THE SUBLETHAL EFFECTS OF PESTICIDE EXPOSURE ON AGING, BEHAVIOR, AND THE POTENTIAL OF IMPROVED DIETARY LIPID SUPPLEMENTATION IN HONEY BEES

by

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Dedication

This dissertation is dedicated to my family, my boyfriend Arth, and my dog daughter Junebug

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Abstract

Honey bees (*Apis mellifera*), the most widely utilized commercial pollinator, have experienced rapid declines over the past two decades due to a synergy of stressors that include poor forage, pathogens, parasites, and pesticides. A major goal of honey bee research is to not only identify the presence and magnitude of these stressors, but to understand how stressors affect bees and to develop ways to mitigate their negative effects. Prior research has focused on identifying which pesticides have the greatest lethality, rather than identifying those that induce subtle, non-lethal effects. Sublethal effects may compromise metabolic homeostasis through continued activation of the stress response, a compounding effect that might eventually lead to colony failure. Here, we focus on insect growth regulators as a possible honey bee stressor. Insect growth regulators are a group of reportedly "pollinator-friendly" pesticides that affect development in target pests by hindering hormonal processes necessary to reach adulthood. While these pesticides are inherently more hazardous to juvenile insects, they may accelerate the degradation of fat body tissue in adult insects, an endocrine organ that acts as the primary internal lipid reserve.

In worker honey bees, fat body degradation is linked to foraging onset. Accelerated fat body degradation is inferred to lower age at foraging onset, a phenomenon known as precocious foraging. Precocious foragers are reported to be less effective, as they exhibit higher mortality rates and complete fewer total foraging trips than non-precocious cohorts. It is unknown whether precocious foragers differ regarding the quantity and nutritional quality of their collected pollen. It is possible that they could be foraging for fattier, more nutritious pollen to compensate for reduced internal nutrient reserves. Understanding the physiology of stressed foragers may provide greater insight as to how endocrine disruption leads to observable changes in food-seeking behavior.

We asked whether two common insect growth regulator pesticides (spirodiclofen, a fatty acid synthesis disruptor, and pyriproxyfen, a juvenile hormone mimic) accelerate fat body

degradation in honey bees and release lipids into the hemolymph to fuel the stress response. I examined how these pesticides affect the survival and lipid metabolism of nurse-aged worker bees reared in laboratory cages (Appendix A). Field-realistic quantities of spirodiclofen did not affect the survival of nurse-aged bees, affirming that this pesticide was indeed non-lethal. Spirodiclofen also accelerated abdominal lipid depletion, whereas pyriproxyfen did not. We then asked whether bees treated with these pesticides would forage earlier and for fattier pollen to compensate for accelerated lipid degradation. I conducted in-hive observations of treated bees to determine if they foraged earlier and for more, fattier pollen (Appendix B). Pyriproxyfen lowered the average age of onset foraging, without accelerating lipid depletion, whereas spirodiclofen accelerated lipid depletion without affecting the average age of onset foraging. Additionally, spirodiclofen-treated bees foraged for less, yet more lipid-rich pollen, suggesting they might be compensating for accelerated lipid loss. Finally, we asked if increasing the lipid component of supplemental pollen diet could mitigate the deleterious effects of pesticide treatment. Lipid supplementation reduced hive weight loss among pesticide-treated hives, suggesting hive biomass might be sustained with improved diet design (Appendix C).

Introduction

Exposure to endocrine disrupting chemicals (EDCs) is a prevailing issue in both public health and wildlife conservation. EDCs, broadly defined as natural and artificial chemicals that damage glandular tissue and hormone production, can have lasting consequences to an organism's health, development and longevity with prolonged exposure (Yang et al., 2015). Honey bees and other pollinating insects are vulnerable to endocrine disruption through exposure to pesticides - especially in commercial agriculture landscapes where pesticides are routinely applied. While not all pesticides are inherently lethal to bees, many present sublethal effects that could act as stressors when used excessively, thereby limiting their longevity and reproduction (Desneux et al., 2007). Understanding the effects of EDCs in honey bees can help minimize industrial and commercial risk to beneficial pollinator populations. Here, I focus on a class of pesticide known as insect growth regulators (IGRs), which affect development in target pests by interfering with insect-specific hormonal pathways (Mulla, 1995). Such pesticides are speculated, yet not confirmed, to act as endocrine disruptors in non-target insect species by accelerating the breakdown of tissues necessary to maintain metabolic homeostasis (Campiche et al., 2006; Fisher et al., 2018; Thompson et al., 2007).

An example of an insect organ that coordinates metabolic homeostasis is the fat body, their primary energetic reserve. Throughout development, the fat body endures cellular remodeling in response to hormonal and nutritional changes in a growing insect (Liu et al., 2009). The adult fat body persists as an amorphous collection of adipose tissue that adheres to the cuticle of the abdomen and thorax (Canavoso et al., 2002). When stressed, endocrine feedback triggers the release of stored nutrients from the fat body into the hemolymph to fuel the physiological and behavioral responses to stress (Even et al., 2012). The hormonally-induced release of nutritional reserves is necessary for energetically demanding behavior, such as predator evasion, but can prove problematic

when energy expenditure outpaces intake. Chronic activation of the stress response to release stored sugar and lipids from the fat body may lead to higher risk of oxidative stress and tissue degradation, common biomarkers of abnormal aging patterns (Sohal, 2002).

The honey bee fat body offers an interesting model to study stress-accelerated aging. In worker honey bees, the fat body steadily declines with age (Ament et al., 2011). This steady decline in total lipid serves as an internal cue for the worker to transition from an in-hive nurse bee that nourishes developing brood to an out-of-hive forager that collects resources for the hive (Ament et al., 2011; Toth et al., 2005; Toth & Robinson, 2005). However, when stressed, workers tend to transition from nurses to foragers at a younger chronological age, indicative of an accelerated aging trajectory. Precocious foragers complete fewer foraging trips and have a higher risk of death, but the physiological explanation as to why they exhibit a reduced foraging efficiency remains unclear (Perry et al., 2015). It is unclear if precocious foragers are less efficient with respect to their lifetime forage yield and if the amount and nutritional quality of their collected pollen differs from non-precocious foragers. Precocious foragers are less capable fliers and have a lower maximal wingbeat frequency and average angular velocity compared to normal-aged foragers, attributed to them being more sensitive to oxidative stress from the aerobic demands of flight (Schippers et al., 2010; Vance et al., 2009). Another hypothesis is that stored brain lipids (not fat body) mobilization is the proximate cause for higher mortality from precocious foraging. Excessive brain lipid mobilization increases oxidative stress and accelerates neurodegeneration, leading to defects in both cognition and spatial reasoning. These effects could compromise a bee's ability to find and recall the location of floral resources (Behrends et al., 2007; Ushitani et al., 2015). Precocious foraging is likely a general response to sudden and significant worker bee die-off, as a variety of stressors have been linked to the onset of precocious foraging such as disease (Higes et al., 2009), drastic seasonal shifts (Watanabe, 2008), and pesticide exposure (vanEngelsdorp et al., 2009).

Hives with a largely precocious foraging workforce are more likely to experience colony failure, a devastating phenomenon wherein the majority of the worker bees perish (Khoury et al., 2011; vanEngelsdorp et al., 2009). Elucidating the dynamics of stress-induced accelerated lipid decline may be an avenue to better understand colony failure and potential ways to control colony breakdown. By understanding significant risk factors that exacerbate nutritional reserves and accelerate aging in bees, we can begin to find ways to minimize their deleterious effects.

The purpose of this dissertation was to investigate the link between the physiological (Appendix A) and behavioral (Appendix B) effects of endocrine-disruptive pesticides, and to see if any of these effects could be mitigated with improved nutritional supplementation (Appendix C). I hypothesize that precocious foraging is a means of compensating for accelerated lipid loss, as stressors implicated in precocious foraging likely invoke a physiological response that utilizes internal lipid reserves at a faster rate. I first established that field-relevant quantities of two different pesticides induce a physiological stress response in young worker bees without increased mortality (Appendix A). Next, I treated newly-emerged bees with the same pesticides and returned them to their source hives to observe both their foraging onset and strategy (Appendix B). I was interested to see if stressed foragers would return with fattier pollen to compensate for accelerated lipid decline. Finally, I examined whether higher fat diets mitigated the deleterious effects of pesticide treatment at the hive level. For my final chapter, I provided a set of 16 hives with supplemental diet containing: 1) additional lipid or no added lipid and 2) IGR or no IGR with 4 hives per combination and observed its effects on overall colony health (Appendix C).

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Appendix A

Acute exposure to insect growth regulator (IGRs) pesticides confer sublethal changes to lipid storage metabolism in worker honey bees

Megan Elizabeth Deeter, Lucy Snyder, Emily Watkins de Jong, Charlotte Meador, Bethany Obernesser, Vanessa Corby-Harris

Abstract

Recent widespread honey bee (*Apis mellifera*) colony loss is attributed to a variety of stressors including parasites, pathogens, pesticides, and poor nutrition. To find creative solutions to mitigate the effects of these stressors, we must first increase our understanding of how honey bees physiologically respond to stressors and at what developmental phases they are most vulnerable to exposure. We found that two pesticides, pyriproxyfen (a juvenile hormone analogue) and spirodiclofen (a fatty acid synthesis inhibitor), trigger the mobilization of fat body lipids into the hemolymph. While the immediate effect of pesticide treatment is the depletion of nutritional reserves, we found the long-term effects of acute pesticide exposure to be an increase in total fat body lipid. This suggests the presence of some compensatory mechanism, wherein stressed bees maintain fat body longer than controls, and that lipid metabolism is important in mediating the honey bee stress response to pesticide treatment.

Introduction

Beneficial pollinators provide both a foundational ecological service and asset for commercial agriculture. Many important pollinators, including the honey bee (*Apis mellifera*), have unfortunately experienced significant population declines over the past two decades due to a synergy of stressors, such as pesticides, pathogens, and pollution (Goulson et al., 2015). Two major goals in honey bee research are to identify the presence and magnitude of these stressors and to develop ways to mitigate their negative effects. In this study, we focus on a particular class of pesticides as a potential stressor in honey bees.

Historically, research efforts have focused on finding which pesticides confer the greatest mortality in beneficial insects to minimize the environmental impact of pesticide usage. More recently, understanding the sublethal effects of pesticide exposure has gained considerable attention

(Chmiel et al., 2020). Sublethal effects are physiological and behavioral changes in response to toxicant exposure that do not immediately increase mortality, but can place limitations on longevity (Desneux et al., 2007). Sublethal effects are often the result of prolonged potentiation of the stress response, which can deplete an insect's energetic reserves and compromise metabolic homeostasis (Even et al., 2012). The primary energetic reserve in insects is the fat body, an amorphous collection of adipose tissue that adheres to the cuticle of both the abdomen and thorax (Arrese & Soulages, 2010). Within the fat body, insects maintain energetic reserves in the form of stored carbohydrates and lipids, which are mobilized and released into the hemolymph to coordinate the stress response (Li et al., 2018). Mobilized energetic reserves are transported through the haemocoel to effector organs to sustain physiological and behavioral adaptations to stress, such as an increase in basal metabolic rate (Even et al., 2012). In addition to nutrient storage, the fat body plays an integral role in various biological systems, including immunity, detoxification, reproduction and development. The fat body is often designated as "the insect liver," by virtue of its diverse functional capacity. For this reason, the fat body is an appropriate focal point for our study detailing the effects of pesticide exposure. In this study, we asked whether sub-lethal quantities of two **insect growth regulator (IGR) pesticides** evoke a stress response in newly-emerged bees by mobilizing abdominal stored lipids into the hemolymph.

IGRs are a class of pesticides that interfere with the growth and development of target pest species. Most IGRs are analogues of hormones that mediate developmental processes unique to insects, such as molting and cuticle formation during metamorphosis (Dhadialla et al., 1998). For this reason, IGRs are safer for the environment because they are insect-specific, have low mammalian toxicity, and do not need to be applied frequently (Desneux et al., 2007). IGR application artificially increases hormone levels to deter developmental progression, such as preventing pupation or adult emergence (Desneux et al., 2007; Johnson et al., 2010; Mullin et al.,

2010). Optimized IGR application factors into account the feeding habits of target organisms, such as the larval stage Lepidopteran pests, and can be applied to crops directly to minimize contact with non-target, beneficial arthropods. While developmental insect stages (egg through pupa) are most vulnerable to IGRs, there is a growing body of evidence that suggests they could also confer some cost to adults, including non-target beneficial species (Carneiro et al., 2022; Chen & Liu, 2002; Desneux et al., 2007; Nijhout et al., 2014; Singh & Kumar, 2011; Tasei, 2001).

In this study, we chose two IGRs with different modes of action that we speculate could affect adult honey bee fat body physiology and confer sublethal effects: pyriproxyfen (a juvenile hormone analogue) and spirodiclofen (a fatty acid synthesis inhibitor). Both are pesticides that honey bee colonies are likely to encounter in an agricultural setting throughout the United States, especially in states where bee pollination is a major commodity in food production (Figure S1). Survey data show that both pesticides are found in apiaries across the United States: pyriproxyfen found in wax and spirodiclofen found in both wax and pollen (Johnson et al., 2010). Since residues of these pesticides are found inside of hives in managed apiaries, the developmental stages of honey bees that are more susceptible to IGRs may be exposed. Therefore, it is paramount to determine whether survey-based, sublethal quantities elicit a stress response. Provided our pesticide-specific rationale in the next two paragraphs, we asked whether these IGRs could elicit a stress response by mobilizing fat body lipid into the hemolymph

Our first pesticide, pyriproxyfen, is a juvenile hormone analogue, a type of IGR that artificially increases juvenile hormone levels beyond the molting stage, therefore arresting the development of immature insects (Ishaaya & Horowitz, 1995). Not until juvenile hormone titers are beneath a certain threshold can a developing insect proceed into the next life stage (Nijhout et al., 2014). Juvenile hormone also plays a prominent role in the reproduction and behavior of adult insects, especially for honey bees. Newly-emerged honey bee workers begin their adult life with low

juvenile hormone titers and nutrient-rich fat bodies (Münch & Amdam, 2010). As they age, an increase in juvenile hormone triggers a signaling cascade that depletes abdominal lipids. These physiological changes prime bees to switch from in-hive tasks to foraging duties (M. Elekonich et al., 2001).

Our second pesticide, spirodiclofen, is a fatty acid synthesis inhibitor. Spirodiclofen is a highly effective miticide against *Varroa destructor*, a parasitic mite that has devastated North American beekeepers for the past few decades (Bahreini et al., 2020). These pesticides limit fatty acid production by inhibiting activity of acetyl-CoA carboxylase, the rate-limiting step in lipid biosynthesis (Lümmen et al., 2014).

We hypothesize that JH analogues (pyriproxyfen) elicit a physiological stress response (an increase hemolymph lipid in bees) by prematurely increasing JH levels and causing a downstream effect of accelerated abdominal lipid depletion. Alternatively, pesticide exposure may still elicit a stress response by prematurely increasing JH levels, increasing hemolymph lipid in bees, but not have the downstream effect of abdominal lipid depletion. Instead, the stress response due to pesticide exposure could be mobilizing nutrients from other lipid-rich organs, such as the hypopharyngeal glands (Corby-Harris & Deeter, 2020). We hypothesize that fatty acid synthesis inhibitors (spirodiclofen) elicit a stress response by reducing endogenous lipid production in young worker bees and increasing their hemolymph lipid due to their reliance on mobilizing existing abdominal lipid reserves. Alternatively, a stress response could be elicited by limiting endogenous lipid production and increasing their hemolymph lipid without mobilizing abdominal lipid but instead mobilizing energetic reserves from other organs, like our alternative hypothesis for the pyriproxyfen-induced stressed response.

In this study, we fed newly-emerged bees a single, field-relevant (Mullin et al., 2010) quantity of one of two pesticides to observe the acute (Experiment 3) and chronic (Experiment 2) effects on

fat body lipid storage in caged bees. For the acute response experiment, we assayed both fat body lipid and hemolymph lipid immediately (1 + 2 hours after pesticide feeding) as a proxy for the magnitude of the stress response. During the stress response, lipid is often mobilized from the fat body and released into the hemolymph to mediate an effector response (Corby-Harris et al., 2020; Zhang et al., 2019). If acute exposure to either pesticide significantly increases circulating hemolymph lipid while decreasing abdominal lipid levels, we can infer that a stress response indeed occurred, supporting our first hypothesis. If acute exposure to either pesticide significantly increases circulating hemolymph lipid without decreasing abdominal lipid levels, we can infer that a stress response still occurred, but it is not coordinated by changes in abdominal lipid.

Materials and Methods

Source bees and Dissection

All experiments were conducted at the Carl Hayden Bee Research Center in Tucson, Arizona. Colonies were headed by *Apis mellifera linguistica* queens from a commercial breeder based in Northern California. For each experiment (1-3), capped brood frames from these colonies were removed and placed in a temperature and humidity-controlled incubator (33°C ± 1°C, 50% humidity) overnight with no light. Adults that emerged within an 18h period were gently brushed from these frames prior to treatment. (see Materials and Methods: Experiment 1-3). Bees for Experiments 1-3 were fed pesticide and subsequently placed in bioassay cages (11.5cm x 7.5cm x 16.5cm) according to their assigned treatment group. Cages were housed in the same incubator and provided with distilled water, 30% sucrose-water solution (by weight), and pollen patty *ad libitum*, consisting of a 1:1:1 ratio (w:w:w) of bee-collected pollen, table sugar, and drivert sugar (8% sucrose, fructose + 92% sucrose). Bee-collected pollen (irradiated and placed in cold storage by supplier to render pesticide residue inactive) was purchased in bulk (Great Lakes Bee Supply, Galesburg, MI, USA).

As a proxy for total abdominal fat body, we assayed only the abdominal lipids, as the majority of fat body is located in the abdomen and the entire fat body is difficult to remove intact. We did not include thoracic fat body tissue because removing the flight muscles from the cuticle wall was too difficult to perform with precision. Given that the fat body is the main lipid storage tissue in the abdomen and adheres well to the cuticle wall without adhering heavily to other abdominal organs (digestive tract, stinger, ovary), abdomen lipid content is likely a good proxy for fat body lipid content. To measure abdominal lipids for lipid analysis in Experiments 2-3, bees were flash frozen in liquid nitrogen and stored in the -80°C freezer until processing. Bees were dissected by removing the abdomen from the thorax. The abdominal carcasses of these bees were dissected by

removing their digestive tract (midgut, hindgut), stinger, stinging gland, ovaries under a light dissecting microscope. After dissection, abdominal carcasses were dried in an oven at 60°C for 48 hours and weighed (mg) individually to measure dry weight (Human et al., 2013). To assay the amount of abdominal lipids, abdominal carcasses were twice subjected to a Folch extraction and assayed for total lipid content per the sulfuric acid-vanillin-phosphoric acid assay (Malagoli et al., 2010).

Pesticide Treatment

Pesticide was administered by hand feeding bees with 2 μL of pesticide solution by pipette. Pesticide solution was concocted by dissolving solid pesticide (Pyriproxyfen (CAS Number: 95737-68-1); Spirodiclofen (CAS Number: 148477-71-8), Sigma Aldrich PESTANAL® analytical standard, St. Louis, MO) in 1mL of 100% acetone solution to achieve a 100X solution. The acetone solution was then diluted with 100mL 30% sucrose-water solution to achieve a 1X solution. Control bees were provided a negative control containing only 1 mL of acetone dissolved in 100mL 30% sucrose-water solution. For the mortality experiment (Experiment 1), bees were fed either a high (42 mg/L), medium (21 mg/L), or low (10.5 mg/L) concentration of pesticide, with the final amount of pesticide being fed to each bee totaling to 84 nanograms (high), 42 nanograms (medium), or 21 nanograms (low). For the lipid mobilization and storage experiments (Experiments 2-3), treatment group bees were fed only the high concentration of either pyriproxyfen or spirodiclofen (42 mg/L). Only bees that consumed the entire 2 μL of treatment without regurgitating or refusing to eat were used for experiments. The number of bees that were excluded from experiments for this reason was negligible (at most 30 on a single day of feeding).

Experiment 1: Does acute IGR exposure increase mortality in caged bees?

We first examined the mortality of bees fed one of the two IGR pesticides at adult emergence. Bees were fed either pyriproxyfen or spirodiclofen solution (low, medium, or high; See

Materials and Methods: Pesticide Treatment) or a negative acetone control (Johnson et al., 2010) once at adult emergence. After feeding, bees were individually isolated in 1.5 mL centrifuge tubes for another hour to prevent them from exchanging treatment through trophallaxis with each other (Fries et al., 2013). After isolation, bees were placed in bioassay cages (Materials and Methods: Source Bees) according to their assigned treatment group and concentration (low, medium, and high for pesticide-fed groups). Three replicate cages (per treatment X concentration) were constructed with exactly 100 bees per cage. All three replicate cages (per treatment) were constructed from newly-emerged bees taken from the same exact hive on two subsequent days. Dead bees were removed and mortality was recorded daily for a minimum of 3 weeks or until the population of surviving bees fell below 10%.

Experiment 2: Does acute IGR exposure at adult emergence (day 0) affect long-term lipid storage in caged bees?

We then examined whether acute exposure to pesticide at adult emergence could later reduce lipid storage capacity throughout the honey bee lifespan. We fed newly-emerged bees the high concentration (42 mg/L) of either pyriproxyfen, spirodiclofen, or an acetone control (see Materials and Methods: Pesticide Treatment). Three replicate cages were constructed, each containing 100 bees per treatment (n=100 control + 100 pyriproxyfen + 100 spirodiclofen = 300 total * 3 cages = 900 bees total). At ages 10, 15, and 30 days, 10 bees per treatment per cage were sampled and dissected (as described in the "Source Bees" section above) All reagents for the sulfuric acid-vanillin-phosphoric acid assay were purchased from Sigma Aldrich (St. Louis, MO). Sample absorbance was measured using a plate reader (BIOTEK Synergy HT™, Agilent: Santa Clara, CA) and absorbances were evaluated against a standard curve of samples containing known amounts of vegetable oil dissolved in chloroform.

Experiment 3: Does acute IGR exposure trigger the release of fat body lipid into the hemolymph?

We then examined whether acute pesticide exposure had an immediate effect on lipid metabolism by quantifying hemolymph and fat body lipid levels in response to treatment. To test this, we assayed hemolymph and abdominal lipid levels in bees fed pesticide on days 0, 3, and 6 at 1h and 2h post-treatment. Day 0 bees were treated and processed immediately. The same day, 4 cages of 100 bees per cage (2 for Day 3, 2 for Day 6) were placed in the incubator until days 3 and 6. Cages for days 3 and 6 were provided pollen patty, water, and sugar water *ad libitum* until their respective treatment day. On days 3 and 6, bees were starved for 2 hours prior to treatment. For each day, we fed newly-emerged bees the high concentration of either pyriproxyfen, spirodiclofen, or an acetone control (see Materials and Methods: Pesticide Treatment). After feeding, bees were placed back in cages in the incubator until processing with only water provided. At 1h and 2h post-treatment, bees were immobilized by being placed in the -20°C fridge for approximately 3-5 minutes (until prior to hemolymph collection.

Hemolymph was drawn by dissecting the abdomen from the thorax, applying gentle pressure to the thorax, and using a 10μL pipette to draw 2 μL of hemolymph from the anterior end of the thorax. Individual hemolymph samples were snap frozen in 1.5 mL centrifuge tubes (by placing in liquid nitrogen for 1 second) containing 20 μL of Folch solution (2:1 chloroform to methanol) and stored at -80°C until the lipid assay was performed. To see if fatter bees mobilize more hemolymph lipid in response to pesticide treatment, we also flash froze the individual bee carcasses, labeled according to individual (sample ID#, treatment, day, and hours post treatment), and stored at -80°C until the lipid assay was performed on their abdominal fat bodies (see Source Bees and Dissection section above).

Statistical analysis

Log-rank survival curve differences between treatment levels of each pesticide were computed and plotted using the survival (https://cran.r-project.org/package=survival) and survminer (https://cran.r-project.org/package=survminer) packages in R. Lipid data were log-transformed prior to analyses. Lipid data (both hemolymph and fat body data) were analyzed using a generalized linear model (GLM) with lipid amount as the dependent variable, three predictor variables (treatment, age of bee (days), hours since treatment), and a gamma link function. Pairwise comparisons were made using Tukey's HSD *post hoc* tests using the R package emmeans (https://cran.r-project.org/package=emmeans).

Results

Experiment 1: Does acute IGR exposure increase mortality in caged bees?

Caged bees fed a high (84 nanograms) concentration of pyriproxyfen once at adult emergence exhibited higher mortality than those fed the control (χ^2=27.2, p<0.0001; n=150 **Figure 1A**), reaching 50% mortality at 408 hours, or 16 days. The control group in the pyriproxyfen mortality cage experiment reached 50% mortality at 648 hours, or 27 days (n=150). Similarly, spirodiclofen-treated bees had no significant differences in median survival time between treatment groups or the negative control (χ^2=4.5, p=0.22; n=150 **Figure 1B**). The control group in the spirodiclofen mortality cage experiment reached 50% mortality at 456 hours, or 19 days.

Experiment 2: Does acute IGR exposure at adult emergence (day 0) affect long-term lipid storage in caged bees?

We assayed abdominal lipid concentration (μg lipid/mg abdominal carcass) in caged bees that were fed either a pesticide or control at adult emergence and sampled on days 10, 15, and 30 (**Figure 2**). We checked for normality using the Shapiro-Wilk test of normality and found our lipid data to be significantly different from a normal distribution (W=0.954, p<0.001, n=269). Pesticide treatment (GLM, $F_{2, 266}$=43.76, p<0.0001) and bee age ($F_{2, 264}$=3.956, p=0.02) had a significant effect on fat body lipid concentration. We found no evidence for a pesticide treatment and age interaction ($F_{4, 260}$=2.120, p=0.07).

Tukey's post hoc comparisons were made to determine where these effects were located. We found that spirodiclofen-treated bees were significantly fatter than controls on days 10 (t_{260}=7.464, p<0.0001), 15 (t_{260}=3.931, p=0.0003), and 30 (t_{260}=4.608, p<0.0001). Pyriproxyfen-treated bees were also significantly fatter than controls on days 10 (t_{260}= 3.524, p=0.0015), 15 (t_{260}= 3.441, p=0.0019), and 30 (t_{260}= 2.922, p=0.0105).

Experiment 3: Does acute IGR exposure trigger the release of fat body lipid into the hemolymph?

Both bee age (GLM, $F_{2,175}$=5.5312, p<0.0001, n=180) and time post-treatment (hours) (GLM, $F_{1,174}$ =47.8259, p<0.0001, n=180) significantly affected the amount of hemolymph lipids. Although treatment did not directly affect hemolymph lipid (GLM, $F_{2,177}$=2.3474, p=0.09, n=180), there was a significant, three-way interaction between treatment, age, and time post-treatment, indicating that (GLM, $F_{4,162}$ =10.22, p<0.0001, n=180; **Figures 3-5**). There was also a significant two-way interaction between treatment and age (GLM, $F_{4,170}$ =7.476, p<0.0001, n=180), treatment and time post-treatment (GLM, $F_{2,168}$ =4.971, p=0.002, n=180), as well as age and time post-treatment (GLM, $F_{2,166}$ =1.426, p<0.0001, n=180). Whether hemolymph lipids increase or decrease between 1- and 2-hours post-treatment depends on the day which bees were treated, with newly-emerged bees having the greatest release at 2 hours post-treatment, whereas 3-day old bees had the greatest release at 1 hour post-treatment. 6-day old bees treated with pesticide had the greatest hemolymph lipid release at 2 hours post-treatment, whereas the control had the greatest release at 1 hour. The effect size of hemolymph release was also far lower in all 6-day old bees compared to the 3-day old and newly-emerged bees.

After collecting hemolymph samples, the abdominal carcasses were also assayed for their lipids to infer whether changes in hemolymph lipid levels correlated with lipid mobilization from the fat body. Treatment ($F_{2,177}$=0.3916, p=0.6766), age ($F_{2,175}$=2.648, p=0.07), and time post-treatment ($F_{1,174}$ =0.0562, p=0.8129) all had no effect on total fat body lipid. However, similar to the hemolymph lipid data, we found a significant three-way interaction between treatment, age, and time post-treatment ($F_{4,162}$ =4.5364, p=0.001; **Figures 3-5**).

In further investigating the interactions effects, we performed Tukey's post-hoc comparisons and found that newly-emerged (day 0) bees treated with either pesticide did not differ from controls

in hemolymph lipid (**Figure 3A**). Both the control and pesticide-treated bees had a significant increase in hemolymph lipid between 1 hour and 2 hours of treatment (Control: t_{162}=4.740, p<0.0001; Pyriproxyfen: t_{162}=4.170, p<0.0001; Spirodiclofen: t_{162}=2.818, p=0.0054).

However, newly-emerged bees treated with either pesticide were significantly fatter than control bees 2 hours post-treatment (Pyriproxyfen: t_{162}=5.230, p<0.0001; Spirodiclofen: t_{162}=5.149, p<0.0001; **Figure 3B**). These increases in fat body lipid cannot be explained by changes in hemolymph lipid titers (**Figure 3A,B**), as neither pesticide treatment differed from controls with respect to hemolymph lipid release.

3-day-old bees treated with pyriproxyfen had significantly less hemolymph lipid at 1 hour after treatment compared to controls (t_{162}=-2.372, p=0.04; **Figure 4A**), but not at 2 hours after treatment (t_{162}=-0.307, p=0.949). 3-day-old spirodiclofen-treated bees did not differ in hemolymph lipid from controls (1 hour: t_{162}=1.048, p=0.548; 2 hours: t_{162}=0.380, p=0.924).

6-day-old control bees had a significant decline in hemolymph lipid between 1 hour and 2 hours of treatment (Control: t_{162}=-2.792, p=0.006), whereas both pesticide-treated groups experienced a significant increase in hemolymph lipid between 1 hour and 2 hours of treatment (Pyriproxyfen: t_{162}=6.922, p<0.0001; Spirodiclofen: t_{162}=3.306, p=0.0012; **Figure 5A,B**). These data suggest that older nurses (6 day-old) are more sensitive to pesticide-treatment than younger nurses (newly emerged and 3 day-old).

The significant influx of hemolymph lipid did not align with a reduction in fat body for pyriproxyfen at either time point (1 hour: t_{162}=-1.316, p=0.3881; 2 hour: t_{162}=0.012, p=0.999; **Figure 5**). However, spirodiclofen-treated bees had significantly reduced fat body lipid 2 hours post-treatment (t_{162}=-2.584, p=0.03). Spirodiclofen was the only treatment in 6-day-old bees to concurrently have an influx in hemolymph lipid and a decrease in fat body lipid.

Discussion

We hypothesized that acute exposure to either pyriproxyfen or spirodiclofen would induce a stress response in young worker bees by mobilizing fat body lipid into the hemolymph, without incurring an immediate cost to survival. This present study partially supported our hypothesis, with some inconsistencies between the long-term lipid storage data in older, caged bees (Experiment 2) and the fat body mobilization data sampled from younger bees shortly after being treated with pesticide (Experiment 3).

We found that spirodiclofen treatment did not affect survival at any concentration. The high concentration (42 mg/L) treatment of pyriproxyfen, however, conferred some cost to survival at approximately 17 days of age. Had these bees not been contained in cages, they would likely be foraging at this age. Because this reduction in survival was not immediate, we inferred that pyriproxyfen induces some effect to ultimately reduce longevity.

The immediate effect of pesticide exposure in newly-emerged bees differed from that of older nurse bees (days 3 and 6). Newly-emerged bees had a gradual, significant increase in hemolymph lipid release for both pesticide treatments, possibly a response to handling stress since controls experienced a similar release pattern, suggesting no effect of treatment in young, newly-emerged bees. Pyriproxyfen and spirodiclofen treated bees had significantly more fat body lipid compared to controls, the latter unexpectedly having a significant decline in fat body between timepoints (1 hour-2 hour). There are likely additional behavioral and physiological factors that promote fat storage in younger bees as a general response to stress that cannot be explained within the scope of this experimental design.

The hemolymph and fat body data for older nurses were more consistent with our hypotheses. We found that 3-day-old pyriproxyfen-treated bees had less fat body lipid 1-hour after treatment compared to controls. However, hemolymph lipid release was lower in pyriproxyfen-

treated bees prior to this fat body reduction. It is possible that the response to pyriproxyfen is so rapid (< 1 hour) that an influx in hemolymph lipid occurred prior to our sampling and that the mobilized lipid from the fat body was already integrated into effector organs, hence the lower hemolymph lipid compared to controls. Spirodiclofen treatment induced no changes in fat body lipid in 3-day-old bees at any time point, suggesting that younger bees are more sensitive to pyriproxyfen than spirodiclofen. Juvenile hormone titers are lowest in newly-emerged bees and gradually increase as they age (M. Elekonich et al., 2001; Malagoli et al., 2010; Münch & Amdam, 2010), so it is reasonable for younger bees to be more susceptible to pyriproxyfen treatment.

Our strongest evidence for pesticide-induced fat body mobilization occurred in 6-day-old bees treated with spirodiclofen. A significant rise in hemolymph lipid 2 hours after treatment occurred in tandem to a reduction in fat body. Here, pyriproxyfen had no reduction in fat body, dissimilar to what we saw in the 3-day-old bees. This supports our previous conclusion that younger bees are more susceptible to pyriproxyfen, whereas older bees are more susceptible to spirodiclofen treatment. Nurse bees housed in cages typically peak in pollen consumption around 5-7 days (Crailsheim et al., 1992), so they may not begin to mobilize fat body lipids immediately if they have a reliable food source.

While our hemolymph and fat body data support our hypothesis of pesticide-induced fat body degradation, our data on the long-term (days 10, 15, and 30) effects of acute pesticide exposure on fat body lipid storage contrast with what was observed in younger bees. Here, we found that bees treated with either pesticide at adult emergence later had significantly greater lipid storage at days 10, 15, and 30. The long-term effects of acute pesticide exposure from our cage experiments suggest that, when provided food and water *ad libitum*, bees could be compensating for pesticide-induced fat body loss with increased consumption. We housed all three treatments in the same cage for this experiment, so it was impossible to infer how pollen was consumed in each group. Increased

consumption could explain why we see the pesticide-treated groups are fatter than the controls. Pollen consumption data from the survival experiments found that there was no significant difference in consumption (Figure S2). Regardless, consumption data from cage experiments should be considered with some degree of caution, as caged bees do not have to forage and are provided resources *ad libitum*. Further investigation is necessary to understand if bees change their food-seeking behavior in the field in response to pesticide treatment (Appendix B), as dietary habits and energetic requirements vary tremendously depending on whether they are performing in-hive tasks or foraging for resources (Crailsheim et al., 1992). Older caged bees might also be mobilizing other nutritional reserves in addition to the fat body. Recently, we found that stressed bees not only mobilize lipid from their fat bodies, but also the nutrient-rich hypopharyngeal glands in the head cavity (Corby-Harris et al., 2020).

The susceptibility of honey bees to pesticide and other xenobiotic factors is comparatively greater than their non-social relatives. Indeed, honey bees have comparatively fewer protein encoding genes for detoxifying enzymes compared to other non-social insect genomes (Barribeau et al., 2015; Claudianos et al., 2006). The cooperative benefits of eusociality undoubtedly are met with some cost to immunity. With this in mind, it is the responsibility of agricultural managers to choose lower-risk pesticides with shorter half-lives to reduce their effect on beneficial insects.

Tables & Figures

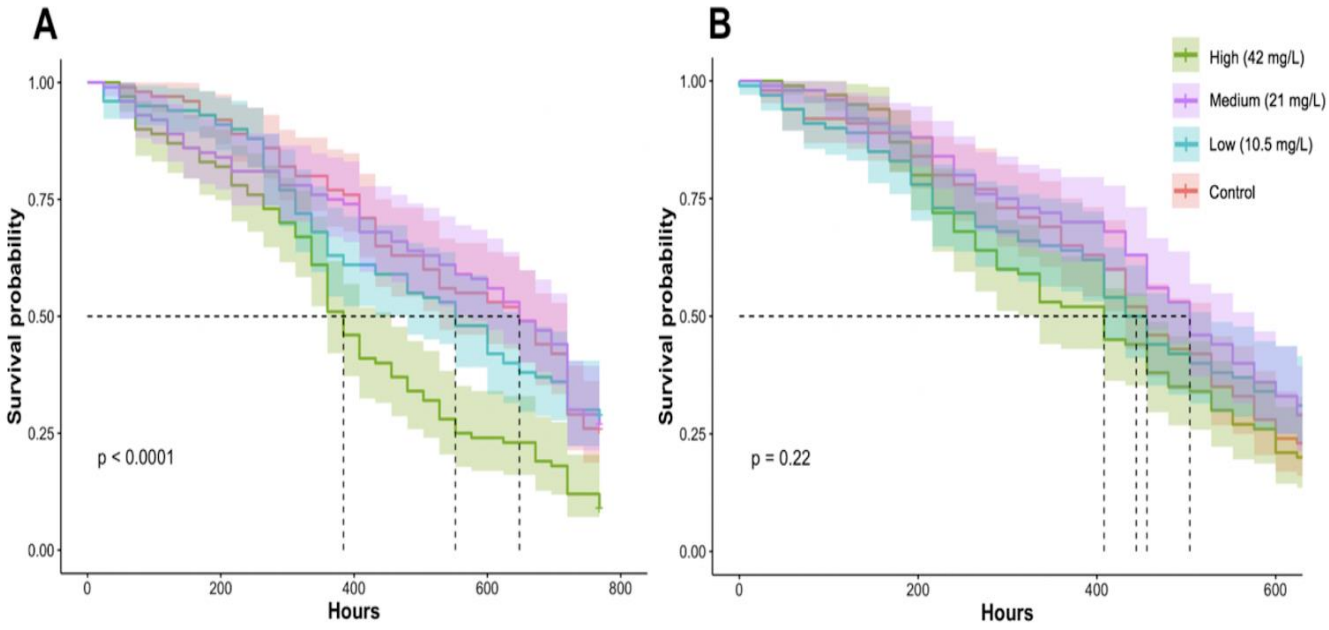

Figure 1. Survival of caged bees orally exposed to (A) pyriproxyfen and (B) spirodiclofen at adult emergence

Survival curves (Kaplan-Meier survival function time-to-event data) indicate the number of caged bees that survived over a (A) 768-hour or (B) 672-hour period with 95% confidence bands constructed by joining the confidence intervals at each time point. For each treatment/dose combination, three replicate cages containing 50 bees each (n=150) were made from single-cohort bees that emerged on the same day. Comparisons between survival cages were conducted using log-rank tests as per Kaplan-Meier analysis. Dashed lines represent the median survival time (when 50% of bees perished) for the corresponding treatment.

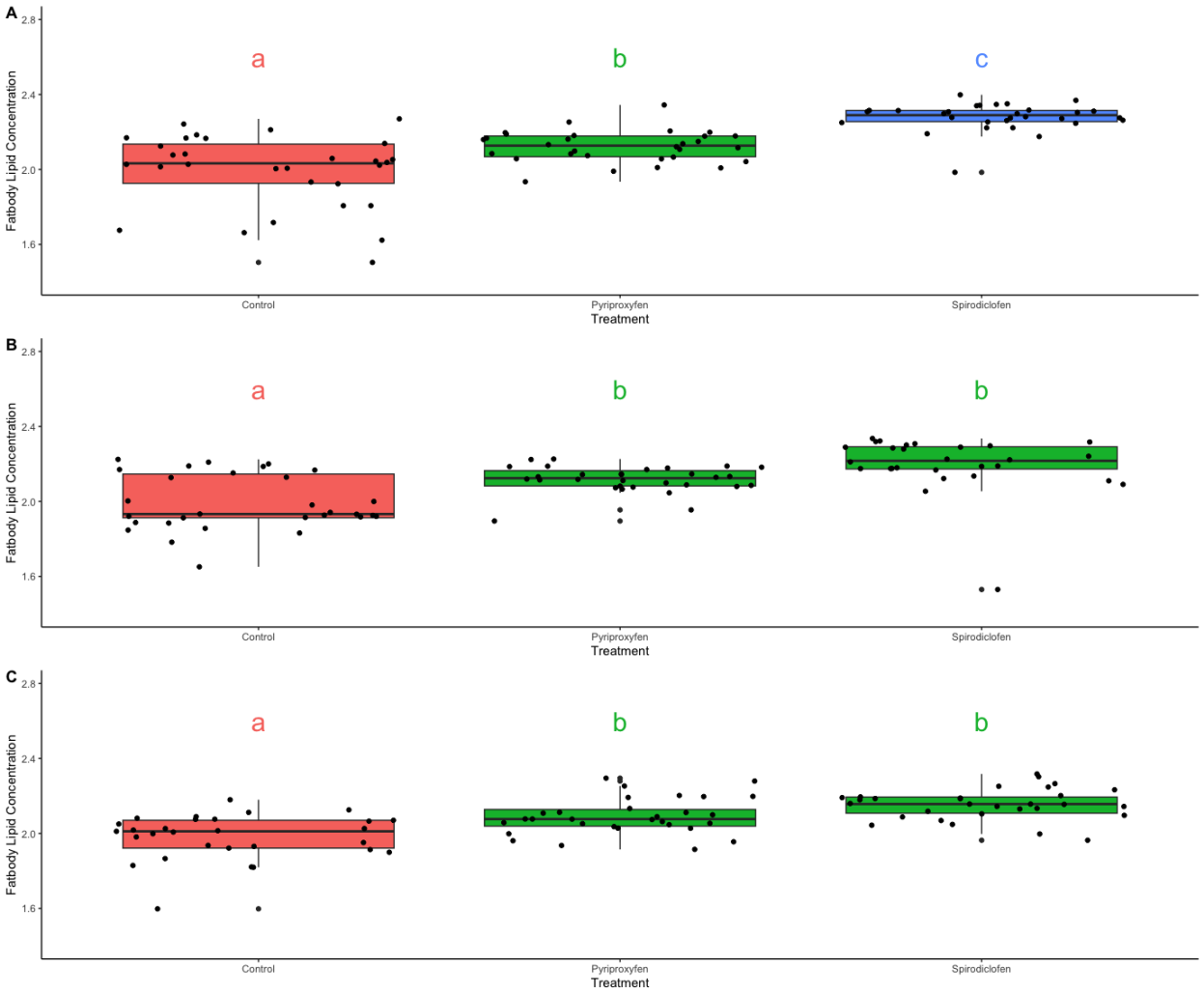

Figure 2. Long-term fat body lipid storage in caged bees treated at adult emergence Abdominal lipid concentration in bees treated with pesticide (pyriproxyfen or spirodiclofen) or an acetone control at adult emergence, caged and sampled for lipid measurement on days: (A) 10, (B) 15, and (C) 30. Boxplots show median (horizontal line within box) abdominal lipid concentration and interquartile range (lower and upper box boundaries) as well as the minimum and maximum values of the distribution. Points indicate a single observation. Outliers are points outside the maximum and minimum of the distribution. Matching letters and colors between boxplots indicate no significant differences between two groups as determined by TukeyHSD multiple comparisons.

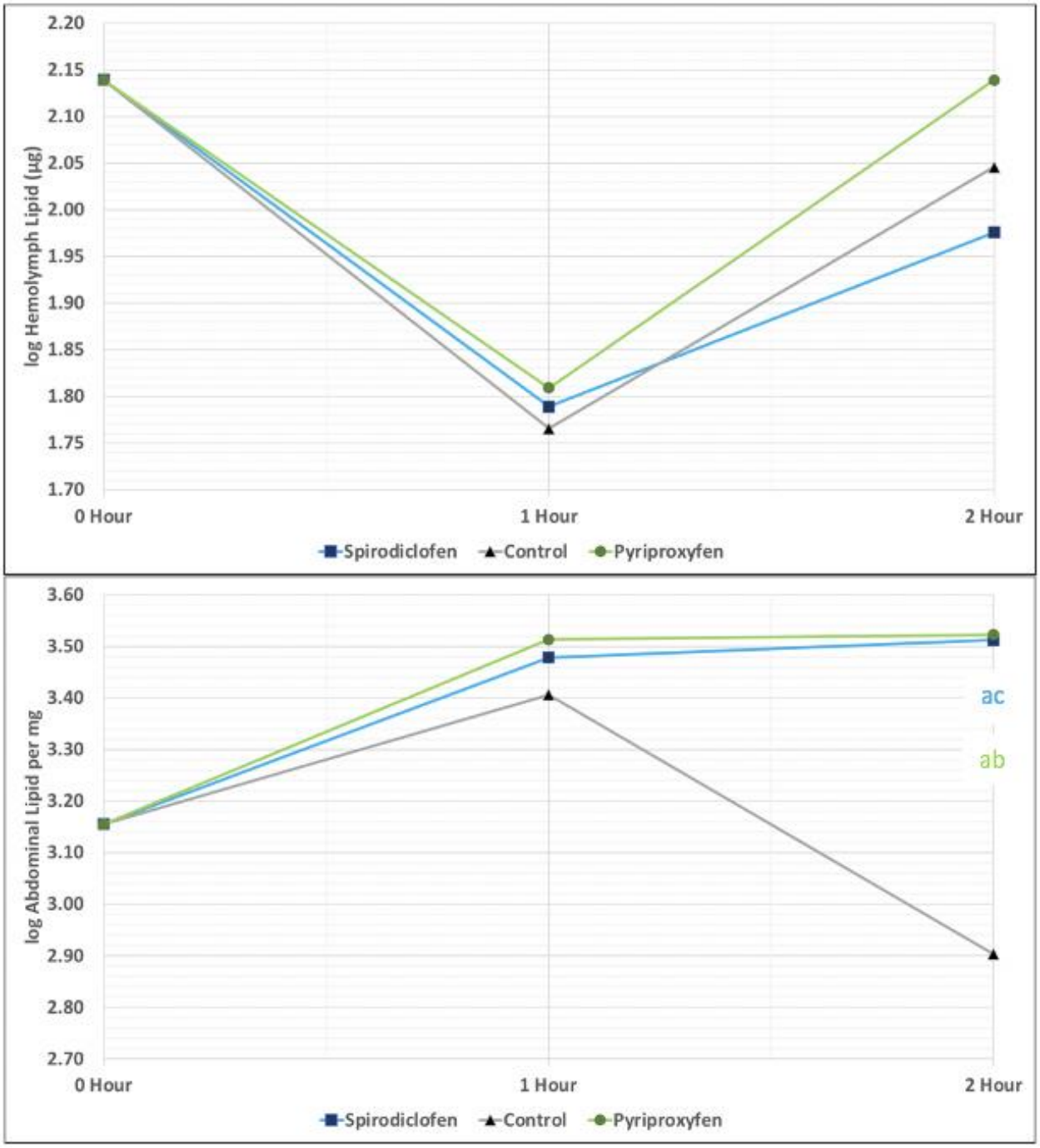

Figure 3: Hemolymph Lipid (A) and Fat body (B) in Newly-Emerged (Day 0) Bees
Line plots depicting average (log): (A) hemolymph lipids (µg) in 2 µL hemolymph sample and (B) total abdominal lipids (µg) per mg abdominal mg in bees (n=10 per timepoint, per treatment) treated with one of two pesticides (spirodiclofen, in blue; pyriproxyfen, in green); "ab" indicates a significant difference between pyriproxyfen and control ($p<0.05$); "ac" indicates a significant difference between spirodiclofen and control ($p<0.05$) (Tukey's post-hoc).

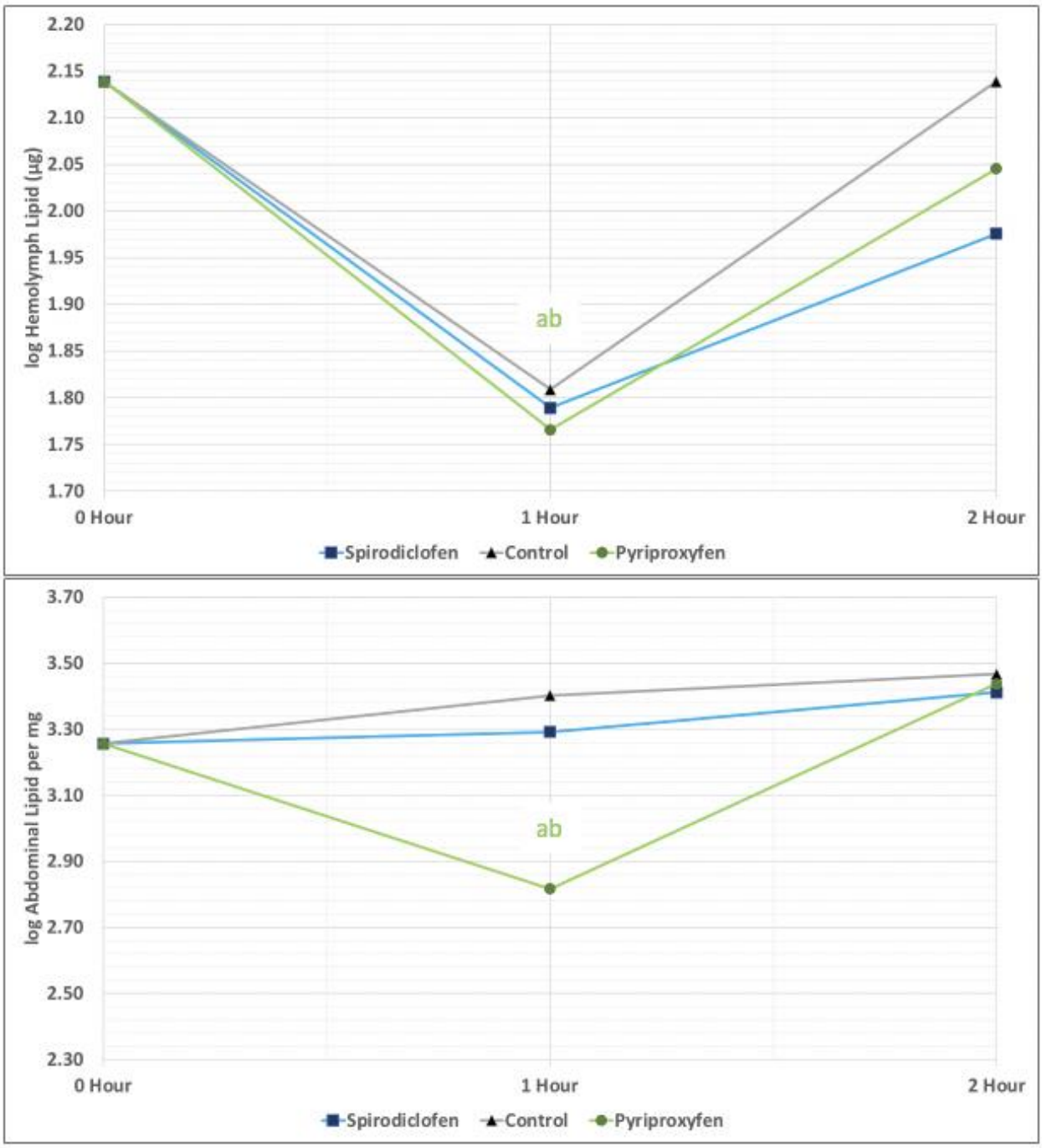

Figure 4: Hemolymph Lipid (A) and Fat body (B) in Newly-Emerged (Day 3) Bees
Line plots depicting average (log): (A) lipids (µg) in 2 µL hemolymph sample and (B) total abdominal lipids (µg) per mg abdominal mg in bees (n=10 per timepoint, per treatment) treated with one of two pesticides (spirodiclofen, in blue; pyriproxyfen, in green); "ab" indicates a significant difference between pyriproxyfen and control ($p<0.05$); "ac" indicates a significant difference between spirodiclofen and control ($p<0.05$) (Tukey's post-hoc).

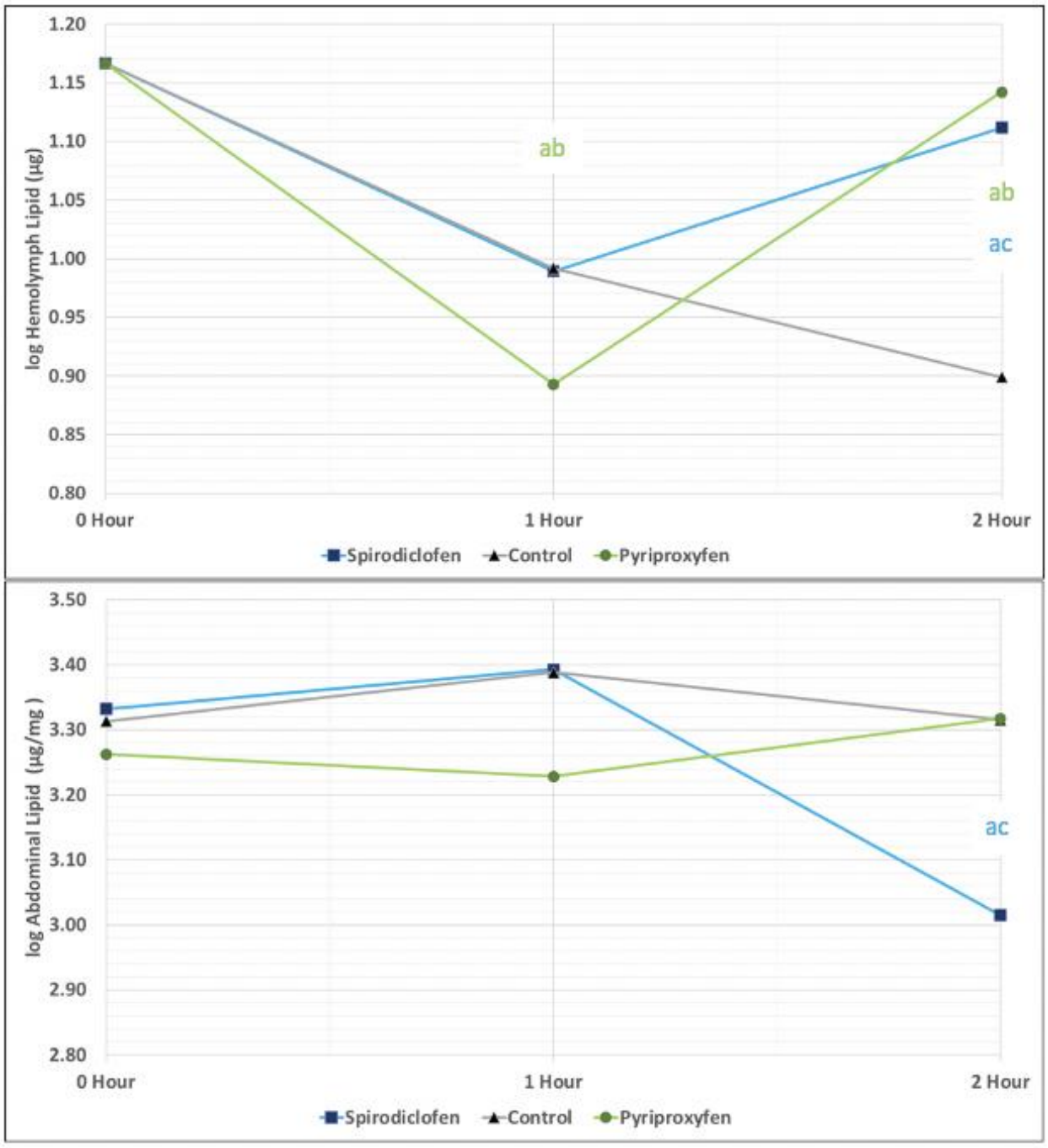

Figure 5: Hemolymph Lipid (A) and Fat body (B) in Newly-Emerged (Day 6) Bees
Line plots depicting average (log): (A) lipids (µg) in 2 µL hemolymph sample and (B) total abdominal lipids (µg) per mg abdominal mg in bees (n=10 per timepoint, per treatment) treated with one of two pesticides (spirodiclofen, in blue; pyriproxyfen, in green); "ab" indicates a significant difference between pyriproxyfen and control ($p<0.05$); "ac" indicates a significant difference between spirodiclofen and control ($p<0.05$). (Tukey's post-hoc).

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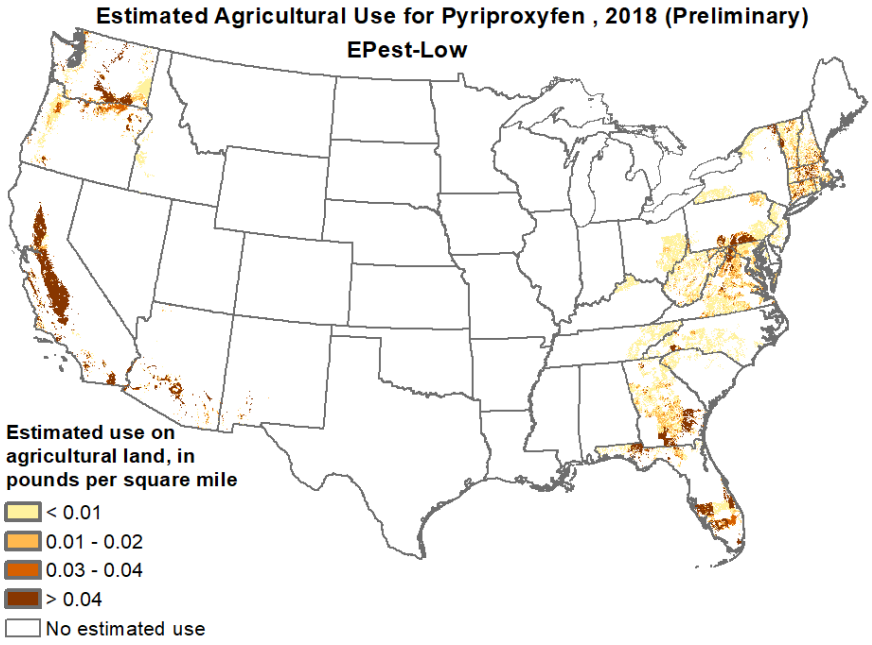
Supplementary Information



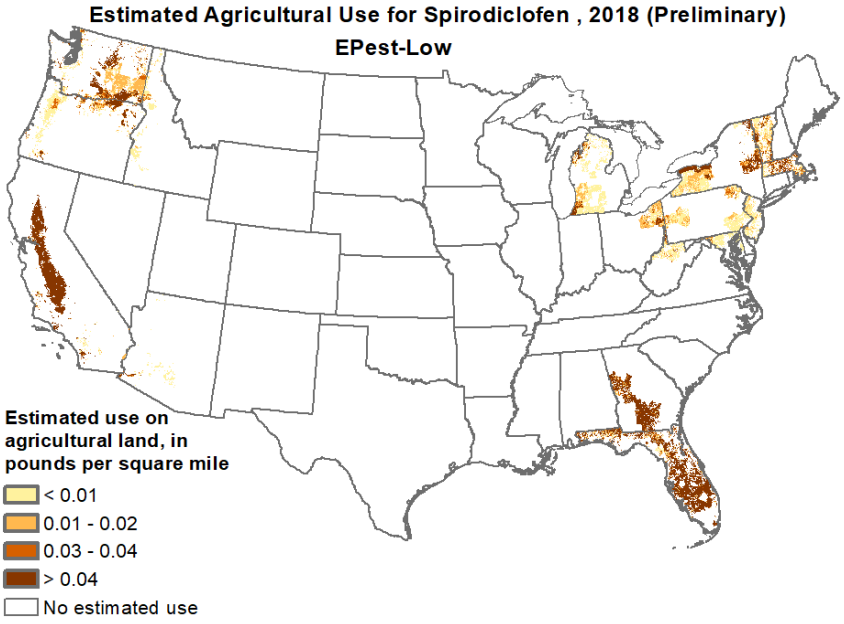


Figure S1: Estimated agricultural use for pyriproxyfen (top) and spirodiclofen from the Pesticide National Synthesis Project (National Water-Quality Assessment (NAWQA) Project)

Data provides preliminary estimates of annual agricultural use of pesticide compounds in United States counties in the year 2018. (Wieben et al., 2021)

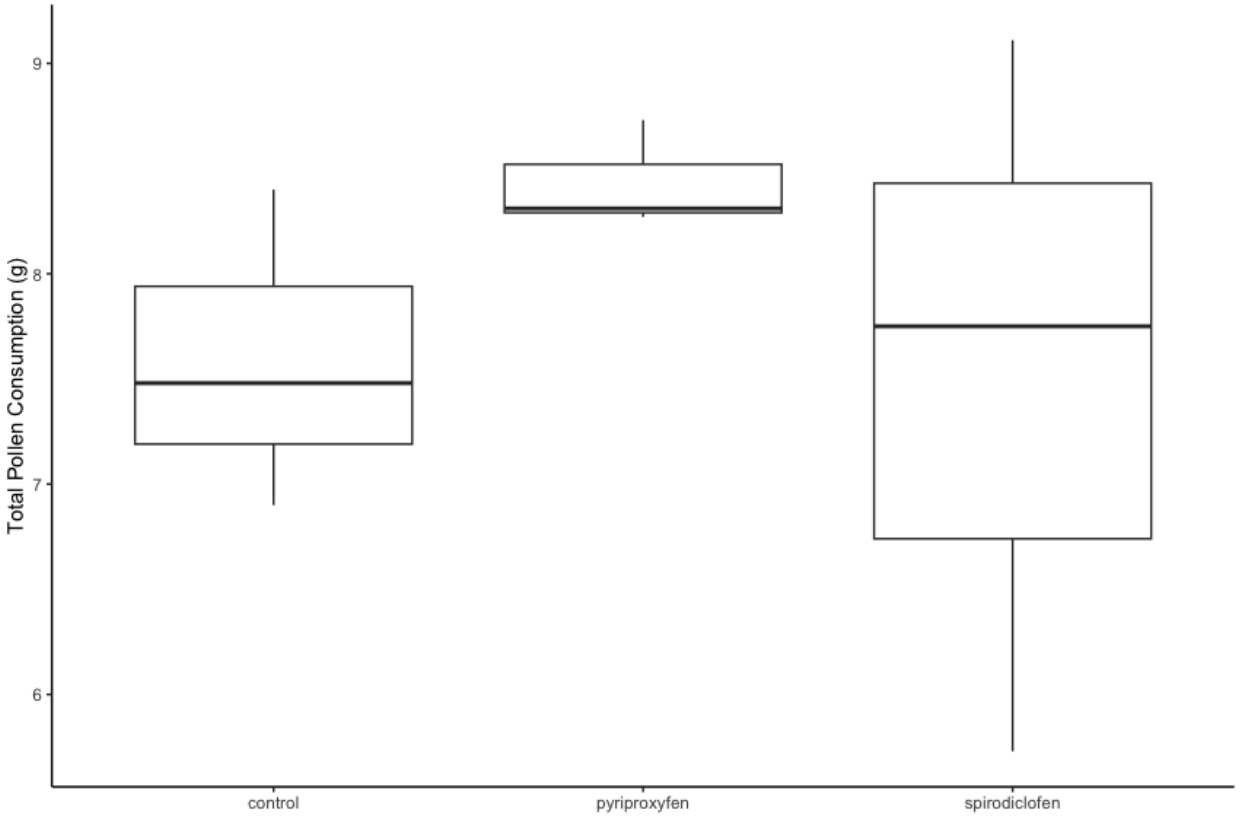


Figure S2: Average pollen consumption from caged bees fed pesticide at adult emergence. Boxplot shows the median (middle line of box) and interquartile range (box boundaries) as well as the minimum and maximum values of the distribution. Outliers are points outside the maximum and minimum of the distribution. There were no significant differences in pollen patty consumption between treatment cages (3 rep per treatment* 100 bees each).

Appendix B
Linking pesticide-induced lipid depletion to changes in foraging behavior

Published as: Deeter et al. (2023) Accelerated abdominal lipid depletion from pesticide treatment alters honey bee pollen foraging strategy, but not onset, in worker honey bees

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Keywords: *Apis mellifera,* adipose tissue, Fat body, foraging, pesticides

Abstract

Honey bee abdominal lipids decline with age, a change thought to be associated with the onset of foraging behavior. Stressors, such as pesticides, may accelerate this decline by mobilizing internal lipid to facilitate the stress response. Whether bees with stressor-induced accelerated lipid loss vary from controls in both the onset and nutritional quality of collected pollen is not fully understood. We asked whether stressors affect foraging behavior through the depletion of abdominal lipid, and whether stress-induced lipid depletion causes bees to forage earlier and for fattier pollen. We tested this by treating newly-emerged bees with one of two pesticides, pyriproxyfen (a juvenile hormone analogue) and spirodiclofen (a fatty acid synthesis disruptor), that may affect energy homeostasis in non-target insects. Bees fed these pesticides were returned to hives to observe the onset of foraging behavior. We also sampled foraging bees to assay both abdominal lipids and dietary lipid content of their corbicular pollen. Initially, spirodiclofen-treated bees had significantly more abdominal lipids, but declined faster compared to controls. These bees also collected less, yet more lipid-rich, pollen. Our results suggest that bees with accelerated lipid decline rely on dietary lipid content and must collect fattier pollen to compensate. Pyriproxyfen treatment reduced the age at first forage but did not affect abdominal or collected pollen lipid levels, suggesting that accelerated fat body depletion is not a prerequisite for precocious foraging.

Introduction

Obesity is a growing public health crisis that threatens the longevity and well-being of those affected. Poor diet, lack of exercise, and chronic stress are the most significant factors contributing to excess lipid accumulation (Blüher, 2019). Although obesity can be treated with exercise and a balanced diet, many patients report tremendous difficulty in losing weight and maintaining weight loss, particularly those with higher BMIs (Carnell and Wardle, 2008). Individuals with higher proportions of body fat, or adipose tissue, oftentimes experience lower levels of satiety, despite

typically consuming a diet of caloric excess (Blüher, 2019; Boutelle et al., 2020). This counterintuitive behavioral response may make humans and non-human animals more vulnerable to obesity (McMillen et al., 2005). Understanding the physiological component behind these seemingly maladaptive feeding behaviors that further the obese state is key to combating this public health crisis.

From an evolutionary perspective, the ability to store excess dietary energy in adipose tissue can be seen as a selective advantage (McMillen et al., 2005). Most organisms have a homeostatic set point that favors lipid storage, so that the energy stored as fat and glycogen can be mobilized during times of nutritional scarcity (McMillen et al., 2005; Arrese and Soulages, 2010; Nelson et al., 2021; Charmandari et al., 2005). External stressors can disrupt homeostasis, triggering a stress response that acts to restore optimal internal conditions (Chovatiya and Medzhitov, 2014). One example of a behavioral response to stress is the increased consumption of palatable, calorically-dense foods (Boutelle et al., 2020; Ortolani et al., 2011). While increased consumption can be beneficial by providing additional energy for the stress response, in excess it can lead to inflammation and other metabolic diseases, such as obesity or insulin resistance (Kershaw and Flier, 2004). Such pathologies have become commonplace in modern human society, as people in industrialized nations experience less nutritional scarcity, an increased availability of heavily-processed and highly palatable foods, and an increase of socioeconomic stress (Blüher, 2019; Capehart and Wisman, 2013).

While subject to conscious control, food-intake behavior is influenced by a variety of physiological signals that can further affect dietary decisions, sometimes at a long-term cost to the individual. Adipose tissue can be a source of such signals and is now recognized as an endocrine tissue in its own right. To understand how body fat influences food-intake behavior, it is helpful to first consider the evolutionary and physiological role of adipose tissue. Adipose tissue not only serves as an energetic reservoir, but also operates in tandem with other effector organs to relay

messages concerning homeostasis (Kershaw and Flier, 2004; Agustí et al., 2018). Adipose tissue synthesizes and secretes appetite-related hormones that relay messages regarding satiety, such as leptin (in vertebrates) and adipokinetic hormone (in invertebrates) (Arrese and Soulages, 2010; Nelson et al., 2021; Kershaw and Flier, 2004; Baile et al., 2000). These signals are received by the central nervous system (CNS) to mediate a physiological and behavioral response to restore homeostasis (Charmandari et al., 2005). Chronic stress can induce lasting changes to appetitive regulation irrespective of an organism's actual energy expenditure (Agustí et al., 2018; Xiao et al., 2020). We are just beginning to understand the physiological mechanisms connecting stress to changes in food-intake behavior. This topic is particularly challenging to study because the links between adipose tissue and behavior are complex and difficult to test and experimentally manipulate within human subjects.

Insect models are valuable tools for elucidating the connection between adipose tissue and behavior. Insects have short generation times compared to vertebrates. Additionally, their nutritive status is far easier to manipulate in laboratory settings, as they can be reared on chemically-controlled diets with relative ease. The majority of insect lipid is stored within the fat body, an amorphous collection of adipose tissue found in both the abdomen and head, although most of the fat body is located in the abdomen (Arrese and Soulages, 2010). In addition to fat storage, the insect fat body is also a dynamic metabolic organ that is highly receptive to endocrine signaling. In *Drosophila*, increased expression of the stress hormone octopamine substantially decreases fat body, in tandem to decreasing food intake, appetite and metabolic activity (Li et al., 2016).

Honey bees (*Apis mellifera*) are an excellent system to understand this stress-fat-behavior axis. Honey bee workers exhibit temporal polyethism, where their role within the colony changes with age (Robinson, 1987; Wilson and Hölldobler, 2005). Workers begin their adult life in the hive, exhibiting brood- and queen-care ("nurse") behaviors within ~2-3 weeks post-emergence. As they

age beyond three weeks old, they leave the hive ("forager") to collect water, nectar, and pollen for the colony (Winston, 1987). During this transition, workers experience a decline in fat body lipid and an increase in juvenile hormone levels (Toth et al., 2005; Toth & Robinson, 2005; Sullivan et al., 2000). Juvenile hormone negatively affects expression of vitellogenin, an egg yolk precursor protein that promotes stress resilience, longevity and reproduction (Corona et al., 2007). Therefore, higher juvenile hormone titers are linked to a reduction in stress resilience and accelerated aging, possibly via depletion of lipid reserves. Honey bees are not the only organism where a decline in internal lipid precedes the transition into foraging behavior. Other Hymenoptera species also exhibit a similar link between fat body and foraging onset, and is common across all Formicidae (ants) (Daugherty et al., 2011; Bernadou et al., 2020).

Although the nurse-to-forager transition is socially regulated, external stressors can prompt workers to forage earlier, a phenomenon known as precocious foraging (Robinson, 1987; Schulz et al., 1998; Toth et al., 2005; Goblirsch et al., 2013; Woyciechowski and Moroń, 2009). While recruitment of precocious foragers immediately increases the food intake for a colony by increasing the foraging workforce, it is associated with higher worker mortality, which can further accelerate colony failure (Perry et al., 2015;). The accelerated development of precocious foragers may also come at a cost to cognition. Precocious foragers display lapses in spatial memory and fine-scale searching behavior (Ushitani et al., 2016), both processes crucial to foraging success. We speculate that these foragers utilize internal nutritional stores at a faster rate and, therefore, have metabolic limitations on the duration of foraging trips.

Previous work indicates that stressed bees forage earlier (Perry et al., 2015; Woyciechowski and Moroń, 2009; Khoury et al., 2011) and experimentally-induced fat body depletion is associated with precocious foraging (Toth et al., 2005), so stress-induced fat body depletion may be the main causative factor linking stress to changes in foraging behavior. Within the eusocial insects, fat body

lipid decline has largely been studied within the context of foraging onset, but whether this expedited rate of decline is linked to changes in foraging behavior remains of interest for future investigation. Whether stressed foragers also shift their foraging strategy towards collecting more nutrient-rich pollen to compensate for abdominal lipid depletion remains unclear. Stressed bees possibly have a lower energetic capacity, due to having fewer stored nutritional reserves after the mobilization of reserves to fuel the stress response (Arrese and Soulages, 2010). Stressed bees are likely more susceptible to the physical demands of foraging and may complete fewer foraging trips (Sullivan et al., 2000). We speculate that stressed colonies may be compensating at the colony level by collecting pollen with a higher lipid content, as it has a higher caloric value per gram than protein.

We examined these questions using one of many stressors that honey bees are likely to encounter in the field - insect growth regulator pesticides (IGRs) (Johnson et al., 2010). IGRs interfere with the development and reproduction of target crop insect pests, such as lepidopteran agricultural pests, and medically-relevant arthropods, such as mosquitos. They are generally regarded as pollinator-safe due to their specificity (Desneux et al., 2007) but may negatively affect hormone signaling and nutrient storage in non-target species (Meola et al., 2000; Rivero et al., 2011). Two IGRs, pyriproxyfen and spirodiclofen, are agriculturally relevant and may affect energetic homeostasis (Johnson et al., 2010). Pyriproxyfen is a juvenile hormone mimic known to accelerate the nurse-to-forager transition, but it is not clear whether it affects lipid loss either directly or indirectly. Spirodiclofen is a fatty acid synthesis disruptor known to inhibit lipid synthesis, but it is unclear if it accelerates the nurse-to-forager transition in bees. Here, we focus on the abdominal fat body as a proxy of nutritional lipid status. We asked whether insect growth regulators (IGRs) induced abdominal lipid loss and altered worker foraging behavior. If stress increases the likelihood of precocious foraging (Toth et al., 2005; Perry et al., 2015; Woyciechowski and Moroń, 2009; Khoury et al., 2011), and if precocious foragers have been shown to be cognitively deficient

(Ushitani et al., 2016), in that they exhibit clear deficits in spatial memory associated with foraging success, then we expect stressed foragers to collect less pollen (smaller corbicular pellets) per foraging trip. Since we expected these stressors to act directly on the fat body, we also expected foragers to collect more, fattier pollen to compensate for stress-induced abdominal lipid reduction.

Materials and Methods

Source bees

All experiments were conducted at the Carl Hayden Bee Research Center in Tucson, Arizona. Colonies were headed by *Apis mellifera ligustica* queens from a commercial breeder in California. Field experiments (Experiments 3-4) used queen-right colonies that had an even brood distribution and no visible signs of disease or stress. Capped brood frames from these colonies were removed and placed in a temperature and humidity-controlled incubator (33°C ± 1°C, 50% humidity) overnight with no light. Adults that emerged within an 18h period were gently brushed from these frames prior to treatment.

Pesticide Treatment

Newly-emerged bees were randomly assigned to one of the three treatment groups: pyriproxyfen, spirodiclofen, or a solvent control. Pesticides (Sigma Aldrich PESTANAL® analytical standard, St. Louis, MO) were dissolved in acetone and then suspended in sugar water. Each bee was fed 2μL of the treatment-sugar water mixture by hand with a micropipette. For all experiments, the bees were fed once at adult emergence, reflecting an acute exposure from contaminated nectar shortly after emergence (Johnson et al., 2010; Fries et al., 2013). We did not test the effects of chronic exposure. Feeding bees once allowed for tight control of the individual pesticide dose, which was important because we observed individual behavior and lipid levels. For both

experiments, pesticide-treatment groups were fed only the high (42 mg/L) concentration of both spirodiclofen and pyriproxyfen (refer back to Appendix A where three different treatment levels (low, medium, high).

Experiment 1: Do IGRs affect the age at first forage?

Field experiments were conducted in August of 2019 (Trial 1, 2 hive replicates) and May of 2020 (Trial 2, 3 hive replicates). Each replicate hive contained 100 bees per treatment (pyriproxyfen, spirodiclofen, or a solvent control), placed into the same hive. The bees were fed a high dose (42 mg/L) of either pyriproxyfen or spirodiclofen, or the solvent control. Bees that successfully consumed the entire treatment were labeled with color-coded (by treatment) and numbered tags in order to track individuals from each treatment group. After the bees were treated and tagged, they were placed into the experimental colony. Hive entrances were extended using a Styrofoam landing platform with a plexiglass cover to better observe and record the ID numbers of foragers. Observations were conducted at the hive entrances every morning (starting at 4 days of age) between 7AM and 12PM. Each hive (n=3) was observed for 15-minutes total per hour, for a total of 6 observational periods each day.

We took note of the treatment group and ID# of every bee observed at the hive entrance and recorded 1) whether they were leaving or returning, 2) the duration of their flight (if seen both leaving and returning to the hive entrance) 3) if returning, whether they had pollen in their corbicula or a distended abdomen, indicative of water and nectar foraging (Chang et al., 2015). Distended abdomen was determined by visual estimate. Prior to the analyses, observations were categorized into orientation flights and foraging trips based whether the trip was shorter or longer than 5 minutes (for detailed criteria see Figure 1). Orientation flights are shorter pre-foraging trips when individuals prepare for the navigational demands of longer foraging trips (Winston, 1987; Capaldi & Dyer, 1999;

Prado et al., 2020). The intention for orientation flights is not necessarily to return with pollen, water, nectar, or other materials for the hive, but rather for the bee to learn to navigate the immediate environment outside of their hive so that they can successfully locate their hive entrance later when they begin foraging. Foraging trips were categorized into two categories: leaving or returning. Individual bees seen leaving the hive for the first time and not returning within 5 minutes were counted as first-time foragers. Returning bees were further separated into two categories: pollen foragers or water/nectar foragers (see above). If any of these criteria were not confirmed within the 15-minute observation period, that individual was removed from the dataset entirely. For example, if a bee was seen leaving the hive 14 minutes into the 15-minute observation period, this observation was discarded, as it could not be confirmed if it went on an orientation flight or a foraging trip, depending on the duration of the flight.

Experiment 2: Do IGR-treated pollen foragers have fewer abdominal lipids and collect less pollen?

Experiments examining the lipids present in the abdomens of returning foragers and their collected pollen were conducted in August of 2020 (Trial 1, 2 hive replicates) and May of 2021 (Trial 2, 3 hive replicates). For each hive replicate, approximately 250 individuals were fed per treatment type. Bees were fed as per Appendix B: Experiment 1 and painted with a water-based marker (POSCA™ queen bee marking pen set; colored by treatment on thorax, colored by hive replicate on abdomen to confirm source hive) prior to being placed into the hive. Collections took place every morning, wherein every pollen forager seen was immediately sampled. Hives (n=3) for this experiment were placed side-by-side so that a single observer could see and collect foragers from all replicates at once between 7AM-12PM. For this experiment, we were focused exclusively on pollen foragers, as dietary lipids come entirely from collected pollen. We did not collect water and nectar

foragers. These returning foragers were collected and flash-frozen in liquid nitrogen and stored at -80°C until they were processed.

For each forager, the corbicular pollen was completely removed from both hind legs under light microscopy and dried in an oven at 60°C for 48 hours and weighed (mg) individually after drying to measure pollen dry weight. To assay pollen lipids, removed pollen was once subjected to a Folch extraction and assayed for total lipid content per the sulfuric acid-vanillin-phosphoric acid assay (Van Handel, 1985; Malagoli et al., 2010).

We assayed only the abdominal lipids, as the majority of fat body is located in the abdomen. We did not include thoracic fat body tissue because removing the flight muscles from the cuticle wall was too difficult to perform with precision. To measure abdominal lipids for lipid analysis in Experiments 2-3, bees were flash frozen in liquid nitrogen and stored in the -80°C freezer until processing. Bees were dissected by removing the abdomen from the thorax. The abdominal carcasses of these bees were dissected by removing their digestive tract (midgut, hindgut), stinger, stinging gland, ovaries under a light dissecting microscope. After dissection, abdominal carcasses were dried in an oven at 60°C for 48 hours and weighed (mg) individually to measure dry weight (Human et al., 2013). To assay abdominal lipids, dissected abdomens were twice subjected to a Folch extraction and assayed for total lipid content per the sulfuric acid-vanillin-phosphoric acid assay (Van Handel, 1985; Malagoli et al., 2010).

All reagents were provided by Sigma Aldrich (St. Louis, MO). Sample absorbance was measured using a plate reader (BIOTEK Synergy HT™, Agilent: Santa Clara, CA) and absorbances were evaluated against a standard curve of samples containing known amounts of vegetable oil dissolved in chloroform. Samples below the limit of detection were discarded prior to the analyses.

Statistical analyses

All statistical analyses were performed using R (https://cran.r-project.org). Graphs were generated using the following R packages: ggplot2, cowplot, ggsignif (ggplot2: https://cran.r-project.org/package=ggplot2; cowplot: https://cran.r-project.org/package=cowplot; ggsignif:https://cran.r-project.org/package=ggsignif). The average age at first forage and orientation flights were analyzed using a generalized linear mixed model (GLMM) with age as the dependent variable, treatment as the fixed predictor variable, hive as a random effect, and a gamma link function. A logistic regression was used to analyze the likelihood of a returning forager carrying pollen or water/nectar, with treatment as the predictor variable and hive as a random effect. Observations where bees were seen leaving the hive, rather than returning, were not included in this logistic model, as it could not be confirmed within the 15-minute observation period what they collected as they were not observed returning. Lipid data were analyzed using a GLM. Total lipid content was the dependent variable, treatment and age were used as fixed predictor variables, and hive was designated a random effect. We tested whether forager abdominal lipids and corbicular pollen lipids were influenced by pesticide treatment and the age of the forager, as well as an interaction between the treatment and forager age. All lipid measurements were log-transformed prior to analyses. Pairwise comparisons were made using Tukey's HSD *post hoc* test using the R package emmeans (https://cran.r-project.org/package=emmeans).

Results

Experiment 1: Foraging onset and behavior

Pesticide treatment did not influence the onset of orientation flights ($F_{2,38}$=0.739, p=0.484; Figure 2A). 41 orientation flights were observed, occurring as early as 5 days and as late as 15 days post-treatment. Pesticide treatment decreased the average age that bees were observed leaving the

nest to forage (GLM, $F_{2,187}$=4.66, p=0.0106; Figure 2B). Pyriproxyfen-treated bees (n=60) foraged significantly earlier than the control bees (n=70) (Tukey's test: t_{187}=-2.97, p=0.0096; Figure 2B). There was no difference between the control (n=70) and spirodiclofen (n=60) groups in the age at first forage (Tukey's t-test: t_{187}=-1.92, p=0.135). Although pyriproxyfen-treated bees foraged significantly earlier than the control, there were no significant differences between any groups in the ages at which foragers first returned with pollen ($F_{2,59}$=0.727, p=0.488).

We also found no significant effect of treatment ($F_{2,108}$=0.210, p=0.811) or age ($F_{2,107}$=0.495, p=0.482) on the likelihood that a forager returned with pollen versus water/nectar (Table 1). Because we found no significant differences in the prevalence of pollen foragers between treatments, we concluded that pesticide treatment was unlikely to influence the type of dietary resource (pollen vs. water/nectar) collected by foragers.

Experiment 2: Forager abdominal and corbicular pollen lipid

We collected pollen foragers to examine the relationship between internal lipid levels and collected dietary lipids. There was a significant effect of pesticide treatment on total abdominal lipids (total µg) ($F_{2,203}$=3.52, p=0.032), with spirodiclofen-treated bees having a higher y-intercept for the average total abdominal lipid in comparison to controls (Tukey's test: t_{203}=-2.479, p=0.037; Figure 3B). Although treatment did not affect average abdominal lipid concentration (total µg lipid/dry weight abdominal carcass mg; $F_{2,203}$=0.736, p=0.480), we noticed a strong effect of day on abdominal lipid concentration (µg/mg; $F_{2,203}$=12.08, p=0.0006; Figure 3A). When looking at the slopes for each treatment regression, we found that spirodiclofen-treated bees experienced a significantly accelerated decline in abdominal lipid concentration (R^2=0.136, $F_{1,75}$= 12.98, p <0.001; Figure 3A), which was not found in those treated with pyriproxyfen (R^2=-0.00359, $F_{1,65}$=0.764, p =0.385) or the control group (R^2=0.0167, $F_{1,63}$=2.09, p =0.153).

Pesticide treatment influenced both the amount of pollen that a forager collected and the lipids in the pollen. The average dry weight of pollen collected by foragers was 8.11 mg ± 7.27 SE, with an average lipid concentration of 85.6 µg lipids/mg ± 161.9 SE. Treatment influenced both the amount of pollen collected (mg; $F_{2,203}$=4.660, p=0.0105) and the pollen's lipid concentration (µg/mg; $F_{2,203}$=3.05, p=0.0496) (Figure 4A-B). Spirodiclofen-treated bees returned with significantly less pollen compared to the controls (p=0.0122). However, the pollen that the spirodiclofen-treated bees collected had a higher lipid concentration (p=0.0383) compared to controls. Forager age did not affect the amount of pollen that each forager collected (mg; $F_{2,203}$=3.46, p=0.0643), but it did affect its lipid concentration (µg/mg; $F_{2,203}$=11.7, p<0.0001). Older bees returned with pollen that was less concentrated in lipids compared to younger bees. Foragers with less concentrated abdominal lipids collected less lipid-concentrated pollen (R^2=0.110, $F_{6,202}$=5.30, p<0.0001; Figure 5A). Total abdominal lipid, however, was not an accurate predictor of total pollen lipid (R^2=0.015, $F_{6,202}$=1.52, p=0.171; Figure 5B), suggesting that abdominal lipid concentration is a more accurate predictor of pollen dietary lipid.

Discussion

We found that pyriproxyfen treatment lowered the average age at first forage, with no changes to the amount or lipid concentration of their collected pollen. We also found that spirodiclofen treatment, while having no significant effect on the onset of foraging behavior, did affect the nutritional quality and quantity of collected forage. Spirodiclofen-treated bees experienced a significantly faster rate of abdominal lipid decline compared to controls. They also collected less, yet fattier, pollen. These results suggest that lipids may be important in the response that bees have to pesticide. Our results provide a new mechanistic basis for the connection between stress, lipid homeostasis, and behavior in honey bees. A single, acute stress exposure early in adulthood altered

behaviors much later in life, as bees fed pesticides once as newly emerged workers exhibited behavioral changes later as foragers (roughly ≥10 days after treatment). The continuation of foraging for higher-value food items is likely a conserved evolutionary response observed seen not only in vertebrates, but in invertebrate models as well. If this response is truly conserved, this insect system might be used to further study the links between stress and pathologies related to altered lipid homeostasis in humans.

Bees treated with an acute stressor, the pesticide pyriproxyfen, a juvenile hormone mimic, before they were then placed into a colony had a lower average age at first forage. This is consistent with existing literature on another juvenile hormone analogue, methoprene, which also decreased the age at first foraging flight, as well as the effective age at death (Chang et al., 2015). Precocious foraging in pyriproxyfen treated individuals did not lead to expedited lipid depletion. Because pyriproxyfen-treated bees were more likely to forage precociously, and precocious foragers are hypothesized to have sub-optimal foraging performance (Woyciechowski and Moroń, 2009; Ushitani et al., 2016), we predicted that precocious foragers would collect less pollen (smaller corbicular pellets) per foraging trip. This was not the case: pyriproxyfen-treated foragers collected the same amount of pollen per trip as the control foragers. This refutes the hypothesis that precocious foraging increases the likelihood of hive failure due to a reduced foraging capacity (as defined by lower individual pollen and dietary lipid yield). Lipid levels in the collected pollen were also equivalent between pyriproxyfen-treated bees and controls. From this study, we also cannot say with certainty whether pyriproxyfen-induced precocious foraging affects colony demography more broadly. Our mortality data (Appendix A) suggests that pyriproxyfen exacts some cost to all-cause mortality. If pyriproxyfen elevates forager mortality above a threshold level at the hive level, colony collapse remains a likely possibility (Perry et al., 2015; Khoury et al., 2011).

The results from the spirodiclofen-treated bees support a link between stress, pollen lipid nutrition, and behavior. Previous literature uses total abdominal lipid to measure changes in fat body lipid integrity (Toth et al., 2005; Anand & Lorenz, 2008). We also wanted to see if lipid levels were affected as a proportion of individual bee weight, so we also analyzed the effect of pesticides on the abdominal lipid concentration (proportion of total bee biomass due to lipid). Spirodiclofen-treated bees had significantly higher total abdominal lipids compared to controls, but it did not affect abdominal lipid concentration. This suggests that, while spirodiclofen treatment may have a significant effect on increasing absolute lipid levels, it does not increase lipid levels as a proportion of total biomass, suggesting that body mass increases along with lipid content. When we incorporate biomass into our model, we find that spirodiclofen-treated bees exhibit a significant decline in abdominal lipid concentration that occurs faster compared to other groups. Spirodiclofen impedes lipid biosynthesis, so this accelerated depletion of abdominal lipids may be due to bees being incapable of replenishing their lipid reserves endogenously It is also possible that fatty acid synthesis inhibitors make younger bees hungrier because they deposit fewer fatty acids in their fat body, and so they over-consume stored nutrients in the hive to compensate for lapses in endogenous lipid biosynthesis. It is possible that stressors with a similar mode of action as spirodiclofen motivate bees to increase dietary lipid intake to maintain energetic homeostasis, but that these efforts are ultimately futile in altering their total proportion of body weight due to lipid stores.

Ultimately, these results support the hypothesis that bee respond to stressors that reduce fat body by regulating lipid intake as foragers in response to stress they were exposed to at adult emergence. Fueling the stress response can be energetically costly (Arrese and Soulages, 2010), so it is reasonable that stressed colonies would collect fattier pollen to restore colony-level lipid homeostasis. Honey bees select pollen with macronutrient ratios that balance nutritional deficiencies (Corby-Harris et al., 2021; Hendriksma and Shafir, 2016) and optimize colony health and survival

(Bouchebti et al., 2022). With this in mind, high-lipid diets may provide a benefit to bees challenged with pesticides or parasites (Annoscia et al., 2017; Crone and Grozinger, 2021). Given our current needs for crop pollination, especially in agricultural landscapes where floral diversity is low and the risk of pesticide or parasite exposure is high (Goodrich et al., 2019), colonies might benefit from additional lipid-rich forage and/or supplemental diets during key periods before and after bloom.

Our study reinforces the hypothesis that stress has lasting consequences on lipid homeostasis and food intake behavior. The nutritional dearth experienced by bee colonies in agricultural landscapes with low floral diversity is analogous to the food scarcity that is experienced by people residing in communities with limited access to affordable, nutritious foods. These communities are typically more vulnerable to a plethora of stressful circumstances, including higher rates of poverty and violence (Walker et al., 2010). These communities also face a higher prevalence of obesity and other metabolic diseases (Ghosh-Dastidar et al., 2014). From a public health perspective, stress management should be an important factor when considering preventative measures against human diseases associated with altered lipid balance, such as obesity. The present study suggests that honey bees provide an experimental framework for addressing such questions on the stress-lipid-behavior axis.

Tables and Figures

Treatment	Total Trips	With Pollen	Without Pollen	Percent Pollen Foragers
Control	**39**	**22**	**17**	**56.4%**
Pyriproxyfen	**42**	**22**	**20**	**52.4%**
Spirodiclofen	**30**	**18**	**12**	**60%**

Table 1: Proportion of returning foragers with or without pollen

A total of 101 returning foragers were observed over the course of 18 days (Experiment 1). Bees with pollen on their corbiculae, visible to the naked eye, were classified as "returning, with pollen" whereas bees seen returning with a distended abdomen (likely nectar or water forager) and no visible pollen were classified as "returning, without pollen." Distended abdomen was determined by visual estimate.

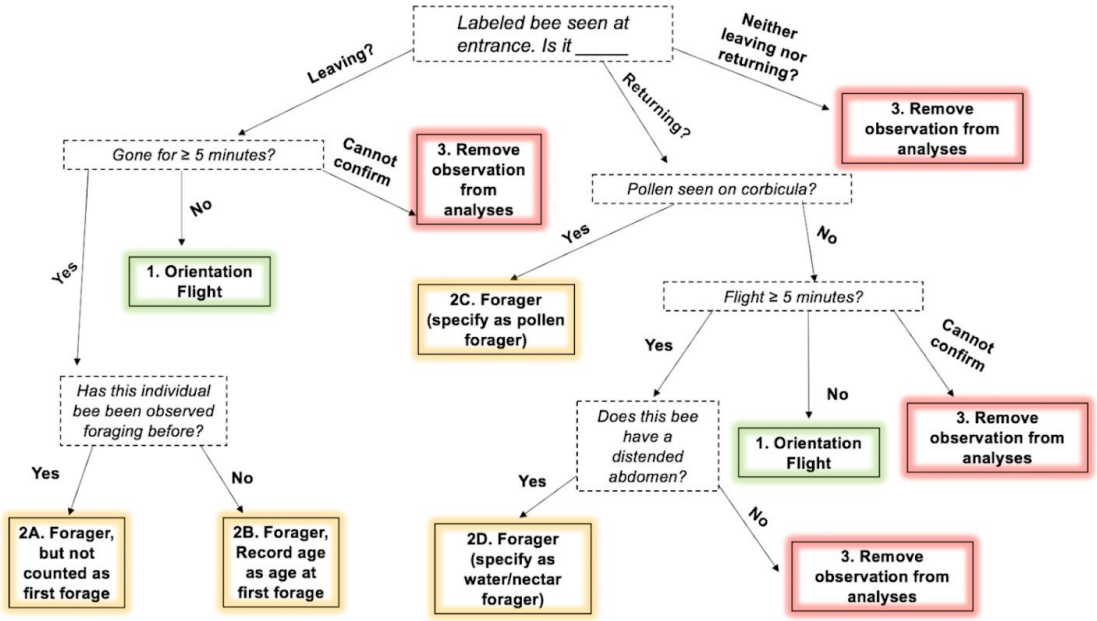

Figure 1. Classification of foraging trips for Experiment 2

The below flow chart depicts the process of classifying foraging trips observed in Experiment 2 as 1) orientation flights 2) foraging trips, including whether a trip was counted as the first recorded age of first forage and whether the forager collected pollen or exclusively water/nectar and 3) the circumstances that an observation was removed from the dataset. Distended abdomen was determined by visual estimate.

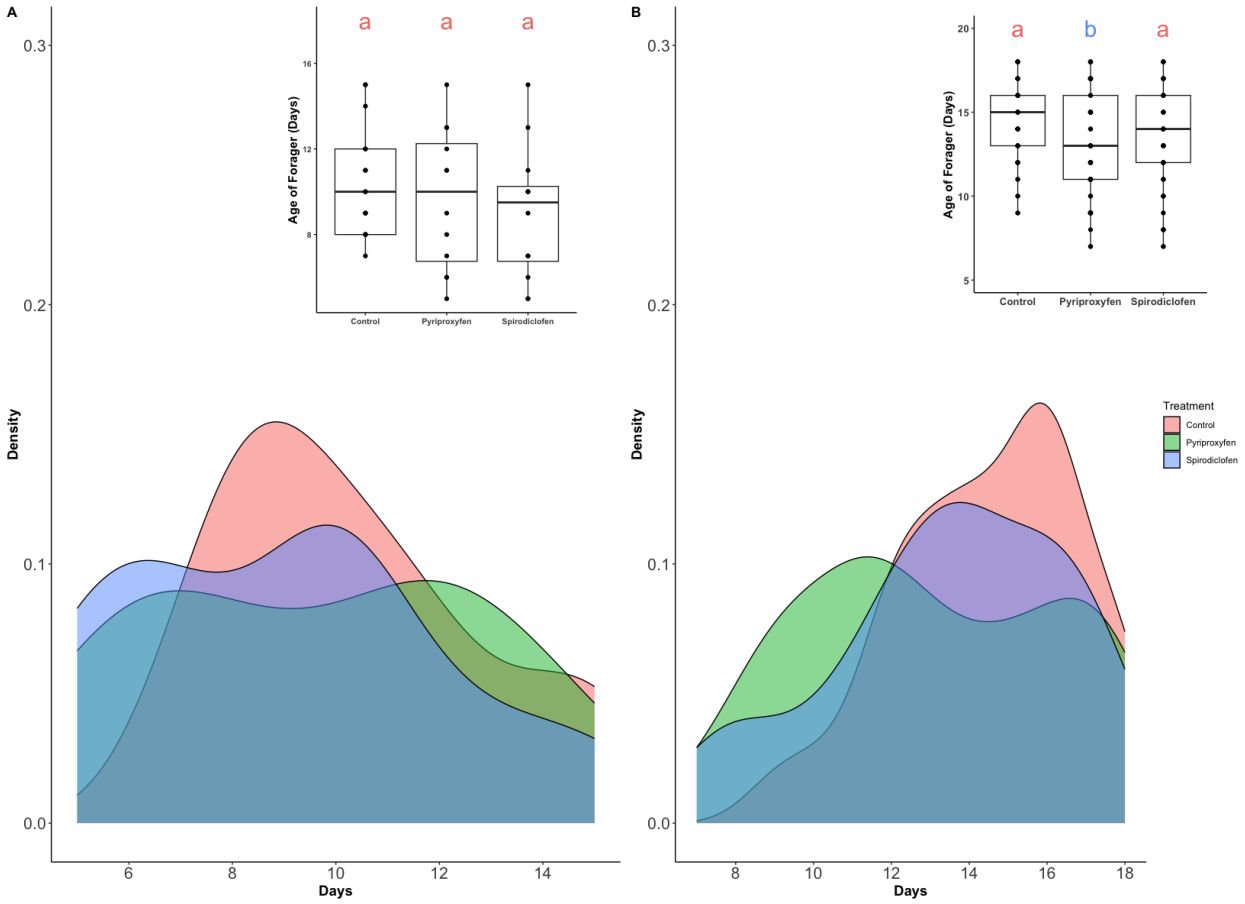

Figure 2. Pyriproxyfen treatment lowers average at first forage, but not onset of orientation flights

Plots indicating the density of observed (A) Orientation Flights (n=41) and (B) First Foraging Trip (as defined by leaving forager) (n=190) for each pesticide on each day following pesticide treatment at adult-emergence. Data were collected from 5 replicate hives. In-set boxplots show median (horizontal line within box) abdominal lipid concentration and interquartile range (lower and upper box boundaries) as well as the minimum and maximum values of the distribution. Points indicate a single observation. Outliers are points outside the maximum and minimum of the distribution. Matching letters and colors between boxplots indicate no significant differences between groups as determined by TukeyHSD multiple comparisons.

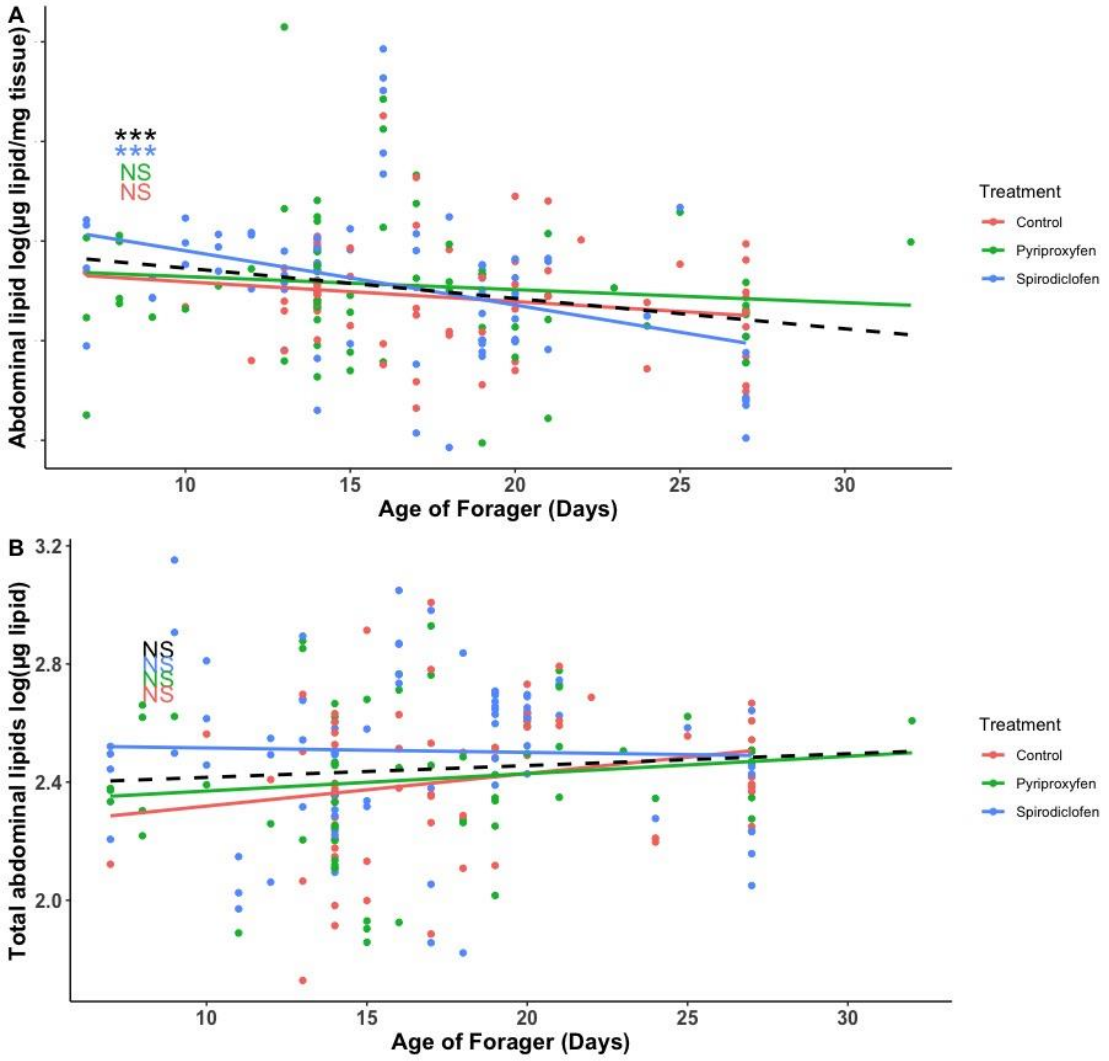

Figure 3. Abdominal total lipids greater, but decline significantly faster, due to spirodiclofen treatment

Scatter plots of (A) abdominal lipid concentration (log(µg total lipid/mg bee total tissue) and (B) total abdominal lipids (log(µg total lipid)) by age of collected forager. Each point represents a single collected forager's age and their corresponding lipid value. Color lines (see legend) indicate stratified linear regression by treatment. Black dashed lines indicate overall linear regression across all treatments. Asterisks and color text on scatter plots indicate summary of linear regression analysis (P<0.0001=***; Not significant=NS).

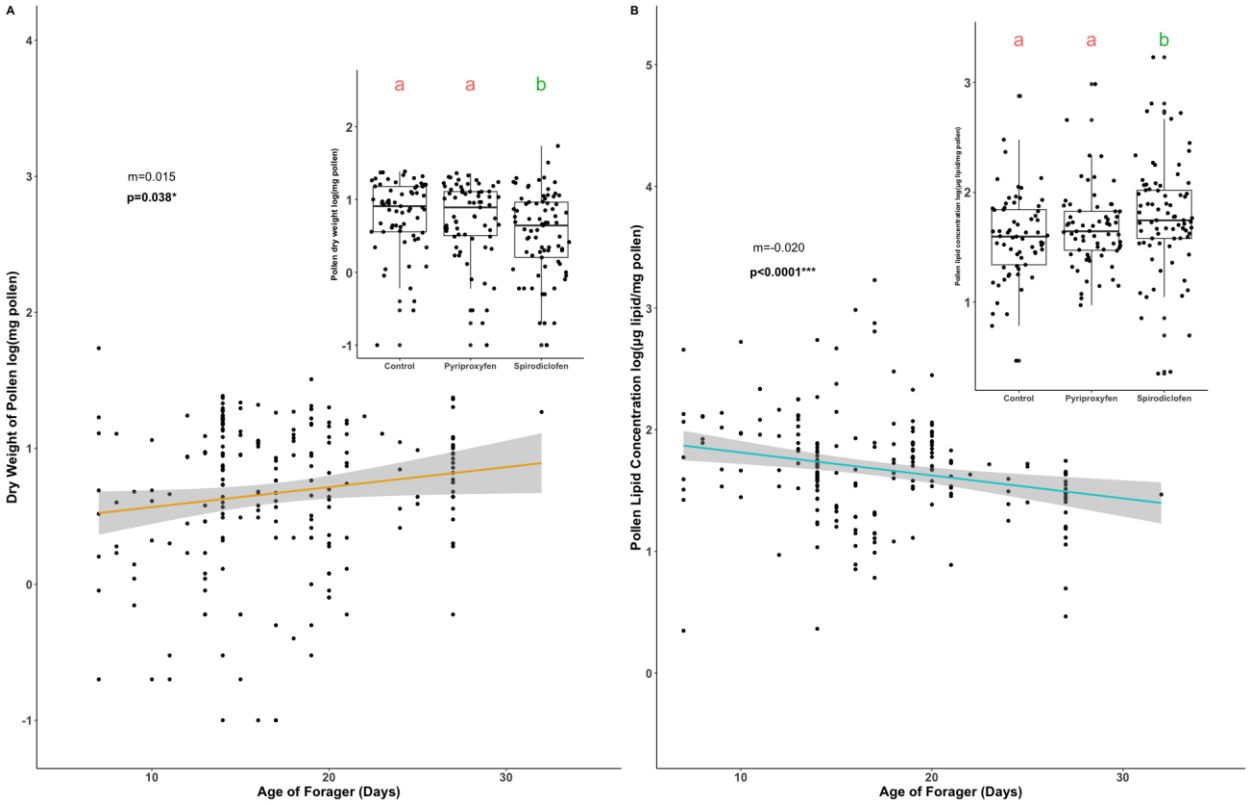

Figure 4. Spirodiclofen treatment increases amount and lipid concentration of collected pollen

Scatterplots of (A) dry weight of collected pollen (log(mg pollen)) and (B) pollen lipid concentration (log(µg pollen lipid/mg pollen)) by age of collected forager. Each point represents corbicular pollen collected from a single forager and their age. In-set box plots represent the median (horizontal line within box) (A) dry weight of pollen and (B) pollen lipid concentration by treatment concentration and interquartile range (lower and upper box boundaries) as well as the minimum and maximum values of the distribution. Outliers are points outside the maximum and minimum of the distribution. Matching letters and colors between boxplots indicate no significant differences between two groups as determined by TukeyHSD multiple comparisons.

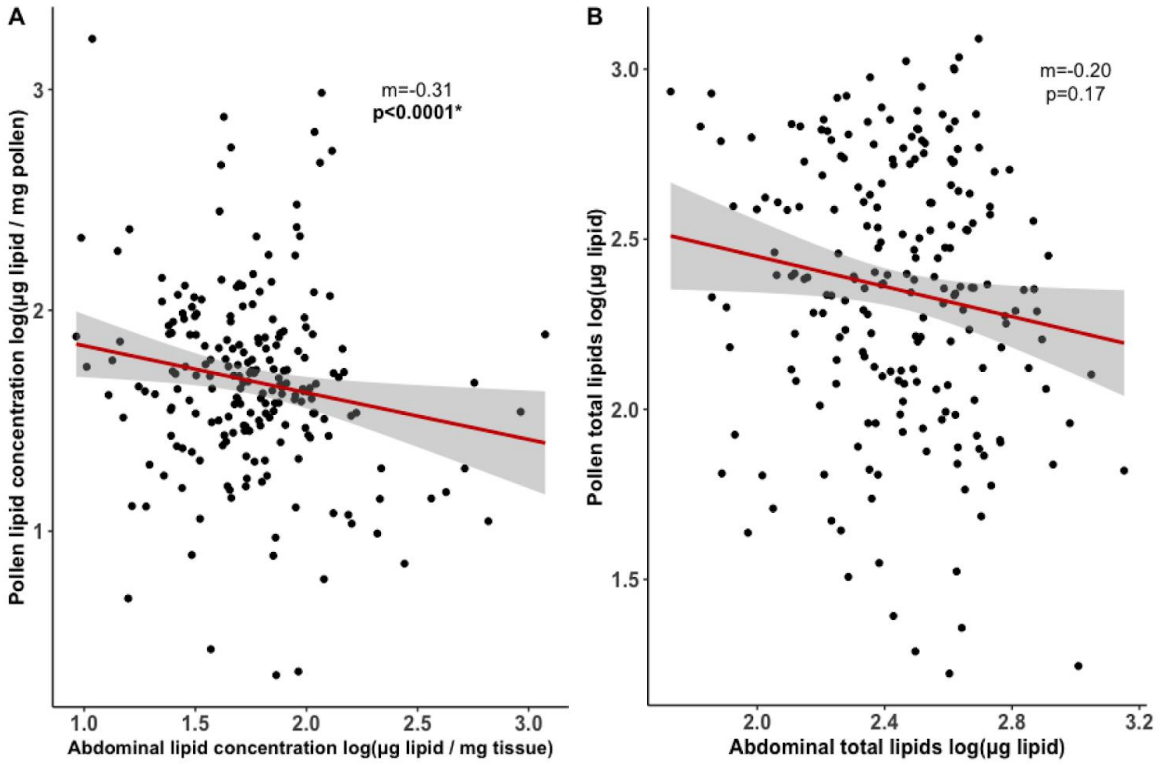

Figure 5. Forager abdominal lipid concentration significantly declines with pollen lipid concentration

Scatterplots of (A) abdominal lipid concentration (log(µg total lipid/mg bee total tissue) by pollen lipid concentration and (B) total abdominal lipid (log (µg total lipid)) by total pollen lipid (log (µg total pollen lipid)). Each point represents a single forager's (n=209) internal lipid state corresponding to the lipid quantity of their collected pollen. Red trend lines indicate best fit linear regression with gray area representing confidence intervals of the slope.

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Appendix C

Increasing pesticide resilience in honey bees through improved lipid supplementation

Megan Elizabeth Deeter, Lucy Snyder, Vanessa Corby-Harris

Abstract

Commercial honey bee colonies in the United States have experienced significant increases in colony failure over the past two decades due to the synergistic effects of multiple stressors, such as pesticides, pathogens, and pollution. The reliance on pesticide usage to control pest insects in agricultural settings has prompted additional research to fully understand their effects on bees and, if possible. Our previous work has found that even a single field-realistic exposure to a common agricultural pesticide, spirodiclofen, capable of accelerating the depletion of abdominal lipids and altering the foraging behavior of bees in that they collect less, yet more lipid-rich, pollen. This suggests that dietary lipid may be important in conferring the response bees have to stress, specifically pesticide stress. One potential solution to mitigate the deleterious effects of pesticide exposure could be to improve the lipid component of pollen patty, a dietary supplement (consisting of natural pollen, sugar, and additional protein) provided by beekeepers. Historically, these supplements have been formulated with minimal consideration for essential dietary lipids. We hypothesize that increasing the lipid component of standard pollen patty may increase stress resilience to pesticides. Further, if dietary lipid caused this increased resilience by counteracting the pesticide-induced abdominal lipid depletion, then pesticide-treated bees fed the supplement should have higher abdominal lipids than pesticide-treated bees that were not fed additional lipid. In this study, we formulated four different pollen patty diets to test the efficacy of flaxseed oil lipid supplementation as a means for increasing honey bee pesticide resilience. We found lipid supplementation with flaxseed oil to be an effective method to reduce weight loss in pesticide-treated hives and improve the body fat composition of nurse bees. Foraging activity was largely unaffected by lipid supplementation until the end of the 2-month treatment phase, when the nurses that were first provided with treatment at the beginning of the 2-month trial had reached foraging age. This suggests lipid supplementation confers the most benefits to bees that directly consume the

treatment (nurses), and that nurses consuming lower fat diets will later forage for fattier pollen when they reach foraging age compared to those provided higher fat diets as nurses. Our results present evidence that lipid supplementation helps mitigate the effects of pesticide treatment and could be a promising method to increase stress resilience in commercial bee hives.

Introduction

Nutritional homeostasis is a necessary biological process that involves adjusting one's feeding behavior in accordance with changing energetic needs (Simpson & Raubenheimer, 2011). Insects require a certain proportion of the major macronutrients (protein, fat, and carbohydrate), along with certain necessary micronutrients (vitamins, minerals, etc.), depending on their life history strategy. Obtaining these nutrients is somewhat complex for the eusocial honey bee, *Apis mellifera*, as foraging is entirely delegated to a sub-population of older workers that collects all resources (pollen, water, nectar, and other plant materials) for their colony.

Workers begin their adult life in the hive as nurse bees that nourish and tend to worker and queen brood (Huang & Robinson, 1996). At roughly three weeks old, they transition into foragers that routinely leave the hive in search of water, nectar, and pollen for the colony (Winston, 1987). In stark contrast to nurses, foragers consume food sources that can be readily degraded on demand to be used as glycolytic fuel during flight, as foraging is an energetically demanding task (Even et al., 2012). The preferred ratio of essential amino acid (EAA) to carbohydrate (by weight) in nurse diets is 1:50, compared to foragers where this ratio reduces to 1:250 (Paoli et al., 2014). Foragers consume sugars in the form of nectar and stored honey, instead of stored pollen (Brodschneider & Crailsheim, 2010; Camazine, 1993). Somewhat paradoxically, foragers are the only specialized demographic tasked with collecting all outside resources for the hive, including pollen, even though only nurses and larvae directly consume it (Brodschneider & Crailsheim, 2010; Wright et al., 2018).

We might expect that this gatherer-consumer disconnect further complicates nutritional homeostasis in hives, since brood, nurses, and foragers have varying nutritional needs from one another. However, a growing body of research suggests foragers can assess both 1) forage value and 2) colony nutritional needs.

For foragers to perceive colony food needs, this implies some level of communication must occur between nurses and foragers. However, hive bees do not seem to consume pollens based on their nutritional quality (Corby-Harris et al., 2018), so it is unlikely that hive bees communicate preference or need directly to foragers. Foragers most likely assay colony nutritional needs using other cues, such as the quantity of pollen storage. Pollen stores can become exhausted during periods of extensive brood rearing, so the presence of brood can act as a signal to recruit more pollen foragers (Dreller et al., 1999). Recent evidence suggests foragers, not nurses, are perceptive to macronutrient deficiencies and forage accordingly. Diet manipulation experiments have demonstrated that foragers will collect pollen that balances nutritional deficiencies in both amino acids (Hendriksma & Shafir, 2016) and fatty acids (Zarchin et al., 2017). Since both experiments were conducted in a controlled-environment enclosed flight arena, the question of whether foragers compensate for these deficiencies by collecting better pollen in the field requires additional investigation. These studies offer exciting insight regarding the capacity of foragers to balance macronutrient levels relative to colony need.

While foraging for fattier pollen could be an excellent strategy to maintain lipid homeostasis, it is not always a viable option. Even if foragers are attuned to hive nutritional needs and have the capacity to forage accordingly in principle, they are still ultimately limited to what floral resources are available in their environment. Total pollen protein and lipid content can be highly variable between flowers, even those within the same environment (Manning, 2001), so finding resources that complement deficiencies can be both time and energy consuming. Additionally, increased

urbanization and agricultural intensification has led to a substantial loss in plant biodiversity that puts additional strain on foragers (Persson et al., 2015). Nutritional dearth due to poor forage quality and quantity is one of the major factors attributed to all pollinator declines over the past few decades, both native and managed non-native pollinators, and is expected only to worsen due to climate change (Potts et al., 2010; Vercelli et al., 2021). Improved land management practices, such as the planting of native habitat, has been shown to mitigate the negative effects of commercial agriculture on bees (Dolezal et al., 2019).

Beekeepers often provide colonies with supplemental feed during times of nutritional scarcity to combat the negative effects of pollen dearth (Lamontagne-Drolet et al., 2019; Paray et al., 2021). Historically, sugar supplementation (such as sucrose or high fructose corn syrup) was provided to minimize forager exhaustion or build comb in preparation for winter, but this was largely ineffective in mitigating the effects of pollen starvation (Lois et al., 2020; Mogren et al., 2018). Most commercial supplements currently available to beekeepers are designed to support brood production by increasing protein consumption, with minimal consideration for lipids (Corby-Harris et al., 2018; Lamontagne-Drolet et al., 2019; Paray et al., 2021; Wright et al., 2018). While effective in supporting brood production, excessive protein intake confers costs that outweigh its benefits, such as increased all-cause mortality and higher pathogen load (Crone & Grozinger, 2021). Soybean and defatted soy flour are the most common bases utilized in commercial supplements, but research suggests soy-based diets tend to be less palatable than natural forage-based diets and overall, less beneficial to honey bees (De Jong et al., 2009).

While the pollen protein is critical for brood development, supplemental diets should also incorporate some aspect of natural forage to increase the palatability and incorporate fat-soluble micronutrients found in plant pollens. Honey bee supplements are often combined with polyfloral pollen mixtures (collected in pollen traps and refrigerated for future use by beekeepers), sugar (to

stimulate appetite), and water to make pollen patties (Branchiccela et al., 2019). Pollen patties are nutritious supplements placed under hive lids, typically over the brood nest, that are readily consumed by hive bees when pollen intake is low. Unfortunately, current commercial supplements available to beekeepers are insufficient in supporting colony lipid needs necessary for growth and development (Corby-Harris et al., 2021, p. 20). Stress depletes individual bee internal lipid reserves (Corby-Harris et al., 2020), so supplementation at the hive level may be an appropriate avenue to compensate for expedited fat loss in stressed bees.

There are multiple nutritional parameters to consider when providing lipid supplementation, such as the protein to lipid (P : L) ratio. Diet studies conducted on caged bees found that those fed pollen patties with 12.5% - 36% lipid had significantly lower mortality and increased immunocompetency (Crone & Grozinger, 2021). Nurses appear to balance their dietary intake to reflect a lower protein to lipid ratio of approximately 1.25 : 1 (by weight) (Stabler, 2020).

Existing commercial supplements are also deficient in essential fatty acids (EFAs), the major dietary lipid component of pollen (Corby-Harris et al., 2021). EFAs are polyunsaturated fatty acids that cannot be synthesized endogenously in insects and must be obtained through food (Kaur et al., 2014). To be optimized, a supplemental diet must not only include EFAs but must also be balanced with respect to which types of EFAs are included. The most common EFAs found in pollen are oleic (ω-9 fatty acid), linoleic (ω-6 fatty acid) and alpha-linolenic (ω-3 fatty) acids (Manning, 2001). Nurse bees consuming high ω-6:3 fatty acid ratios in their diet have smaller hypopharyngeal glands and decreased brood-rearing ability (Arien et al., 2015, 2020) Additionally, bees deficient in ω-3 fatty acids exhibited a reduced capacity for olfactory memory and learning (Arien et al., 2015; Bennett, 2022).

In this study, we tested the efficacy of flaxseed oil as an additive to our standard pollen patty diet in terms of whether it could mitigate the negative effects of the pesticide spirodiclofen

(Appendices A-B). We chose flaxseed oil as our supplemental lipid because it consists of mostly polyunsaturated fatty acids and has a balanced ω-6:3 fatty acid ratio that the bees were more likely to consume compared to similar, less-balanced lipids.

Materials and Methods

Source bees

16 small, 5 frame nucleus hives were constructed from frames of capped brood with adult worker bees from source colonies at the Carl Hayden Bee Research Center. Source colonies were determined to have low *Varroa destructor* mite levels and were visibly disease-free (no chalkbrood larvae, deformed wings). Each hive was provided with *Apis mellifera ligustica* queen provided by a commercial breeder based in Northern California and requeened with a replacement from the same genetic line whenever necessary (Table 2). Hives were constructed in late May, 2022. Colonies were all fed the same, unsupplemented pollen patty as described below (Control, Low Fat Diet) for 4 weeks (growth period) prior to the start of the 2-month treatment phase.

Diet Preparation

We determined the P : L ratio of our unsupplemented pollen patty (2 : 1 sugar : pollen + water added *ad libitum* to get fondant-like consistency) using the sulfo-phosphoric-vanillin assay to measure total lipid (Van Handel, 1985) and BCA protein assay (Thermo Fisher Scientific, Pierce(™) BCA Protein Assay Kit). We measured the total lipid and total protein in 0.5 gram (500 mg) samples of the unsupplemented pollen patty (no added lipid, no added pesticide), repeated 6 times (12 total: 6 times for lipid assay, 6 times for protein assay) to determine μg lipid or protein per gram of pollen patty. We determined the P : L ratio of the unsupplemented pollen patty to be 12.2 : 1 (μg protein : μg lipid per gram), or 8.2% lipid per weight.

From this batch of pollen patty, we added 155 μL of flaxseed oil per gram of pollen patty to achieve a protein : lipid ratio of 6 : 1 by weight. The unsupplemented pollen patties fed during the growth phase contained neither added pesticide nor supplemental lipid (flaxseed oil) and was later fed to our double-control group hives (Control, Low Fat Diet) during the 2-month treatment phase.

For pesticide treated pollen patties, the initial low fat diet and high fat diet batches were each divided in half (by weight) with spirodiclofen treatment being applied to one half and the other receiving an acetone control. An appropriate amount of spirodiclofen was added to two of the treatment groups: Pesticide/Low Fat Diet and Pesticide/High Fat Diet to achieve a final concentration of 2ppb, or 2 μg spirodiclofen per kg pollen. This concentration was based upon maximum pesticide incidence survey data found in apiary pollen stores (Johnson et al., 2010).

Growth Period & Treatment Phase

During the 4-week growth period, all sixteen hives were provided roughly 150 grams of unsupplemented, pesticide-free (Corby-Harris et al., 2018) pollen patty each week, a 1:1:1 ratio (by weight) of bee-collected polyfloral pollen (Spring pollen, Tucson AZ), table sugar, and drivert sugar (8% sucrose, fructose + 92% sucrose). After the 4-week growth period, hives were randomly assigned to 1 of 4 diets for the duration of the experimental phase (2 months): (Group 1) Pesticide/Low Fat Diet; (Group 2) Pesticide/High Fat Diet; (Group 3) Control/Low Fat Diet and (Group 4) Control/High Fat Diet.

The pollen patty was placed underneath the hive box lid over the center frame where it could be consumed by nurse workers, as brood production tends to be concentrated in the middle frames. After each week, the remaining pollen patty (if any) was removed and weighed to determine total consumption. Altered diet experiments ran from late June to late August 2022. We collected the following weekly (pollen patty consumption, hive weight, brood nest bee and pollen forager

samples) and monthly (hive demographics & queen status) metrics to measure the overall health and nutritional status of our hives (**Table 1**).

Data Collection

Metric	Method	Purpose
Pollen Patty Consumption (g)	**Weekly.** Record weight of pollen patty before adding to hive. Remove and weigh remaining pollen patty	Determine if pesticide and/or lipid supplementation causes changes in consumption at the hive level.
Total Hive Weight (kg)	**Weekly.** Place hives on postal scale before dawn (5AM local time)	Proxy measure of total hive biomass, measured before dawn to include foragers.
Nurse bee abdominal lipid	**Weekly.** Observe brood nest frames and collect ~30 nurse bees. Store in the -80°C fridge until processing. Perform vanillin assay on nurse bee abdomens.	Assay nurse bee abdominal lipid to determine effect of pesticide treatment & lipid supplementation on brood-rearing parameters.
Forager pollen intake	**Weekly;** Collect at least 6 foragers (between 6AM-12PM) with intact corbicular pollen; Weigh and assay total lipid content of collected pollen; Refer to Appendix B: Materials & Methods for details on dissection and assay.	Determine the effect of pesticide treatment and lipid supplementation on forager pollen intake; Do bees collect less but fattier pollen to compensate for pesticide stress?
Hive demography	**Monthly;** Check frames and record the percent surface area for each of the following cell storage types: brood, pollen, honey, and empty. The percent surface area for each of the following cell storage types were visually estimated with the same observer for all three hive evaluations.	Determine the effect of pesticide treatment and lipid supplementation on how hives allocate their resources.
Queen status	**Monthly;** Check for queen while performing hive evaluations for demography (see above)	Confirm that hive remains queen right and producing brood; Find patterns for when queen failure events occur. Queen failure is declared when the queen is no longer observed in the hive and eggs are no longer seen in the brood nest area (Winston, 1987).

Table 1: Data collection metrics throughout experiment

Statistical Analyses

All statistical analyses were performed using R. We compared the consumption of unsupplemented, pesticide-free pollen patty prior to treatment between hives using the Kruskal-Wallis test. Treatment phase metric data were analyzed using Repeated Measures ANOVA with hive (n=16) as a random effect. *Post-hoc* analysis was done using pairwise comparisons (Tukey's HSD) using the R package emmeans. Graphs were generated using the following R packages: ggplot2, cowplot, ggsignif.

Results

Pollen Patty Consumption & Hive Weight

The average weekly pollen patty consumption per hive during the 4-week growth phase was 108.69 grams (Standard Deviation: 18.63 grams, n=64). At the beginning of treatment, the average hive weight was 16.145 kg (Standard deviation of 1.23, n=16). All hives had at least 50% of frame coverage by bees in the brood nest, the middle frames, which is indicative of a healthy worker bee demographic. The total amount of unsupplemented, pesticide-free pollen patty consumption across the entire 4-week growth phase did not vary between hives, indicating that all hives had similar energy intake prior to being assigned treatment groups at the beginning of the 2-month treatment phase (Kruskal-Wallis, $H(15)$=0.6838, p=0.877; **Figure 1A**).

The average weekly pollen patty consumption per hive during the 2-month treatment phase was 127.44 grams (Standard Deviation: 33.25 grams, n=128). Weekly pollen patty consumption changed significantly across the 2-month treatment phase in all four treatment groups (Friedman,

2(2)= 12.5, p=0.002; **Figure 1B**). Post-hoc comparisons found a significant increase in patty consumption between the beginning of the treatment phase (week 1) and the halfway point (week 4) [weeks 1-4, p=0.0001], followed by a significant decrease in consumption between the halfway point (week 4) and end of the treatment phase (week 8) [weeks 4-8, p=0.036)].

The total amount of pollen patty consumed during the 2-month treatment phase did not vary between treatment groups (ANOVA, $F_{3,12}$=0.319, p=0.811). Hives provided with supplemental lipid did not differ in weekly pollen patty consumption from those which received no lipid supplementation (Two-way ANOVA, $F_{1,128}$=0.8423, p=0.3683). Hives provided with pesticide-treated pollen patty also did not differ in total pollen patty consumption from controls ($F_{1,128}$=0.2328, p=0.6340). We found a significant two-way interaction between pesticide treatment and week ($F_{1,128}$=4.8842, p=0.02921) on patty consumption (**Figure S1**). Post-hoc analysis revealed that hives treated with pesticide (Pesticide, High Fat Diet & Pesticide, Low Fat Diet) gradually consumed less pollen patty over time during the 2-month treatment phase ($t_{1,108}$=2.210, p=0.03).

Hive weight declined with time during the 2-month treatment phase (ANOVA, $F_{1,13}$=121.39, p<0.0001; **Figure 2A**). The average percent weight loss was roughly 11.73% between the beginning and final week of the treatment phase. Neither pesticide treatment (ANOVA, $F_{1,13}$=1.2661, p=0.2800; **Figure 2B**) nor lipid supplementation ($F_{1,13}$=0.4896, p=0.4959) had a significant effect on hive weight. The total amount of pollen patty consumed during the treatment phase was not an accurate predictor of hive weight, indicating that hives which consumed more pollen patty did not necessarily weigh more at the end of the treatment phase ($F_{1,14}$=0.889, p=0.362).

Although hive weight declined across all groups during the experimental phase, we found that lipid supplementation had a significant effect on worker bee population size ($F_{1,14}$=7.015, p=0.01193; **Figure 2C**). Hives provided with the high fat diet had more frame coverage of worker

bees than those that did not received supplementation (t_{36}= -2.649, p=0.0119; **Figure 2D**), suggesting that high fat diet may increase the worker bee population.

Nurse Bee Abdominal Lipid & Brood Area

Pesticide treatment (ANOVA, $F_{1,470}$=11.39, p=0.0008017), lipid supplementation ($F_{1,469}$=6.899, p=0.008912), and week ($F_{1,467}$=44.004, p<0.0001) all had a significant effect on brood nest bee abdominal lipid concentration (log ug lipid/mg bee tissue), as well as an interaction between pesticide treatment and week ($F_{1,464}$=9.278, p=0.0001), lipid supplementation and week ($F_{1,462}$=29.37, p<0.0001), as well as a three-way interaction between pesticide treatment, lipid supplementation and week ($F_{1,460}$=23.07, p<0.0001) on brood nest bee abdominal lipids.

Pairwise contrasts showed that brood nest bees from Pesticide, High Fat Diet hives were significantly fatter than those from Control, High Fat Diet hives after 4 weeks of treatment, although no such difference was found between Control, Low Fat Diet and Pesticide, Low Fat Diet (Control, High Fat Diet < Pesticide, High Fat Diet: t_{460}=5.197, p<0.0001; Control, Low Fat Diet = Pesticide, Low Fat Diet: t_{460}=1.834, p=0.2588, **Figure 3A**). After 8 weeks of treatment, there was no significant difference in nurse bee fattiness between Control, High Fat Diet and Control, Low Fat Diet hives (Control, High Fat Diet = Control, Low Fat Diet: t_{460}=0.064, p=0.9999). However, there was a significant difference in nurse bee fattiness between Pesticide, High Fat Diet and Pesticide, Low Fat Diet, with Pesticide, High Fat Diet hives having significantly fatter nurses (Pesticide, High Fat Diet > Pesticide, Low Fat Diet; t_{460}=-4.717, p<0.0001).

Forager Pollen Intake

Pollen samples below the range of detection were removed from analyses (6 total: 1 in Control, High Fat Diet; 4 in Control, Low Fat Diet; 1 in Pesticide, High Fat Diet; 0 in Pesticide,

Low Fat Diet). Pesticide exposure ($F_{1,226}$=0.4213, p=0.5170), lipid supplementation ($F_{1,225}$=0.7971, p=0.3729), and weeks of treatment ($F_{1,223}$=2.7214, p=0.06804) had no effect on the amount of pollen (log mg) brought into the hive by returning foragers.

Lipid supplementation ($F_{1,225}$=15.3577, p=0.0001) and weeks of treatment ($F_{1,223}$=70.99, p<0.0001) both had a significant effect on the total amount of lipid (ug) in forager corbicular pollen. Pesticide treatment alone had no such effect ($F_{1,226}$=2.813, p=0.09495) on the total amount of lipids (ug). However, we found evidence for a strong significant interaction between pesticide treatment and weeks of treatment ($F_{1,220}$=4.939, p=0.007993). At the beginning of treatment (week=0), foragers from hives pesticide-treated hives (Pesticide, High Fat Diet + Pesticide, Low Fat Diet) collected pollen with greater total lipids compared to their matching controls that received the same diet (Control, High Fat Diet and Pesticide, Low Fat Diet, respectively) (t_{216}=2.588, p=0.0103). After 4 and 8 weeks of treatment, foragers from hives treated with pesticide did not differ from controls with respect to their total amount of collected lipid per forager (4 weeks: t_{216}=-1.906, p=0.06l; 8 weeks: t_{216}=1.651, p=0.1001; **Figure 4A**). These results suggest a possible, immediate response to the pesticide.

Corbicular pollen lipid concentration (ug lipid/mg pollen) decreased with time (weeks) across all hives ($F_{1,223}$=28.0685, p<0.0001) and was not influenced by pesticide treatment ($F_{1,226}$=0.0034, p=0.9537) or lipid supplementation ($F_{1,225}$=0.5421, p=0.4624). We found an interaction term between pesticide treatment and weeks of treatment ($F_{1,220}$=4.293, p=0.01485). After 4 weeks of treatment, foragers from hives treated with pesticide collected corbicular pollen that was less concentrated with fatty acids than that collected from control hives (4 weeks: t_{216}=2.193, p=0.0294; **Figure 4**). We saw no such effect at the end of treatment (8 weeks; t_{216}=-1.952, p=0.0522).

Hive Demography

We performed hive evaluations at the beginning (week=0), middle (week=4), and end (week=8) of the treatment phase by recording the percent frame surface area of brood, pollen, honey, and empty space for each of the 5 frames per hive. We also recorded visual estimates for the percent of each frame covered by adult worker bees to roughly determine their population, as the postal scale from our hive weight data were unlikely to detect subtle changes in worker demography. The same observer made these estimates for each of the three hive evaluations to avoid between observer discrepancies.

Empty space (frame surface area not filled with brood, pollen, or honey) was unaffected by pesticide treatment ($F_{1,46}$=3.127, p=0.0855) and lipid supplementation ($F_{1,45}$=0.1754, p=0.6779), but declined with time ($F_{1,43}$=7.1460, p=0.002435; **Figure 5B**).

Pesticide treatment ($F_{1,46}$=0.2691, p=0.6071), lipid supplementation ($F_{1,45}$=0.3874, p=0.5376), and time (weeks of treatment; $F_{1,45}$=1.109, p=0.5376) had no effect on brood frame coverage (**Figure 5A**). We found an interaction between pesticide treatment and lipid supplementation ($F_{2,42}$=6.460, p=0.01548). Post-hoc contrasts revealed that, in the absence of lipid supplementation, hives treated with pesticide had greater brood coverage than controls (brood area: Pesticide, Low Fat Diet > Control, Low Fat Diet; t_{36}= -2.164, p=0.0372). There were no significant differences in brood coverage in groups that were provided lipid supplementation (Pesticide, High Fat Diet = Control, High Fat Diet t_{36}=1.430, p=0.1612).

Food storage parameters were largely unaffected by treatment. Neither pesticide treatment ($F_{1,46}$=2.076, p=0.1583) nor lipid supplementation ($F_{1,45}$=1.589, p=0.2156) had an effect on pollen storage (percent of total hive surface area containing pollen). However, pollen storage increased in response to greater pollen intake from foragers (mg corbicular pollen/forager; ($F_{1,46}$=8.444,

p=0.0005821;). Pollen storage decreased as the treatment phase progressed, except for one replicate (weeks 0-8; $F_{1,44}$=21.73, p<0.0001; **Figure 5B**).

Like pollen, honey storage was unaffected by pesticide treatment ($F_{1,46}$=3.635, p=0.06457) and lipid supplementation ($F_{1,45}$=0.0449, p=0.8334). We did, however, find an interaction between pesticide treatment and lipid supplementation ($F_{1,42}$=9.7644, p=0.003507). Post-hoc contrasts revealed that, for hives provided with lipid supplementation, pesticide-treated hives dedicated significantly more space for honey storage compared to controls (honey: Pesticide, High Fat Diet > Control, High Fat Diet; t_{36}=-3.558, p=0.0011; **Figure 5C**). There were no significant differences in honey storage surface area between hives without lipid supplementation (honey: Pesticide, Low Fat Diet = Control, Low Fat Diet; t_{36}=0.861, p=0.3947). Honey storage (percent of total hive surface area containing honey) was also not influenced by pollen intake (mg corbicular pollen/forager; $F_{1,46}$=0.0991, p=0.7545), but also declined significantly between the beginning and end of the treatment phase (weeks 0-8; t_{42}=3.007; p=0.0121).

Queen Status

During our hive evaluations at the beginning, middle, and end of treatment we also recorded whether each hive still contained a labeled and egg-laying queen. Hives that were deemed queen less were immediately requeened following evaluations. Of the 48 total hive evaluations conducted over the course of the treatment phase, there were only 6 instances where hives exhibited queen failure (**Figure 5**). Pesticide-treated hives (Pesticide, High Fat Diet + Pesticide, Low Fat Diet) exhibited a combined total of 5 queen failure events, whereas control hives (Control, Low Fat Diet) had only 1 queen failure event (**Table 2**). Control, High Fat Diet hives had no queen failure events.

Discussion

In this study, we found that high fat diet was effective in reducing pesticide-induced weight loss. We also found that high fat diet directly confers benefits to the bees consuming it, as brood nest bees consuming the supplemented diet were fatter than those unsupplemented. Finally, we found that foragers from pesticide-treated hives collected fattier pollen if not provided with high fat diet, suggesting that pesticide stress combined with a comparatively low amount of supplemental lipid causes foragers to compensate with fattier pollen. This is similar to what we had seen in Appendix B, but this time at the hive-level.

The functional significance of lipid nutrition in the insect diet has been historically underrepresented in literature when compared to that of protein and carbohydrate nutrition. Our results indicate that high fat supplemental diets demonstrate some potential in improving the overall health and longevity of commercial honey bee hives, especially hives already exposed to other stressors such as pesticides.

The first challenge in creating an effective supplemental lipid diet is ensuring it is both palatable and readily consumed by the workers. Lipid supplementation had no effect on consumption, suggesting that consumption of lipid supplemented diets did not differ from unsupplemented diet. Similar studies found that targeted protein supplements and unsupplemented polyfloral pollen were also equivalent in palatability (DeGrandi-Hoffman et al., 2008). The influx in pollen consumption between the beginning and middle of the treatment phase was likely an effect of increased brood rearing characteristic for mid-summer hives, although brood production patterns were largely inconsistent and not accurately predicted by consumption patterns.

Lipid supplementation could help maintain a stronger worker bee demographic but is rendered less effective when hives are exposed to additional stressors, such as pesticide treatment. The older worker bee demographic, the foragers, is less likely to benefit from improved lipid

supplementation. If high fat diet were to confer a benefit to the foraging workforce, the earliest we could expect to see any changes due to dietary supplementation would be at least one month after treatment began, since the nurses that consumed the patty at the beginning of the treatment phase would require some time before they reached foraging age, especially if their internal lipid status is greater and prolongs the onset of foraging. Pollen storage declined over time across all treatment groups. With the amount of pollen as a proxy for pollination services, we can infer that providing bees with supplemental lipids did not lead to a reduction in foraging over time. Pesticide treatment also had no effect on the amount of pollen brought back by foragers. Lipid supplementation did, however, influence the lipid content of pollen. Reducing a symptom of pesticide exposure by bolstering bee lipid content in nurse-aged bees may not be a permanent fix for an alteration in behavior due to pesticide exposure but can at least provide some benefit the bees that immediately consume it. There is some literature to suggest that pesticide exposure decreases pollen collection, but the mechanism that causes this shift is unknown (Barraud et al., 2020).The decline in corbicular pollen lipid concentration seen in all treatment groups is likely more of a seasonal effect. With the seasonal transition from summer to fall, floral bloom decreases and bees are left to forage for whatever is still available.

Hive weight loss is expected during the summer-to-autumn seasonal transition, as the worker bee demographic gradually decreases in preparation for winter (DeGrandi-Hoffman et al., 2018). While providing pesticide-treated hives with lipid supplementation did not influence the worker bee population, we saw an effect of supplementation on their final percent weight loss in hives exposed to pesticide. This is very encouraging, as the accumulation of resources into the fall season is necessary for winter survival (overwintering), when forage is no longer available (Anderson et al., 2014; DeGrandi-Hoffman et al., 2016).

Another challenge in creating an effective supplemental diet is designing one that confers some benefit to the young worker bees that directly consume the pollen patty. Pesticide treatment, lipid supplementation and time all demonstrated a significant effect on the abdominal lipid stores of nurse bees. Fat body mass increases from fall to winter and the longevity of winter bees is directly attributed to these changes in body composition (Brejcha et al., 2023).

Further investigation is required to understand the effects of supplemental diet and pesticide treatment on queen fecundity. One study found insect growth regulators (IGRs) to negatively affect honey bee larval survival rates, without impacting egg production rates, suggesting queens were largely unaffected by pesticide exposure (Fine, 2020). Another study found neonicotinoids to decrease egg-laying and locomotor activity in queens, but colony population size also increased (Wu-Smart & Spivak, 2016). Larger colony populations may act as a buffer to pesticide exposure, so we collected weight and population data to observe the effect of lipid supplementation on hive size.

Lipid supplementation offers great promise in the management of commercial hives frequently exposed to agricultural pesticides. Further measures should be taken to increase the palpability of diet to increase consumption and, perhaps, increase the efficacy of targeted lipid supplementation. Replicating this study using other ω-6:3 balanced oils is also of value. The addition of phagostimulatory elements found in natural pollen could facilitate higher consumption of supplemental diet. This includes, but is not limited to, microbes, secondary compounds, odors, or enzymes that have evolved in plants to increase resource value to pollinators. Research on phagostimulatory properties is limited and requires further research.

The promise of lipid supplementation should not, however, overshadow the importance of increasing the availability of natural forage. Efforts to improve natural forage availability should also consider the behavior of native pollinators and how they differ from honey bee foragers. Whereas honey bees prefer high P:L ratios, bumble bees and other native pollinators show preference for

high P:L ratios (Vaudo et al., 2020). Planting forage that considers dietary preferences of different pollinators can help minimize competition and disease crossover between *Apis* and native pollinators (Purkiss & Lach, 2019; Thomson, 2004). Improved lipid supplementation offers a temporary, yet effective, bee management strategy until further action can be taken to improve and maintain floral diversity patterns amidst global climate change.

Tables and Figures

For Table 1, see **Materials and Methods (above)**

Group	Total Queen Failure/Unmarked Queen Events
Pesticide, High Fat Diet	4
Control, High Fat Diet	0
Pesticide, Low Fat Diet	1
Control, Low Fat Diet	1

Table 2: Queen Failure Events

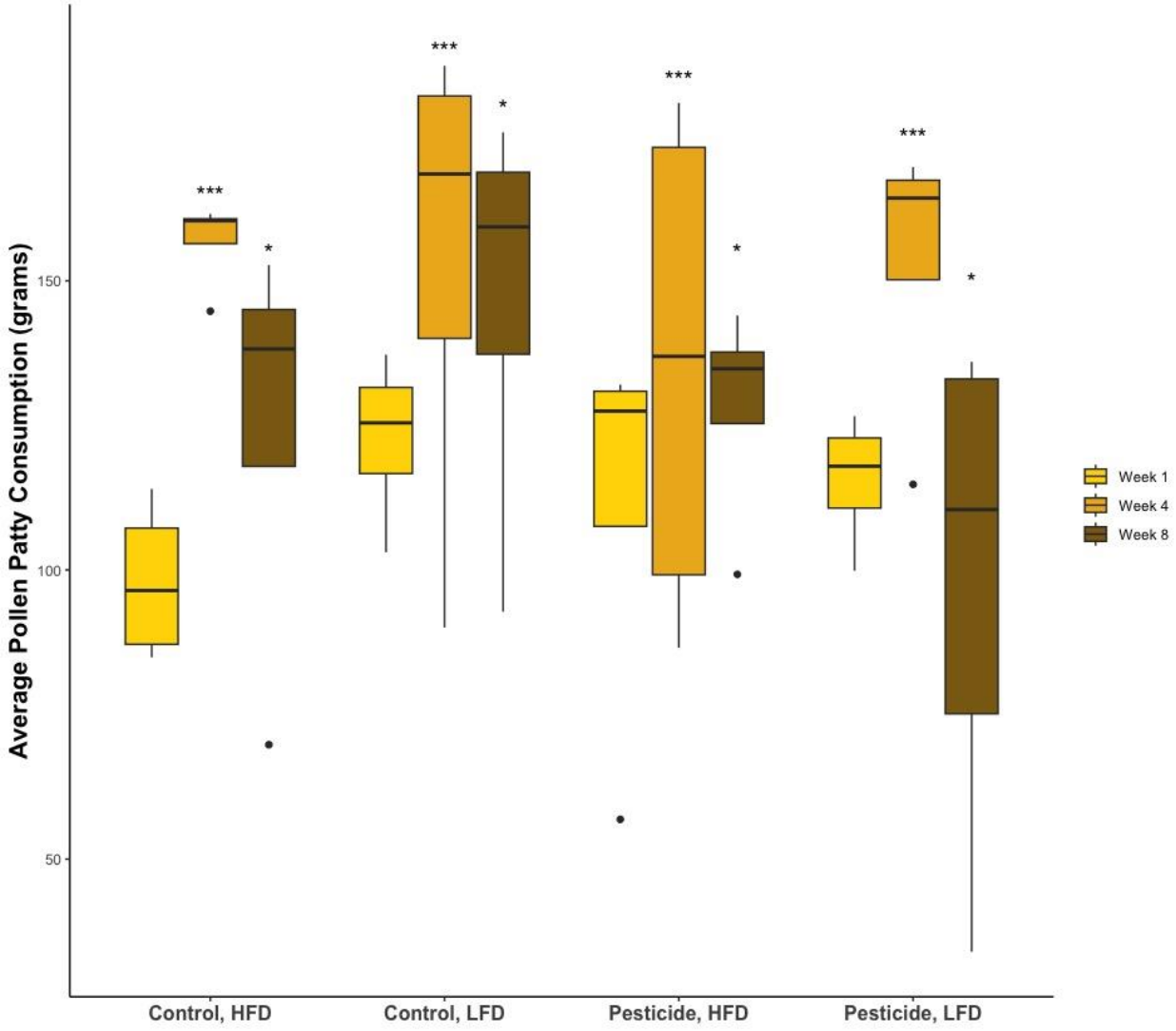

Figure 1: Average weekly pollen patty consumption
Pollen patty consumption for each treatment group (Control, High Fat Diet, Control, Low Fat Diet, Pesticide, High Fat Diet and Pesticide, Low Fat Diet) for weeks 1, 4 and 8 (beginning, middle and end of the treatment phase, respectively). Significant differences determined by nonparametric repeated measures ANOVA (Friedman test). Asterisks denote a significant difference in average pollen patty consumption compared to the previous period (for example, an asterisk over a "week 4" box indicates a significant change in consumption between week 4 and week 1) ($p \leq 0.0001$ = ***, $p < 0.05$= *, No asterisk=not significant ($p > 0.05$)).

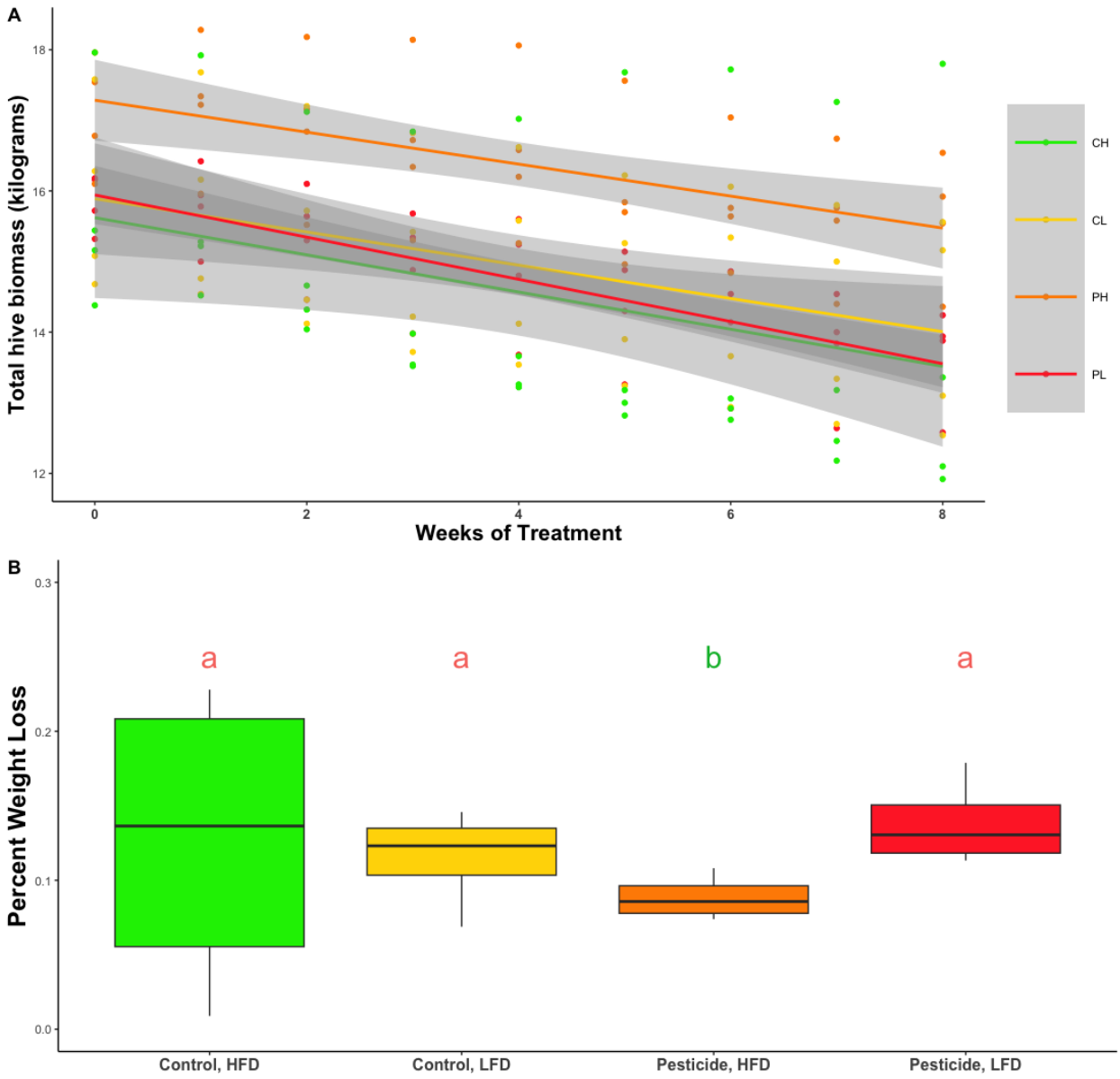

Figure 2: Colony size over course of treatment
All 16 hives were weighed immediately before sunrise (~5AM, weekly) when all foragers were in the hive. **A)** Total hive biomass (kg) over the course of treatment. **B)** Average percent weight loss over treatment period. Boxplots show median (horizontal line within box) abdominal lipid concentration and interquartile range (lower and upper box boundaries) as well as the minimum and maximum values of the distribution. Points indicate a single observation. Outliers are points outside the maximum and minimum of the distribution. Matching letters and colors between boxplots indicate no significant differences between two groups as determined by TukeyHSD multiple comparisons.

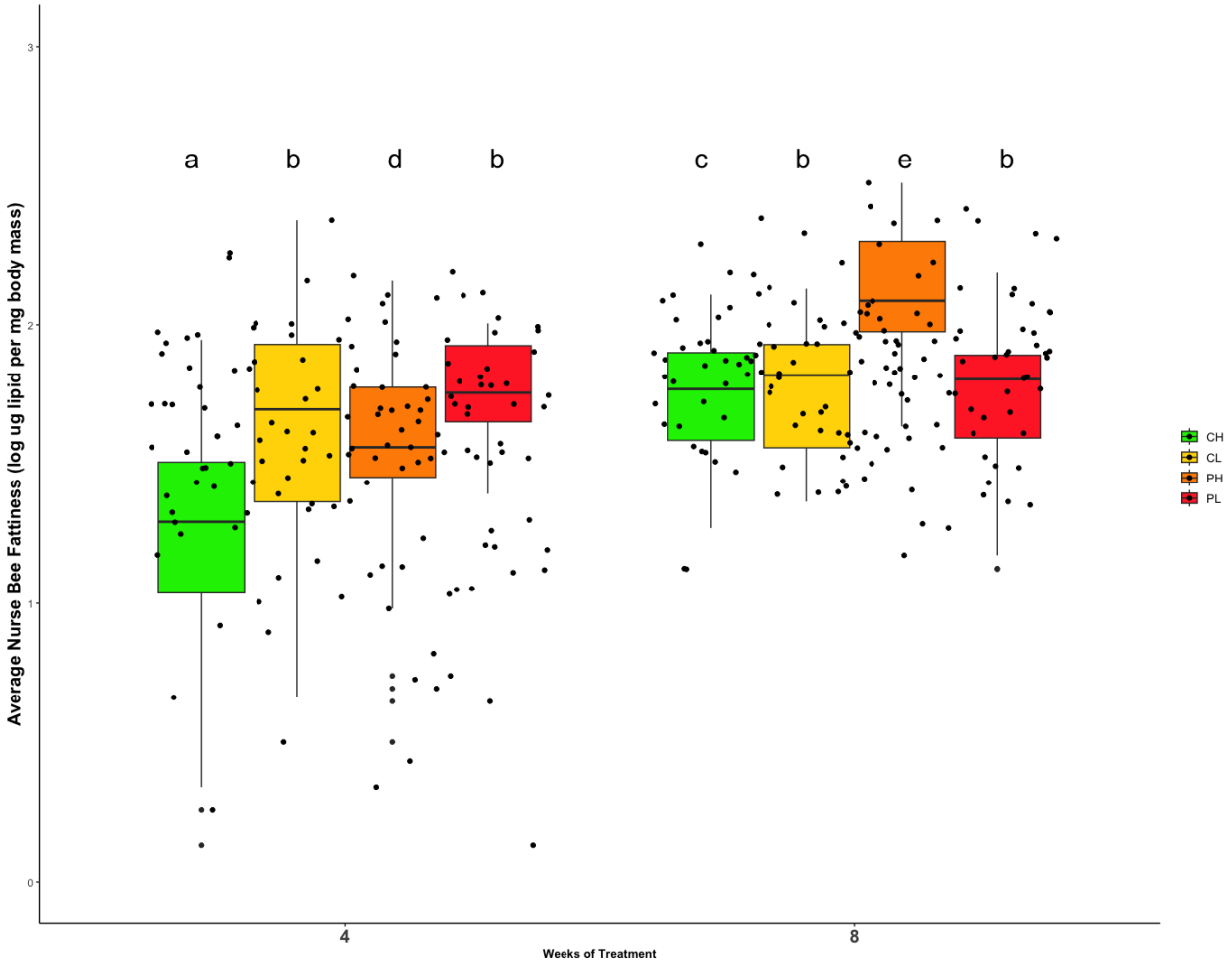

Figure 3: Nurse bee abdominal lipid concentration by diet
Nurse bees were sampled from each hive at 4and 8 weeks into the treatment phase (n=10 per hive * 4 replicate = 40 per each boxplot). Boxplots show median (horizontal line within box) abdominal lipid concentration and interquartile range (lower and upper box boundaries) as well as the minimum and maximum values of the distribution. Points indicate a single observation. Outliers are points outside the maximum and minimum of the distribution. Matching letters and colors between boxplots indicate no significant differences between two groups as determined by TukeyHSD multiple comparisons.

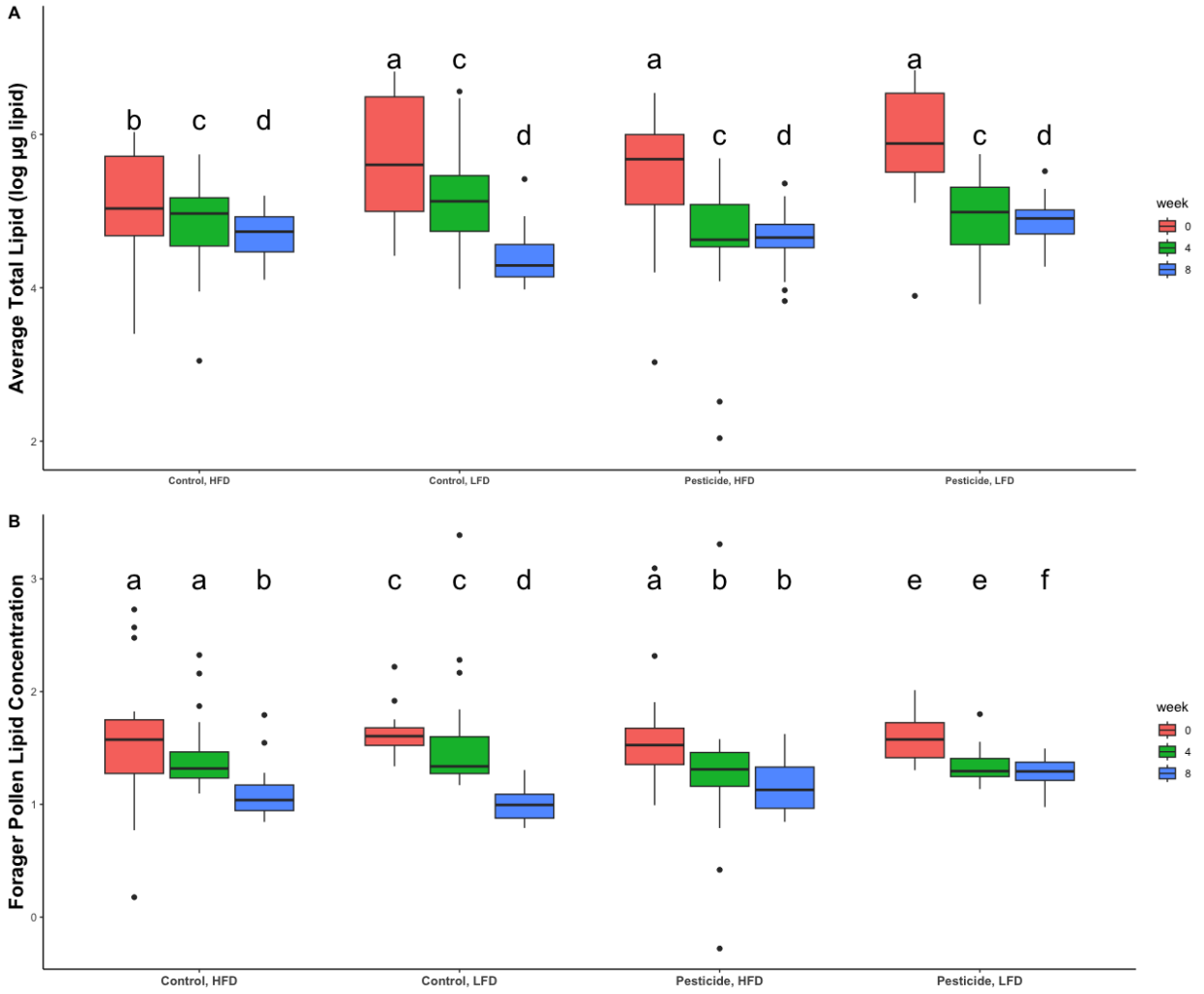

Figure 4: Forager lipid yield
Foragers (n=5 per hive * 4 per treatment = 20 per boxplot) were collected outside of hive entrances between sunrise and noon on weeks 0, 4, and 8 after weighing the hive. Corbicular pollen was removed by microscopy and assayed using the vanillin-phosphoric acid assay to measure **A)** total lipids (µg lipids) **B)** lipid concentration (µg lipids /mg pollen). Boxplots show median (horizontal line within box) abdominal lipid concentration and interquartile range (lower and upper box boundaries) as well as the minimum and maximum values of the distribution. Points indicate a single observation. Outliers are points outside the maximum and minimum of the distribution. Matching letters and colors between boxplots indicate no significant differences between two groups as determined by TukeyHSD multiple comparisons.

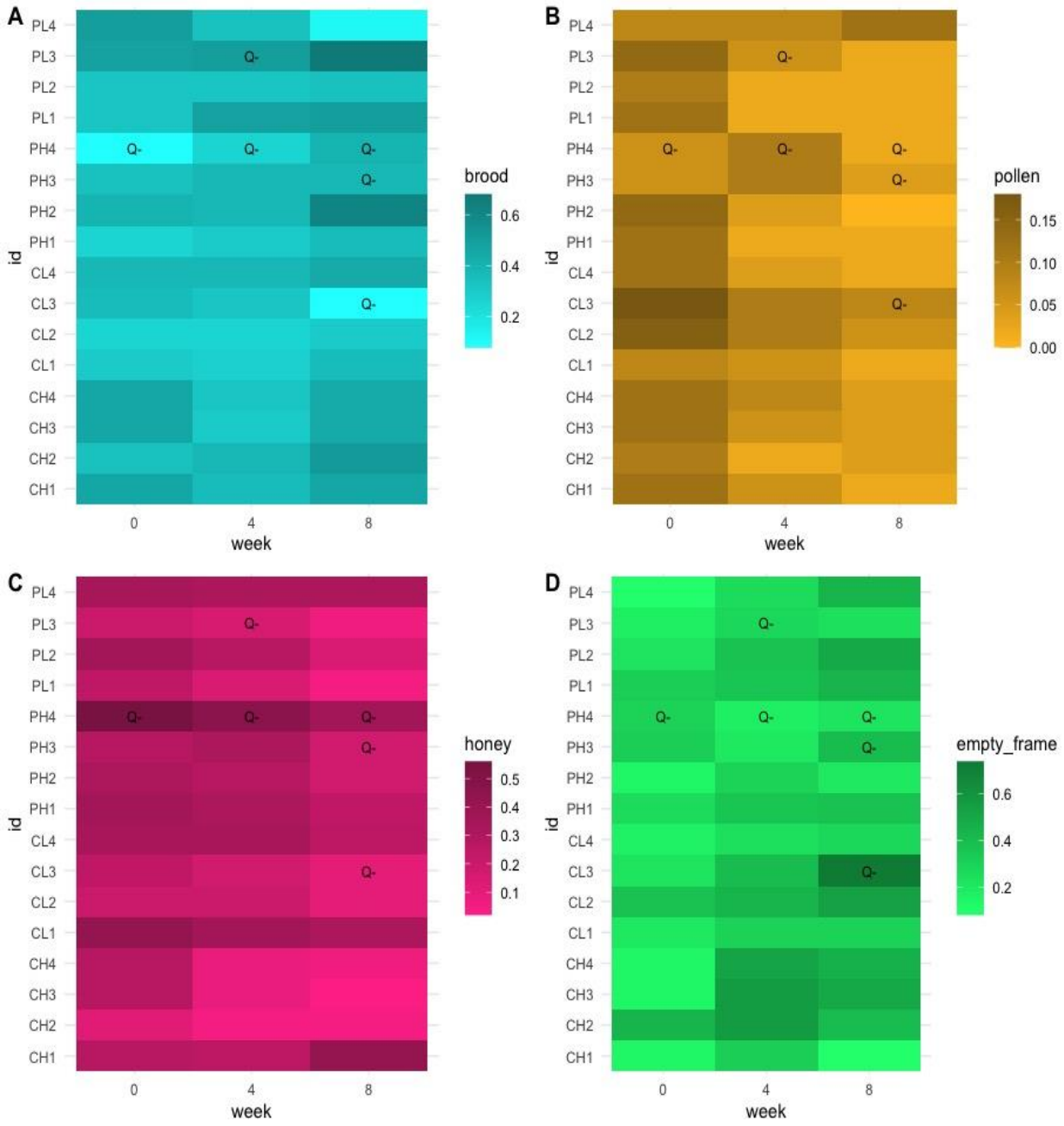

Figure 5: Hive Demography

Heatmaps indicating the percentage of frame coverage with: **A)** brood **B)** pollen **C)** honey or **D)** empty frame for each hive at weeks 0, 4, and 8. id: PH= Pesticide High Fat Diet, PL= Pesticide Low Fat Diet, CH= Control High Fat Diet, and CL= Control, Low Fat Diet. Number by id indicates replicate number. (Q-) indicates a week where a queen failure event occurred.

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