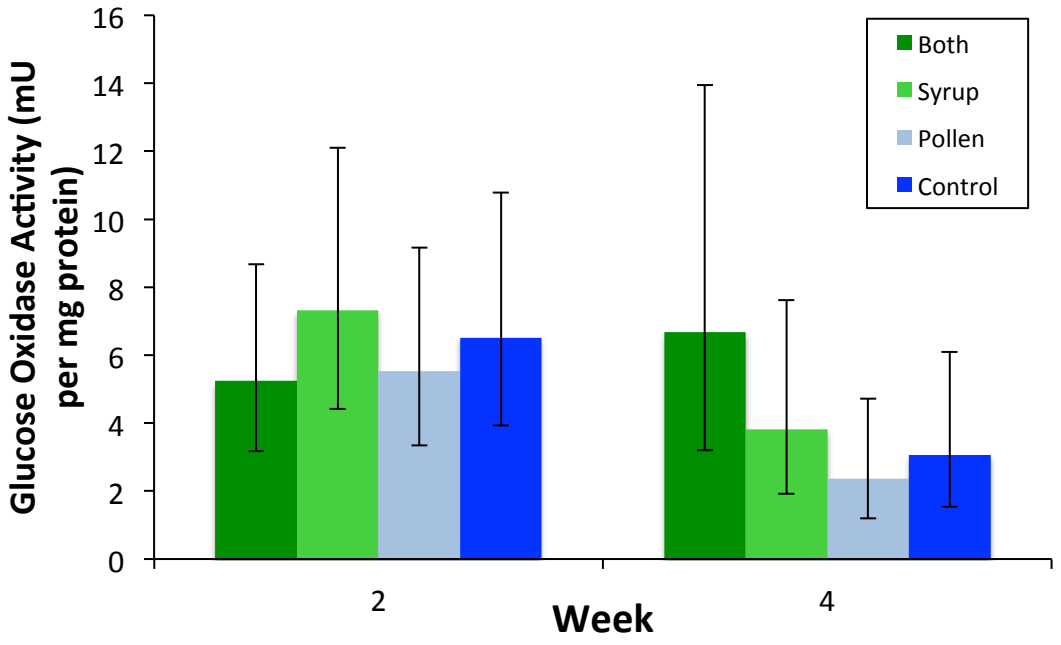

Figure 3.5 Median glucose oxidase activity (+ 95% confidence intervals) of bees exposed to imidacloprid through different routes, with outliers removed excluded.

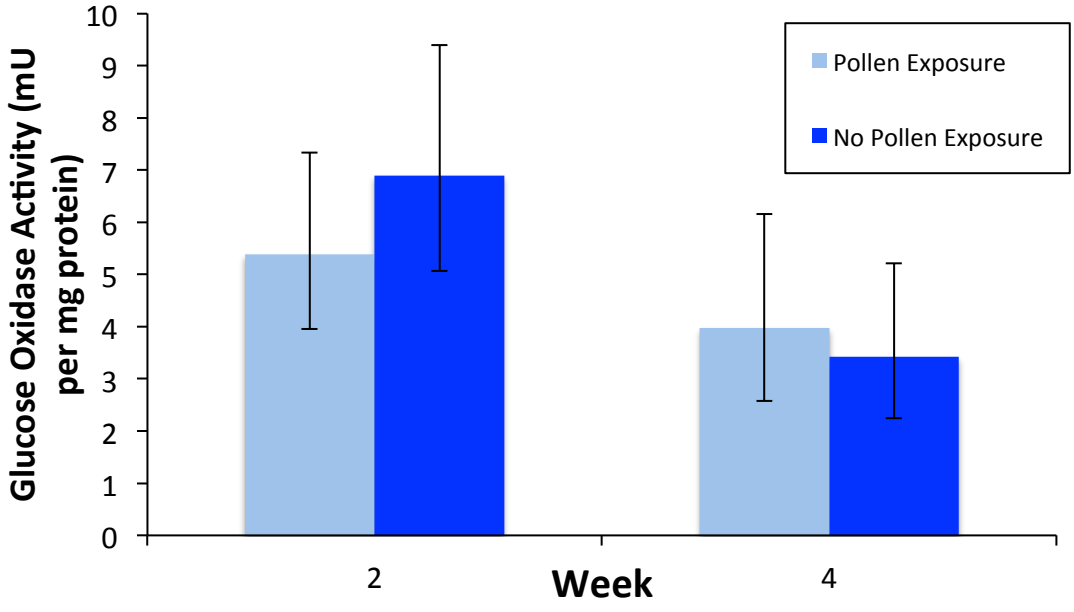

Figure 3.6 Median glucose oxidase activity (+ 95% Tukey-adjusted confidence intervals) for bees exposed to imidacloprid through pollen diet and bees not exposed to imidacloprid through pollen diet, outliers excluded.

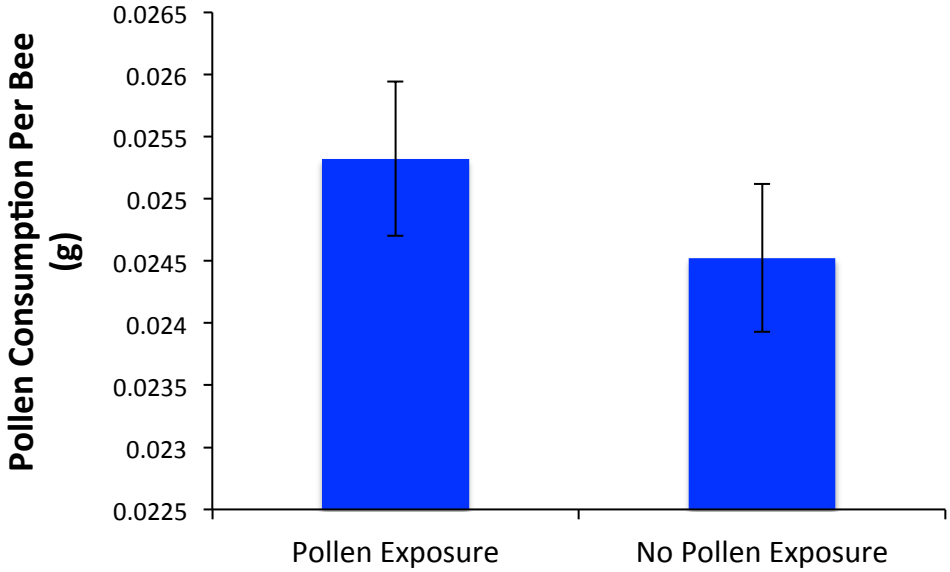

Figure 3.7 Median pollen consumption (+90% confidence intervals) of bees exposed to imidacloprid through pollen diet and those not exposed to imidacloprid through pollen diet, averaged across the duration of the experiment.

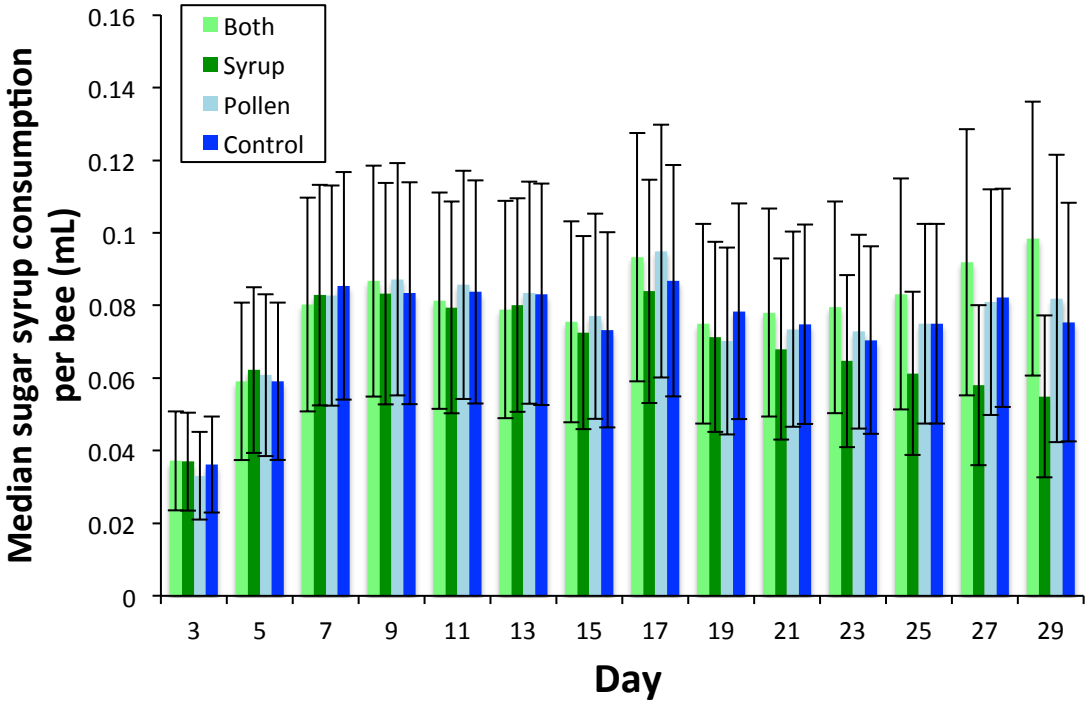

Figure 3.8 Median sugar syrup consumption (+90% confidence intervals) of bees in all treatment groups over each 2-day period, with outliers excluded.

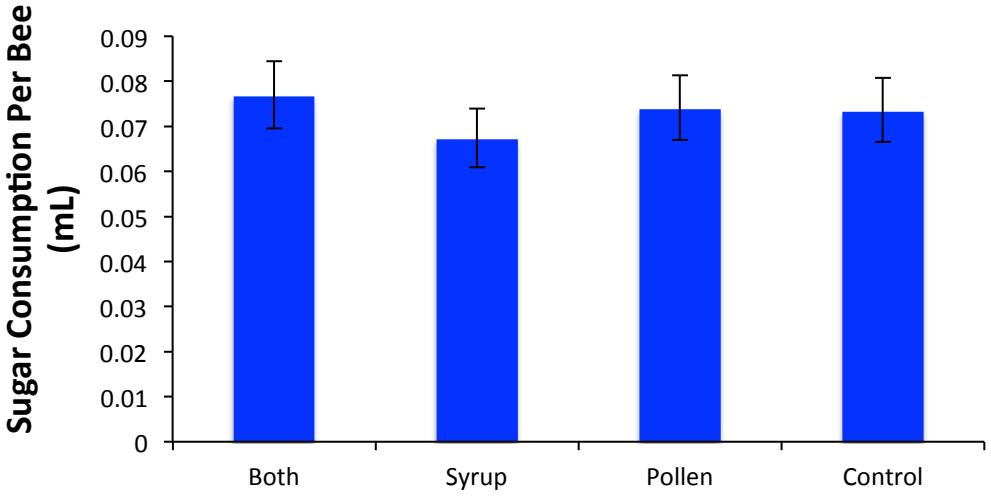

Figure 3.9 Median sugar syrup consumption (+95% Tukey-adjusted confidence intervals) of bees exposed to imidacloprid through different routes, averaged across the duration of the experiment, with outliers excluded.

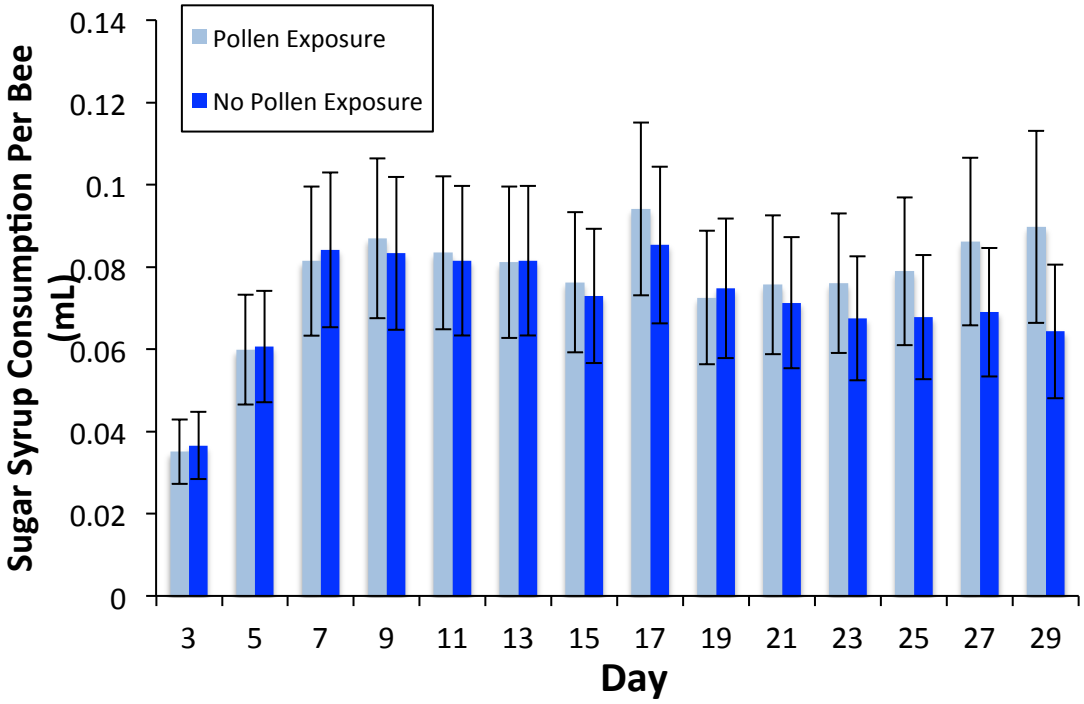

Figure 3.10 Median sugar syrup consumption (+ 90% confidence intervals) of bees exposed to imidacloprid through pollen diet and bees that were not exposed through pollen diet over each 2-day period.

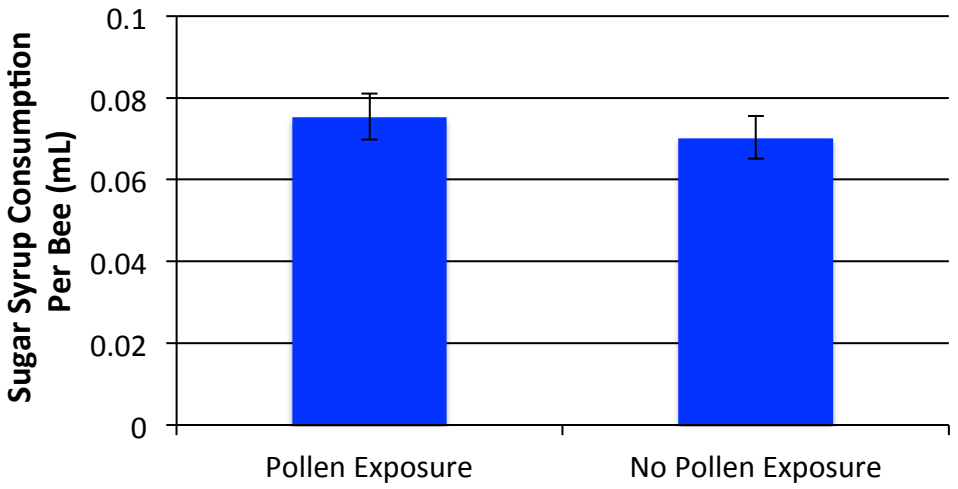

Figure 3.11 Median sugar syrup consumption (+ 95% Tukey-adjusted confidence intervals) of bees that were exposed to imidacloprid through pollen diet and those that were not exposed through pollen diet, averaged across the duration of the experiment.

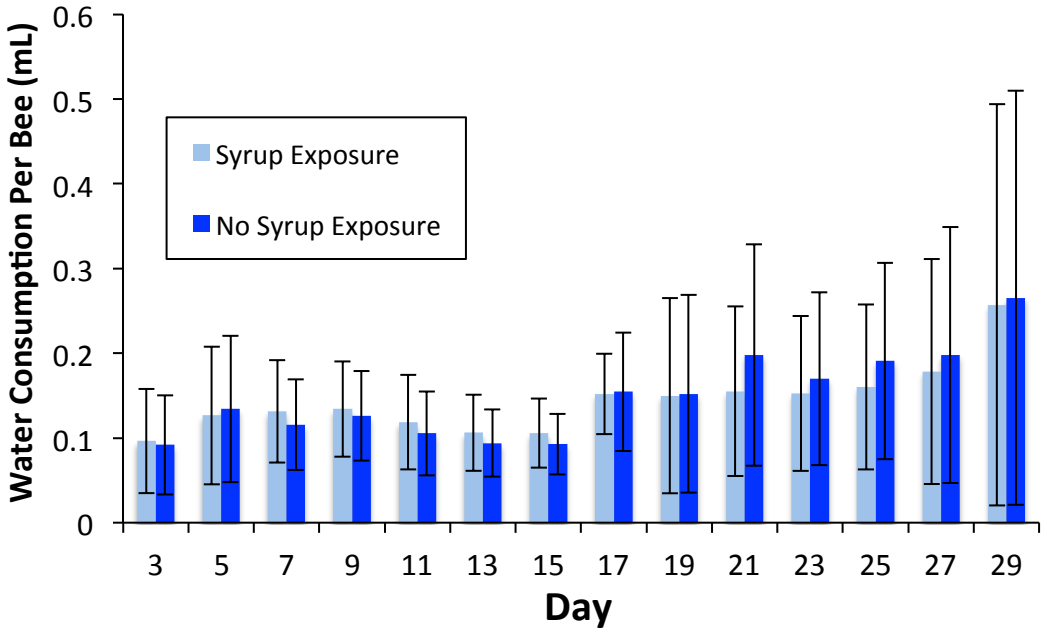

Figure 3.12 Median water consumption (+90% confidence intervals) of bees exposed to imidacloprid through sugar syrup and those that were not exposed through sugar syrup over each 2-day period, with outliers included.

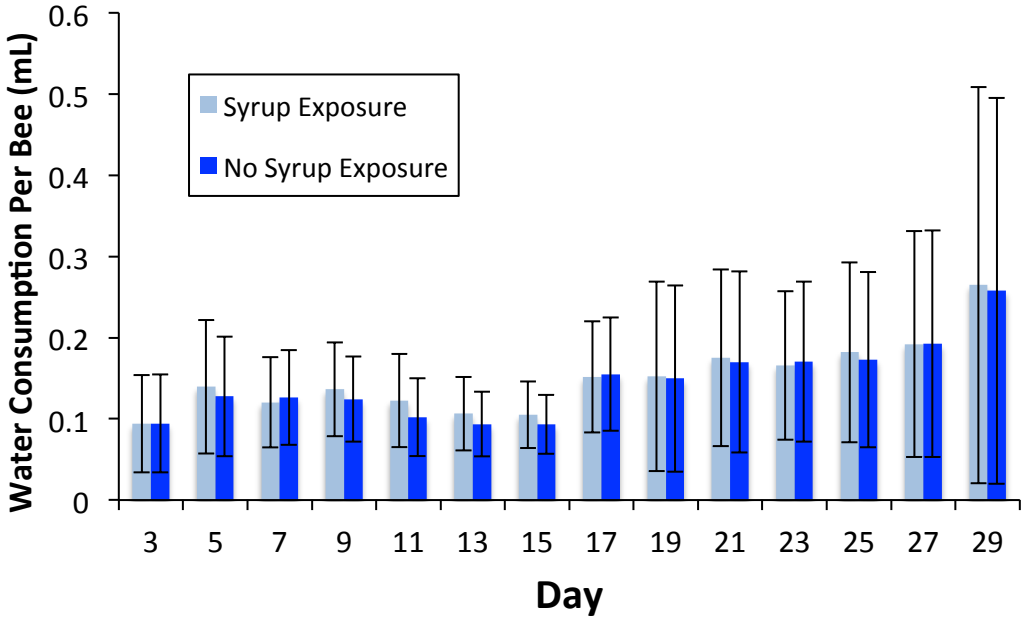

Figure 3.13 Median water consumption (+95% confidence interval) of bees exposed to imidacloprid through sugar syrup and bees not exposed through sugar syrup over each 2-day period, excluding outliers.

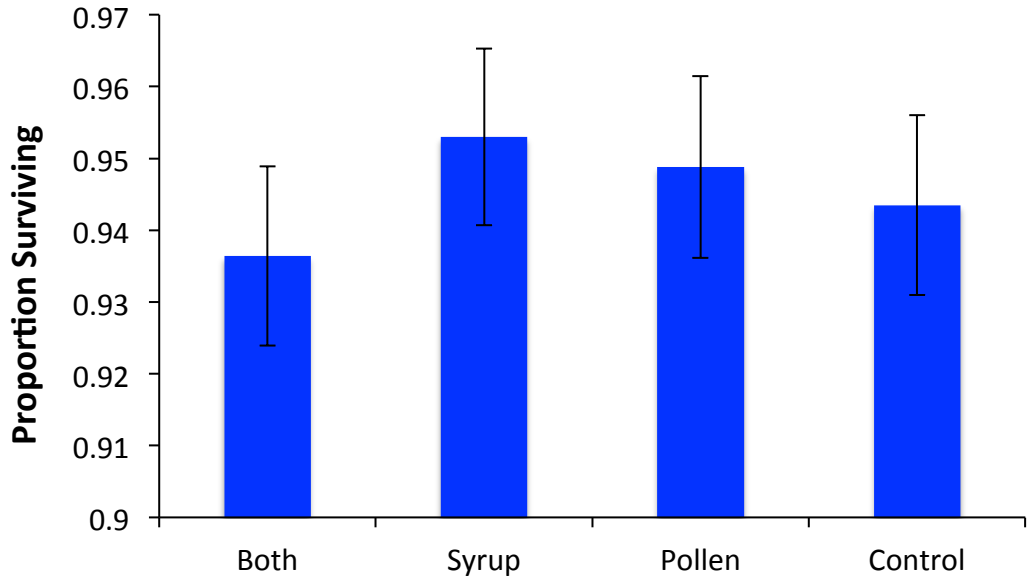

Figure 3.14 Mean proportion of bees surviving (+ 95% confidence intervals) in all treatments averaged across all 2-day intervals throughout the duration of the experiment.

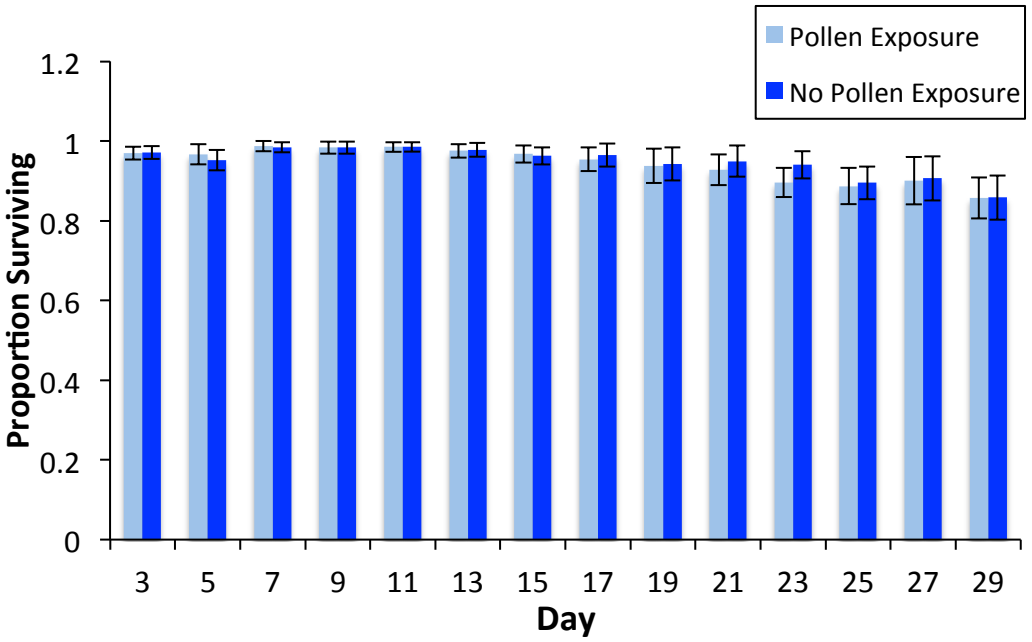

Figure 1.15 Mean proportion of bees surviving (+95% confidence intervals) over each 2-day period for bees exposed to imidacloprid through pollen diet and those not exposed to imidacloprid through pollen diet.

Chapter 4: Summary

Honey bees play a critical role in American agriculture [1], and honey bee colony losses pose a persistent threat to pollination and crop production. While researchers agree that honey bee colony losses are due to a number of different interacting factors [2-6], the impacts of pesticide on colony health and mortality are still poorly understood. Neonicotinoid insecticides in particular have been scrutinized for their impacts on bees [7-8], although most studies have not directly linked neonicotinoid exposure to colony mortality [6, 9-10]. Although researchers have largely dismissed the role of neonicotinoids in colony losses, there are still many gaps in our understanding regarding the impacts of these chemicals on long-term colony health. In this thesis, i sought to determine how field-realistic exposure of honey bees to a neonicotinoid insecticide, both in combination with a fungicide and alone, and through multiple routes, impacts colony health, foraging, worker physiology, and potentially susceptibility to pathogens over a more extended period of time than previous studies.

Honey bees are commonly exposed to multiple chemicals at once [11-14], but most research has largely investigated the impacts of single-chemical exposure. Other pesticides, such as fungicides, may also interact with neonicotinoids to increase colony mortality, though many of these interactions have not yet been investigated [15-18]. The objective of Chapter 2 was to determine whether a commonly used neonicotinoid (imidacloprid) and a widely used fungicide (chlorothalonil) interact to affect various parameters pertaining to colony health and immunity. These two chemicals are widely used in various agricultural crops, co-occur in pollen samples [12], and have thus far not been investigated for their potential interactive effects. We observed the effects of field-realistic concentrations of these chemicals in pollen diet on colony size, foraging behavior, food stores, and nurse bee physiology. We detected no significant main effects or interactive effects on the adult bee population or brood area of colonies throughout the duration of the experiment, nor did we detect effects on the relative changes in adult bee population and brood area throughout the

experiment. We also did not detect any effects of these chemicals or their interaction on midgut proteolytic enzyme activity, hypopharyngeal gland protein content, abdominal lipid stores, or glucose oxidase activity. The results for the prophenoloxidase analysis differed when outliers were excluded from the analysis. We did not detect any effects on prophenoloxidase activity when outliers were included in the analysis, but there was a significant interaction between chlorothalonil and time when the two outliers were excluded. In this interaction, colonies not exposed to chlorothalonil exhibited higher prophenoloxidase activity than those exposed to chlorothalonil prior to the exposure period, but lower prophenoloxidase activity than exposed colonies at the end of the exposure period. These results concur with previous findings that chlorothalonil exposure causes prophenoloxidase genes to be upregulated [19], but it is still somewhat unclear whether this is helpful or detrimental to colony immunity. Chlorothalonil has been significantly correlated with higher *Nosema ceranae* infection in a previous experiment [12], but prophenoloxidase activity also did not change in response to *Nosema ceranae* infection [20]. Chlorothalonil exposure may therefore increase honey bee immunocompetence against wounding [21-22] and less prevalent bacterial pathogens [23], but this may not necessarily mean that these bees are less susceptible to the most problematic honey bee pathogens. Further research should determine the implications of the impacts of chlorothalonil on the prophenoloxidase cascade, and how these changes may relate to susceptibility or resistance to various honey bee parasites and viruses.

Finally, while we did not detect any effects of these chemicals or their interaction on the number of pollen foragers returning to the colonies (both when outliers were included and excluded), we found that both the number of total foragers and the number of non-pollen foragers returning to the colony were significantly affected by both chemicals. When outliers were excluded from the analysis, we found that imidacloprid and chlorothalonil both caused a significant reduction in the number of non-pollen foragers returning to the colonies seven weeks after the end of exposure period. At that time, the number of non-pollen foragers returning to colonies exposed

to both chemicals was half that of colonies exposed to neither chemical. These results suggest that young bees were effected by the presence of these chemicals or their metabolites in pollen or brood food at later stages, which has been suggested in previous studies [24-26]. Further, because these effects were not necessarily consistent for all generations of bees that were exposed to these chemicals, we suggest that agrochemical exposure reaches a critical threshold after a certain period of time, which ultimately reduces the number of foragers returning to colonies. Because of these findings, we hypothesize that these two chemicals may pose a greater risk to bee colonies during the pollination of crops that bloom for a month or longer. Further, while the total number of foragers was lower for both colonies exposed to imidacloprid and those exposed to chlorothalonil over the duration of the experiment, the chemicals did not have a synergistic effect on the number of total returning foragers. These differences were likely driven by the reduction in non-pollen foragers.

While we detected a significant interaction between these chemicals that impaired the success of non-pollen foragers long after exposure, we did not detect a similar interaction on the amount of honey stores in honey bee colonies. It is possible that honey stores were not impaired despite reductions in non-pollen foragers, because the number of foragers were reduced long after the end of the honey flow, when most nectar had already been collected. These results may have been different if our colonies had been exposed to these chemicals during the spring months, when more nectar collection was under way. Because crop pollination and pesticide exposure occurs mostly during the spring months, and pesticide sensitivity differs widely between seasons [27-28], we recommend that future field experiments should attempt to quantify how these results may differ when colonies are exposed to agrochemicals during the spring. In most field studies, colonies are exposed to agrochemicals during the months of June and July [29-33], rather than earlier months. The effects of pesticide exposure during the spring months have received little to no attention from the scientific community.

Finally, our analyses of prophenoloxidase activity, non-pollen foragers, and total foragers in Chapter 2 show that results may differ drastically when outliers are included or excluded from the analyses. The presence of outliers in the data significantly reduces statistical power and increases the probability of Type II errors [34]. Outliers may have significantly reduced the power of previous field studies pertaining to imidacloprid, of which 50% have not been statistically powerful enough to detect effects [9]. Despite this possibility, researchers have neither mentioned the presence or influence of outliers in previous field data. Failure to detect significant effects in experiments without addressing statistical power and outliers may lead researchers to incorrectly conclude that agrochemicals have no impacts on honey bee colonies. We emphasize the need to conduct experiments that maximize replication, minimize variation between subjects, and thoroughly discuss how the data meet, or violate, the assumptions of the chosen statistical model. The accurate determination of interactions between pesticides, as well as between pesticides and pathogens, will require thorough consideration of appropriate statistical analyses and violations of the model assumptions.

The objective of Chapter 3 was to investigate whether bee mortality and physiology (abdominal lipid stores, hypopharyngeal gland protein content, prophenoloxidase activity, midgut proteolytic enzyme activity, and glucose oxidase activity) were significantly affected by imidacloprid exposure through pollen or sugar syrup, and to investigate if these effects were significantly exacerbated by exposure through both routes. Although imidacloprid and other neonicotinoids have been detected in multiple matrices in previous experiments, including pollen [14, 16, 35-37], nectar [35-39], and water [35, 40], the potential for exposure through multiple routes to exacerbate the impacts of exposure has not been investigated. Most studies expose bees or colonies to neonicotinoids solely through pollen diet [30, 32] or sugar syrup [28, 33, 41-43]. There are currently two laboratory studies that have investigated some physiological effects when bees are exposed to imidacloprid through both pollen and sugar syrup [44-45], but these experiments neither determined if exposure

through pollen diet and exposure through sugar syrup interacted with one another to exacerbate the effects of imidacloprid, nor did they analyze differences in mortality.

We found that exposure through one or both routes did not affect abdominal lipid stores, hypopharyngeal gland protein content, or prophenoloxidase activity of worker bees. We did, however, find that midgut proteolytic enzyme activity was significantly higher in bees exposed to imidacloprid through sugar syrup than those not exposed through sugar syrup, although the effect size was relatively small (equivalent to 0.05 OD_{440}). This effect size may not necessarily be biologically significant to the long-term health and survival of the colony. Because bees exposed to imidacloprid through sugar syrup did not survive at significantly higher rates than those that were not, and because bees exhibiting similar differences in proteolytic enzyme activity did not have significantly different rates of survival in a previous laboratory experiment [46], we conclude that though our analysis detected a statistically significant difference between these groups, the difference was unlikely to be biologically significant.

We also found that glucose oxidase activity was affected differently by imidacloprid exposure when outliers were included or excluded from the analysis. When outliers were included in the analysis, we detected both an interaction between exposure through sugar syrup and time, as well as a main effect of exposure through sugar syrup. There were no differences between groups after two weeks of exposure, but after four weeks of exposure, glucose oxidase activity was higher in bees exposed to imidacloprid through sugar syrup than it was in bees not exposed through sugar syrup. The main effect of sugar syrup showed that overall, bees exposed through sugar syrup had higher glucose oxidase activity than those that were not exposed through sugar syrup. When outliers were excluded from the analysis, the interaction between exposure through sugar syrup and time remained the same, but there was also an interaction between both exposure routes and time, as well as an interaction between exposure through pollen and time (although insignificant after the Tukey multiple comparisons adjustment).

In the interaction between exposure routes and time, glucose oxidase activity was not significantly different between treatments after two weeks of exposure, but was significantly different between treatments after four weeks of exposure. After four weeks of exposure, bees exposed to imidacloprid through both pollen diet and sugar syrup exhibited higher glucose oxidase activity than either control bees or bees exposed to imidacloprid through pollen. At the same time, bees in the control group, the group exposed through only sugar syrup, and the group exposed through only pollen were not different from each other. These results differ greatly from Alaux et al. [41], in which bees exposed to various concentrations of imidacloprid did not differ in glucose oxidase activity after ten days of exposure. Our results demonstrate the need for experiments that investigate the effects of imidacloprid when it is present in both pollen and sugar syrup, as well as experiments that investigate chronic exposure over a period longer than two weeks.

Our analysis of mortality in Chapter 3 determined that mortality was higher in bees exposed to imidacloprid through both pollen and sugar syrup than those exposed to imidacloprid through only sugar syrup. These differences were probably due to increased imidacloprid intake, since these bees consumed more sugar syrup than bees exposed through only syrup, and consumed more pollen than bees not exposed through pollen. Further, this was the only group of bees that had no access to imidacloprid-free food throughout the experiment. At the same time, mortality in bees exposed through both matrices was not significantly different from control bees or the bees exposed to imidacloprid only through pollen. We hypothesize that because these bees consumed more pollen and more sugar syrup than other bees, increased nutrient intake may have buffered the detrimental effect of imidacloprid. However, we also emphasize the need for a more robust survival analysis that accounts for the random effects of cages and rows within the incubator, and determines differences in time to death between treatment groups. The results from our mortality analyses indicate the need for longer term chronic toxicity studies, although the effects we detected were not as dramatic as in previous chronic toxicity studies [47-48].

Overall, these studies have provided new insights on impacts of pesticide exposure to honey bee colonies. Future research should continue to investigate interactions between agrochemicals, effects of multiple exposure routes on entire colonies, effects on nurse bees and bee foraging in the long term, and interactions between pesticides and pathogens.

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APPENDICES

Appendix X1. Details of Protocols and Statistical Methods for Chapter 2

1. Chemical Mixing Protocol and Calculations

Chemicals were mixed into pollen on a weekly basis. Each treatment group was assigned a 1-liter HDPE bottle for its corresponding acetone solution. Each bottle contained enough solution for 11 pollen patties (990 mL) in order to account for potential loss of solution during the mixing process. The amount of active ingredients added to dry pollen was based on the target concentration and the total mass of one pollen patty: 501 g pollen and 280.5 g (255 mL) of sugar syrup. The mass and concentration of each chemical in each treatment is shown in **Table 1.**

Treatment Group	Concentration (ppb)	Mass Per Patty (mg)	Mass Per Bottle (mg)	Concentration in acetone solution (mg/ml)
Control	Imidacloprid: 0 Chlorothalonil: 0	Imidacloprid: 0 Chlorothalonil: 0	Imidacloprid: 0 Chlorothalonil: 0	Imidacloprid: 0 Chlorothalonil: 0
Imidacloprid	Imidacloprid: 39 Chlorothalonil: 0	Imidacloprid: 0.030 Chlorothalonil: 0	Imidacloprid: 0.335 Chlorothalonil: 0	Imidacloprid: $3.4x10^{-5}$ Chlorothalonil: 0
Chlorothalonil	Imidacloprid: 0 Chlorothalonil: 3014.8	Imidacloprid:0 Chlorothalonil: 2.356	Imidacloprid: 0 Chlorothalonil: 25.9	Imidacloprid: 0 Chlorothalonil: 0.0262
Imidacloprid + Chlorothalonil	Imidacloprid: 39 Chlorothalonil: 3014.8	Imidacloprid: 0.030 Chlorothalonil: 2.356	Imidacloprid: 0.335 Chlorothalonil: 25.9	Imidacloprid: $3.4x10^{-5}$ Chlorothalonil: 0.0262

Table 1. Intended concentrations and total mass of imidacloprid and chlorothalonil in pollen patties and in acetone solutions.

Chlorothalonil was added directly to each final solution bottle prior to mixing. Imidacloprid was applied by adding 100 µL of more concentrated stock solution (3.3 mg/mL, prepared beforehand) to the final solution. The stock solution was vortexed before each time it was used in order to maintain homogeneity of the active ingredient. All bottles containing the final solution were shaken vigorously to produce a homogenous solution.

A set of utensils, containers, bowls and glassware was dedicated for each treatment group, and was used during both the mixing of pesticides and the mixing of pollen with sugar syrup. The pollen for each colony was mixed individually to ensure the appropriate mass and concentration of active ingredient was present in the patty for each colony. We measured 90 mL of acetone solution per colony, and sprayed the solution gradually into the dry pollen using an HDPE spray bottle. The pollen was sprayed and mixed vigorously with a whisk until the pollen took a uniform damp tone. Afterward, the pollen was transferred to two pie tins per colony and left to dry overnight.

The following day, the pollen for each colony was combined with 255 mL of 33% (v/v) sugar syrup in the appropriate bowl. The mixture was mixed thoroughly with a spatula until the texture of the patty was homogenous.

After a 2 g of each patty was removed for analytical testing, the rest was transferred to an aluminum pie tin labeled for the appropriate colony, and the mass of the patty was recorded as follows:

Mass of pollen patty in pie tin – Mass of pie tin = Mass of the pollen patty

Afterward, each patty was covered with a sheet of waxed paper and stored at -20˚C until the day of feeding.

2. *Varroa* and *Nosema* quantification

Mites were counted in a double alcohol wash. The number of Varroa mites and the number of bees were recorded for each sample. *Varroa* infestation was recorded as % bees parasitized (# of mites / # of bees * 100%).

To quantify *Nosema* infection, abdomens were macerated using a mortar and pestle and mixed with 100 ml (1 mL per bee) of deionized water. A drop of this solution was added to a hemocytomer (Hausser Scientific, PA, USA) and *Nosema* spores were counted according to the light microscopy techniques described by Cantwell [1].

3. Hypopharyngeal Gland Protein Content

Samples were thawed on ice, homogenized with one 3-mm tungsten carbide bead at 20 oscillations/second for two rounds of 45 seconds in a Qiagen® Tissue Lyser II, and then centrifuged at 4˚C and 13,300 rpm for 4 minutes. We added 10 µL of the resulting supernatant to 90 µL of chilled PBS to create a $1/10^{th}$ dilution. Following the microplate procedure of the Pierce Biotech BCA Assay Kit Instructions (Thermo-Scientific), 10 ul of each sample dilution and each of seven Bovine Serum Albumin standards (125-2000 ug/ml, diluted in PBS) were plated in duplicate on a chilled 96-well plate. After adding 200 µl of working reagent to all wells, the plate was shaken for 30 seconds and incubated for 30 minutes at 37 C, followed by 4 minutes at room temperature. Absorbance at 562 nm was then read on a BioTek Synergy 2 plate reader at 25 C, and total protein concentration was calculated. HPG protein per bee was then calculated using the following formula:

$$Protein\ per\ bee\ (mg) = \frac{[protein] \times\ Sample\ Volume \times Dilution\ Factor}{\#\ of\ bees}$$

Where:
[protein] = protein concentration (mg/ml) reported by the assay
Sample Volume = the total volume of the homogenized sample (here, 0.125 ml)
Dilution Factor = amount by which the original sample was diluted (here, 10)
# of bees = # of pairs of hpg glands in the sample (here, 10)

4. Prophenoloxidase Activity

To create a workable concentration of L-Dopa (which is only marginally soluble in water), and thus to yield the maximum reaction velocity possible with this substrate, we made a saturated solution by vortexing 10 mg L-Dopa per 1 ml distilled, autoclaved water (water temperature 20-21ºC) on the maximum setting for 10 minutes. We then filtered the solution through a sterile 0.45 µm PTFE syringe filter to remove the L-Dopa that had not dissolved. This saturated solution had a final concentration between K_m (5.75 mM) and half K_m [2]. The solution was protected from light by a tinfoil wrapper at all times.

Twenty microliters of each hemolymph sample were mixed with 135 µl of distilled, autoclaved water, and 20 µl PBS in each of two replicate wells of a 96-well plate (Greiner Bio-One non-binding, chilled). Sodium cacodylate was used as a blank. Five microliters of chilled α-chymotrypsin were then added to all wells. The plate was shaken for 30 seconds, and subsequently incubated at room temperature (21 $\pm$ 0.5 ºC) for 4.5 minutes. Finally, we added 20 µl of L-Dopa to all wells. Absorbance at 490 nm was read every 31 seconds for 1 hour at 30 ºC on a BioTek Synergy 2 plate reader. Maximum linear velocity of the reaction was then calculated from the reaction progress curve.

5. Glucose Oxidase Calculation

The assay measured change in absorbance (ΔAbs_{430}) in a given amount of time. We chose to exclude from the reaction time the first several minutes of the assay, while plates reached optimal reaction temperature and reactions were mixed. Reaction ending time was chosen such that the value of a sample did not exceed the value of the highest standard.

From ΔAbs_{430}, by way of the H_2O_2 standard curve, we calculated the average amount of H_2O_2 (nmol) produced in the two replicate reaction wells of a sample. We then calculated the units of glucose oxidase activity—where one unit of GOx activity is equal to the amount of the enzyme required to generate 1.0 μmole of H_2O_2 per minute at 37 °C—using the following equation:

$$\text{Glucose Oxidase Activity (mU/ml)} = \frac{\text{nmoles of } H_2O_2 \times \text{Dilution Factor}}{\text{Rxn Time} \times \text{Volume}}$$

Where:

nmoles of H_2O_2 = amount (in nmoles) of H_2O_2 generated between $T_{initial}$ (starting minute for ΔAbs_{430} calculation) and T_{final} (ending minute for ΔAbs_{430}).

Dilution Factor = amount by which the original sample was diluted (here, 5)

Rxn Time = $T_{final} - T_{initial}$ (in minutes)

Volume = sample volume (in ml) added to well (here, 0.01)

ΔAbs_{430} of sample blanks were subtracted from that of the samples before the amount of H_2O_2 generated in that sample was calculated.

6. Midgut Proteolytic Enzyme Activity

We transferred 350 µL of each sample and blank into plastic 1-cm cuvettes containing 200 µL of 50% ethanol. We vortexed each cuvette gently and then measured absorbance at 440 nm on a Beckman® DU spectrophotometer. For each sample, the spectrophotometer was blanked, using the sample blank, before absorbance of the two duplicate sample reactions were measured and averaged. Total MEA was expressed as mean OD_{440}.

7. Abdominal Lipid Stores

Abdomens were dissected from ten nurse bees. An incision was made down the ventral side of each abdomen to maximize ventilation during the drying period. Abdomens were dried ventral-side-up at room temperature for 72 hours in a fume hood. Following the first drying period, samples were weighted and then soaked (fully submerged) in 3 ml of ethyl ether in sealed glass vials, for 24 hours. Following the ether bath, samples were rinsed once with another 3 mL ether to remove residual lipids. Abdomens were then left to dry in the fume hood for another 72 hours, and subsequently weighed once more. Abdominal lipid stores were recorded as the percent change in dry abdominal mass after the ether bath.

7. Statistical Analysis Details

The explanatory variables in each model were as follows: imidacloprid (indicator variable of 1 or 0), chlorothalonil (indicator variable of 1 or 0), each time period (indicator variable of 1 or 0), and all combinations of the three factors. Time period was set as “Week” for foraging data (indicator variable of 1 or 0 for weeks 0-12) and “Month” (indicator variable of 1 or 0 for months 0-3 for colony evaluations and pathogen data, indicator of 1 or 0 for Months 0-2 total of 3 months for percent change in adult bee population/brood, and all physiological analyses) for all other responses. The baseline for each (Week or Month 0) indicates the baseline measurement. For

each model, the random effect of Hive (a factor denoted by the colony's identifying number) was nested in the random effect of Block (a factor based on the colony's assigned block).

The models that were run on log-transformed data are as follows: pollen foragers, nectar foragers, total adult bee population, total frames of honey, prophenoloxidase activity, glucose oxidase activity, *Nosema*, and *Varroa*. The models that allowed unequal variances between time points were adult bee population, percent change in brood, total brood, total foragers, total frames of honey, hypopharyngeal gland protein content, midgut proteolytic enzyme activity, glucose oxidase activity, abdominal lipid stores, *Varroa*, and *Nosema*.

For all analyses, we used the "anova" function in R to compare models incorporating an autoregressive 1 correlation structure, a compound symmetry correlation structure, and a general correlation matrix. We chose the model with with the lowest BIC value to select the correlation structure of the model.

If two models had similar and low BIC values, we compared residuals plots to determine which model best met the normality and constant variances assumptions. The following responses incorporated no additional correlation between time points: percent change in adult bee population, total adult bee population, percent change in brood, total brood, total frames of honey, *Varroa*, *Nosema*, glucose oxidase activity, and midgut proteolytic enzyme activity. The following models incorporated an autoregressive 1 correlation structure: hypopharyngeal gland protein content, prophenoloxidase activity, abdominal lipid stores, total foragers, non-pollen foragers, pollen foragers, and total foragers.

In some cases, the assumptions of the model were not met while these outliers were included in the model, and in these cases the model was run only on data excluding outliers. We excluded outliers from data sets in which the assumptions of the model could not be met when outliers were included. For data sets that appeared to meet the

assumptions of the model both with and without outliers, we performed the analysis in both cases. Data sets that excluded outliers to meet the assumptions of the model include percent change in adult bee population (2 outliers), midgut proteolytic enzyme activity (3 outliers), and glucose oxidase activity (4 outliers). In cases when assumptions appeared to be met both with and without outliers, the model was run for both scenarios. For analyses that were different with outliers than without outliers, we report results for both analyses. For those that were not different, we only report results for the analyses including outliers. Models that were run both with and without outliers are as follows: adult bee population (6 outliers), percent change in brood (2 outliers), total brood (2 outliers), prophenoloxidase activity (3 outliers), pollen foragers (13 outliers), non-pollen foragers (20 outliers out of 384 data points), abdominal lipid stores (1 outlier), total foragers (20 outliers of 384 data points), and total frames of honey (5 outliers).

When zero values were included in the models for the *Varroa* and *Nosema* analyses, residuals indicated a downward trend with increasing fitted values, which violated the assumptions of the model. While these variables could have been analyzed with a generalized linear mixed model, the alternate model would not have been able to account for the nested random effects of colonies within blocks.

Appendix X1 References

1. Cantwell GE. Standard methods for counting Nosema spores. Amer Bee J. 1970; Available: http://agris.fao.org/agris-search/search.do?recordID=US201301199366

2. Laughton AM, Siva-Jothy MT. A standardised protocol for measuring phenoloxidase and prophenoloxidase in the honey bee, Apis mellifera. Apidologie. 2011;42: 140–149. doi:10.1051/apido/2010046

Appendix X2. List of F-statistics and P-values Not Given in the Results Section for Chapter 2

Interaction	$F_{3, 62}$	p-value
Imidacloprid*Month	2.030	0.140
Chlorothalonil*Month	1.976	0.147
Imidacloprid*Chlorothalonil*Month	1.852	0.166

Table X1. F-statistics and p-values for the analysis of the percent change in adult bee population.

Interaction	$F_{3,96}$	p-value
Imidacloprid*Month	0.092	0.964
Chlorothalonil*Month	1.152	0.332
Imidacloprid*Chlorothalonil*Month	0.297	0.828

Table X2. F-statistics and p-values for analysis of total brood area, outliers included.

Interaction	$F_{3,94}$	p-value
Imidacloprid*Month	0.411	0.745
Chlorothalonil*Month	1.778	0.157
Imidacloprid*Chlorothalonil*Month	0.991	0.401

Table X3. F-statistics and p-values for analysis of total brood area, outliers excluded.

Interaction	$F_{3, 95}$	p-value
Imidacloprid*Month	0.497	0.686
Chlorothalonil*Month	0.533	0.661
Imidacloprid*Chlorothalonil*Month	0.166	0.919

Table X4. F-statistics and p-values for the analysis of honey stores with outliers included.

Interaction	$F_{12, 384}$	p-value
Imidacloprid*Month	1.051	0.401
Chlorothalonil*Month	1.216	0.270
Imidacloprid*Chlorothalonil*Month	0.973	0.474

Table X5. F-statistics and p-values for the analysis of non-pollen foragers with outliers included in the analysis.

Interaction	$F_{12, 384}$	p-value
Imidacloprid*Week	1.163	0.308
Chlorothalonil*Week	0.893	0.555
Imidacloprid*Chlorothalonil*Week	0.850	0.599

Table X6. F-statistics and p-values for the analysis of total foragers with outliers included.

Interaction	$F_{12, 365}$	p-value
Imidacloprid*Week	1.488	0.126
Chlorothalonil*Week	1.383	0.171
Imidacloprid*Chlorothalonil*Week	0.836	0.613

Table X7. F-statistics and p-values for the analysis of total foragers with outliers excluded.

Interaction	$F_{3, 44}$	p-value
Imidacloprid*Month	0.935	0.432
Chlorothalonil*Month	0.178	0.911
Imidacloprid*Chlorothalonil*Month	0.756	0.525

Table X8. F-statistics and p-values for the analysis of *Varroa* mite infestation with outliers included.

Effect	F statistic	Degrees of freedom	p-value
Imidacloprid	1.195	1, 24	0.285
Chlorothalonil	0.385	1, 24	0.541
Imidacloprid*Chlorothalonil	0.281	1, 24	0.601
Imidacloprid*Month	1.078	3, 43	0.369
Chlorothalonil*Month	0.154	3, 43	0.927
Imidacloprid*Chlorothalonil*Month	0.776	3, 43	0.514

Table X9. F-statistics and p-values for the analysis of *Varroa* mite infestation with outliers excluded.

Interaction	$F_{3, 31}$	p-value
Imidacloprid*Month	1.558	0.219
Chlorothalonil*Month	0.857	0.474
Imidacloprid*Chlorothalonil*Month	0.200	0.895

Table X10. F-statistics and p-values for the analysis of *Nosema* infection.

Interaction	$F_{2, 64}$	p-value
Imidacloprid*Month	1.243	0.295
Chlorothalonil*Month	0.159	0.854
Imidacloprid*Chlorothalonil*Month	0.791	0.458

Table X11. F-statistics and p-values for the analysis of hypopharyngeal gland protein content.

Interaction	$F_{2, 61}$	p-value
Imidacloprid*Month	1.527	0.225
Chlorothalonil*Month	0.589	0.558
Imidacloprid*Chlorothalonil*Month	0.721	0.491

Table X12. F-statistics and p-values for the analysis of midgut proteolytic enzyme activity.

Interaction	F	Degrees of Freedom	p-value
Imidacloprid*Chlorothalonil	0.012	1, 24	0.915
Imidacloprid*Month	1.397	2, 64	0.255
Chlorothalonil*Month	1.634	2, 64	0.203
Imidacloprid*Chlorothalonil*Month	0.433	2, 64	0.651

Table X13. F-statistics and p-values for the analysis of glucose oxidase activity.

Effect	Degrees of freedom	F statistic	P-value
Imidacloprid	1, 24	1.119	0.301
Chlorothalonil	1, 24	0.032	0.860
Imidacloprid*Chlorothalonil	1, 24	0.255	0.618
Imidacloprid*Month	2, 61	0.024	0.976
Imidacloprid*Chlorothalonil*Month	2, 61	0.849	0.433

Table X14. F-statistics and p-values for the analysis of prophenoloxidase activity with outliers excluded.

Interaction	$F_{2, 63}$	p-value
Imidacloprid*Month	1.475	0.237
Chlorothalonil*Month	0.025	0.976
Imidacloprid*Chlorothalonil*Month	1.576	0.215

Table X15. F-statistics and p-values for the abdominal lipid stores analysis.

Appendix Y1: Details for Protocols and Statistical Analyses for Chapter 3

1. Pollen Treatment Procedure

1.1. Stock Solution

The mass of each pollen patty was 38 g, as determined prior to the start of the experiment. For a target concentration of 39 ppb, we calculated that 0.000148 mg of imidacloprid should be present in each patty. Because this mass is too low to be detected by a laboratory balance, we made a 50-mL stock solution from which we could pipette a smaller mass of imidacloprid. We used the following formula to determine the appropriate volume and concentration of imidacloprid stock solution that would produce the target mass of imidacloprid:

$$\text{Volume (mL)} \times \text{concentration (mg/mL)} = \text{mass (mg)}$$

Ultimately, we chose to make a 0.07 mg/mL stock solution using 3.5 mg of imidacloprid and 50 mL of acetone. This concentration was the most ideal for measuring an accurate mass of imidacloprid on the balance. It was also ideal for producing a stock solution dilute enough to extract a volume that was not immeasurably small.

1.2. Mixing Procedure

For each imidacloprid-treated pollen patty, we transferred 2.1 uL of this stock solution a high-density polyethylene eye-dropper bottle containing an additional 6 ml of acetone, and then distributed the entirety of this solution into the 25 g of dry pollen, as further described below. We used two stainless steel bowls to make the pollen patties for this experiment: one for imidacloprid-treated patties and one for patties not treated with imidacloprid. If the pollen patty was not assigned to

imidacloprid treatment, we added 6 mL of plain acetone to the pollen gradually using the control eye dropper. For the imidacloprid-treated pollen patties, we added the acetone solution to the pollen after transferring the 2.1 µL of the stock solution and swirling the solution to evenly distribute the chemical.

All acetone was gradually but vigorously whisked into the pollen until the mixture was evenly damp throughout the pollen. We then transferred the pollen into a petri dish labeled for a specific cage (lined with aluminum foil to prevent degradation by the acetone) to dry. All pollen was allowed to dry overnight, and 13 mL of 33% sugar syrup was mixed in the following day.

1.2. Sugar Syrup

The mass of 13 mL of 33% sugar syrup was consistently 13 g. Sugar syrup was mixed on a magnetic stir plate in multiple 1-liter batches, each containing 300 mL (119 g) of sucrose, and 600 mL water.

2. Sugar Syrup Treatment Procedure

Sugar syrup for the syrup treatment was prepared in multiple 1-L batches, each containing 500 mL (419 g) of sucrose and 500 mL of DI water. The syrup was mixed thoroughly on a magnetic stir plate. The mass of sugar syrup in a single vial (100 mL) was consistently 119 g.

Prior to the experiment, an acetone stock solution of imidacloprid (8 mg dissolved in 50 mL, concentration 0.16 mg/mL) was prepared in an amber glass vial, which was stored in the dark at room temperature throughout the experiment. 2.2 µL (for a target mass of 0.000352 mg imidacloprid) of this solution was added to every fresh 100 ml allotment of sugar syrup for each cage assigned to sugar syrup imidacloprid exposure.. The syrup for cages not receiving imidacloprid-treatedsugar syrup was given 2.2 µL of plain acetone.

After the appropriate acetone solution was added to the sugar syrup for a given cage, the vial was capped with a treatment-specific lid and shaken vigorously to create a homogeneous solution. Both the sugar syrup and water vials were covered with two layers of cheesecloth. They were subsequently secured, inverted, to the top of each cage to allow workers to feed ad libitum.

3. Mortality Counts During Pollen Removal

On a weekly basis, pollen patties and cage bottoms were removed and replaced, and dead were counted and removed. The number of dead bees on the cage bottom was counted regardless of whether that day fell at the end of a 2-day mortality interval. If mortality was being recorded that day, the dead bees were discarded after the mortality count was complete. If mortality had been recorded on the day prior to pollen patty replacement, we added the number of bees that had died over the 24-hour period to the count on the following day.

4. Abdominal Lipids

The abdomens from ten sampled bees were removed and dried in small tin weigh boats in a room-temperature fume hood for 4 days, and their mass was subsequently recorded. After all samples were weighed, they were submerged in 3 mL of ethyl ether for 24 hours. After the ether bath, abdomens were rinsed twice in ethyl ether to remove any residual lipids, and left to dry for another 4 days. Abdominal lipid stores were recorded as the percent change in abdominal mass.

During the initial drying period for the samples from the first sampling event, one of the abdomens from Cage 32 was determined missing. The abdominal lipid stores for this cage were therefore calculated on 9 abdomens. Assuming that the percent lipid weight in each cage was even for all bees, we assumed that this would not significantly change the measurement of abdominal lipid stores.

5. Details of Statistical Analyses

The models that were performed on log-transformed data are as follows: hypopharyngeal gland protein content, prophenoloxidase activity, glucose oxidase activity, pollen consumption, sugar syrup consumption, and water consumption. The fitted means for these responses were back-transformed after the analysis.

The following models allowed for unequal variances among time points: hypopharyngeal gland protein content, abdominal lipid stores, midgut proteolytic enzyme activity, prophenoloxidase activity, glucose oxidase activity, and proportion of bees surviving over each 2-day period.

Correlation structures for each model were determined using the "anova" function. The models for abdominal lipid stores, hypopharyngeal gland protein content, prophenoloxidase activity, and glucose oxidase activity did not account for any additional correlation between time points. The models for sugar syrup consumption and water consumption incorporated an autoregressive 1 correlation structure, while the models for pollen consumption and midgut proteolytic enzyme activity incorporated a general correlation matrix. The model for the proportion of bees surviving over each 2-day period incorporated a compound symmetry correlation structure.

Several of our analyses contained data points that were outliers (absolute value of residual > 2). Some of our models did not meet the assumptions when these outliers were included in the analysis, and the outliers were therefore removed. These models include abdominal lipid stores (1 outlier), midgut proteolytic enzyme activity (1 outlier), and proportion surviving at the end of each 2-day period (20 outliers out of 448 data points). Other models appeared to meet the assumptions of the model when outliers were included or excluded. For these models, we ran the model under both conditions: pollen consumption (5 outliers), sugar syrup consumption (16 outliers out

of 447 total data points), water consumption (6 outliers out of 446 data points), glucose oxidase activity (1 outlier), and the proportion surviving over the entire experiment (1 outlier). If the results differed between the models, we reported results on both. If the results did not differ, we reported the results only for the model including outliers.

The prophenoloxidase activity model only met the assumptions of the model when outliers were included (3 outliers total). Therefore we ran the model only with outliers included.

The model analyzing the proportion of surviving bees overall excluded Cage 30, 31, and 4 in order to meet the assumptions of constant variance in the fitted versus residuals plot. These cages, all of which were in the treatment group exposed through both syrup and pollen, had abnormally high mortality. These cages also fell under approximately the same fitted value but all had residuals lower than the fitted mean, thus violating the assumptions of the model.

Appendix Y2. Tables of F-Statistics and P-Values Omitted from Chapter 3

Interaction	$F_{1,27}$	p-value
Pollen x Week	0.0001	0.991
Syrup x Week	1.417	0.244
Pollen x Syrup x Week	0.089	0.768

Table Y1. F-statistics and p-values from the abdominal lipid stores analysis.

Interaction	$F_{1,27}$	p-value
Pollen x Week	0.072	0.791
Syrup x Week	0.094	0.761
Pollen x Syrup x Week	0.171	0.683

Table Y2. F-statistics and p-values from the hypopharyngeal gland protein content analysis.

Interaction	$F_{1,28}$	p-value
Pollen x Week	0.223	0.641
Syrup x Week	1.383	0.250
Pollen x Syrup x Week	0.041	0.841

Table Y3. F-statistics and p-values from the prophenoloxidase activity analysis.

Interaction	$F_{13,363}$	p-value
Pollen x Day	0.851	0.606
Syrup x Day	0.450	0.950
Pollen x Syrup x Day	1.002	0.448

Table Y4. F-statistics and p-values from the glucose oxidase activity analysis, outliers included.