Mitigating Adverse Effects of Pesticides on Honey Bees Through Dietary Phytochemicals

Project No.: **POLL18-Berenbaum**
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A. Summary

Pesticides are generally ingested by bees along with dietary phytochemicals in nectar and pollen, as well as with adjuvants; these chemicals can all interact to influence bee health. We have shown that consuming p-coumaric acid and quercetin increases worker longevity ingested in concentrations naturally occurring in honey. Moreover, chronic exposure to the fungicide propiconazole reduces worker longevity at field concentrations (0.9 ppm). Propiconazole (Tilt) interacts with the insecticide chlorantraniliprole (Altacor) when ingested, consistent with the results from our Ohio State collaborators. Our data also show that nurses consuming pesticide-contaminated pollen provide sub-optimal care of queen larvae. Nurses ingesting propiconazole and chlorantraniliprole with an adjuvant tended queen larvae less assiduously than those consuming uncontaminated pollen. These behavioral responses were not observed in bees provided with quercetin-supplemented sugar water.

Additionally, via electroantennography, we found pesticide-induced alterations in olfactory responsiveness to brood pheromones, a larval signal pheromone (ocimene), and the alarm pheromone 2-heptanone. Our preliminary data, from one hive, show that, in the presence of the adjuvant Dyne-Amic, nurse bees ingesting Altacor and Tilt together exhibit reduced sensitivity to olfactory stimuli. However, response patterns varied with hive identity; additional replicates of these trials may reduce this variation.

To characterize phytochemicals of almond honey/pollen, we collected first batch almond honey/pollen from a California almond orchard and farmers market this Spring. Via HPLC-MS, we identified as abundant constituents in almond honey abscisic acid and the flavanone galangin. Surprisingly, amygdalin, assumed to occur in almond honey, was not detected. Other constituents are currently being identified. Almond honey was also evaluated for its antioxidant capacity relative to other monofloral honeys. Almond honey exhibited the second-highest antioxidant capacity of all tested monofloral honeys, exceeded only by buckwheat honey. We completed a longevity assay of adult bees evaluating the effect of propiconazole- chlorantraniliprole ingestion along with the almond honey and are analyzing the results.

B. Objectives

- **Objective 1: Determine effects of pesticide/adjuvant/phytochemical interactions on queen quality**
Progress: The manuscript reporting the effects of the fungicide propiconazole, the insecticide chlorantraniliprole and two phytochemicals, quercetin and p-coumaric acid, interactions on worker longevity has been prepared and will be submitted soon. Our nursing behavior evaluation system and electroantennography system have been tested and are now ready to evaluate the effects of pesticide/phytochemical interactions on queen cell nursing behavior and on olfactory impairment. The insecticide Altacor, the fungicides Pristine and Tilt and the phytochemical quercetin have been evaluated with and without the adjuvant Dyne-Amic in 2019 with noted alterations in pesticide toxicity in the presence of the adjuvant. Although we have completed a number of behavior trials, testing with other

adjuvant chemistries and phytochemicals and completing additional replicates remain to complete in 2020.

- **Objective 2: Evaluate impacts of almond phytochemicals**

  Progress: We collected almond honey and pollen in 2019 and we have a preliminary analysis of antioxidant and phytochemical characteristics of almond honey. Additional replicates of LC-MS analysis will be performed in 2020. We also evaluated effects of amygdalin and intact almond honey on worker longevity. Additional replicates are needed to evaluate statistically the effect of almond honey on honey bee longevity in 2020.

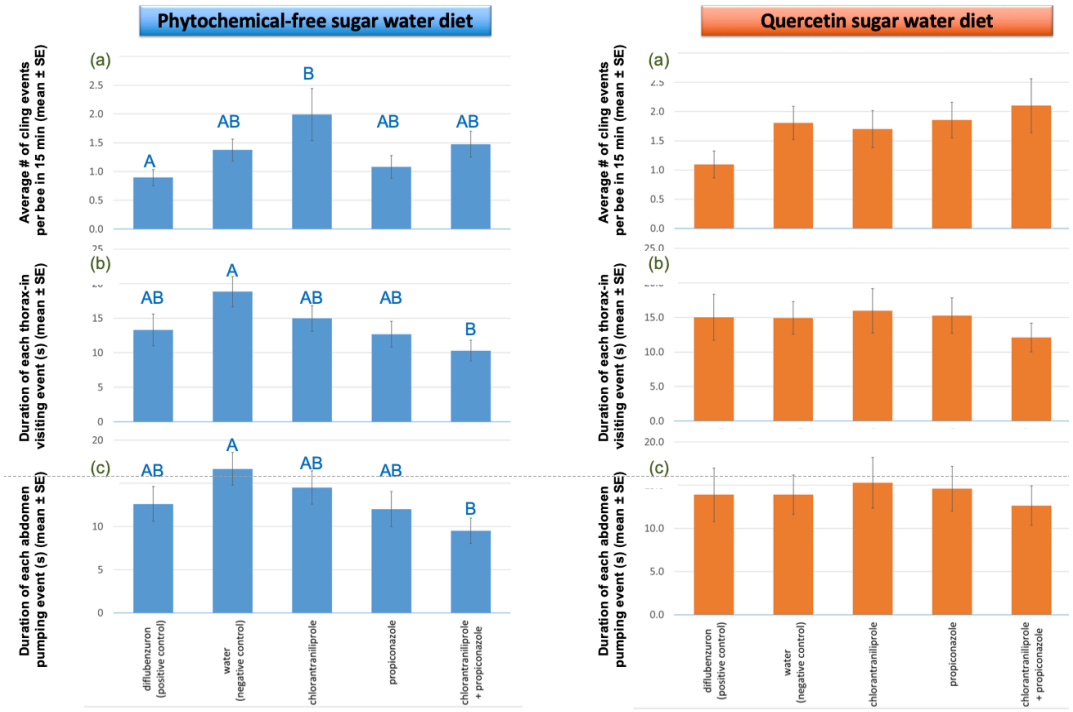
C. Annual Results and Discussion

Based on the California Department of Pesticide Regulation's Pesticide Information Portal and combined with conversations with stakeholders, the potential for interactions among six fungicides, three insecticides and four spray adjuvants when applied alone, in combinations (139 total treatments) to affect adult workers and larval workers was evaluated by our collaborator Reed Johnson and his Ohio State colleagues. Based on their results, we tested for effects of several high-risk candidate pesticides individually, interactively, and in combination with phytochemicals on honey bee health: the insecticides Altacor and Intrepid, the fungicides Tilt and Pristine, and the adjuvant Dyne-Amic were selected as representative pesticides for combination testing.

- **Objective 1: Determine effects of pesticide/adjuvant/phytochemical interactions on queen quality**

1.1 Effects of Pesticides/Phytochemical Interactions on Queen Cell Nursing Behaviors

Mixed propiconazole and chlorantraniliprole reduced the duration of visiting and abdomen pumping behaviors of nurse bees consuming phytochemical-free sugar water.



Consuming 250µM quercetin-supplemented sugar water neutralized all impaired nursing behaviors with all pesticide treatments. We detected no significant difference between any treatments.

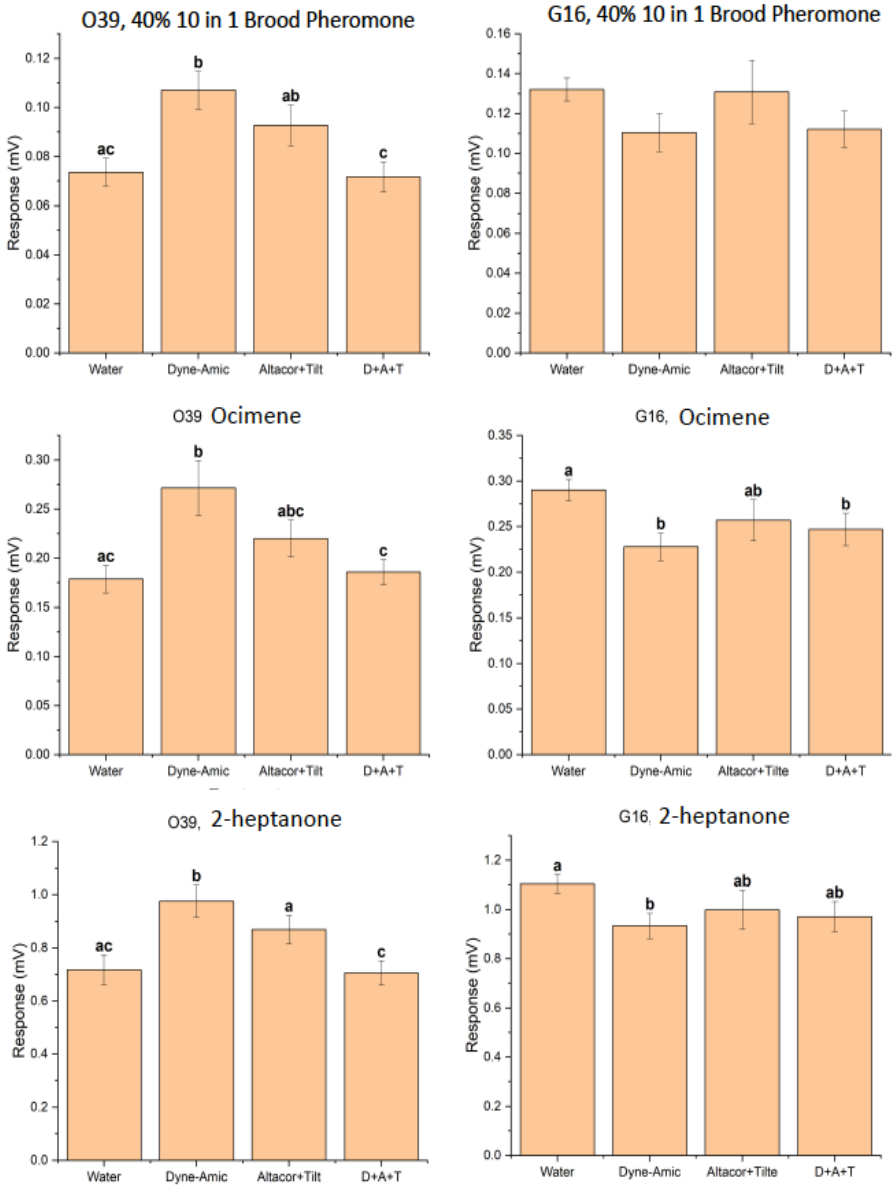
1.2 Effects of Pesticides on Olfactory Impairment via Electroantennography (EAG)

We conducted assays to assess pesticide-induced alterations in olfactory responsiveness to brood pheromone as a possible mechanism underlying nursing behavior changes.

We measured nurse bee EAG responses to serial concentrations (5%, 10%, 20%, 40% and 100%) of synthetic non-volatile brood pheromone and a volatile brood pheromone, ocimene, to detect signs of olfactory impairment. In addition to the non-volatile brood pheromone, we also tested mineral oil as a negative control and 2-heptanone, an alarm pheromone, as a positive control.

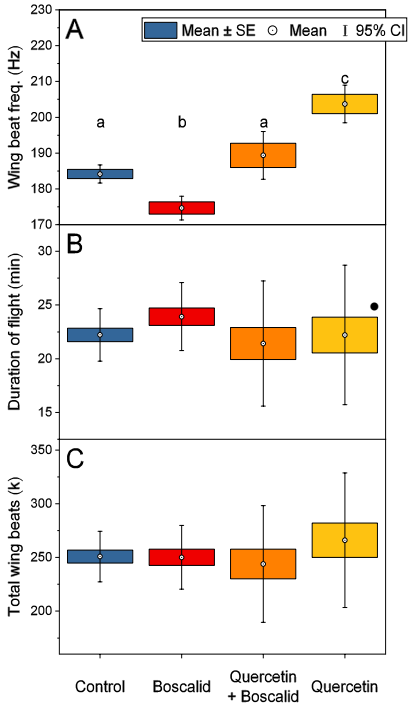
The mixture of the fungicide Tilt and the insecticide Altacor did not alter the olfactory responses of nurses. However, the adjuvant Dyne-Amic may alter the olfactory response of nurse bees to brood pheromones and the alarm pheromone 2-heptanone.

Our data, from one hive (hive O39), also show that, in the presence of the adjuvant Dyne-Amic, nurse bees ingesting Altacor and Tilt together exhibit reduced sensitivity to olfactory stimuli. Nevertheless,due to differences in responses between hives, no consistent pattern could be detected. Further analysis is ongoing.



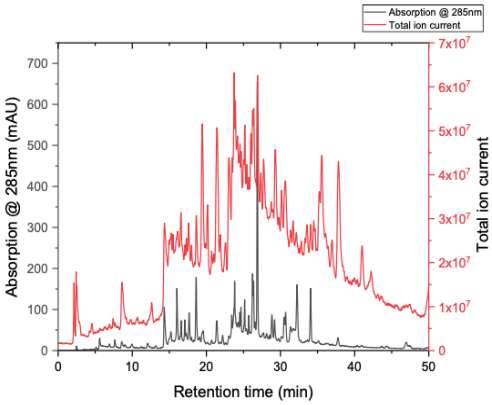
1.3. Effects of Fungicide Boscalid and Phytochemical Quercetin interation on Flight Performance of Workers

The fungicide boscalid inhibits mitochondrial complex II, part of the ATP-generating system that provides energy to cells and tissues. When bees consume boscalid with sugar water, their wingbeat frequency declines, possibly impairing their foraging ability. However, pollen and honey contain quercetin, a phytochemical that boosts ATP levels in flight muscles. When bees consumed quercetin with the fungicide boscalid, wingbeat frequencies were restored to normal levels. Thus, their natural diet may protect bees against fungicide toxicity.



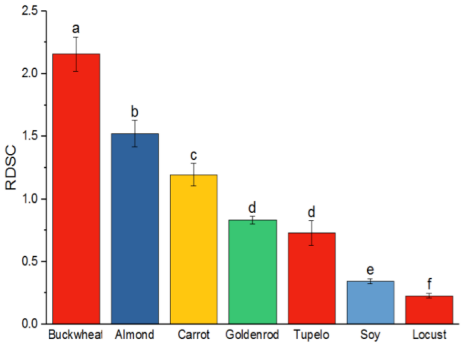
(Figure to the left): Effects of a phytochemical (quercetin) and fungicide (boscalid) individually and in combination on the flight performance of foragers. A) Wingbeat frequency was affected by treatments ($df = 3$, $\chi = 88.59$, $p < 0.001$). B) duration of flight and C) total wingbeats per flight bout were not affected by treatment. Different lower-case letters indicate significant differences between treatments ($p < 0.05$, generalized estimating equation).

Objective 2: Evaluate impacts of almond phytochemicals 2.1 Characterize phytochemical composition of almond honey



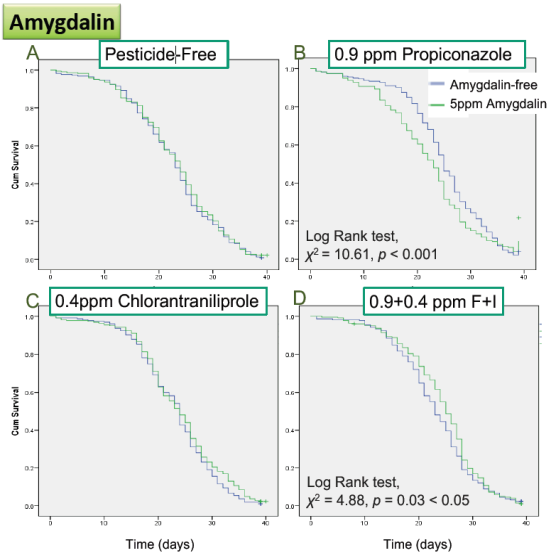
Two constituents, abscisic acid and the flavanone galangin, were identified in substantial quantities from almond honey. The figure (right) depicts the UV absorption (285 nm) chromatogram (black line) and the total ion current chromatogram (red line) of an almond honey sample analyzed by HPLC-MS.

2.2 The antioxidant capacity of almond honey



In addition to almond honey, we also measured the antioxidant capacity of several other monofloral honeys using the relative DPPH radical scavenging capacity (RDSC) assay. We found that antioxidant capacity of buckwheat honey is highest, followed by antioxidant capacity of almond honey and carrot honey. The antioxidant capacity of goldenrod honey was comparable to that of tupelo honey. Soy and locust honey (both of which are legumes) have the lowest antioxidant capacity. (Different lower-case letters indicate statistical differences between honeys; Games-Howell test, $p < 0.0071$ after Bonferroni correction.)

2.3 Evaluate effects of almond-characteristic phytochemicals on worker longevity



Kaplan–Meier plots of adult bee survivorship on different pesticides with (green line) or without (blue line) amygdalin, a cyanogenic glycoside in almonds. The hive source significantly affected the longevity of bees. Thus, we used hive identity as an adjusted stratum in the statistical analysis.

Amygdalin, a cyanogenic glycoside in almonds, when ingested at the field concentration of 5 ppm, did not affect the survival of bees consuming it in unamended sugar

water diet (Fig. A) or sugar water with 0.4 ppm chlorantraniliprole (Fig. C). However, amygdalin reduced the longevity of bees in diets with 0.9 ppm fungicide propiconazole (Fig. B). Surprisingly, amygdalin neutralized the impaired survival caused by the mixed propiconazole and chlorantraniliprole diet (Fig. D).

D. Outreach Activities

June 25, 2019 Raleigh, NC "Honey bees and the four Ps—P450s, p-coumaric acid, pathogens, and pesticides, Society for Toxicologic Pathology 38th Annual Symposium,

October 3, 2019 Chicago, IL "Honey bees are in trouble, Entomological end-times, Peggy Notebaert Museum, Chicago, IL October 3, 2019;

October 23 2019 Washington, DC "The insect apocalypse", North American Pollinator Protection Campaign, Department of Interior

November 17 - 20, 2019， St Louis，MO， "Impact of Almond Insecticides, Fungicides, and Phytochemicals on Behavioral Responses of Honeybee Nurses", Entomology Society of America Annual Meeting

December 10 - 12, 2019， California State Fair, Sacramento, CA, "Mitigating adverse effects of pesticides on honey bees through dietary phytochemicals", The Almond Conference

E. Materials and Methods *(500 word max .)*:

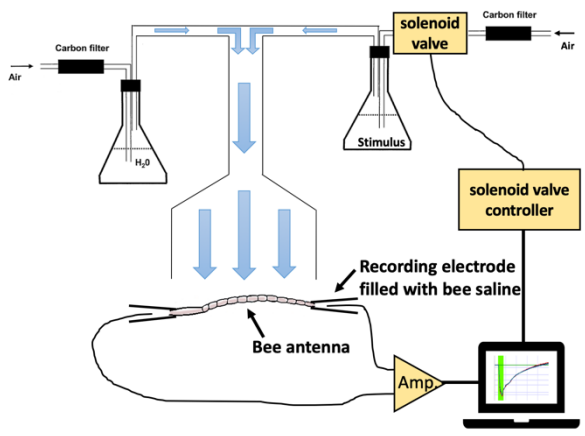
- **Objective 1: Determine effects of pesticide/adjuvant/phytochemical interactions on queen quality**

1.1 Nursing Behaviors

The general arrangements were modified from the research of Shpigler and Robinson (2015). Ten adult one-day-old bees were marked, each with an unique color for individual recognition, and were kept in vertically oriented Petri dishes (100X20 mm) with a beeswax foundation sheet placed on the base of the dish to simulate in-hive conditions. Dishes were supplied with one tube of 50% phytochemical-free/quercetin-supplemented sucrose water (2ml) and one g of treated pollen. Before the behavior analysis, the groups of workers in Petri dishes were held in a incubator room (34°±1°C, 45%±10% RH) for seven days.

On Day 7, a four-day-old queen cell was provided in each arena with seven-day-old workers to document their behavior associated with queen cell care. Each observation session lasted 15 min. The associated behaviors were recorded with a customized LabView program**.**

1.2 Electroantennography (EAG)



We fed workers pesticide-treatment pollen aliquots to adult bees for one week and then measured their EAG responses to serial concentrations of synthetic non-volatile brood pheromone and a volatile brood pheromone, ocimene, to detect signs of olfactory impairment. We also tested mineral oil as a negative control and 2-heptanone, an alarm pheromone, as a positive control. Each antenna was tested with sequential exposure to the negative control, the serial concentrations of non-volatile brood pheromones, the volatile brood pheromone, and the positive control for each odor trial. We performed six odor trials per antenna.

1.3. Flight Performance of Workers

The experimal method has been published in Liao et al. (2019).

- **Objective 2: Evaluate impacts of almond phytochemicals**

2.1 and 2.2 Characterize antioxidant and phytochemical composition of almond honey

The sample preparation and LC-MS analysis were adapted from the methods published by Gheldof et al. (2002) as well as Michalkiewicz et al. (2008). Ten g almond honey were dissolved in 100 mL of deionized acid water (pH 2.0) and stirred for 30 min. The samples were extracted by solid-phase extraction (SPE, Oasis PRiME HLB) cartridges. After the first extracton, the SPE was washed with 100 mL acidified water to remove sugars and polar compounds and then eluted with 50 mL methanol to recover the adsorbed phenolic acids and flavonoids. The methanol extract was concentrated to 0.5 mL under reduced pressure in a rotary evaporator at 30°C. The supernatant of concentrated extract was used for LC-MS analysis.

The evaluation of antioxidant capacity of almond honey was adapted from the method of Chen et al. (2006). One gram of honey was dissolved in 1 mL methanol for the relative 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging capacity (RDSC) assay. The sample was diluted into various concentrations, which were then tested to obtain the best result from an optimized dilution range. Eight dilution concentrations in the linear range of their concentration-antioxidant capacity curve were selected and tested in triplicate to calculate the reported RDSC value.

2.3 almond-characteristic phytochemicals on worker longevity

The experimental arrangements were as described in Liao et al. (2017).

F. Publications that emerged from this work

Berenbaum, M. R., and L.-H. Liao. 2019. Honey bees and environmental stress: Toxicologic pathology of a superorganism. Toxicol. Pathol. **47**:1076-1081.

Liao, L.-H., W.-Y. Wu, A. Dad, and M. R. Berenbaum. 2019. Fungicide suppression of flight performance in the honeybee (*Apis mellifera*) and its amelioration by quercetin. Proc. R. Soc. B 286: 20192041.

Liao, L.-H., W.-Y. Wu, D. J. Pearlstein, A. G. Kelley, W. M. Montag, E. M. Hsieh and M. R. Berenbaum. Amelioration of xenobiotic toxicity by honey phytochemicals in the honey bee *Apis mellifera*. (*in preparation*)

G. Literature Cited

Cheng, Z., J. Moore, and L. Yu. 2006. High-throughput relative DPPH radical scavenging capacity assay. J. Agric. Food Chem. **54**:7429-7436.

Gheldof, N., X.-H. Wang, and N. J. Engeseth. 2002. Identification and quantification of antioxidant components of honeys from various Floral Sources. J. Agric. Food Chem. **50**:5870-5877.

Liao, L.-H., W.-Y. Wu, and M. R. Berenbaum. 2017. Impacts of dietary phytochemicals in the presence and absence of pesticides on longevity of honey bees (*Apis mellifera*). Insects **8**:22.

Liao, L.-H., W.-Y. Wu, A. Dad, and M. R. Berenbaum. 2019. Fungicide suppression of flight performance in the honeybee (*Apis mellifera*) and its amelioration by quercetin. Proc. R. Soc. B **286**:20192041.

Michalkiewicz, A., M. Biesaga, and K. Pyrzynska. 2008. Solid-phase extraction procedure for determination of phenolic acids and some flavonols in honey. J. Chromatogr. A **1187**:18-24.

Shpigler, H. Y., and G. E. Robinson. 2015. Laboratory assay of brood care for quantitative analyses of individual differences in honey bee (*Apis mellifera*) affiliative behavior. PLoS ONE **10**:e0143183.