ABSTRACT

Title of Thesis:	POLLEN NUTRITION, PESTICIDES, AND PATHOGENS: INTERACTIVE EFFECTS ON HONEY BEE HEALTH
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Thesis Directed By:	Associate Professor, Dennis vanEngelsdorp, Department of Entomology

While a variety of stressors influence honey bee (*Apis mellifera*) health, it is the additive and interactive effects of these factors on bee health that have been driving modern research. We devised a set of two experiments to test the effects of multiple stressors on honey bee health. First, we grew sunflowers to test the effects of drought stress and seed treatment on sunflower pollen. We fed the pollen collected from these sunflowers to cohorts of bees that were either infected or uninfected with the microsporidian pathogen *Nosema ceranae* to find that drought stressed pollen leads to increased mortality in infected bees. Next, we fed 37 experimental pollen diets of different floral varieties and pesticide loads to honey bees infected with *N. ceranae*, but we were unable to find a connection between diet variety and pesticide exposure on bee health.

POLLEN NUTRITION, PESTICIDES, AND PATHOGENS: INTERACTIVE EFFECTS ON HONEY BEE HEALTH

by

Andrew Garavito

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2017

Advisory Committee:
Associate Professor Dennis vanEngelsdorp, Chair
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Associate Professor David Hawthorne

Dedication

I would like to dedicate this work to my family. Specifically, I would like to thank my Mom and Dad. Thank you for always supporting my interest in biology: from the Magic School Bus, fossil collecting trips, and terrifying books about germs, to your encouragement over the past few months. I could not have gotten here without all your help. I must also thank all the scientists in my family that came before me, for inspiring me.

Acknowledgements

There are many people I need to thank. First, I must thank Dr. Dennis vanEngelsdorp for being a great PI and employer. I am excited to see what the next few years bring for the UMD Apiary! Next, I need to thank Dr. Galen Dively for introducing me to my career in Entomology and more specifically, honey bees and beekeeping. Looking back, I am a bit surprised that I managed to become so fascinated with honey bees because you had some of the meanest bees I have ever worked with. I would also like to thank Dr. David Hawthorne for my first undergraduate research opportunity to learn about the lab side of honey bee work (thanks Grace Kunkel for letting me help with your research!) and for helping me with all of the little things leading up to my Defense.

I need to thank everyone who helped me collect data for this project: Todd Waters, Mike Gladchuck, Rachel Fahey, Sid Ganesam, Katie Reding, Nathalie Steinhauer, Meghan McConnell, Stephanie Bosch, Shayne Madella, Tyler Connine, Heather Eversole, Ashley Jones, Sue Boo, Austen Bendik, Anthony Nearman, Ian Lacey, Lexi Dolegui, Byron Mariani, & Bayley Perry (hopefully I did not miss anyone). Thank you for helping plant sunflowers, collect pollen, measure plants, inoculate honey bees, set up cage studies, sort pollen, and count spores. Three of you deserve special thanks: Bayley, it was great having your help during that long summer of cage studies! Todd and Mike, between the three of us we counted 669,374 *Nosema* spores for my second chapter. You guys are amazing, and good at things other than counting spores.

Last, but not least, I need to thank the bees.

Table of Contents

List of Tables

List of Figures

Chapter 1: Assessing the Impact of Seed Treatment and Drought Stress on Sunflower Pollen, and the Resulting Effects on Honey Bee Health.

Abstract

Honey bee colonies are often placed in sunflower fields for pollination and honey production. In the summer of 2012, beekeepers in North Dakota reported dramatic losses of honey bee colonies in sunflower fields. Commercially grown sunflowers are often seed treated with a variety of systemic pesticides. Beekeepers believed that the drought that year caused more pesticides from these seed treatments to accumulate in the nectar and pollen that bees were collecting. We grew untreated and CruiserMaxx® treated sunflower seeds of the same hybrid under three different watering regimens to simulate well-watered, moderate drought, and severe drought conditions. The thiamethoxam applied to treated seeds was detected in 10.7, 25.3, and 33.9 ppb concentrations in the pollen collected from well-watered, moderately, and severely drought stressed plants respectively. This shows that drought leads to increasing levels of seed treatment pesticides in pollen. Upon feeding the pollen collected from severely drought stressed sunflowers grown from untreated or treated seeds to adult honey bees, we found that bees infected with *Nosema ceranae* died faster than uninfected bees. This indicates that the effect of drought stress on pollen serves as a stressor that can negatively affect honey bees.

Introduction

Pesticides are one of top three most frequently self-reported causes of honey bee colony death in the United States (Lee et al., 2015; Steinhauer et al., 2014). There is growing concern among beekeepers about one particular class of insecticides, the neonicotinoids, which target the nervous system of insects. While all neonicotinoids are highly toxic to honey bees, some – like imidacloprid (LD_{50} = 0.0179 μg/bee), clothianidin (LD_{50} = 0.0218), and thiamethoxam (LD_{50} = 0.0299) –are more toxic than others (e.g. acetamiprid (LD_{50} = 7.07) and thiacloprid (LD_{50} = 14.6)) (Iwasa et al., 2004). Neonicotinoids are neurotoxins that mimic acetylcholine and bind to the nicotinic acetylcholine receptors in honey bee brains, leading to tremors, erratic movements, and hyperactivity in exposed honey bees (Blacquiere et al., 2012; Johnson, 2015). Individual worker bees exposed to sub-lethal doses of neonicotinoids suffer from impaired learning, memory loss, decreased responsiveness to sucrose, and are less likely to forage (Aliouane et al., 2009; Bortolotti et al., 2003; Eiri and Nieh, 2012). Further, sub-lethal doses of neonicotinoids negatively impact the functions of honey bee immune systems (Brandt et al., 2016), which helps explain why worker bees that had sub-lethal exposures to neonicotinoids also had increased susceptibility to pathogens such as *Nosema* sp. and Deformed Wing Virus (Di Prisco et al., 2013; Pettis et al., 2012). Queens heading colonies that are exposed to sub-lethal levels of neonicotinoids laid fewer eggs, had decreased motor activity, and had lower proportions of viable sperm stored in their spermathecas when compared to control queens (Williams et al., 2015; Wu-Smart and Spivak, 2016).

Neonicotinoids are commonly applied as seed treatments directly onto the seed coats of a variety of crops (Elbert et al., 2008). As the seed germinates and the plant grows, the neonicotinoids are systemically taken up by the plant and expressed in a variety of tissues, including the pollen and nectar collected by honey bees (Krupke et al., 2012; Pilling et al., 2013; Sanchez-Hernandez et al., 2016). While bees foraging on crops that were grown from treated seed do collect pollen and nectar that have detectable levels of seed treatment neonicotinoids, these residues have no discernable effect on colony health (Cutler and Scott-Dupree, 2007; Cutler et al., 2014; Pilling et al., 2013; Pohorecka et al., 2012). However, commercial beekeepers operating colonies in North Dakota during 2012 were particularly adamant that proximity to fields of seed treated sunflowers caused the high rates of colony losses they experienced that summer and fall. The summer of 2012 was particularly dry and beekeepers theorized that the drought was causing seed treatment products to amass in toxic levels in the pollen and nectar of the blooming sunflowers. Sunflowers grown under drought conditions have significantly decreased biomass, height, diameter of head and stem, seed weight and quantity, number of filled seeds, oil yield, and uptake of CO_2 (Alza and FernandezMartinez, 1997; Ghani et al., 2000; Human et al., 1990; Soleimanzadeh et al., 2010). The smaller biomass of drought stressed sunflowers may potentially result in higher concentrations of systemic seed treatments expressed in the pollen and nectar of sunflowers grown from treated seeds.

Beekeepers in other countries have also expressed concern over the use of imidiacloprid, thiamethoxam, and clothianidin as sunflower seed treatments. These products (and their metabolites) are repeatedly detected in samples of pollen and

honey collected from colonies foraging on sunflower crops grown from treated seed (Laurent and Rathahao, 2003; Sanchez-Hernandez et al., 2016). Since the early 1990s, French beekeepers keeping colonies near fields of blooming sunflowers that were grown using treated seeds have reported seeing symptoms suggestive of neonicotinoid poisoning; bees foraging on sunflower blooms behaving oddly, large numbers of lost foragers, and peculiarly low honey yields (Chauzat et al., 2009; Laurent and Rathahao, 2003). However, nucleus colonies caged over sunflowers grown from either imidacloprid treated or untreated seeds did equally well, and no aberrant symptoms were observed (Schmuck et al., 2001). Work conducted to assess the effects of sunflowers themselves on bees failed to demonstrate differences in colony health matrices when comparing full colonies placed adjacent to untreated sunflower fields and colonies placed between 1.5 and 3 km away from any sunflower fields (Charriere et al., 2010; Chauzat et al., 2009). Sunflower pollen is low in protein, a protein diet of solely sunflower pollen leads to poorer ovary and hypopharyngeal gland development in workers when compared to workers fed other monofloral pollen diets (Pernal and Currie, 2000). It is conceivable that the deleterious effects beekeepers report when bees forage on sunflowers is independent of the seed treatment, and rather a result of the poor nutritional quality of the pollen itself.

Here we set out to explore the effects, if any, drought conditions have on the concentration of seed treatment products in sunflower pollen. Specifically we wished to determine if drought leads to higher levels of neonicotinoids in sunflower pollen. Additionally we wanted to assess whether or not the pollen collected from plants

grown using treated or untreated seeds grown under various watering regimens had any adverse effects on health when fed to honey bees. To investigate these questions, a set of two experiments was devised to test the interactive effects of pollen quality due to drought stress, pesticide exposure, and pathogen exposure on honey bee health. First, we grew seed treated and untreated sunflowers under three drought treatments to determine if drought had an effect on the amount of seed treatment products that could be detected in pollen. Then we fed the pollen from the sunflowers grown under drought stress to newly emerged honey bees to see if it had any effects on bee health.

Materials and Methods

Growing Sunflowers

Sunflower seeds were planted and grown in the Research Greenhouse Complex of the University of Maryland, College Park. Plants were provided with a 16 hour photoperiod and an ambient temperature of 70^{o}F, to ensure optimum growth. OSF5633-CLDM hybrid sunflower seeds were used for all experimental plantings. Seeds were either untreated (untreated seeds) or treated with CruiserMaxx® (treated seeds); a seed treatment made up of the neonicotinoid thiamethoxam and the fungicides azoxystrobin, fludioxonil, and mefenoxam. Syngenta (Greensboro, NC) provided both the untreated and treated seeds needed for this project, as untreated seed is not commercially available.

Guided by two preliminary growing trials conducted in 2013, a final sunflower growth trial was conducted in the summer of 2014 using 360 plants grown from 180 treated seeds and 180 untreated seeds. Seeds were planted in 6.8 gallon pots

to give the sunflowers sufficient room to grow. Pots were filled with Turface, an artificial substrate, chosen to ensure a homogeneous growth medium with regards to nutrient content. Natural substrates, such as soil and peat, can have varying levels of nitrogen, phosphorus, and potassium. Due to its absorptive nature, Turface was soaked in a 1:100 Hoagland's fertilizer solution for 24-48 hours prior to potting. Pots were placed on three 3" tall PVC rings and kept in saucers to permit the detection of any leachate and to prevent leachate from being reabsorbed by the substrate. Treated and untreated plants were divided evenly amongst three different drought treatments: well-watered, moderate drought, and severe drought (Table 1). Each drought treatment was regulated by an individual hose, split into five feeder lines which were arranged in an alternating fashion across the greenhouse to control for any gradient of abiotic factors that was present (Figure 1). Feeder lines were equipped with one water emitter per pot and watering nodes were positioned a few inches from the seed. Pots were organized in an alternating fashion, by seed type, down each side of a feeder line (Figure 1).

For the first week after planting, seeds received a 10 second pulse of water (ca. 61 mL) two to three times per day. By the second week after planting, seeds had germinated and watering was reduced to a five second pulse between two and three times per day. Plants were fertilized once a week with 100 mL of 1:100 Hoagland's solution between the third and seventh week post planting. Foliar applications of the insecticidal soap M-Pede (2% rate), were conducted on the 29th (week 4) and 48th days (week 6) after planting, in order to control for plant stressing levels of thrips, whiteflies, and spider mites. Experimental drought treatments were enacted 30 days

after planting (week 4), when plants were robust and established. The irrigation of each drought treatment was controlled using an nR5 node (Decagon Devices: 2365 NE Hopkins Court Pullman, WA 99163) that monitored 10HS soil moisture sensors (Decagon Devices) that were placed in between three and five individual pots of plants in each drought treatment. Drought treatments were regulated by using the 10HS soil moisture sensors (Decagon Devices) to maintain the average volumetric water content at 31%, 29%, and 27% for the well-watered, moderate drought, and severe drought treatment substrates respectively. Plants began blooming 54 days (week 7) after planting.

Sunflower heads that appeared close to bloom (Figure 2) were bagged using brown paper bags and binder clips (Figure 3), in order maximize pollen collection. Pollen was collected by carefully removing the paper bag while holding a piece of clean 8”x11” printer paper under the bloom and catching any pollen grains that had accumulated in the bag. Once the bag was removed, the sunflower head was positioned face down over the paper and was tapped to dislodge any mature pollen (Figure 4). Because sunflower florets bloom over time, this process was repeated every 2-4 days between days 54 and 69 (weeks 7 to 10) for each plant, in order to collect as much pollen as possible. Pollen collected from all flowers within a treatment was aggregated and stored in 50 ml Falcon tubes (Corning, Inc.: One Riverfront Plaza, Corning, NY, 14831) that were wrapped in tin foil and kept frozen at ca. -20^{o}C. The individual height and head diameter for each plant was measured on the last day of pollen collection, 69 days after planting (week 10). One gram subsamples of pollen from each treatment group were sent to the USDA-AMS NSL

in Gastonia, NC for pesticide analysis using methods described by (Mullin et al., 2010).

Cage Studies

Frames of sealed brood were collected from four different honey bee colonies located at the Central Maryland Research and Education Center (CMREC)- Beltsville Facility. Brood frames were selected by opening a few capped cells to inspect for pupae with dark eyes, an indicator that emergence will occur in 1-3 days (Williams et al., 2013). Frames were placed in wire mesh cages, and stored overnight in an incubator at 34.5^{o}C, 70% relative humidity; the optimum conditions for brood development (Williams et al., 2013). Newly emerged adults were collected off of brood frames after approximately 24 hours and placed into colony specific hoarding cages. Bees from each hoarding cage were removed, fed, and placed into one of sixteen 16 oz. Solo cup cages (after Evans et al., 2009) until each cage had 20 bees, five from each of the four source colonies. Cages were randomly assigned to one of two inoculum feeding treatment groups – control or *Nosema* – those bees assigned to control cages were fed 10 uL of 50% (w/v) sucrose solution, while those bees assigned to *Nosema* cages were fed 10 uL of 50% (w/v) sucrose solution containing ca. 10,000 spores of *Nosema ceranae*.

Nosema inoculums were developed following the procedures by Fries et al., 2013. Presence of *N. ceranae* was confirmed for all honey bees used to produce *Nosema* inoculums using qPCR methods (Forsgren and Fries, 2010). *Nosema* solutions were prepared by homogenizing 10-20 infected bees from several source colonies. The *Nosema* load in millions of spores per mL for each raw solution was

then quantified using haemocytometer counts (Human et al., 2013). Raw *Nosema* solutions were diluted with 50% (w/v) sucrose solution into experimental *Nosema* inoculums with a concentration of one million spores per mL (10,000 spores per 10 uL). Lines of infected bees were propagated and maintained in the lab by providing excess *Nosema* solution to newly emerged bees.

Each Control and *Nosema* treatment cage was randomly assigned to one of eight experimental diets: one of six experimental sunflower pollen diets, a protein control diet (Megabee), or a protein-free control diet (Table 2). Sunflower pollen diets were created by preparing a paste in a 1008 Falcon petri-dish (Corning, Inc: One Riverfront Plaza, Corning, NY, 14831) made of 0.38 mL of 50% (w/v) sucrose solution and 0.25g of pollen from collected from one of the six sunflower treatment groups. Approximately 0.63 g of Megabee, prepared according to label instructions, was placed in a 1008 Falcon petri-dish to serve as the protein control diet; while an empty petri dish served as the protein-free diet. Diets were placed in the bottom of each cage, and were replaced with an identical formulation on the fourth day of the experiment. Each cage was provided with access to clean 50% (w/v) sucrose solution *ad libitum*.

Cages were maintained in an incubator at 30^{o}C and 70% relative humidity (Williams et al., 2013) for 12 days, dead bees were removed daily (Pettis et al., 2013). After 12 days, all surviving bees were killed and stored in 90% ethanol for *Nosema* spore quantification using haemocytometer counts (Human et al., 2013). This cage study experiment was replicated three times.

Statistical Analysis

Sunflower plant growth measures (height and diameter of bloom head) were compared between treatment groups using the Proc Mixed procedure in SAS 9.4 to conduct ANOVAs followed by Tukey multiple mean comparisons after data normality was confirmed. Seed treatment and drought treatment were treated as fixed effects, while feeder line (rep) was treated as a random effect. *Nosema* load in millions of spores per bee (msb), was analyzed using the SAS 9.4 Proc Mixed procedure to conduct an ANOVA. *Nosema* load data was log transformed to ensure normality. However, we report treatment means that are back transformed to ease interpretation. Diet treatment was treated as a fixed effect, and cage rep was treated as a random effect.

A full parametric survival model was analyzed using JMP Pro 10 statistical software to test the effect that the 3-way factorial treatment structure of the 12 cage treatments using experimental sunflower pollen (three drought treatment levels x two seed treatment levels x two inoculation treatment levels) had on bee survival. Survival curves for bees in each of the 16 cage treatments (eight diets x two inoculation treatments) were plotted and analyzed using the SAS 9.4 Proc Lifetest procedure. Log rank comparisons were used to compare survival curves.

Results

Growth Metrics

Drought treatment had an effect on height ($F_{2, 23} = 78.38$; $p < 0.0001$). Well-watered plants were significantly taller than moderately ($T_{23} = 11.80$; $p < 0.0001$) and

severely drought stressed plants (T_{23} = 9.52; p < 0.0001; Figure 5). Seed treatment had no effect on height ($F_{1, 22}$ = 0.03; p = 0.8601). Drought treatment had a similar effect on head diameter ($F_{2, 23}$ = 20.75; p < 0.0001). Well-watered sunflowers had significantly larger heads than those that were moderately (T_{23} = 5.50; p < 0.0001) and severely drought stressed (T_{23} = 5.65; p < 0.0001; Figure 6). Seed treatment had no effect on sunflower head diameter ($F_{1,22}$ = 0.15; p = 0.7043). On average we collected 10.39 ± 0.62 g of pollen from each sunflower treatment group, providing enough pollen for pesticide analysis and cage feeding trials (Table 1). Well-watered plants also appeared fuller and heartier when visually compared to moderate and severely drought stressed plants.

Pesticide Analysis

Thiamethoxam loads were 10.7, 25.3, 33.9 ppb for the treated seed groups in the well-watered, moderate drought, and severe drought treatments respectively (Figure 7). Neither azoxystrobin, fludioxonil, nor mefenoxam were detected in any treated samples. There were no pesticides detected in any of untreated sunflower pollen samples.

Nosema Infection

Of the 236 surviving honey bees collected from *Nosema* cages for *Nosema* quantification, only two were uninfected. These two uninfected bees were removed from analysis. *Nosema* loads (msb) were not different between individual sunflower pollen treatment groups, nor did loads differ in bees from the protein control (Megabee) and the protein-free control (Syrup) treatments ($F_{7, 14}$ = 2.03; p = 0.1234;

Figure 8). When comparing only the bees fed experimental sunflower pollen diets, we found that the interaction between drought and seed treatment had no effect on *Nosema* load, nor did drought itself. However, bees fed on pollen coming from seed treated plants had *Nosema* loads that tended to be higher than those of bees fed on pollen from plants grown using untreated seeds ($F_{1, 14} = 4.35$; $p = 0.0557$).

Survivorship

A full parametric survival model analysis on the survival of bees in the 12 sets of cages fed sunflower pollen treatments showed that our three way factorial cage treatments (three drought treatments x two seed treatments x two inoculation treatments) had an effect on overall survival ($\chi^2_{11} = 117.0243$; $p < 0.0001$). There was a three way interaction between drought, seed treatment, and inoculation ($\chi^2_2 = 24.4990$; $p < 0.0001$).

Survival curves were plotted for each of the 16 cage treatments (two inoculums x two different diets x three different water regimes, one positive control, and one negative control) (Figure 9). Treatment had an effect on bee survivorship ($\chi^2_{15} = 151.8622$; $p < 0.0001$). We then conducted log-rank comparisons between survival curves of *Nosema* infected bees to those of uninfected bees fed each experimental sunflower pollen diet treatment. *Nosema* inoculated bees that were fed pollen originating from plants grown from treated seeds under moderate or severe drought stress died faster than uninfected bees that were fed similar diets ($\chi^2_1 = 24.7735$; $p < 0.0001$; Figure 10; and $\chi^2_1 = 18.8978$; $p < 0.0001$; Figure 11 respectively). *Nosema* infected bees fed pollen from severely drought stressed plants

grown from untreated seed also had a reduced lifespan when compared to their similarly fed but uninfected counterparts ($\chi^2_1 = 23.7363$; $p < 0.0001$; Figure 12).

When comparing the survival curves of *Nosema* infected bees fed sunflower pollen to our protein control, we found that bees fed Megabee survived longer than bees fed pollen from well-watered plants grown from untreated seeds, as well as both moderately and severely drought stressed plants, regardless of seed treatment ($\chi^2_7 = 43.9416$; $p < 0.0001$; Figure 13). Bees fed our protein-free control diet of solely sugar syrup survived longer than bees fed pollen from both moderately drought stressed untreated and treated plants, and pollen from severely drought stressed plants grown using treated seeds ($\chi^2_7 = 43.9416$; $p < 0.0001$; Figure 13).

Discussion

As expected, sunflowers grown under drought stress were shorter (Figure 5) and had smaller heads (Figure 6) than well-watered plants. This finding is consistent with past work on drought stress (Human et al., 1990) and helps assure us that the pollen derived from the moderately and severely drought stressed treatment groups was indeed representative of pollen that would be produced by plants grown under drought conditions. Thiamethoxam, the neonicotinoid in the seed treatment applied to sunflower seeds, was found at higher concentrations in pollen as drought stress on the plants increased (Figure 7). While clearly suggestive, this result should be interpreted with caution as it represents the results from one growth trial. Regrettably, attempts to repeat this experiment and thus collect pollen from additional sunflower growth trials failed because of constraints on the flowers and technical issues with the automatic watering system. Limited resource prevented further attempts.

Our pre-trials in the summer and fall of 2013 did not yield enough pollen for both cage studies and pesticide analysis. In the summer of 2013 an average of 0.42 ± 0.05 grams of pollen was collected for each experimental sunflower treatment preventing us from sending out any pollen for pesticide analysis, as the minimum requirement for analysis is one gram per sample. The fall of 2013 trial yielded an average of 5.20 ± 0.73 grams of pollen, providing us only with enough pollen to conduct pesticide analysis. Thiamethoxam loads were 18.5, 8.2, and 9.5 ppb for the pollen collected from treated sunflowers grown under well-watered, moderate drought stress, and severe drought stress respectively. We attributed the low pesticide load for the moderately drought stressed pollen to a faulty watering node in that treatment. The node malfunctioned one evening and was left on, resulting in continuous watering for 24 hours. This flooded all of the pots in the moderate drought treatment which likely washed seed treatments off of treated seeds. The low level of thiamethoxam detected in the severely drought stressed pollen was either the result of the very small amount of pollen that was collected from the plants, the poor condition the plants were in, or a combination of both. The sunflowers in the severe drought treatment were very stressed and wilted due to lack of water, so watering was increased towards the end of the experiment in order to keep the plants from dying.

Our original intent was to calculate risk ratios by comparing the prevalence rates in *Nosema* inoculated bees (e.g. (Pettis et al., 2013) that were fed different diets. However since nearly all (99.15%) bees inoculated with *Nosema* became infected, this analytical approach was not possible. Instead we compared *Nosema* load (msb) across treatment groups. Our high infection rate was possibly the result of

individually dosing bees, rather than group feeding them a *Nosema* inoculum (Pettis et al., 2013) which does not ensure equal exposure for all bees. The effects of neonicotinoid exposure on *Nosema* loads are inconsistent. Some studies link exposure to higher *Nosema* loads, while others show that exposure leads to lower *Nosema* loads (Pettis et al., 2012; Retschnig et al., 2014; Vidau et al., 2011). We found no evidence of differing parasite loads in bees feeding on pollen containing thiamethoxam residues when compared to those feeding on clean pollen. In fact, the presence of pollen in our provisioned diets may have influenced *Nosema* replication as pollen is of great importance to *Nosema* replication in bees (Fleming et al., 2015; Jack et al., 2016).

There is growing consensus that the drivers of elevated colony losses are multiple and interactive (Goulson et al., 2015). In this study we uncovered evidence that tri-factor interactions (climate (drought), pesticide exposure (seed treatment), and pathogen (*Nosema*) exposure) can influenced individual bee survivorship. We have documented evidence that the consumption of pollen produced by stressed plants (e.g. drought) has a negative effect on worker longevity when combined with exposure to a pathogen. Notably, bees inoculated with *Nosema* and fed a diet of pollen collected from severely drought stressed plants died faster than their uninfected counterparts. Elevated mortality rates were the same, regardless of pesticide residues in the pollen they fed on (e.g. treated (Figure 11) and untreated seeds (Figure 12)). Additionally, bees fed pollen from moderately drought stressed plants grown from treated seeds died faster when infected with *Nosema* (Figure 10), indicating that moderate stress is

not enough to induce harm unless it is coupled with another stressor (e.g. neonicotinoid exposure).

These findings indicate that bees exposed to one stressor have an increased susceptibility to other stressors, resulting in a shorter lifespan. The additive effect between pathogen infection and neonicotinoid exposure that we observed has been shown to cause decreased survival in honey bees (Alaux et al., 2010a; Aufauvre et al., 2012; Doublet et al., 2015; Vidau et al., 2011). In this study, and for the first time, we document evidence pollen produced by stressed plants (e.g. drought) and feed to bees has a negative effect on worker longevity when combined with exposure to a pathogen. Our work confirms that the synergistic effects of multiple stressors on honey bee health warrant more investigation as bees today are faced with a plethora of pathogens, pesticides, and poor nutrition in the environment.

Tables and Figures

Table 1. Factorial treatment design for sunflower growth trial.

Drought Treatment	Seed Type	Treatment Code	# Plants	Pollen Yield (g)
Well-watered (A)	Untreated	AU	60	12.6
Well-watered (A)	Treated	AT	60	11.33
Moderate drought stress (B)	Untreated	BU	60	8.59
Moderate drought stress (B)	Treated	BT	60	10.4
Severe drought stress (C)	Untreated	CU	60	10.6
Severe drought stress (C)	Treated	CT	60	8.8

Table 2. Factorial treatment design for the cage feeding study of experimental sunflower pollen.

Cage Treatment Code	Drought Treatment of Sunflower Pollen	Seed Type	*Nosema* spores per bee
AU.no	Well-watered	Untreated	0
AU.yes	Well-watered	Untreated	10000
AT.no	Well-watered	Treated	0
AT.yes	Well-watered	Treated	10000
BU.no	Moderate drought stress	Untreated	0
BU.yes	Moderate drought stress	Untreated	10000
BT.no	Moderate drought stress	Treated	0
BT.yes	Moderate drought stress	Treated	10000
CU.no	Severe drought stress	Untreated	0
CU.yes	Severe drought stress	Untreated	10000
CT.no	Severe drought stress	Treated	0
CT.yes	Severe drought stress	Treated	10000
Megabee.no	-	-	0
Megabee.yes	-	-	10000
Syrup.no	-	-	0
Syrup.yes	-	-	10000

Figure 1. A diagram of the experimental design for the sunflower growth trial.

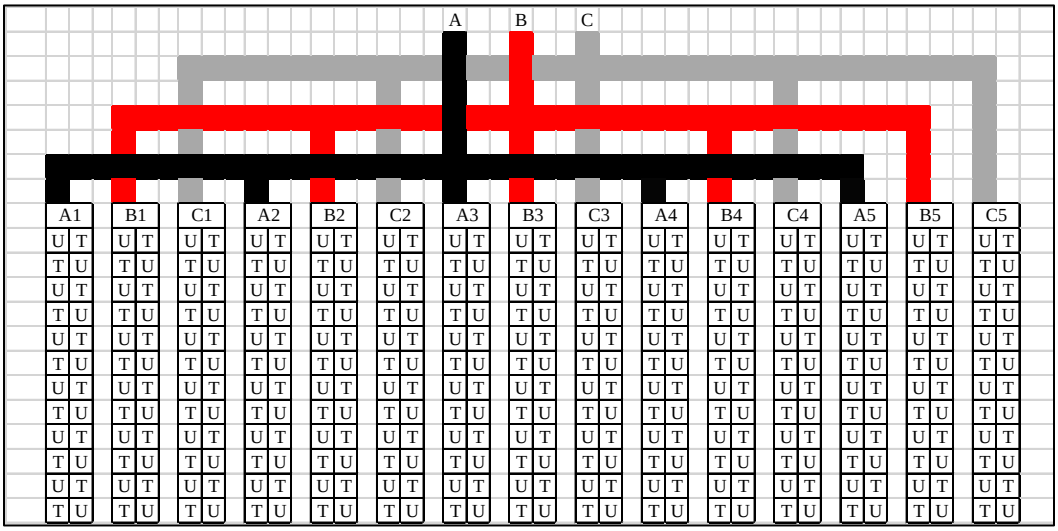


Figure 2. A sunflower head that appears close enough to bloom to be bagged.

Figure 3. A bagged sunflower head. Heads were bagged with a small paper bag, and secured with a small binder clip.

Figure 4. Collecting pollen from a sunflower head. After the brown paper bag was removed, the heads were tapped over a sheet of paper to gather pollen.

Figure 5. Mean (± SE) height (cm) of greenhouse reared sunflowers. There was a significant effect of Drought Treatment on Height ($F_{2, 23} = 78.38$; $p < 0.0001$). Means that differ significantly are indicted by different letters. Well-watered plants were significantly taller than moderately ($T_{23} = 11.80$; $p < 0.0001$) and severely drought stressed plants ($T_{23} = 9.52$; $p < 0.0001$).

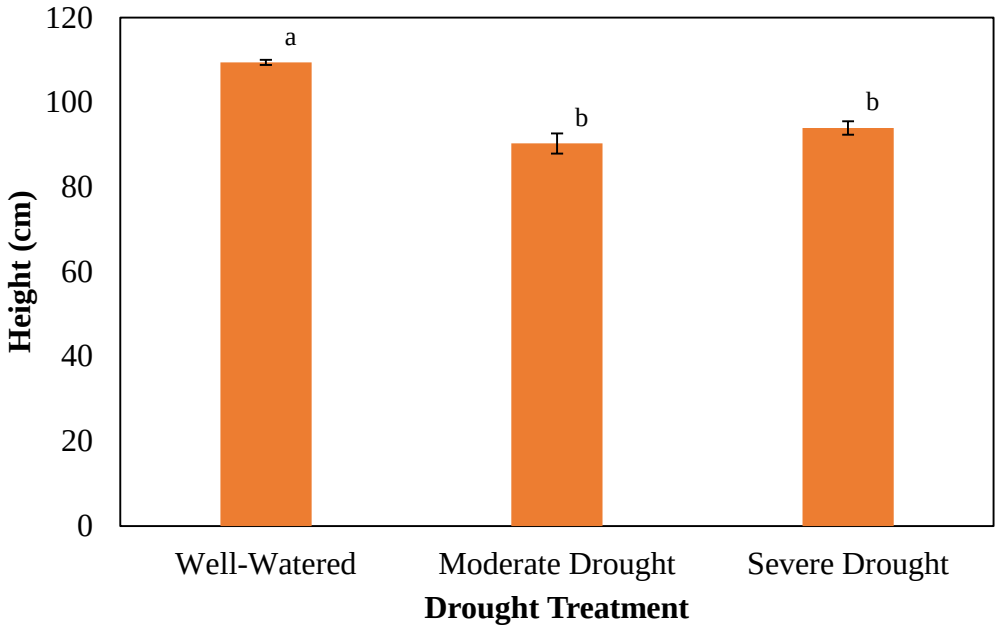


Figure 6. Mean (± SE) head diameter (cm) of greenhouse reared sunflowers. Drought treatment had a significant effect on height ($F_{2, 23} = 20.75$; $p < 0.0001$). Means that differ significantly are indicted by different letters. Well-watered sunflowers had significantly larger heads than those that were moderately ($T_{23} = 5.50$; $p < 0.0001$) and severely drought stressed ($T_{23} = 5.65$; $p < 0.0001$).

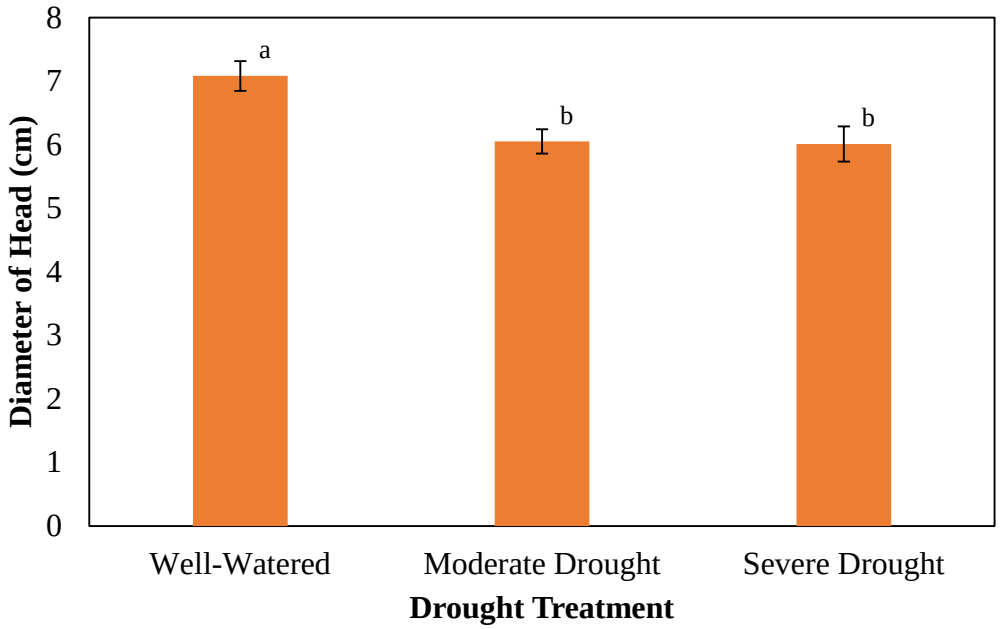


Figure 7. Pesticide load in pollen collected from experimental sunflowers grown using treated seeds. Thiamethoxam was detected in 10.7, 25.3, and 33.9 ppb concentrations in pollen collected from plants grown under well-watered, moderate drought, and severe drought conditions respectively. Thiamethoxam was not detected in untreated seeds grown under any of the three drought treatments.

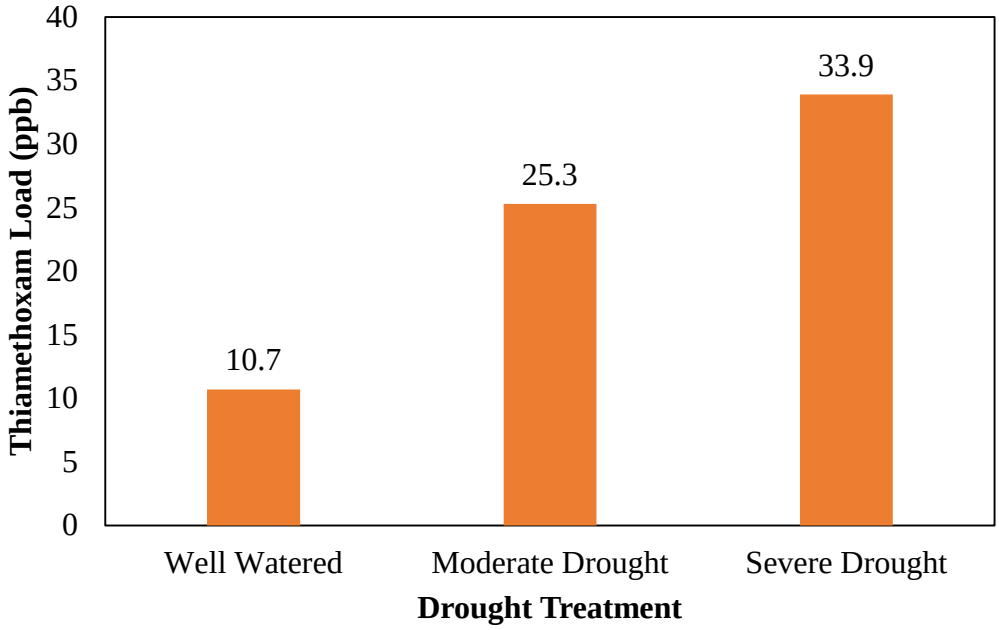


Figure 8. Mean (± SE) *Nosema* load (millions of spores per bee) for bees fed each diet. Diet treatment did not have a significant effect on *Nosema* load ($F_{7, 14} = 2.03$; $p = 0.1234$).

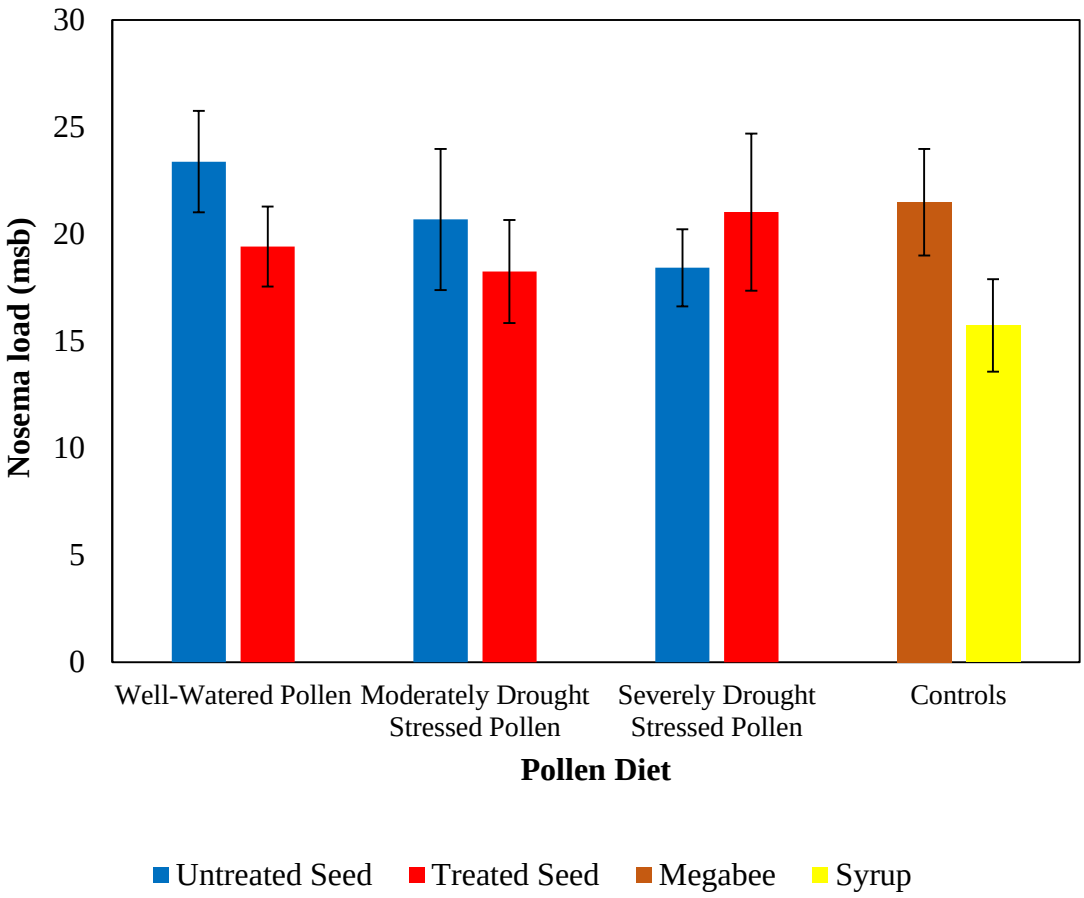
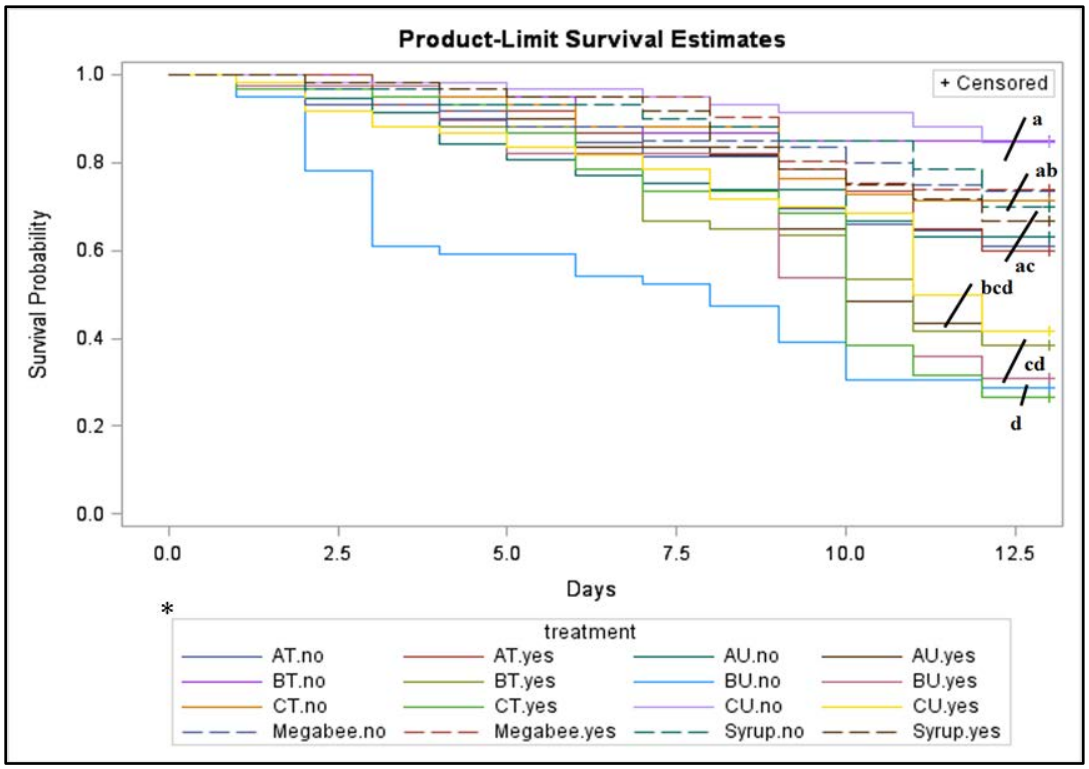


Figure 9. Survival curves for bees fed each of the 16 experimental cage study treatments. There was a significant effect of treatment on survival ($\chi^2_{15} = 151.8622$; $p < 0.0001$). Survival curves that differ significantly are indicated by different letters.



* **AT.no**: Uninfected bees fed pollen from well-watered seed treated plants

AT.yes: *Nosema* infected bees fed pollen from well-watered seed treated plants

AU.no: Uninfected bees fed pollen from well-watered untreated plants

AU.yes: *Nosema* infected bees fed pollen from well-watered untreated plants

BT.no: Uninfected bees fed pollen from moderately drought stressed seed treated plants

BT.yes: *Nosema* infected bees fed pollen from moderately drought stressed seed treated plants

BU.no: Uninfected bees fed pollen from moderately drought stressed untreated plants

BU.yes: *Nosema* infected bees fed pollen from moderately drought stressed untreated plants

CT.no: Uninfected bees fed pollen from severely drought stressed seed treated plants

CT.yes: *Nosema* infected bees fed pollen from severely drought stressed seed treated plants

CU.no: Uninfected bees fed pollen from severely drought stressed untreated plants

CU.yes: *Nosema* infected bees fed pollen from severely drought stressed untreated plants

Megabee.no: Uninfected bees fed Megabee

Megabee.yes: *Nosema* infected bees fed Megabee

Syrup.no: Uninfected bees fed sugar syrup

Syrup.yes: *Nosema* infected bees fed sugar syrup

Figure 10. Survival curves for bees fed pollen collected from moderately drought stressed sunflowers grown using treated seeds. Survival curves that differ significantly are indicated by different letters. Bees infected with *Nosema* died faster than those who were uninfected ($\chi^2_1 = 24.7735$; $p < 0.0001$).

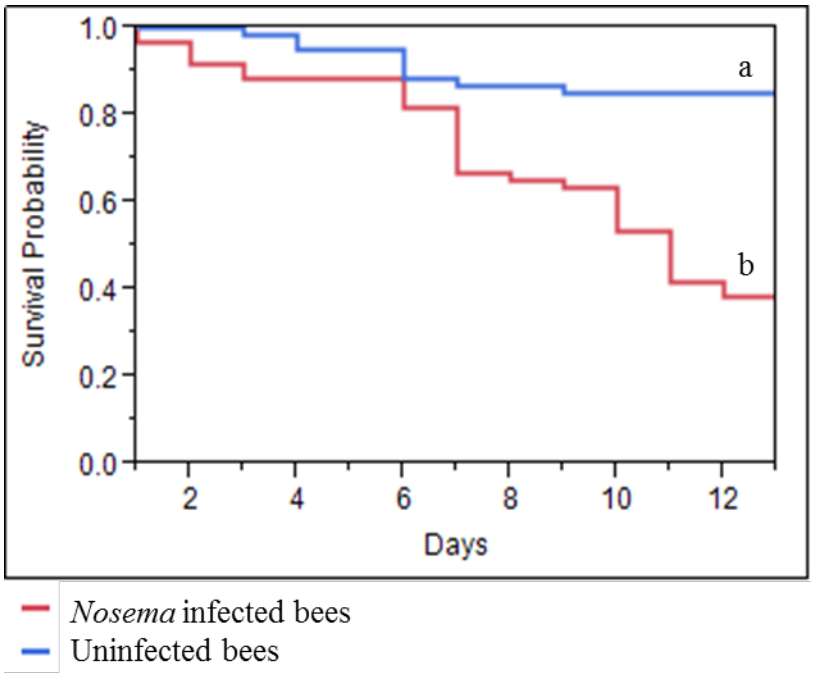


Figure 11. Survival curves for bees fed pollen collected from severely drought stressed plants grown using treated seeds. Survival curves that differ significantly are indicated by different letters. Bees infected with *Nosema* died faster than those who were uninfected ($\chi^2_1 = 18.8978$; $p < 0.0001$).

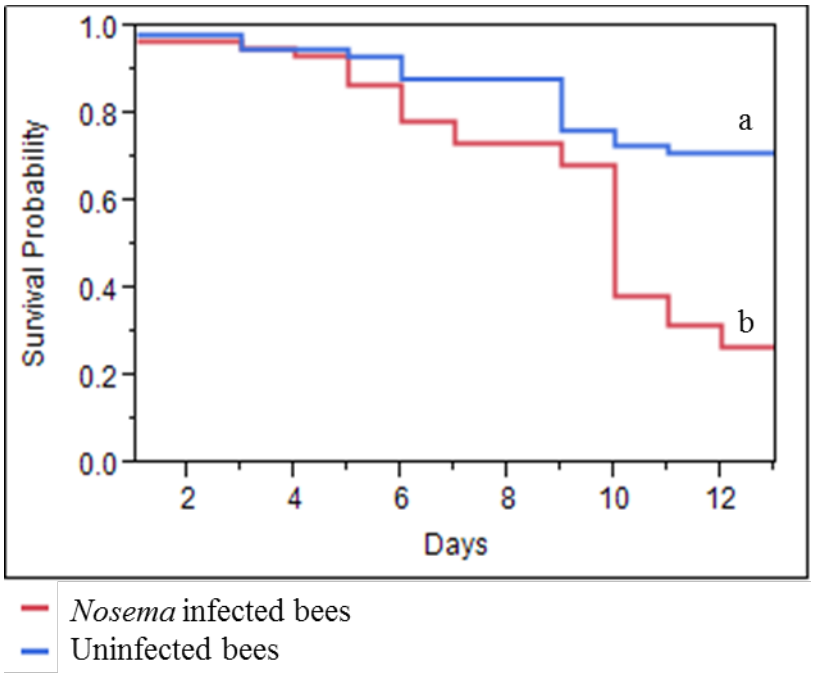


Figure 12. Survival curves for bees fed pollen collected from severely drought stressed plants grown using untreated seeds. Survival curves that differ significantly are indicated by different letters. Bees infected with *Nosema* died faster than those who were uninfected ($\chi^2_1 = 23.7363$; $p < 0.0001$).

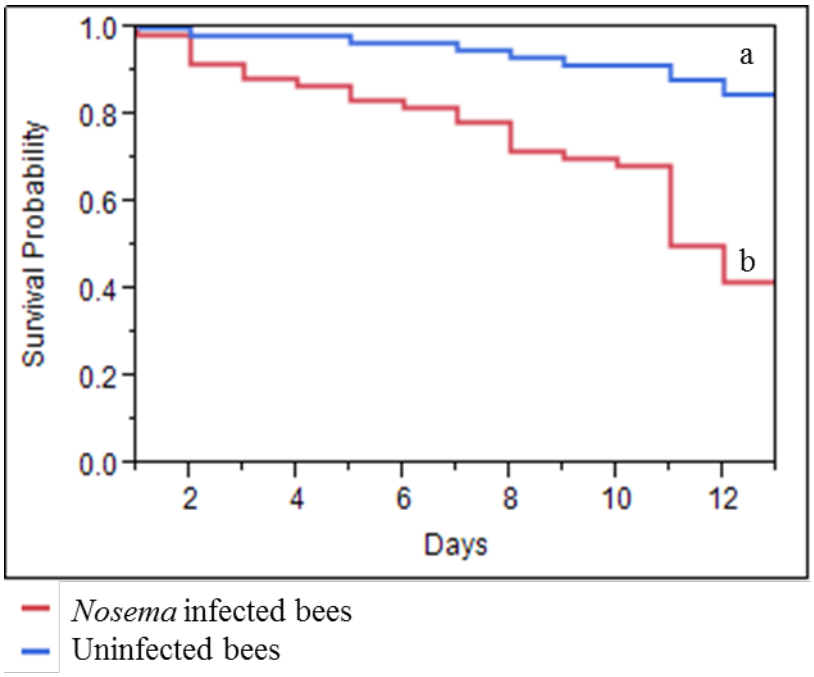
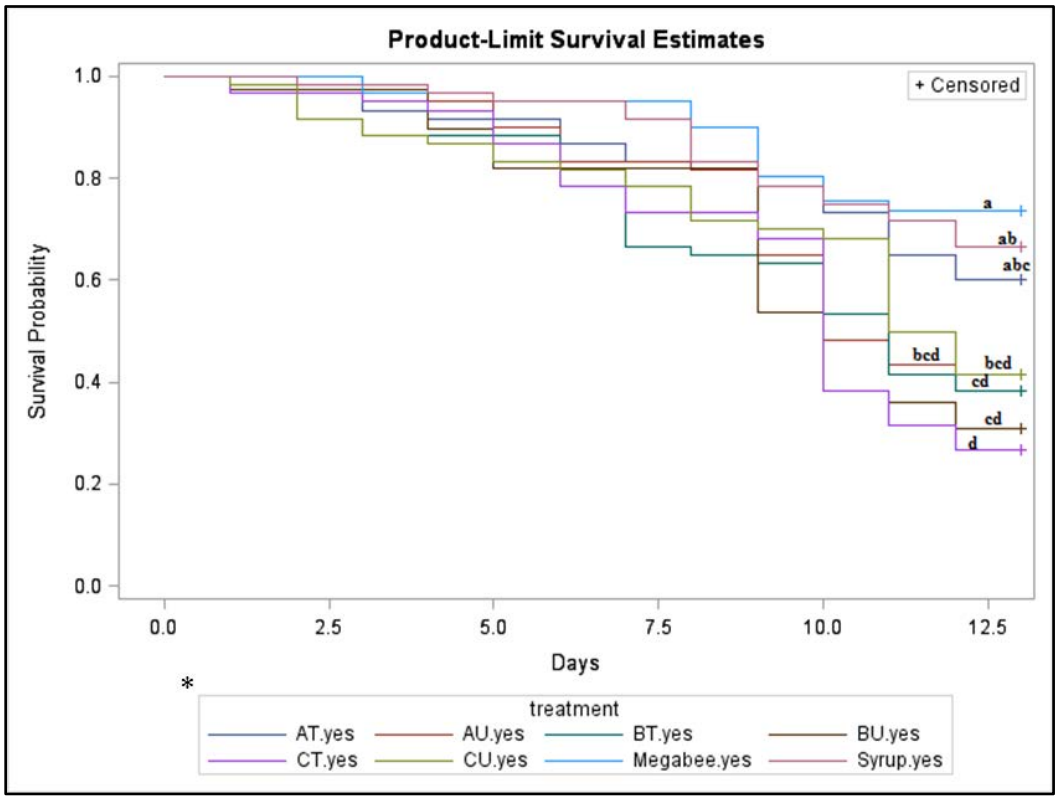


Figure 13. Survival curves for bees inoculated with *Nosema* and fed each different diet treatment. Diet treatment had a significant effect on survival (χ^2_7 =43.9416; p < 0.0001). Survival curves that differ significantly are indicated by different letters. Bees fed Megabee lived the longest, outliving bees fed bees fed pollen from well-watered plants grown from untreated seed (χ^2 = 11.0691; p = 0.0061), moderately drought stressed plants (untreated seed: χ^2 = 18.3569; p = 0.0001, and treated seed: χ^2 = 14.8275; p = 0.0008), and severely drought stressed plants (untreated seed: χ^2 = 10.0625; p = 0.0105, and treated seed: χ^2 = 23.347; p < 0.0001) Bees fed our protein-free control diet of solely sugar syrup survived longer than bees fed pollen from both moderately drought stressed untreated (χ^2 = 12.5554; p = 0.0028) and treated plants (χ^2 = 10.0777; p = 0.0105), and pollen from severely drought stressed plants grown from treated seeds (χ^2 = 17.3356; p = 0.0002). Bees fed pollen from well-watered plants grown from treated seed survived longer than bees fed pollen from severely drought stressed treated plants (χ^2 = 12.2612; p = 0.0129).



* **AT.yes**: Bees fed pollen from well-watered seed treated plants

AU.yes: Bees fed pollen from well-watered untreated plants

BT.yes: Bees fed pollen from moderately drought stressed seed treated plants

BU.yes: Bees fed pollen from moderately drought stressed untreated plants

CT.yes: Bees fed pollen from severely drought stressed seed treated plants

CU.yes: Bees fed pollen from severely drought stressed untreated plants

Megabee.yes: Bees fed Megabee

Syrup.yes: Bees fed sugar syrup

Chapter 2: Elucidating the Effects of Real World Pesticide Load and Diet Variety on Honey Bee Health.

Abstract

Polyfloral pollen diets provide more nutritional variety than monofloral pollen diets, leading one to believe that a polyfloral diet would help honey bees mitigate the effects of environmental stressors more effectively than a monofloral diet. However, pollen collected by honey bees is often found to be contaminated with agricultural pesticides. Here we set out to determine if pesticide exposure via consumed pollen varies based on floral source. We tested pollen collected from honey bee colonies in four different crop systems: Black Cap Raspberry, Meadowfoam, Crimson Clover, and Almond. Experimental pollen diets were prepared by using portions of the polyfloral pollen mix collected by bees in each field, while monofloral diets were prepared by sorting pollen mixes by floral source. We found that polyfloral pollen diets and monofloral diets consisting of pollen from a target crop had higher amounts of pesticides than monofloral diets prepared from non-target pollens. When our experimental pollen diets were consumed by adult bees infected with *Nosema ceranae* we found no differences in pesticide or pathogen susceptibility between bees fed polyfloral diets and those fed monofloral pollen diets.

Introduction

Honey bees that feed on pollen early in their adult lives survive longer than honey bees feeding solely on carbohydrates (de Groot, 1953). Pollen consumption is

an important facet of honey bee colony health, as pollen is the colony's primary source of proteins, amino acids, minerals, lipids, and vitamins (Brodschneider and Crailsheim, 2010). Adult honey bees that consume adequate amounts of pollen live longer and have large and well developed hypopharyngeal glands, fatbodies, and ovaries (Brodschneider and Crailsheim, 2010; Haydak, 1970; Pernal and Currie, 2000). The beneficial effect of pollen consumption on hypopharyngeal gland development is of particular note because it facilitates the production of brood food secretions which are the primary source of protein that nurse bees use to feed young larvae (Brodschneider and Crailsheim, 2010). Well fed larva result in healthier adult bees, which leads to healthier colonies.

The consumption, and thus preferences, of monofloral pollens by young adults is directly correlated with total protein content (Schmidt and Johnson, 1984). Young adult honey bees also prefer polyfloral pollen blends over monofloral pollen diets (Schmidt, 1984; Schmidt and Johnson, 1984). A mixed diet of several pollens increases the likelihood bees will receive adequate amounts of all 10 essential amino acids required for proper honey bee development (de Groot, 1953), as pollen from different plants contain varying quantities and types of amino acids. The drivers of the preference for polyfloral diets are unknown, but several theories have been postulated. Combining pollens can dilute adverse textures, deterrents, and toxins while spreading benefits from more desirable pollens, such as phagostimulants and better nutrition, across less desirable pollens (Schmidt, 1984). The benefits of a varied diet are intuitive, but very little work has been done to assess them. Adult honey bees parasitized with *Nosema ceranae* tend to survive longer when fed a diet of polyfloral

pollen compared to those fed monofloral diets, while uninfected bees survive at the same rate regardless of diet variety (Di Pasquale et al., 2013). This is evidence that polyfloral pollen diets are better suited to help bees fight off other stressors, but are not essential for the survival of healthy adult bees.

Honey bees foraging for pollen in modern agricultural ecosystems are not only collecting an important food source, they are also indirectly collecting the agricultural products used on or around their food sources. Pollen contaminated with pesticides can have negative, sub-lethal effects on individual and colony health. For instance, when pollen mixes with real world levels of fungicide contaminants were fed to bees, they became more susceptible to *N. ceranae* infection (Pettis et al., 2013). Pollen is a vast repository of agricultural chemicals, with an average of 7.1 pesticides detected in pollen samples collected from honey bee colonies in the United States (Mullin et al., 2010). Pettis et al., 2013 found an average of 9.1 pesticides in pollen samples collected from colonies being used to pollinate seven different fruit and nut crops. When colonies are rented out for pollination or otherwise placed in a high intensity agricultural area, pollen foragers tend to bring in more pollen from non-cultivated plants than that of the crops being grown in the area (Long and Krupke, 2016; Pettis et al., 2013). This indicates that non-target species are significant drivers of the pesticide exposure from foraged pollen in agricultural areas.

While there is a large body of work indicating that pesticides are present in the polyfloral mixes of non-cultivated pollen and crop pollen collected in high intensity agricultural areas (Long and Krupke, 2016; Pettis et al., 2013), very little work shows the differences in pesticide exposure caused by these different sources of pollen

(Krupke et al., 2012). For this reason, we decided to survey polyfloral pollen mixes collected from honey bee colonies rented by growers to pollinate fields of four food and seed crops: Black Cap Raspberry Meadowfoam, Almond, and Crimson Clover. The polyfloral mixes collected in three fields of each crop were analyzed for pesticide residues, providing a snapshot of real world pesticide loads encountered by foraging honey bees. From the 12 polyfloral mixes we isolated 37 monofloral pollens that were also analyzed for pesticide residues and were identified as coming from the target crop, or a non-target plant.

These polyfloral pollen mixes and monofloral pollens were then fed to bees infected with *N. ceranae* in order to examine the effects of diet variety on honey bee health, while also allowing us to take into account the effects of pesticides present in the pollen consumed. Our goals were three fold. First, we wanted to see if contamination levels and their relative risks (HQ) were similar between pollen collected from the target crop and non-target plants. Next, we wanted to see if polyfloral pollen diets mediated the negative effects of pesticide or pathogen exposure more effectively than monofloral pollen diets. Finally, we wanted to see if pollen contaminated with real world pesticide loads would negatively affect bee health.

Materials and Methods

Pollen Diet Preparation

The pollen used in this experiment was collected by the Bee Informed Partnership's (BIP) Pacific Northwest tech-transfer team as part of a study designed

to observe pesticide exposure events in colonies used for the commercial pollination of various seed, fruit, and nut crops. In order to track these exposure events, corbicular pollen was sampled and analyzed for pesticide residues. Pollen trap samples were collected from 12 honey bee colonies, evenly distributed across three different fields, for 17 different crops. These pollen samples were sent to the BIP Diagnostic lab at the University of Maryland for preparation to be sent to the EPA for pesticide residue analysis. Residue analysis was conducted at the field level by combining a subsample of pollen collected from all four colonies in a field.

We chose the pollen trap samples collected from the Black Cap Raspberry, Meadowfoam, Crimson Clover, and Almond fields to assess the impact of pollen variety on honey bee's susceptibility to *N. ceranae* because of the abundance and variety of pollen collected in those fields. For each field of Black Cap Raspberry, Meadowfoam, Crimson Clover, and Almond, we prepared a field level composite pollen mix by aggregating pollen collected from the four colonies in each field. This process provided us with 12 field level pollen mixes, three fields per crop. A 2 g subsample of each field mix was sorted by color (using color as an indicator of floral source) to determine the floral diversity in each sample. Each color was weighed, to determine the proportion per sample, and was identified as being the target crop or a non-target plant. Twelve experimental polyfloral pollen diets were created by isolating 10 g of pollen from each field mix.

Next, we attempted to isolate 10 g of the three most predominant colors (floral sources) from each field mix to create monofloral diets for our cage feeding trials. However we were only able to isolate three monofloral diets from seven of the 12

field mixes. The remaining field mixes lacked enough pollen to sort, or the vast majority of the pollen in the sample came from less than three sources. In total, 25 experimental monofloral pollen diets were created by sorting out 10g of the most predominant pollen colors from each of the field level samples. Subsamples (1.5-3g) of each of the 37 experimental pollen diets were individually packaged in 15 mL Falcon tubes (Corning, Inc.: One Riverfront Plaza, Corning, NY, 14831) and shipped on dry ice to the USDA-AMS National Science Laboratories in Gastonia, NC where pesticide analysis was conducted using modified methods similar to those described by Mullin et al., 2010. When we received the results, we summed the total number of pesticides in each sample and the number of products detected in each class of pesticides (Fungicides, Herbicides, Insecticides, and Varroacides) (Tables 3-4). The overall Hazard Quotient (HQ) was calculated for each sample (Tables 3-4). Hazard Quotient was calculated as the sum of each chemical detection in parts per billion (ppb) divided by the LD_{50} for that chemical (Stoner and Eitzer, 2013; Traynor et al., 2016).

Cage Studies

Newly emerged bees were collected by retrieving frames of sealed brood from four different colonies at the Central Maryland Research and Education Center (CMREC) - Beltsville Facility. Frames were caged and stored overnight in an incubator at 34.5^{o}C, 70% relative humidity (Williams et al., 2013). After 24 hours, bees were removed from the frames and placed into colony specific hoarding cages. Five bees from each hoarding cage were placed into one of 41 disposable 16 oz. Solo cup cages modeled after Evans et al., 2009. 37 cups of bees were provided a 1008

Falcon petri dish (Corning, Inc., One Riverfront Plaza, Corning, NY 14831) with a paste made up of 0.5 g of one of the 37 pollen diets mixed with 0.25 mL of 50% (w/v) sucrose syrup (Pettis et al., 2013). Each of the 37 cages was fitted with a pipette bulb feeder containing 2 mL of 50% (w/v) sucrose syrup inoculated with ca. 1 million spores of *Nosema ceranae*. The *Nosema* inoculum was created using methods described by Fries et al., 2013. The remaining four cups of bees served as controls. Two cups were provided with a 1008 Falcon petri dish containing 0.75 grams of MegaBee pollen substitute, prepared as a patty using label instructions, to serve as protein controls. The other two cups were fitted with an empty 1008 Falcon petri dish to serve as protein free controls. One of each set of control cups was provided with a pipette bulb feeder containing ca. 1 million spores of *Nosema* in 2 mL of 50% syrup, while the remaining two cups were provided a pipette bulb containing 2 mL of 50% syrup.

All 41 cages were maintained in an incubator set at 30^{o}C and 70% relative humidity (Williams et al., 2013). Dead bees were removed daily and 50% syrup was added to any empty feeders. On the twelfth day, all surviving bees were killed (Pettis et al., 2013). Bees were stored in 90% ethanol until they could be processed. *Nosema* load in millions of spores per bee (msb) was individually quantified for each surviving bee using haemocytometer counts (Human et al., 2013). This cage study experiment was replicated three times.

Statistical Analysis

ANOVAs were used to assess any differences in the mean number of products detected and the mean HQ scores detected in pollen mixes from each of the four crop

systems studied. Before comparisons, HQ scores were log transformed to meet normality assumptions, means were back transformed for graphical presentations. ANOVAs were also used to assess any differences in the proportion of honey bees fed each experimental diet that survived until the end of the experiment. Finally, ANOVAs were used to analyze *Nosema* load (msb) for bees fed different pollen diets. Analysis was conducted on the average spore load of all surviving bees per cage, and cage rep was treated as a random variable. The Proc Mixed procedure in SAS 9.4 (SAS Institute, Inc., Cary, NC) was used to conduct ANOVAs followed by Tukey multiple mean comparisons.

Eighty-six variables pertaining to pesticide exposure were compared to the average *Nosema* load (msb) for each of the 37 experimental pollen diet cage treatment (3 reps per cage) to assess for correlations. Correlations were analyzed using the Proc Corr procedure in SAS 9.4. First we screened the total number of products detected, the number of fungicides, herbicides, varroacides, and insecticides. Next, we screened the total Hazard Quotient (HQ), the number of products that yielded an HQ over 50, and the HQ pertaining to fungicides, herbicides, varroacides, and insecticides. Then we screened the number of detections and the HQ for products grouped into 14 groups based on unique Mode of Action (MOA) (Ache, IGR 0, EcRs, ORA, NaCh, MBC, SDHI, QoI, AP, MAP, DMI, Multisite, PPG, and Tubulin) and the total number of different MOAs detected in a sample. Finally, we screened the number of detections and the load (in ppb) for each of the 23 pesticides detected in a sample (2,4 dimethylphenyl formamide (DMPF), azoxystrobin, bifenthrin, boscalid, captan, carbaryl, carbendazim (MBC), chlorothalonil, chlorpyrifos, coumaphos,

cyprodinil, diflubenzuron, fluvalinate, iprodione, methoxyfenozide, oxyfluorfen, pendimethalin, pronamide, pyraclostrobin, tebuconazole, thiabendazole, THPI, trifloxystrobin).

Results

Composition of Field Level Pollen Samples

Pollen from the target crop was detected in each field sample at varying concentrations. Most of the pollen brought back by honey bees placed in Meadowfoam, Crimson Clover, and Almond fields was from the target crop, while Black Cap Raspberry pollen was only collected by bees in small amounts (Table 3). Each of the 25 monofloral pollen diets prepared for cage studies was identified, using light microscopy, as being the target crop or a non-target plant (non-targets were identified as being in the Fabaceae or Rosaceae family but not from the crop plant) (Table 3).

Pesticide Detections

There were 95 detections of 23 different pesticides in the 37 experimental pollen diets. The 23 chemicals detected had 14 different Modes of Action (MOA). Neonicotinoids were not detected in any samples. Up to 8 products were detected in a sample, with an average of 2.57 ± 0.34 pesticide detections per sample. No pesticides were detected in the sample of our Megabee protein control that was sent for analysis.

All polyfloral pollen diets (n = 12) had at least one product detected, with an average of 3.33 ± 0.66 products detected per sample (Table 4). Polyfloral diets made from Almond field mixes had more products than those made from the pollen mixes

collected in Meadowfoam and Crimson Clover fields; more products were also found in Black Cap Raspberry polyfloral diets than in Meadowfoam (Figure 19; $F_{3, 8}$ = 16.22; p = 0.0009). The average HQ score over all polyfloral samples was 59.94 ± 37.91.There was no difference in HQ between pollen mixes from different crops (Figure 20; $F_{3, 8}$ = 3.4; p = 0.074).

With regards to monofloral diets, pesticides were detected in 21 of the 25 diets. No products were detected in two diets made of pollen from target crops (Meadowfoam), and two diets made of non-target pollen (one from a Black Cap Raspberry field, and one from a Crimson Clover field). Collectively, all monofloral diets with product detections (n = 21) had an average of 2.62 ± 0.38 products and an HQ of 126.03 ± 42.56. Contaminated monofloral diets made up of the target crop (n=11) had an average of 3.27 ± 0.63 products and an HQ of 189.12 ± 73.60, whereas contaminated monofloral diets made up of non-target plant species (n = 12) had an average of 1.58 ± 0.31 products and an HQ of 56.63 ± 28.44. Of the 12 fields investigated, only four fields yielded monofloral diets of pollen from the target crop and a non-target plant. Different pesticides were detected in the three types of pollen diets (polyfloral, monofloral target, and monofloral non-target) created from the pollen collected within each of these four fields (Figures 14-17). The total number of products detected in the polyfloral, monofloral target, and monofloral non-target diets made from these four fields did not differ ($F_{2, 23}$ = 1.21; p = 0.3304). However, when comparing the total number of products detected based on diet variety across all 37 experimental pollen diets, we found that monofloral non-target diets were less

contaminated than polyfloral diets and the monofloral diets made from target crops ($F_{2, 24} = 8.44$; $p = 0.0011$; Figure 18).

Survival Rates

There was no significant difference in overall survival between bees fed different diet treatments ($F_{38, 76} = 0.94$; $p = 0.5723$). On Average 89.13 ± 1.31 % of bees fed each experimental diet survived until harvest on day 12.

Nosema Infection

Nosema was detected in 98.7% of the 2,075 bees that survived until the twelfth day of the cage studies. There was no difference in *Nosema* load between bees fed experimental polyfloral diets from the four different crop systems, however bees fed the polyfloral diets had higher *Nosema* loads then those fed either Megabee or syrup (Figure 21; $F_{5, 34} = 9.18$; $p < 0.0001$). Bees fed monofloral pollen diets made up of one of the four target crops tended to have higher *Nosema* loads than controls (Figure 22; $F_{5, 37} = 11.04$; $p < 0.0001$). Of particular note, bees fed Meadowfoam pollen had lower *Nosema* loads than those fed Crimson Clover pollen ($T_{37} = 3.17$; $p = 0.0031$). For each crop system, *Nosema* loads did not differ between bees fed the polyfloral mixes, monofloral diets of the target crop, or monofloral diets made of non-target plants, however bees fed an experimental pollen diet tended to have higher *Nosema* loads than bees fed either control (Black Cap Raspberry: Figure 23; $F_{4, 35} = 6.57$; $p = 0.0005$; Crimson Clover: Figure 24; $F_{4, 35} = 18.5$; $p < 0.0001$; Meadowfoam: Figure 25; $F_{4, 17} = 5.89$; $p = 0.0037$; Almond: Figure 26; $F_{3, 21} = 10.98$; $p = 0.0002$). Lastly, there was no difference in *Nosema* load between bees fed polyfloral diets or

monofloral diets but, regardless of floral variety, *Nosema* loads were higher than those of bees fed either control (Figure 27; $F_{3, 111} = 12.44$; $p < 0.0001$).

Correlations between Nosema and Pesticides

We found a significant, negative correlation between Nosema load (msb) and presence of thiabendazole ($r = -0.22132$, $p = 0.0196$) and load of thiabendazole ($r = -0.22132$, $p = 0.0196$). All other correlations were insignificant.

Discussion

Pollen from the target crop was detected in pollen trap samples collected from all three fields for each of the four crops we investigated. Only 16 (± 9.17) % of the pollen that honey bees collected in Black Cap Raspberry fields came from Black Cap Raspberry plants. While over 50% of the pollen collected by bees in Meadowfoam, Crimson Clover, and Almond fields came from the target crop (Table 3). Prior work showed that bees used to pollinate crops originating from the New World (Blueberry, Cranberry, Watermelon, and Pumpkin) do not bring back much – if any – pollen from the target crops, while the pollen collected by bees used to pollinate old world crops (Almond and Apple) was predominately from the target crop (Pettis et al., 2013). Our findings confirm this work, showing that honey bees are not efficient at collecting pollen from crops originating from the New World (Black Cap Raspberry), but are excellent pollinators of the Old World crops (Almond and Crimson Clover) with which they likely coevolved (Pettis et al., 2013).

Our survey of real world pesticide residues found in pollen collected from honey bees in agricultural settings was done to answer several questions. First, we

wanted to determine if there was a consistent pesticide exposure across different fields of the same crop. At over $300 a sample, pesticide analysis is cost prohibitive. For this reason, we were only able to analyze one subsample from each field level mix which prevented us from assessing any statistical difference between the pesticide detections across the three field mixes of pollen analyzed from each different crop. Regardless, there were noticeable differences in the number of products, and the amount of each product (HQ), across the different fields sampled within each crop. Next, we wanted to determine if there is a difference in pesticide exposure across polyfloral pollen mixes from different crops. With this survey we do show a difference in the number of products detected in polyfloral pollen mixes collected from honey bee colonies across different crops (Figure 19).

While pesticides are consistently found in samples of aggregated pollen taken from the honey bee colonies used to pollinate commercial crops (David et al., 2016; Krupke et al., 2012; Long and Krupke, 2016; Mullin et al., 2010; Pettis et al., 2013; Traynor et al., 2016), little work has been done to show from what floral source these pesticides originate (Botias et al., 2015; David et al., 2016; Krupke et al., 2012). We compared the number of pesticides detected in all 37 experimental pollen diets created for cage feeding trials to determine if pesticides are more commonly found in pollen collected from the target crop, non-target crop, or the overall pollen mix brought into a colony. We found that non-target crop monofloral pollen diets had fewer products than target crop monofloral pollen diets and polyfloral pollen diets containing both target and non-target pollens ($F_{2, 24} = 8.44$; $p = 0.0011$; Figure 18). This confirms prior work showing that more pesticides are found in target crop pollen

collected directly from cultivated crops than in the wild-flowers growing adjacent to fields (Botias et al., 2015; David et al., 2016). The types and quantity of pesticides detected in the polyfloral and monofloral diet mixes coming from the same field were not consistent. This is surprising as we would not expect that monofloral diets derived from a polyfloral mix would have unique products not detected in the polyfloral mix; yet we saw this in several instances (Figures 14-17). This hints at the inherent flaw of subsampling pollen for pesticide analysis, it would seem that several subsamples should be analyzed to get an accurate count on the amount and types of pesticides in pollen sample. Finally, we wanted to determine if there were differences between product detections and relative risks between the three diet types (polyfloral, target, and non-target pollen) at the crop level. Regrettably, we were unable to conduct this comparison due to the low sample size of monofloral diets that some pollen mixes yielded (Table 3).

We found no evidence that polyfloral pollen diets mitigate the effects of pesticide or pathogen exposures on adult honey bees when compared to monofloral pollen diets. Adult honey bees infected with *Nosema* survive at equal rates regardless of pollen diet variety for at least the first 20 days of life, but after 50 days it is evident that bees fed polyfloral diets live longer than those fed monofloral diets (Di Pasquale et al., 2013). We found no difference in overall survival between bees fed each of the 37 different experimental pollen diets ($F_{38, 76} = 0.94$; $p = 0.5723$). However, our analysis of adult bee survival across bees fed different diets was truncated as all bees surviving on day 12 were sacrificed for *Nosema* quantification. Studies similar to ours, have shown that bees exposed to pesticides, specifically neonicotinoids, die

faster if they have also been infected with *Nosema* (Aufauvre et al., 2012; Doublet et al., 2015; Retschnig et al., 2014; Vidau et al., 2011). While there were 23 different pesticides detected across all 37 experimental diets we tested, none of them were neonicotinoids. Differences in adult bee longevity based on diet variety or pesticide exposure may have been detected had we tested survival over a greater period of time.

We found no differences in *Nosema* load (msb) when comparing bees fed polyfloral diets prepared from the pollen mixes of each crop (Figure 21). Had pesticides played a role in increasing susceptibility to *Nosema,* we would have expected to see higher *Nosema* loads in bees fed the pollen diets with higher pesticide loads. While others have found such a relationship (Pettis et al., 2013), we failed to. *Nosema* loads also did not differ between bees fed monofloral diets of different target crop pollens suggesting all crops studied had equal or no effect on bee health (Figure 22).

Finally, there was no difference in *Nosema* load between bees fed polyfloral, monofloral target, and monofloral non-target pollen diets prepared from pollen trapped in each of the four different crop systems we investigated (Figures 23-26) While a diverse pollen diet can increase the imunocompetence and longevity of healthy honey bees (Alaux et al., 2010b; Schmidt et al., 1987), we found that diet variety did not increase inoculated bees susceptibility to *Nosema* infection. In fact, the consistent infection across bees fed any sort of pollen is likely the result of the pollen consumption itself, as a pollen diet increases *Nosema* replication in bees (Fleming et al., 2015; Jack et al., 2016). While we did not detect a relationship between pesticide

exposure and *Nosema* load, this should not be interpreted as pesticides having no effect. Different pesticides have different effects on *Nosema* load, some products cause an increase in *Nosema* loads while others cause a decrease (Vidau et al., 2011). For these reasons, future investigations into the impacts of nutritional variety and real world pesticide loads on *Nosema* susceptibility and longevity of adult honey bees would do well to focus on survival rates, rather than *Nosema* load, as longevity has already been documented to alter depending on diet variety, pathogen infection, and pesticide exposure (Alaux et al., 2010b; Aufauvre et al., 2012; Di Pasquale et al., 2013; Doublet et al., 2015; Retschnig et al., 2014; Schmidt et al., 1987; Vidau et al., 2011).

Tables and Figures

Table 3. Floral composition and quantity of monofloral pollen diets prepared for cage studies, as well as the mean (± SE) number of product detections and mean (± SE) relative risk (HQ) for each type of monofloral diet.

Crop	Pollen Type	n	% of pollen in mix	Fungicides Detected	Herbicides Detected	Varrroacides Detected	Insecticides Detected	Total Products Detected	Total HQ
Black Cap Raspberry	Crop	1	16 ± 9.17	5	0	0	0	5	21.05
Black Cap Raspberry	Non-target	8	84 ± 9.17	1.25 ± 0.31	0.13 ± 0.13	0	0.5 ± 0.27	1.88 ± 0.4	70.61 ± 34.07
Meadowfoam	Crop	2	74 ± 6.66	0	0	0	0	0	0
Meadowfoam	Non-target	1	26 ± 6.66	2	0	0	0	2	1.24
Crimson Clover	Crop	6	54.67 ± 15.34	1.5 ± 0.22	0.67 ± 0.21	0.17 ± 0.17	0.5 ± 0.22	2.83 ± 0.6	221.99 ± 99.43
Crimson Clover	Non-target	3	45.33 ± 15.34	0.67 ± 0.33	0	0	0	0.67 ± 0.33	0.07 ± 0.04
Almond	Crop	4	92.67 ± 1.76	2.5 ± 0.87	0.25 ± 0.25	0	0.75 ± 0.48	3.5 ± 1.55	181.83 ± 148.42
Almond	Non-target	0	7.33 ± 1.76	-	-	-	-	-	-

Table 4. Mean (± SE) number of product detections and mean (± SE) relative risk (HQ) for polyfloral pollen diets.

Crop	Pollen Type	n	Fungicides Detected	Herbicides Detected	Varrroacides Detected	Insecticides Detected	Total Products Detected	Total HQ
Black Cap Raspberry	Mix	3	2.67 ± 1.2	0.67 ± 0.33	0.67 ± 0.67	0	4 ± 0.58	4.42 ± 2.16
Meadowfoam	Mix	3	0.67 ± 0.67	0	0.67 ± 0.33	0	1.33 ± 0.33	1.89 ± 0.92
Crimson Clover	Mix	3	1 ± 0.58	0	0.33 ± 0.33	0.33 ± 0.33	1.67 ± 0.33	12.37 ± 11.48
Almond	Mix	3	3.33 ± 0.33	1.67 ± 0.33	0	1.33 ± 0.88	6.33 ± 0.88	221.09 ± 118.89

Figure 14. Venn diagram comparing the products detected in the three different types of pollen diets prepared from pollen trap samples collected in Field 3 of Black Cap Raspberry. Azoxystrobin and THPI were detected in the polyfloral pollen mix (n = 1), as well as both types of monofloral diets (Black Cap Raspberry pollen (n = 1) and non-target pollen (n = 2)) prepared from the field 3 polyfloral mix. Boscalid, captan, and cyprondil were detected in the polyfloral mix and the monofloral diet made from Black Cap Raspberry pollen. Tebuconazole was only detected in the monofloral diets made from non-target pollen.

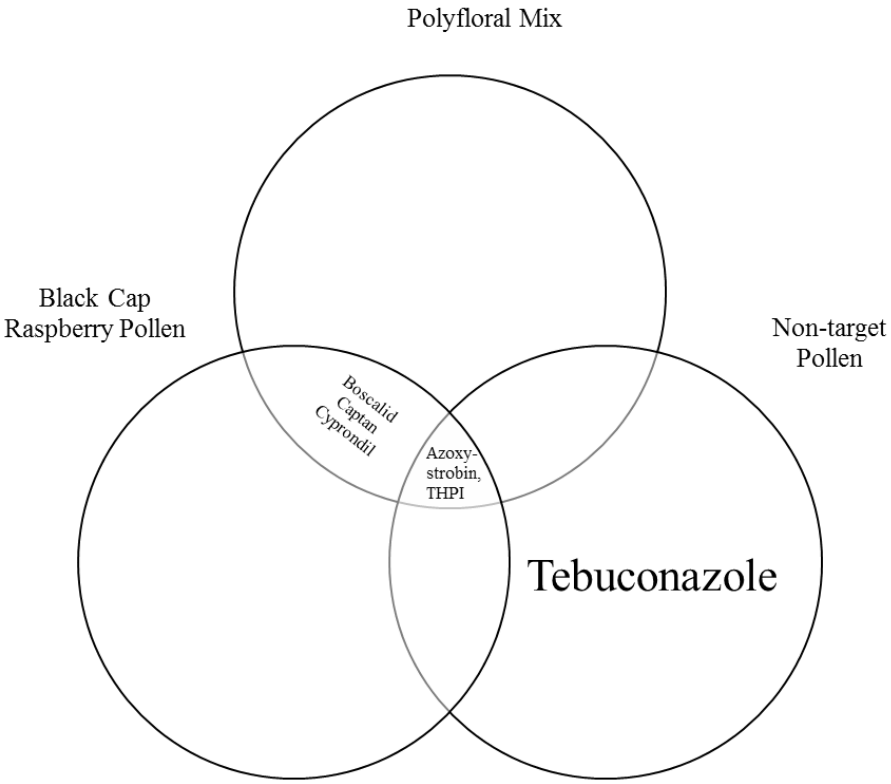


Figure 15. Venn diagram comparing the products detected in the three different types of pollen diets prepared from pollen trap samples collected in Field 1 of Meadowfoam. Boscalid and chlorothalonil were only detected in the polyfloral mix diet (n = 1) collected in Field 1 of Meadowfoam. Captan and cyprodinil were only detected in the monofloral diet made from non-target pollen (n = 1). There were no products detected in the monofloral diets made from Meadowfoam pollen (n = 2).

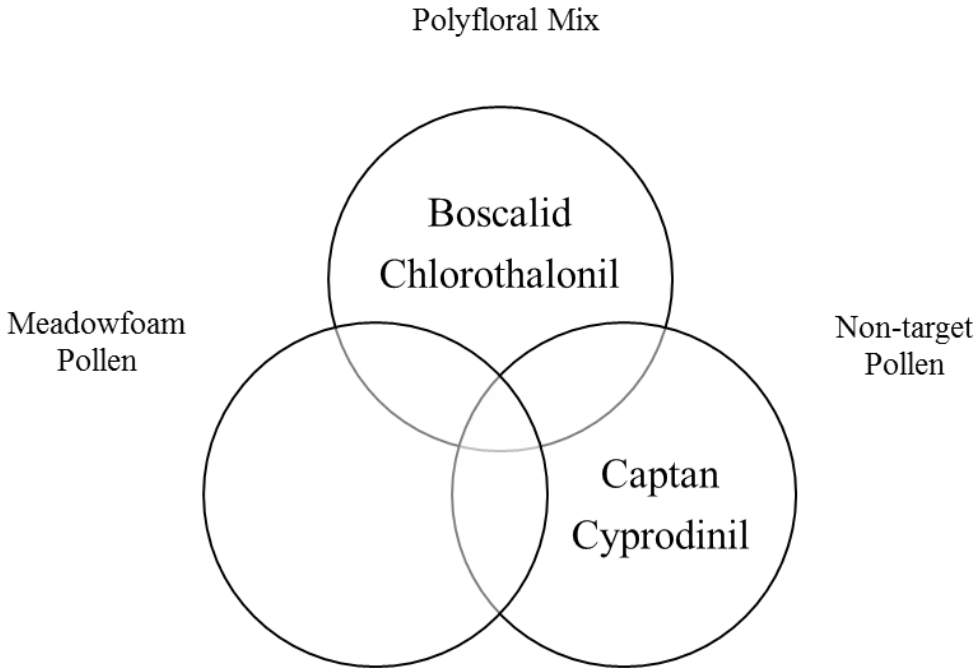


Figure 16. Venn diagram comparing the products detected in the three different types of pollen diets prepared from pollen trap samples collected in Field 1 of Crimson Clover. Carbaryl and fluvalinate were only detected in the polyfloral mix (n = 1), Azoxystrobin was only detected in the monofloral diets made of Crimson Clover pollen (n = 2), and trifloxystrobin was only detected in the monofloral diets made from non-target pollen (n = 1).

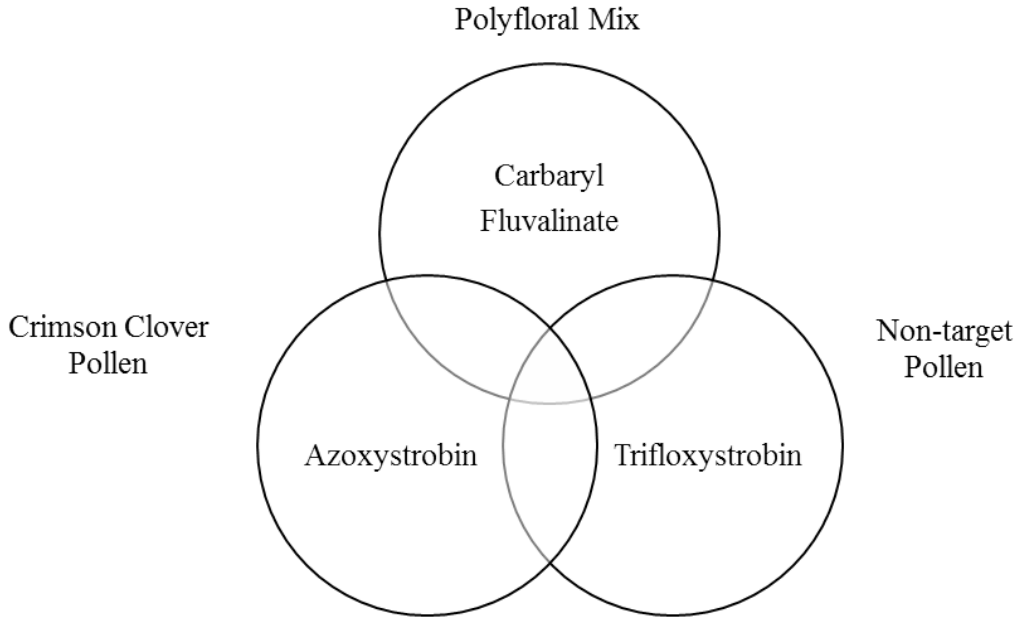


Figure 17. Venn diagram comparing the products detected in the three different types of pollen diets prepared from pollen trap samples collected in Field 2 of Crimson Clover. Azoxystrobin was detected in the polyfloral mix (n = 1), the monofloral diets made from non-target pollen (n = 2), and the monofloral diets made from Crimson Clover pollen (n = 1). THPI was only detected in the polyfloral pollen diet. Bifenthrin, coumaphos, and pronamide were only detected in the monofloral diet made from Crimson Clover pollen.

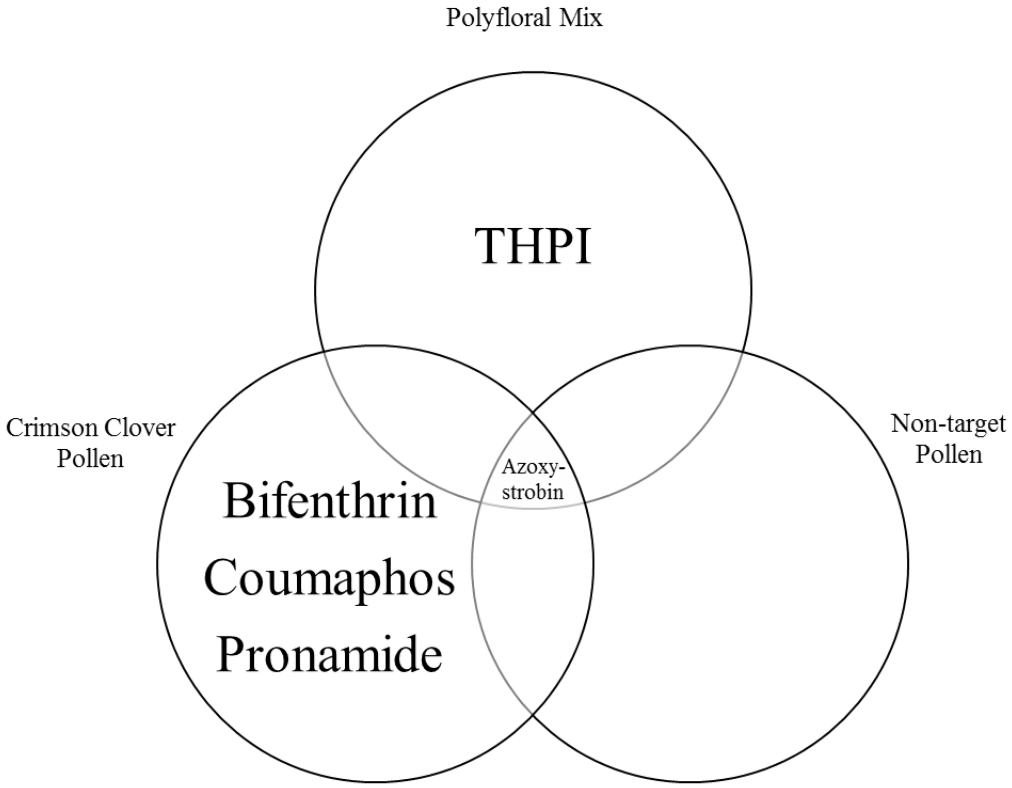


Figure 18. Mean (± SE) number of products detected based on diet variety ($F_{2, 24}$ = 8.44; p = 0.0011). Significantly different means are indicated by letters. Polyfloral pollen diets and monofloral diets made of target crop pollen had more product detections than monofloral diets made up of pollen from non-target plants.

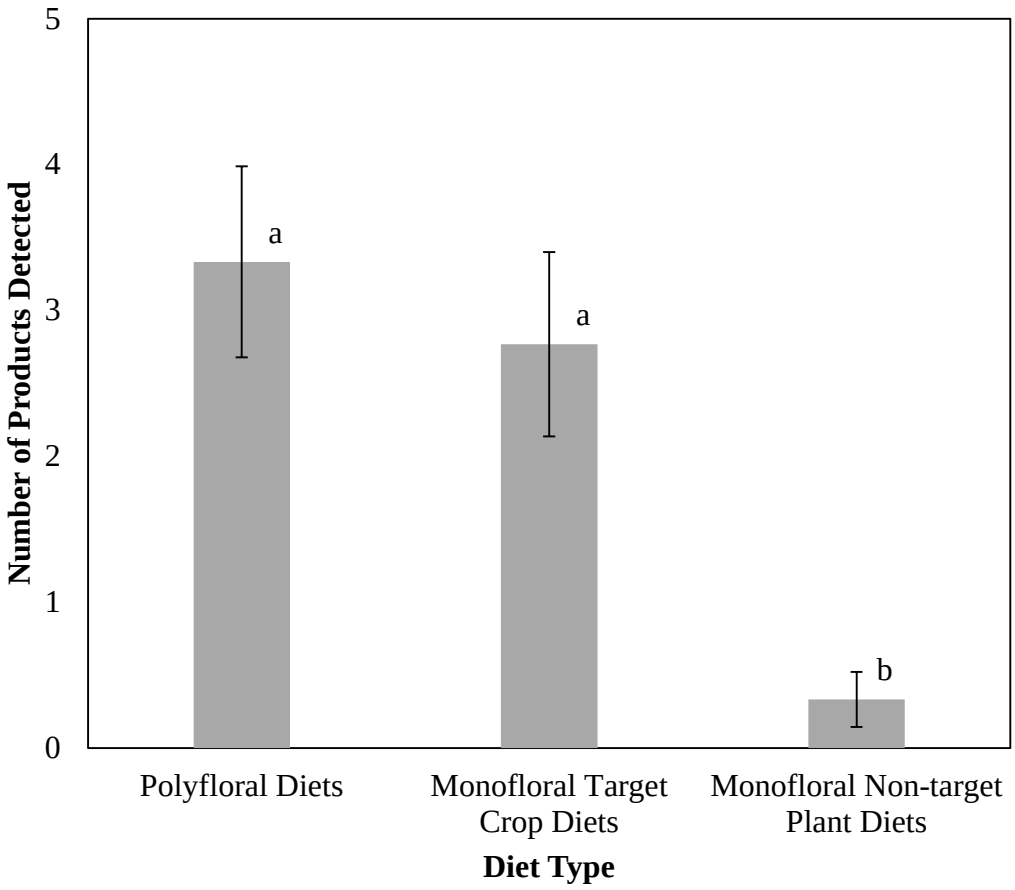


Figure 19. Mean (± SE) total number of products detected in polyfloral pollen mixes from each of the four crops ($F_{3, 8} = 16.22$; $p = 0.0009$). Significantly different means are indicated by letters. Polyfloral diets prepared from pollen trap samples taken in almond fields were more contaminated than those prepared from Meadowfoam fields or Crimson Clover fields. Polyfloral diets prepared from pollen trap samples taken in Black Cap Raspberry fields were more contaminated than those made from Meadowfoam fields.

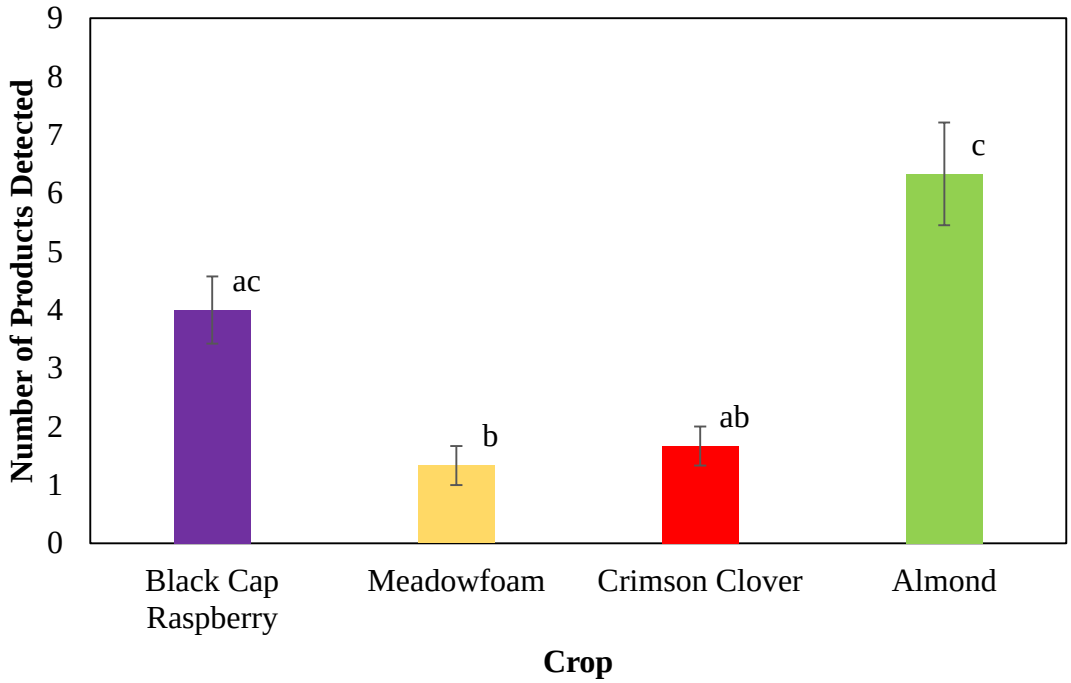


Figure 20. Mean (± SE) total Hazard Quotient for the polyfloral pollen mixes collected from each crop. There was no significant treatment effect ($F_{3, 8} = 3.4$; $p = 0.074$).

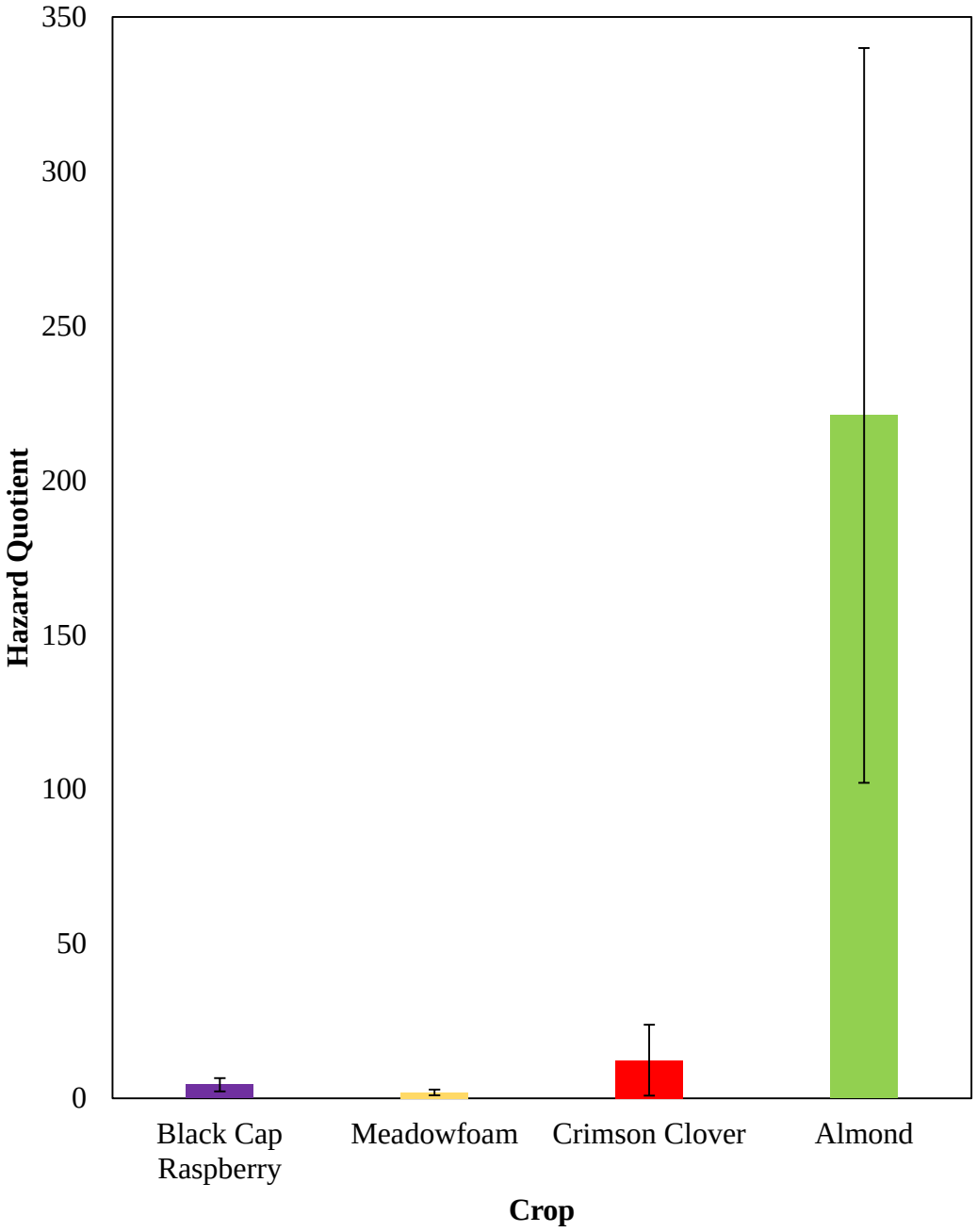


Figure 21. Mean (± SE) *Nosema* load (millions of spores per bee) for honey bees fed polyfloral diets from each of the four different crops ($F_{5, 34} = 9.18$; $p < 0.0001$). Means that differ significantly are indicted by different letters. There was no difference across experimental pollen treatments, however bees fed any of the pollen diet treatments had significantly higher levels of *Nosema* infection that bees fed either control.

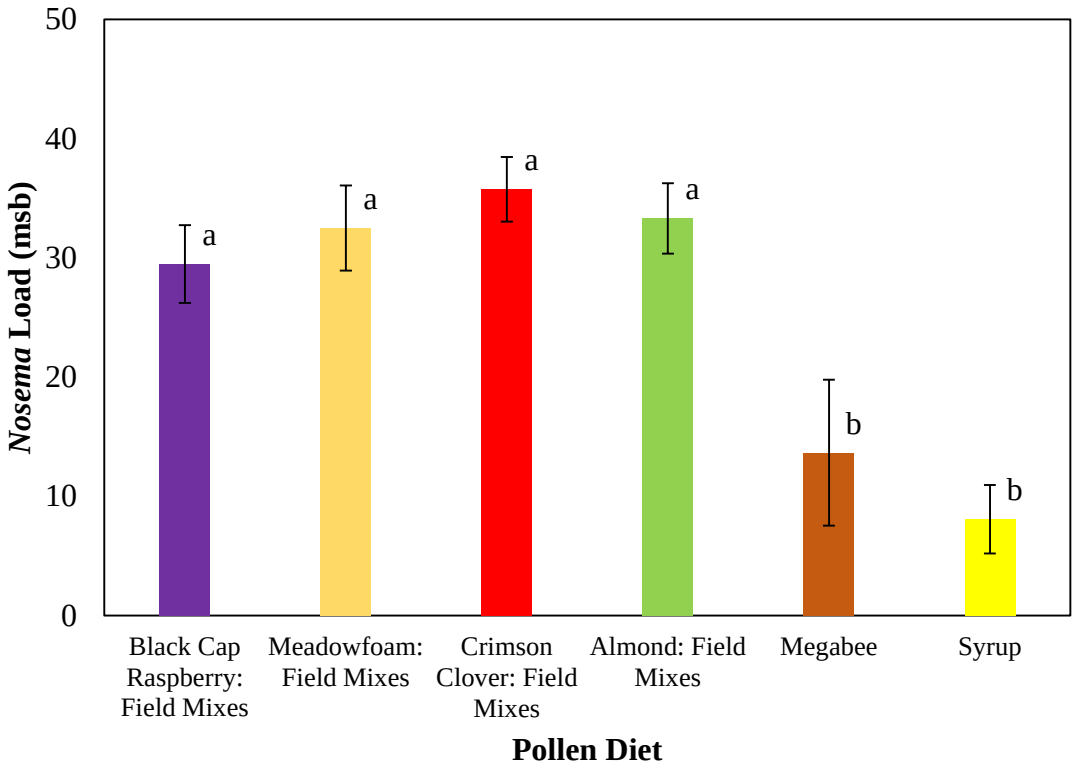


Figure 22. Mean (± SE) *Nosema* load (millions of spores per bee) for honey bees fed monofloral pollen diets made up of the target crop ($F_{5, 37} = 11.04$; $p < 0.0001$). Means that differ significantly are indicted by different letters. Bees fed monofloral diets of Meadowfoam pollen had significantly lower *Nosema* infection levels than those fed Crimson Clover pollen ($T_{37} = 3.17$; $p = 0.0031$).

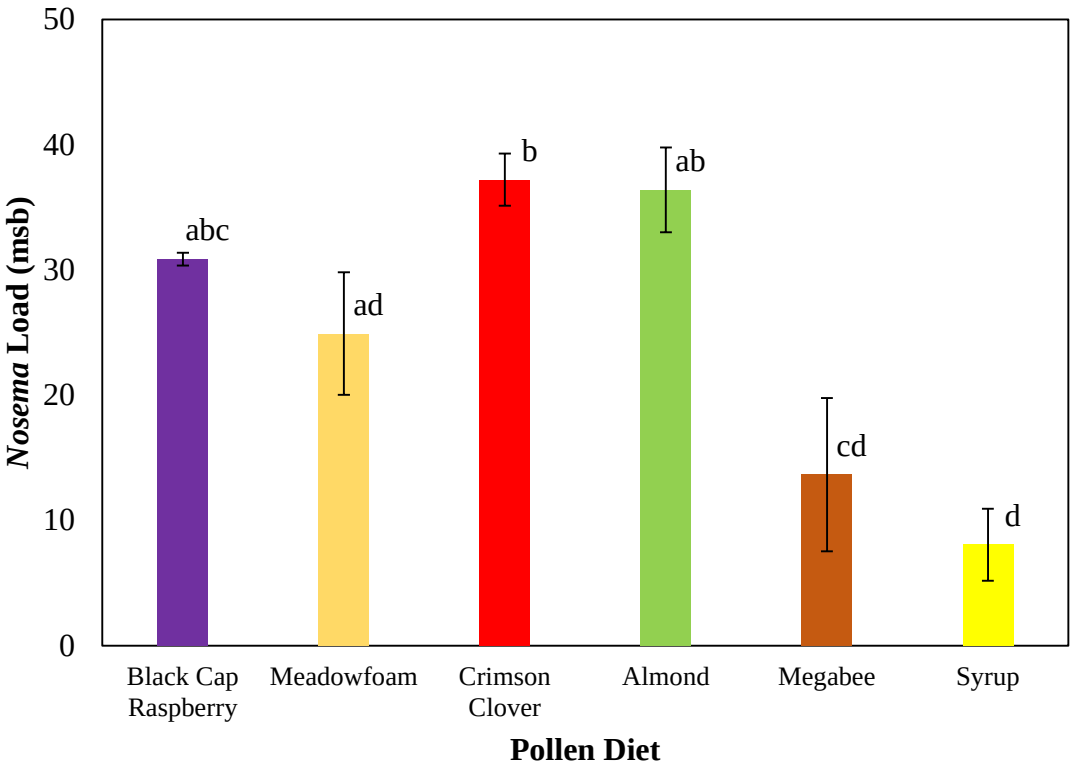


Figure 23. Mean (± SE) *Nosema* load (millions of spores per bee) for honey bees fed pollen diets prepared from pollen collected in Black Cap Raspberry fields ($F_{4, 35}$ = 6.57; p = 0.0005). Means that differ significantly are indicated by different letters.

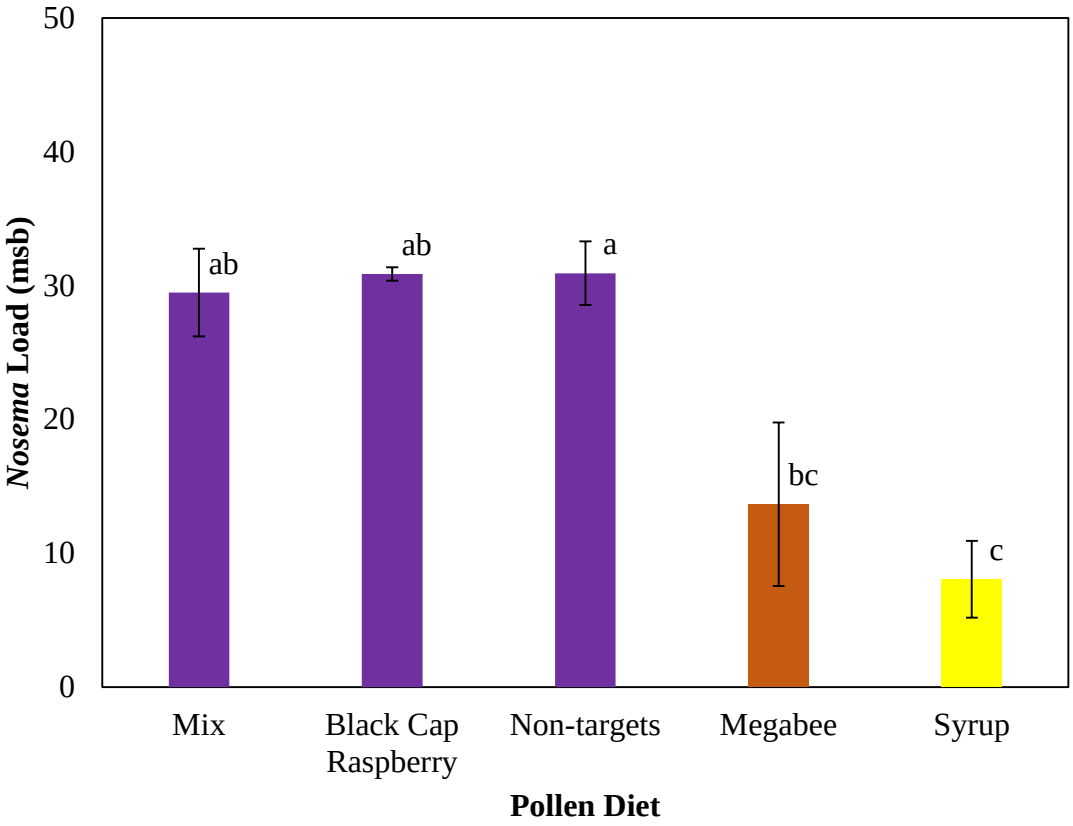


Figure 24. Mean (± SE) *Nosema* load (millions of spores per bee) for honey bees fed pollen diets prepared from pollen collected in Crimson Clover fields ($F_{4, 35} = 18.5$; $p < 0.0001$). Means that differ significantly are indicated by different letters.

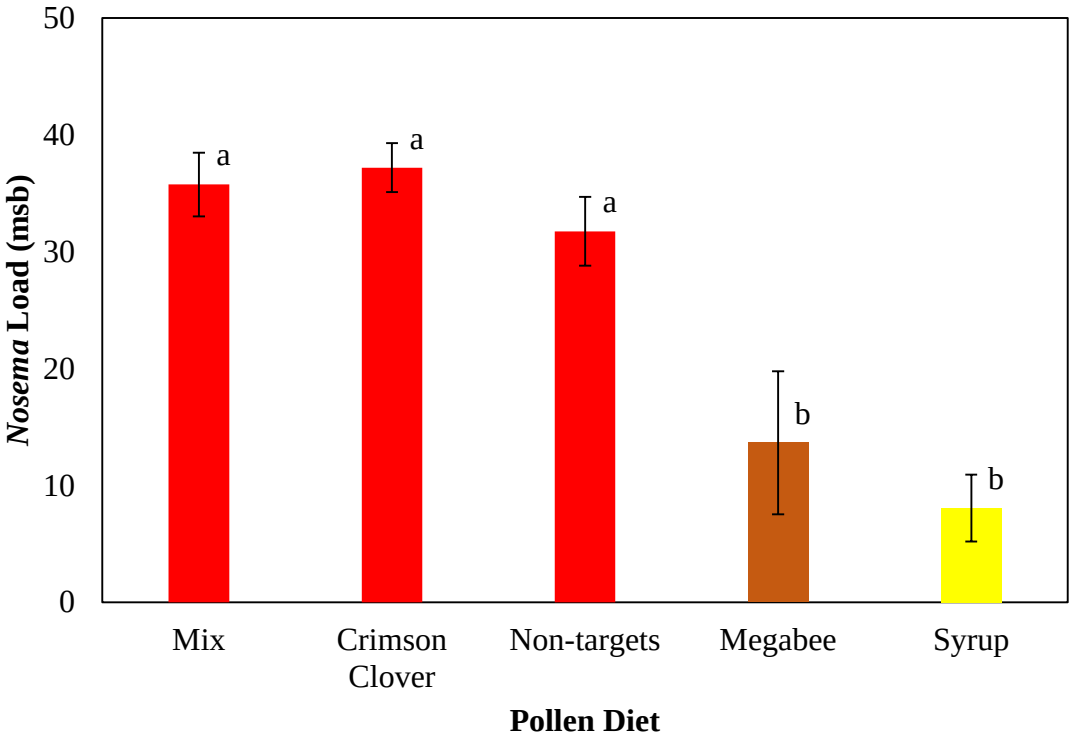


Figure 25. Mean (± SE) *Nosema* load (millions of spores per bee) for honey bees fed pollen diets prepared from pollen collected in Meadowfoam fields ($F_{4, 17} = 5.89$; $p = 0.0037$). Means that differ significantly are indicated by different letters.

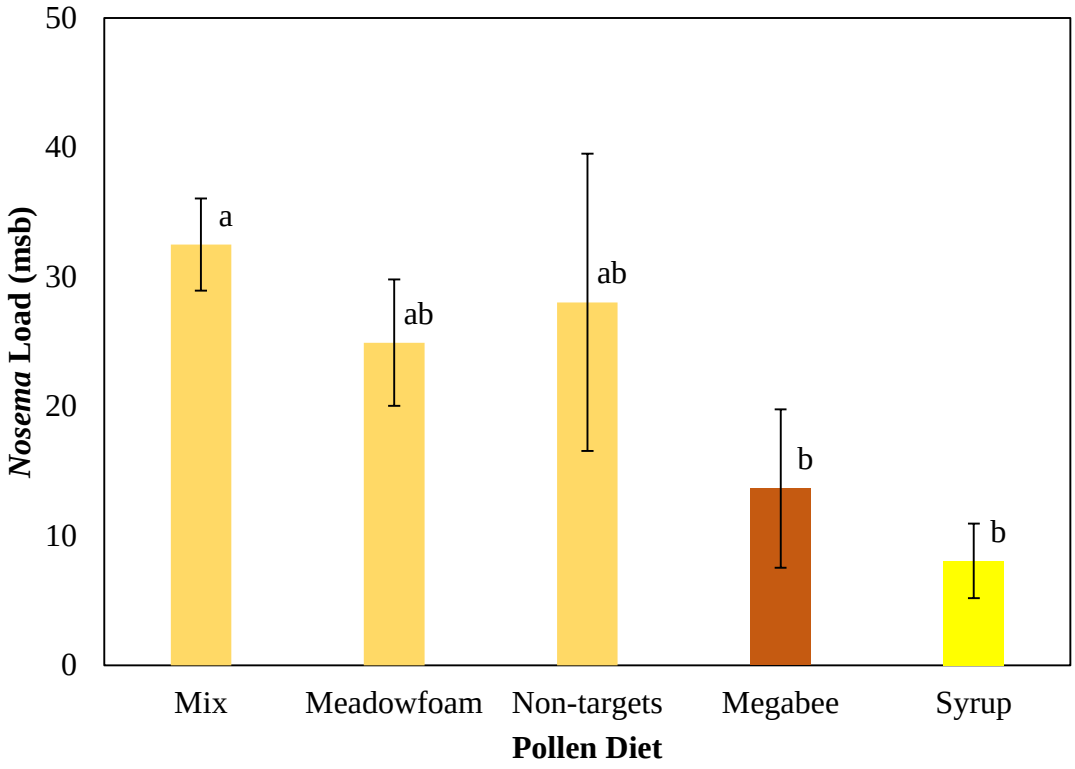


Figure 26. Mean (± SE) *Nosema* load (millions of spores per bee) for honey bees fed pollen diets prepared from pollen collected in Almond fields ($F_{3, 21} = 10.98$; $p = 0.0002$). Means that differ significantly are indicated by different letters.

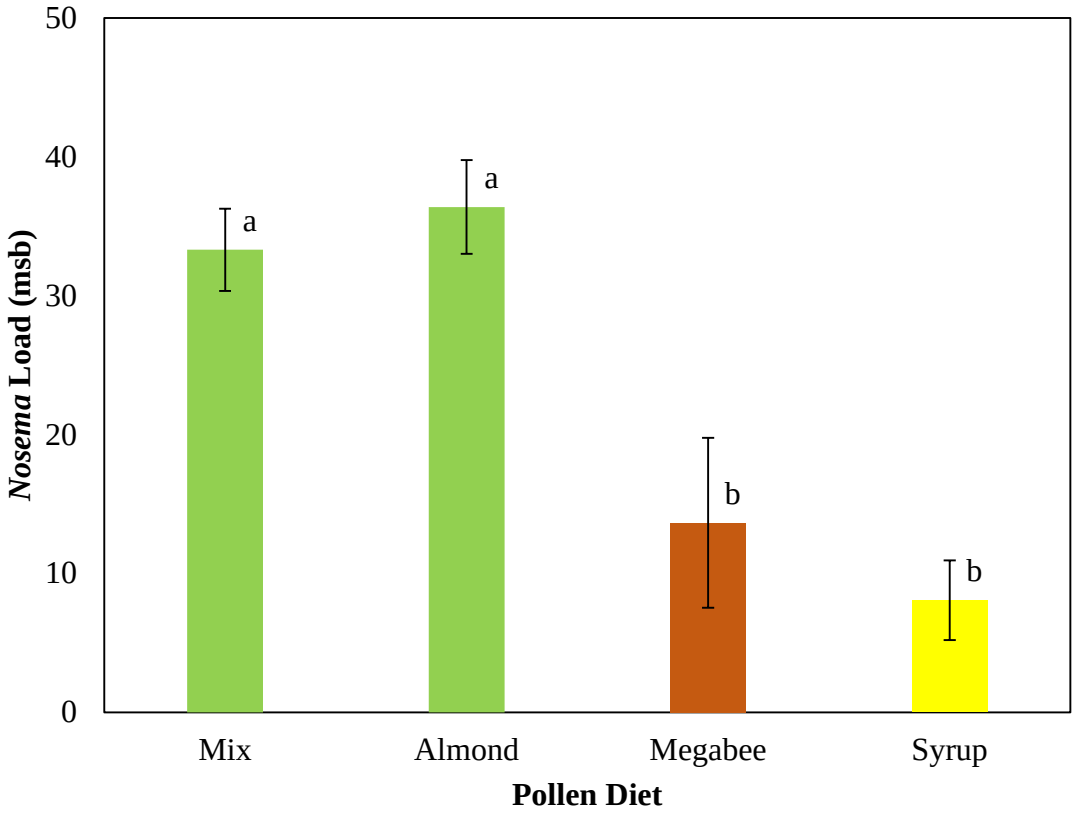
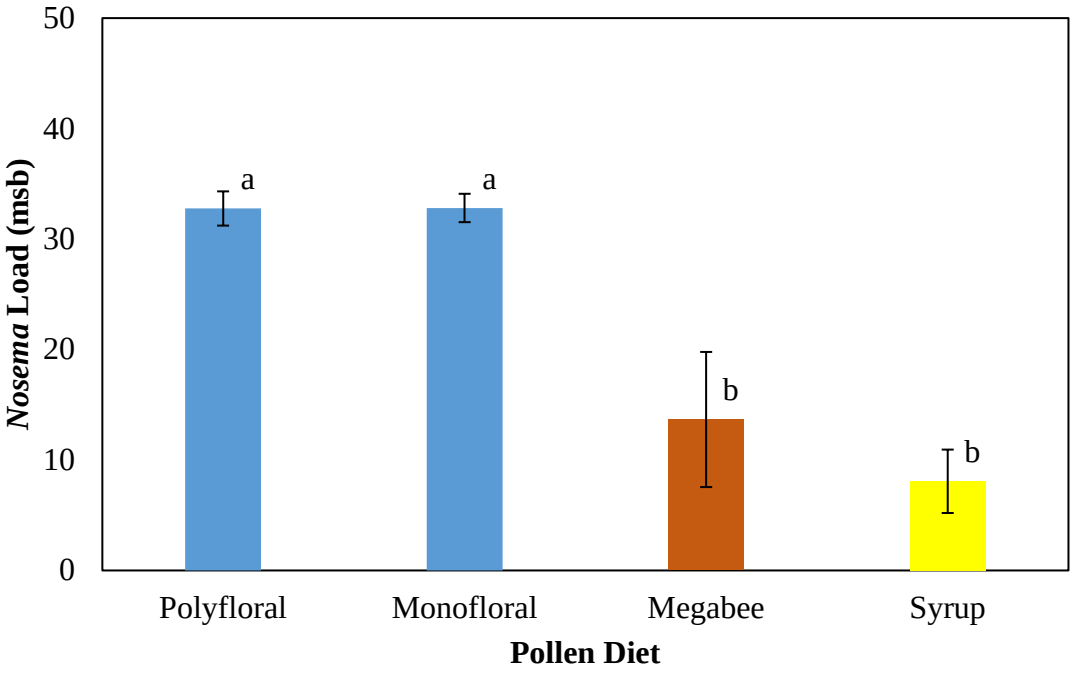


Figure 27. Mean (± SE) *Nosema* load (millions of spores per bee) for honey bees based on diet variety ($F_{3, 111} = 12.44$; $p < 0.0001$). Means that differ significantly are indicated by different letters.



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