Final Research Report to the National Honey Board

Ramesh Sagili and Stephanie Parreira

Department of Horticulture, Oregon State University, Corvallis, OR 97331-7304

Project title: **Effects of commonly used agrochemicals and their interactions on honey bee colony health**

Pesticides are presumed to play a significant role in honey bee colony declines, but impacts of pesticides on honey bee health are not well understood. Unfortunately, there is a gap in understanding the colony-level impacts of agrochemicals in order to make conclusive interpretations, except the fact that many pesticide residues have been documented in honey bee hive matrices. The majority of existing studies have focused on effects on individual worker bees, determined LD50 toxicities, or only examined the lethal or sub-lethal effects of a single pesticide exposure. Further, most of these studies were conducted in a laboratory set-up in small cages that do not represent a realistic colony environment, and many times the pesticide concentrations tested are not realistic. Also, there is a huge gap in understanding the additive and synergistic effects of agrochemicals on bees.

Our goal here was to understand the impact of field realistic concentrations of two predominantly used agrochemicals and their interactions on honey bee colony health. Specifically, we examined the independent and interaction effects of a fungicide chlorothalonil and a neonicotinoid insecticide imidacloprid, on honey bee colony health for a period of one year. Both these chemicals are commonly found in honey bee colonies in relatively high concentrations. The fungicide Chlorothalonil is used ubiquitously on a variety of crops that are pollinated by honey bees. VanEngelsdorp et al. (2009) found that entombed pollen (sealed to prevent consumption) contained high concentrations of chlorothalonil. Imidacloprid is the second most widely used insecticide in the world (Pettis et al. 2012) and is used in a number of bee pollinated crops.

OBJECTIVES

The overall goal of this project is to gain comprehensive understanding of the impact of field realistic concentrations of predominantly used agrochemicals and their interactions on honey bee colony health. Following were the specific objectives of this study:

Evaluate independent and interaction effects of imidacloprid and chlorothalonil on honey bee colony physiology, colony health and foraging behavior.

MATERIALS AND METHODS / EXPERIMENTAL DESIGN

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

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

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

Pollen Patty Diet and Pesticide Mixing

Colonies were exposed to imidacloprid and/or chlorothalonil via a pollen patty diet. Bee collected pollen from Yukon, Canada (The Pollen Man®) was finely ground and used for making the pollen patties. The pollen was analyzed for pesticide content by the USDA-AMS-NSL

laboratory in Gastonia, NC. Only trace levels of few pesticides were detected in the pollen with no detectable levels of imidacloprid or chlorothalonil.

Pollen patties were made fresh every week for 4 weeks. Imidacloprid and chlorothalonil (pure active ingredients, Sigma Aldrich®, MO, USA) were infused to pollen through acetone solutions. The target concentrations of the two chemicals in the pollen patties were chosen based on the mean concentrations of these chemicals in stored pollen, as reported by Mullin et al. [58]: 39 ppb imidacloprid and 3014.8 ppb chlorothalonil. These concentrations are results from the only extensive documentation of pesticide concentrations in stored pollen in North America. The concentrations used here represent field-realistic concentrations in bee bread, and provide a balance between the lowest and worst-case scenario concentrations that may occur in the field. From each pollen patty in a given treatment group, we removed a 2-g sample of pollen patty. All patty samples in each treatment group were combined and sent to the USDA National Science Laboratory in Gastonia, NC to confirm concentrations.

Pollen Patty Feeding and Consumption

Pollen patties were removed and replaced weekly for four weeks (June 30, July 7, July 14, July 21). During this time, pollen traps were placed on all colonies during the exposure period to prevent the consumption of incoming pollen and induce feeding on the pollen patty diet. Patties were placed between hive bodies for each colony using a 4.5-cm spacer. Pollen consumption for each week was calculated as the change in pollen mass, accounting for the weight of the pie tin. Samples of the remaining pollen patties were also sent to the USDA analytical laboratory to determine whether the concentrations changed or remained stable over the course of each week.

Colony Evaluations

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

Forager Counts

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

Hypopharyngeal Gland Protein Content

We quantified hypopharyngeal gland protein content using a standard BCA assay (Pierce Biotech BCA Assay Kit, Thermo Scientific, IL, USA). Hypopharyngeal glands (HPG) were dissected from 10 nurse bees per colony. All glands from the bees of one colony were pooled together in 125 µL of phosphate-buffered saline (PBS; 10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4, Sigma Aldrich), and stored at -80˚C until time of analysis. The protocol was adapted from Jack et al. [178], and modified for protein quantification in the glands of 10 heads instead of 1.

Hemolymph Extraction

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

Prophenoloxidase Activity (ProPO)

Prophenoloxidase activity (ProPO) was measured as maximum linear rate (Max V) of the substrate (L-Dopa) conversion per milligram of hemolymph protein. Prophenoloxidase activity

was determined using a protocol adapted from Laughton and Siva-Jothy, Laughton and Siva-Jothy, Wilson-Rich et al. We quantified the amount of hemolymph protein per sample with a standard BCA assay similar to that used to quantify hypopharyngeal gland protein content. Hemolymph BCA assays differed from hypopharyngeal gland BCA assays only in that the BSA standards were diluted in 1:1 NaCac:PBS, and samples were plated as ½ dilutions (in PBS).

Glucose Oxidase Activity

We measured glucose oxidase (GOX) activity in pooled samples of 10 whole heads, using a colorimetric kinetic assay. These heads were immediately frozen in dry ice and then stored at -80°C until time of homogenization and GOX analysis. Each sample was homogenized in 500 µl of sodium phosphate lysis buffer (80 mM sodium phosphate monobasic/sodium phosphate dibasic pH 7.4, 20 mM sodium chloride, 1% Triton X-100, 1X Halt™ EDTA-free protease inhibitor (Thermo Scientific, IL, USA)), with one 3-mm tungsten carbide bead in a Qiagen® TissueLyser II at 30 oscillations/second for 2 rounds of 1.5 minutes. The resulting homogenates were centrifuged at 14,000 rpm for 15 minutes, 4 °C. Supernatants were then pipetted into clean tubes and centrifuged again for 4 minutes at 14,000 rpm, 4 °C to further pellet any remaining cellular debris. From those supernatants we made $1/5^{th}$ dilutions in lysis buffer.

The assay was performed on a BioTek® Synergy 2 plate reader, with a hydrogen peroxide standard curve (nmoles H_2O_2 produced/microplate well). Ten microliters of each standard or sample dilution were combined with 180 µl of sample reaction mix (100 µl distilled, autoclaved water, 50 µl 0.5 M potassium phosphate pH 7, 20 µl 0.5 M D-glucose, and 2.5 U horseradish peroxidase (10 µl)) in each of two replicate wells in a chilled Greiner Bio-One® non-binding 96-well plate. For sample blanks, 10 µl of each diluted sample were combined with 180 µl of a similar mixture—differing only in that glucose was replaced with an additional 20 µl of water. After shaking the plate for 2 minutes at 37 °C, 20 µl of o-dianisidine dihydrochloride (0.95 mg/ml, Sigma-Aldrich®, MO, USA) were added to each well. Absorbance at 430 nm, 37 °C, was measured every 34 seconds for 1.5 hours, with continuous shaking. We then calculated the units of glucose oxidase activity present in each sample. One unit of GOX activity is denoted as the amount of the enzyme required to generate 1.0 µmole of H_2O_2 per minute at 37 °C.The final glucose oxidase values were calculated as milliunits per mg of protein in each sample. We

quantified the protein concentration of the samples using a standard BCA assay, as previously described. Samples were analyzed as $1/20^{th}$ dilutions (in PBS).

Abdominal Lipid Stores

A pooled sample of 10 nurse bees per colony were used to quantify abdominal lipid stores using the protocol of Wilson-Rich et al.

Midgut Proteolytic Enzyme Activity

Midgut proteolytic enzyme activity was measured by recording absorbance at 440 nm with a Beckman® DU spectrophotometer as in Jack et al.2016.

RESULTS

Pesticide Concentrations

The results from the USDA analytical laboratory confirmed that imidacloprid concentrations used in this study were consistently near the intended concentration, averaging 35 ppb. Our chlorothalonil concentrations were, however, only 3.6% of the intended concentration, averaging 108 ppb. Potential reasons for these discrepancies are outlined in the Discussion section. Imidacloprid concentrations also remained stable in our pollen patties throughout each week, as whereas there were no longer detectable levels of chlorothalonil in pollen patties by the end of the week.

Pollen Patty Consumption

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

Adult Bee Population

Adult bee population was not significantly different between levels of imidacloprid ($F_{1, 24} = 0.090$, $p = 0.767$), chlorothalonil ($F_{1, 24} = 1.806$, $p = 0.193$), or the combination of both chemicals ($F_{1, 24} = 0.295$, $p = 0.592$). Adult bee population was significantly affected by time ($F_{3, 96} = 99.351$, $p < 0.0001$). Neither the effect of imidacloprid ($F_{3, 96} = 0.517$, $p = 0.672$), chlorothalonil

($F_{3, 96} = 0.368$, $p = 0.777$) nor was there an interaction between both chemicals and time ($F_{3, 96} = 0.337$, $p = 0.798$).

Percent Change in Adult Bee Population

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

Brood Area

In the model containing all data points, brood area was not affected by imidacloprid ($F_{1,24} = 0.281$, $p = 0.601$) or chlorothalonil ($F_{1,24} = 0.0008$, $p = 0.9775$). The interaction between chlorothalonil and imidacloprid was nearly significant at the 0.10 alpha level ($F_{1,24} = 2.886$, $p = 0.102$). Brood area was also significantly different between months for all groups ($F_{3,96} = 269.021$, $p < 0.0001$). There were no significant interactions between any of the treatments and time.

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

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

Percent change in Brood Area

Percent change in brood area was not significantly affected by imidacloprid exposure ($F_{1, 24} = 0.030$, $p = 0.863$) or chlorothalonil exposure ($F_{1, 24} = 0.128$, $p = 0.723$). The interaction between both chemicals also did not significantly affect change in brood area ($F_{1, 24} = 1.012$, $p = 0.325$).

Neither the effect of imidacloprid ($F_{2, 64} = 0.1091$, $p = 0.897$) nor the effect of chlorothalonil ($F_{2, 64} = 1.272$, $p = 0.287$) had a significant interaction with time. There was also no significant interaction between imidacloprid, chlorothalonil, and time ($F_{2, 64} = 0.384$, $p = 0.683$).

Pollen Foragers

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

The median number of pollen foragers in the colonies was different between different weeks ($F_{12, 371} = 14.866$, $p < 0.0001$). However, neither the effect of imidacloprid ($F_{12, 371} = 0.991$, $p = 0.457$) nor chlorothalonil ($F_{12, 371} = 0.967$, $p = 0.480$) interacted with time. There was also no interaction between both chemicals and time ($F_{12, 371} = 0.918$, $p = 0.529$).

Non-Pollen Foragers

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

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

The interaction between imidacloprid exposure and time indicated that colonies exposed to imidacloprid had different numbers of returning non-pollen foragers than colonies not exposed to imidacloprid prior to treatment (at week 0), and 7 weeks after the conclusion of the exposure

period (week 11). Prior to treatment, the median number of returning non-pollen foragers in colonies assigned to imidacloprid exposure was nearly 1.5 times greater than colonies not assigned to imidacloprid exposure. At Week 1, these differences no longer existed, indicating that the number of returning non-pollen foragers increased significantly more in colonies that were not exposed to imidacloprid than colonies that were during the first week of exposure. Further, seven weeks after the exposure period ended, the median number of returning non-pollen foragers for colonies not exposed to imidacloprid was 1.4 higher than that of colonies exposed to imidacloprid.

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

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

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

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

Total Foragers

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

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

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

The effect of imidacloprid was such that overall, colonies not exposed to imidacloprid had an overall greater number of returning foragers than colonies exposed to imidacloprid. The same was true for the effect of chlorothalonil. We estimate that there were approximately 8.6 more foragers returning every 3 minutes to colonies not exposed to imidacloprid than those colonies exposed to imidacloprid, and 8.1 more foragers returning every 3 minutes to colonies not exposed to chlorothalonil than for those exposed to chlorothalonil. Furthermore, based on pairwise comparisons, these differences did not exist between these groups prior to treatment.

Hypopharyngeal Gland Protein Content

Hypopharyngeal gland protein content did not differ significantly between levels of imidacloprid ($F_{1, 24}$ = 1.050, p = 0.316) or chlorothalonil ($F_{1, 24}$ = 2.066, p = 0.164). It was also unaffected by

the combination of both chemicals ($F_{1, 24} = 0.071$, $p = 0.792$). Furthermore, there were no interactions between either of the chemicals, their combination, and time. Furthermore, hypopharyngeal gland protein content was not significantly different between months ($F_{2, 64} = 0.372$, $p = 0.691$).

Prophenoloxidase Activity

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

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

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

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

Glucose Oxidase Activity

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

Abdominal Lipid Stores

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

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

Midgut Proteolytic Enzyme Activity

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

DISCUSSION

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

In our experiment, honey bee colony size and brood area were not significantly affected by exposure to imidacloprid, cholorothalonil or the combination of both chemicals. These results are similar to several other field studies, especially with respect to imidacloprid. We did not observe any periods in which there were no brood in the colonies. This observation differs from the patterns previously observed at concentrations of 20 ppb and 100 ppb. Considering that our experimental concentration was within this range, it is possible that the concentration of imidacloprid was not the sole reason for breaks in the brood cycle in the previous experiment. For example, the subspecies used in that study was not disclosed. This may be potentially because of diet differences, but may also be due to differences in the experimental subspecies.

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

The potential for subspecies to influence the outcome of a pesticide experiment has been documented in some recent studies. Sandrock et al. found that long-term changes in colony size

and brood area in response to neonicotinoid exposure differed between *A.m. carnica* and *A.m. mellifera*. Effects on *A.m. ligustica* were not examined in that study. Another previous study (Danka et al. 1986) also demonstrated that pesticide sensitivity varies between both honey bee subspecies, though this varies between pesticides. Otherwise, studies determining how different honey bee subspecies respond to pesticide exposure are rare. Further research should be conducted to determine whether specific subspecies are more robust to long-term pesticide exposure than are others.

The results of our physiological assays support the findings of some previous studies, while contradicting others—even when field realistic concentrations of chlorothalonil were used. Similar to a previous experiment, we found that chlorothalonil exposure does not affect the lipid stores of nurse bees in experimental colonies. The findings for hypopharyngeal gland-related analyses, however, may contradict previous findings that chlorothalonil reduces total protein content and increases glucose oxidase activity of nurse bees. Previous data on the effects of chlorothalonil on prophenoloxidase activity are also mixed. Our results differ from the findings in which chlorothalonil did not affect prophenoloxidase activity in nurse bees or foragers bees in the field, but are in agreement with a laboratory experiment in which chlorothalonil increased transcription of prophenoloxidase-activating enzymes. The contradiction of the field experiment may be due to our higher experimental concentration (~100 ppb instead of 10 ppb [142], and exposure through pollen diet instead of sugar syrup. Because nurse bees consume more pollen than they do sugar syrup (Rortais et al. 2005), pesticides in pollen may pose a greater risk for nurse bees than do pesticides in nectar. Furthermore, because chlorothalonil concentrations are well-documented for pollen but virtually unknown in nectar, we can be more certain that studies pertaining to exposure through pollen are field-realistic. However, we recognize the need to quantify chlorothalonil concentrations in nectar and honey in order to gain a clearer understanding of field-realistic exposure through multiple exposure routes. Future research should also focus on determining the impacts of chlorothalonil when concentrations are at the higher end of the field-realistic spectrum.

We found that imidacloprid had no significant effect on activity of the immune enzymes, glucose oxidase, prophenoloxidase, and hypopharyngeal gland protein content. In a laboratory experiment, Alaux et al. demonstrated a similar lack of effect on glucose oxidase activity, but

previous findings on effects of imidacloprid on prophenoloxidase activity are mixed. Very few studies have examined the protein content of hypopharyngeal glands as it relates to imidacloprid exposure, but some research has investigated how the effects of imidacloprid exposure might manifest in hypopharyngeal gland size—with mixed results. It may be possible that smaller hypopharyngeal gland size does not necessarily translate into lower hypopharyngeal gland protein content in honey bees, but this is unlikely based on previous research. We also may not have detected effects on hypopharyngeal gland protein content because our data is from an open-field experiment, not a laboratory experiment. The social component of honey bee biology that is incorporated into open-field experiments—including the presence of brood and a queen, which both alter worker bee behavior and physiology may buffer the effects of pesticide exposure in a way that has not been discovered. Previous experiments explored effects at much lower concentrations than used in this experiment, but all were conducted in the laboratory, thereby leaving out critical elements of sociality that may mitigate such effects. Furthermore, we exposed our colonies to contaminated pollen instead of contaminated sugar syrup. Because worker bees tend to consume different amounts of pollen and sugar syrup based on their age, and because the times at which they consume different resources also coincides with changes in physiology, the potential for pesticides to affect bees differently through different exposure routes should not be overlooked in experiments and risk assessments. Multiple exposure routes are frequently ignored in the current literature. With the exception of one experiment (Alburaki et al. 2015), laboratory experiments have largely determined the impacts of imidacloprid exposure through sugar syrup only [222-224]. Lastly, none of these experiments focused solely on *A.m. ligustica*, and only one included this subspecies (mixed with *A.m. mellifera*). Because hypopharyngeal gland size and number of secreted brood food proteins differ widely between specific honey bee strains and subspecies, it is important to note that the impacts of pesticide exposure on hypopharyngeal gland protein content may also differ between these subspecies.

Though the impact of imidacloprid exposure on abdominal lipid stores of nurse bees is not thoroughly explored in previous research, a previous experiment found that different genes involved in lipid metabolism were significantly upregulated and downregulated in larvae from colonies that were exposed to an even lower concentration of imidacloprid in sugar syrup. Low concentrations of imidacloprid also alter levels of antimicrobial peptides, which are produced in the abdominal lipid stores (the fat body). However, how these changes translate into actual

changes in lipid stores is not yet clear. Additionally, another previous experiment determined that two metabolites of imidacloprid, which are more likely to impact brood in this experiment (see the discussion of effects on foraging), caused precocious foraging in bees as young as 8 days old. Because precocious foraging is associated with reduced longevity, and because precocious foraging occurs in conjunction with the early loss of abdominal lipids, further work is needed to confirm whether these effects take place at field-realistic concentrations.

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

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

The effects we detected in non-pollen foraging, seven weeks after the end of the exposure period suggests that larvae fed with chemical contaminated pollen were affected as adults. This is consistent with previous findings that imidacloprid significantly impacts the olfactory behavior of adults exposed to imidacloprid as larvae, and significantly alters the expression of genes related to flight performance, metabolism and longevity in exposed larvae. Imidacloprid itself has only been detected in brood food when imidacloprid is at much higher concentrations—100 ppb—in the pollen diet of the nurse bees. Based on the rapid pace at which imidacloprid is metabolized in adult honey bees and the presence of its metabolites in the honey bee head soon after oral exposure, it is more likely that metabolites, rather than the active ingredient, are causing these effects. Dively et al. did not detect any imidacloprid metabolites in brood food after exposing colonies to protein diet spiked with imidacloprid, but noted this may be due to the high level of detection for those metabolites. Although previous work has estimated how much imidacloprid different workers consume within a specified period of time, there are currently no such estimations for the amount of metabolites consumed by worker larvae, and no data on the presence of chlorothalonil metabolites in brood food.

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

While our results pertaining to the effects of imidacloprid on foraging contribute to existing knowledge, the findings regarding negative effects of chlorothalonil on foraging are novel. Because chlorothalonil is relatively non-toxic to bees, it is possible that its effects on foraging result indirectly from its effects on pathogens such as *Nosema*. Previous findings have linked both chlorothalonil and imidacloprid exposure to higher *Nosema* infection, which also impairs foraging abilities and worker survival. We did not detect such patterns in this experiment, but

this may be due to the sampling methods we used to quantify *Nosema* infection. We quantified *Nosema* in bees that were collected from comb area with open brood, and hence were more likely to be nurse bees. Because forager bees typically have higher *Nosema* loads than nurse bees, our findings may have been different if we had specifically sampled forager bees from our experimental colonies. Further research is needed to determine effect of these chemicals on *Nosema* loads specifically in forager bees to capture interactions impacting foraging success.

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

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

large outliers may mask the true long-term effects of pesticide exposure, which likely leads to misleading conclusions and ineffective management recommendations.

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

Statistical power also may have played a critical role in our ability to detect the effects of these field realistic concentrations of chemicals on honey bee colony health. Cresswell found that only 50% of field experiments had high enough statistical power to detect adverse effects of pesticides. While our experiment utilized higher replication than most other field experiments and minimized variation present in other experiments by keeping all colonies in the same apiary and using sister queens to minimize genetic variability, our replication is still lower than is typically recommended to detect effects. Statistical power may not have been high enough to detect effects on one or more of these response variables, especially if such effects led to slight differences. Our statistical power may have prevented us from detecting significant effects and interactions for responses where p-values were considerably low, but not low enough to be considered statistically significant at either the 0.10 or 0.05 alpha level. We suggest that researchers conduct further studies that maximize replication, minimize variation and background noise. It is possible that an experiment with higher replication would have more precisely estimated the distribution of non-pollen foragers, reduced the presence of outliers in the data, and more accurately estimated differences between groups. Future studies should seek to identify an optimal number of replicates to detect these effects. However, it is also important to note that experiments with higher replication are near impossible to do if it is critical to take all measurements and samples on the same day, or within the same time frame. Some of the parameters we estimated, such as colony size and food stores, took up to 2 days to survey all 36 experimental colonies.

PUBLICATIONS AND PRESENTATIONS

Research finding will be published in the online journal PLoS ONE. Further the research results will also be disseminated at American Beekeeping Federation (ABF) annual conference, Entomological Society of America (ESA) annual meeting, and also at the Oregon State Beekeepers conference.

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