Effects of Insecticide-Fungicide-Adjuvant Combinations Commonly Applied to Almonds During Bloom on Honey Bee Development and Survival

Project No.: **17-POLL17-R. Johnson**

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Objectives:
1. Adult bee survival in laboratory bioassays using new insecticide/fungicide/adjuvant combinations
2. Larval worker bee survival and development using *in vitro* larval rearing with select insecticides, fungicides and combinations
3. Queen development, reproductive success and prevalence of bee viruses following exposure to select pesticides and combinations

Interpretive Summary:

Honeybees are exposed to fungicides and fungicides mixed with insecticides during almond pollination. We have tested the effects of the most common insecticides, fungicides and insecticide-fungicide combinations on honey bee worker larval survival and adult longevity as well as effects on queen larval development and subsequent reproductive health. Additionally, we have tracked the movement of pesticides in contaminated pollen through nurse bees and into the royal jelly provided to developing queens and workers. The insecticide diflubenzuron, formulated for use in almonds as Dimilin, is capable of killing both worker and queen larvae and was found to be present in royal jelly at higher concentrations than other pesticides. The insecticide chlorantraniliprole, formulated as Altacor, appears to be relatively safe for larvae and adults when applied alone, but significantly higher larval and adult mortality were observed when combined with fungicides, including propiconazole (Tilt) and iprodione (Rovral). The addition of a spray adjuvant did not appear to alter the toxicity of pesticides or combinations to adults. Together, these results highlight the potential harm to pollinators associated with insecticide application during bloom and support the recommendation that all insecticide use should be avoided when bees are present.

Materials and Methods:

Objective 1. Adult worker bee laboratory bioassays

Changes in adult worker honeybee longevity following oral exposure to insecticide-fungicide-adjuvant combinations were assessed through a pollen feeding assay in which newly emerged bees were fed pollen artificially contaminated with field-relevant combinations. Insecticides tested include Dimilin 2L (diflubenzuron), Intrepid (methoxyfenozide) and Altacor (chlorantraniliprole). Fungicides tested include Pristine (boscalid and pyraclostrobin), Tilt (propiconazole) and Rovral (iprodione). These represent the most widely used insecticides and fungicides applied to almonds during bloom (**Figure 1**). Combinations were made by mixing the two pesticides at a ratio based on the maximum label rate with insecticide concentrations ranging from 0.4 to 4000 ppm. A representative spray adjuvant (Dyne-Amic) was added at to the insecticide-fungicide mix at a rate of 0.8% in the final pollen mixture.

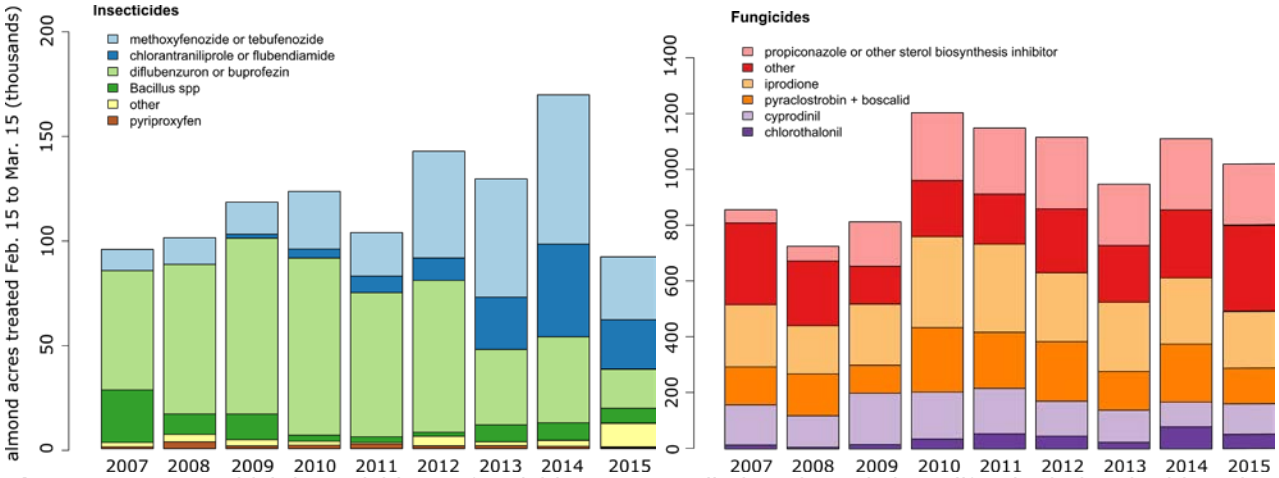

Figure 1. Area to which insecticides or fungicides were applied to almonds in California during the blooming period. Data from https://calpip.cdpr.ca.gov/.

To set up these trials, adult worker bees less than 24 hours post-emergence were collected from an isolated frame and placed in groups of 20 inside 6 oz. paper ice cream cups with a cheesecloth cover. Bees were provided approximately 1 g of contaminated pollen and 1:1 (w/w) sucrose syrup *ad libitum* and maintained at 33°C and 60-80% relative humidity. Mortality was scored daily for seven days. Each trial was replicated at least 3 times and survival curves compared to control bee survival in a pairwise manner, working down from the top concentration, until a significant difference was no longer observed.

Testing of additional fungicides that are frequently used in almonds, including Bravo (chlorothalonil) and Vanguard (cyprodinil) is ongoing in summer 2018.

Objective 2. Larval *in vitro* rearing bioassays

Very young honey bee worker larvae were grafted and reared *in vitro* (**Figure 2**) and fed with a semi-synthetic diet consisting of royal jelly, sugar and yeast extract (Schmehl et al. 2016). After 72 hours acclimation, groups of 16 healthy larvae were selected to receive treatments. At 96 hours after grafting larvae were fed with 30 µl of diet contaminated with technical grade insecticide (1.02 µg chlorantranliprole, 2.28 µg diflubenzuron or 2.25 µg methoxyfenozide)

and/or fungicide active ingredients (2.24 µg propiconazole, 5.04 µg iprodione or the combination of 4.68 µg boscalid and 2.37 µg pyraclostrobin) dissolved in acetone. Doses and dose combinations were set based on the maximum label rate. Diets with 2% acetone were used as the negative control and dimethoate (5.1 µg) as the positive control. Twelve replicates were performed for each insecticide, fungicide or combination (N=144 bees / treatment). The proportion of treated of individuals ultimately developing into adults was recorded and analyzed using ANOVA.

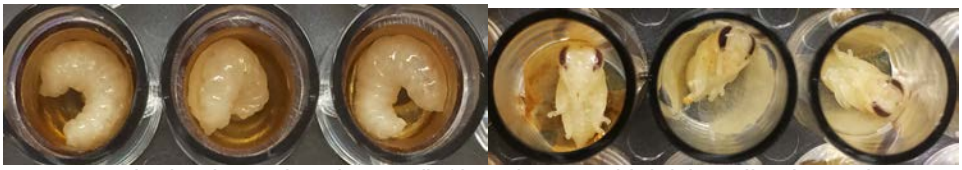

Figure 2. Developing honeybee larvae (left) and pupae (right) in cell culture plates.

For insecticides and insecticide-fungicide combinations found to affect larval development (chlorantraniliprole-propiconizole, methoxyfenozide-iprodione, and diflubenzuron) trials are underway to test a variety of dose levels to generate dose response curves. Additionally, a dose-response curve for the spray adjuvant Dyne-Amic is being generated to identify a maximum sublethal concentration that can be combined with all insecticides and fungicides to determine the potential for elevated toxicity of a spray application when an adjuvant is used.

Objective 3. Queen development and health

Closed swarm boxes, capable of rearing dozens of queens at a time (Spivak and Reuter 1994, Johnson and Percel 2013), were used to evaluate the effect of insecticides and fungicides on queen development (**Figure 3**). Thirty larvae (< 24 hours old) from one colony were grafted into plastic queen cell cups and placed in each swarm box containing ~3 pounds of young worker bees shaken and homogenized from multiple OSU colonies. Pesticide-free pollen (200g) was artificially contaminated with the formulated insecticide Altacor (40 ppm chlorantraniliprole), the fungicide Tilt (90 ppm propiconazole), a combination of Altacor and Tilt, the insecticide, Dimilin 2L (100 ppm diflubenzuron) or plain water as a negative control. Treated pollen and clean sucrose syrup (50% w/w) were fed to worker bees in the swarm box, which produced royal jelly to provision the queen larvae. After preparation, the swarm boxes were stored in a dark, well-ventilated space for four days. Queen cells were then removed from swarm boxes and incubated inside a healthy colony until adult emergence.

After blending pollen with formulated pesticides, 5 g of the treated pollen was collected for pesticide residue testing to determine the final concentrations of each active ingredient. Approximately 5 g of nurse bees and royal jelly were extracted from 4 - 6 queen cells taken from each swarm box on day four. All samples of pollen, nurse bees, and royal jelly were stored at -20°C and shipped frozen to the Agriculture and Food Laboratory at University of Guelph to quantify the level of chlorantraniliprole, propiconazole, and diflubenzuron residues using a GC/LC-MS/MS.

The total number of queen cells capped, adults emerged and adults that survived for more than 7 days were recorded. Five queen cells per treatment from each trial were installed in

small mating colonies containing one frame of worker bees and food. Each treatment was replicated six times (N = 180 queen larvae).

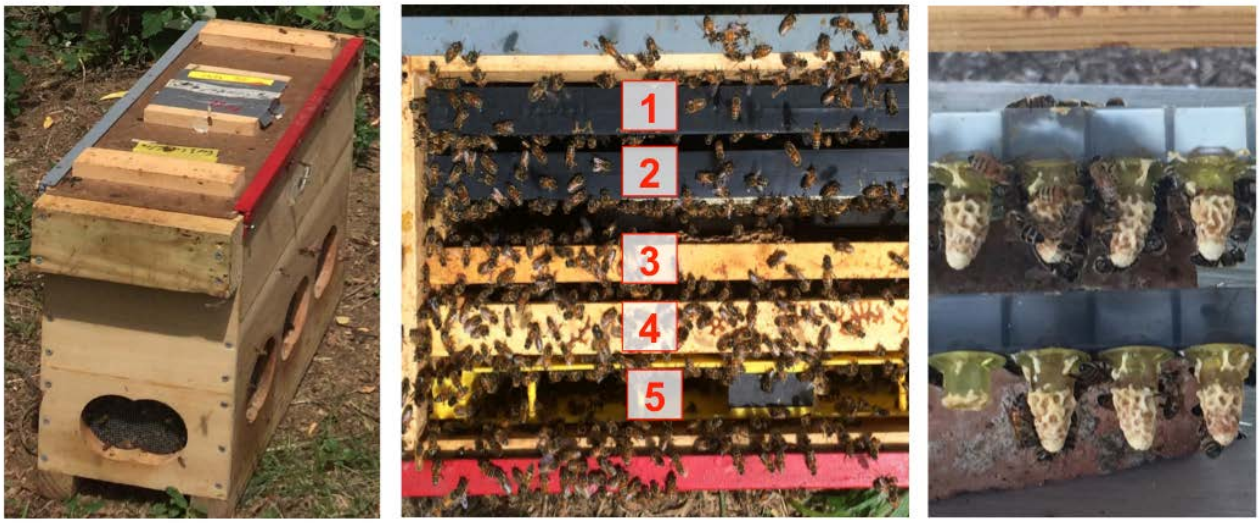

Figure 3. A "closed swarm box" (left) and frame arrangement inside the box (center), and queen cells removed from the swarm box 4 days after installation (right). Each box contains five items as pictured: (1) an empty plastic foundation (dummy frame) to hold items in place, (2) a pollen frame, (3) a queen cell frame holding 30 artificial queen cups, (4) a frame with empty drawn comb, and (5) a plastic in-hive feeder with sugar syrup.

Additional queen-rearing trials with the Altacor-Tilt combinations and Altacor-Tilt with a spray adjuvant (Dyne-Amic) are ongoing in summer 2018. We will collect 2 - 5 virgin queens from each treatment per trial to evaluate the abundance of common RNA viruses (DWV, BQCV, LSV2 and SBV) using quantitative real-time PCR (Evans 2012).

Thirty grams of the treated pollen have been shipped to May Berenbaum's lab group at the University of Illinois-Champaign to evaluate the nursing behaviors of working bees tending to queen cells after consuming the contaminated pollen. Virgin queens (~10 days old) not collected for the viral assays are also being shipped to the Berenbaum lab to evaluate the flight performance of queens reared under pesticide exposure.

Results and Discussion:

Objective 1. Adult worker bee laboratory bioassays

The presence of the spray adjuvant (Dyne-Amic) had no effect on adult worker bee survival over 7 days by itself and did not alter the survival of bees exposed to field-relevant insecticide-fungicide combinations. The combination of Altacor and Tilt, which was found to reduce bee survival in the absence of spray adjuvant (**Table 1**), was also found to reduce bee survival in the presence of a spray adjuvant (**Table 2**). It should be noted that the highest concentrations of pesticides used in this trial were much higher than would likely be encountered in almond orchards, which is probably 10 ppm or less. However, the reduction in bee survival observed at 4 ppm chlorantraniliprole (Altacor) in combination with 8.8 ppm propiconazole (Tilt) lies within a range that bees have encountered for other sprayed pesticides (Mullin et al. 2010).

Table 1. Minimum concentration of insecticide, in field-relevant combination with fungicides causing a reduction in adult worker bee survival over 7 days.

	Insecticide alone	boscalid + pyraclostrobin (Pristine)	iprodione (Rovral)	propiconazole (Tilt)
Fungicide alone		> 4000 ppm	> 4000 ppm	> 4000 ppm
chlorantraniliprole (Altacor)	> 4000 ppm	> 4000 ppm	4000 ppm	4 ppm
methoxyfenozide (Intrepid)	> 4000 ppm	4000 ppm	> 4000 ppm	> 4000 ppm
diflubenzuron (Dimilin)	> 4000 ppm	4000 ppm	4000 ppm	4000 ppm
thiamethoxam (positive control)	0.4 ppm			

Table 2. With addition of 0.8% Dyne-Amic adjuvant, the minimum concentration of insecticide, in field-relevant combination with fungicides causing a reduction in adult worker bee survival over 7 days.

	Insecticide alone	boscalid + pyraclostrobin (Pristine)	iprodione (Rovral)	propiconazole (Tilt)
Fungicide alone		> 4000 ppm	> 4000 ppm	> 4000 ppm
chlorantraniliprole (Altacor)	> 4000 ppm	4000 ppm	4000 ppm	4 ppm
methoxyfenozide (Intrepid)	4000 ppm	> 4000 ppm	4000 ppm	4000 ppm
diflubenzuron (Dimilin)	> 4000 ppm	4000 ppm	> 4000 ppm	4000 ppm

Objective 2. Larval *in vitro* rearing assays

The insecticide diflubenzuron was found to significantly reduce survival of honeybee worker larvae, but other insecticides and fungicides, when applied to larvae alone, did not affect survival (**Figure 4**). The insecticide chlorantraniliprole combined with either propiconazole or iprodione was found to reduce larval survival relative to control. The insecticide diflubenzuron remained highly toxic to larvae regardless of fungicide combination.

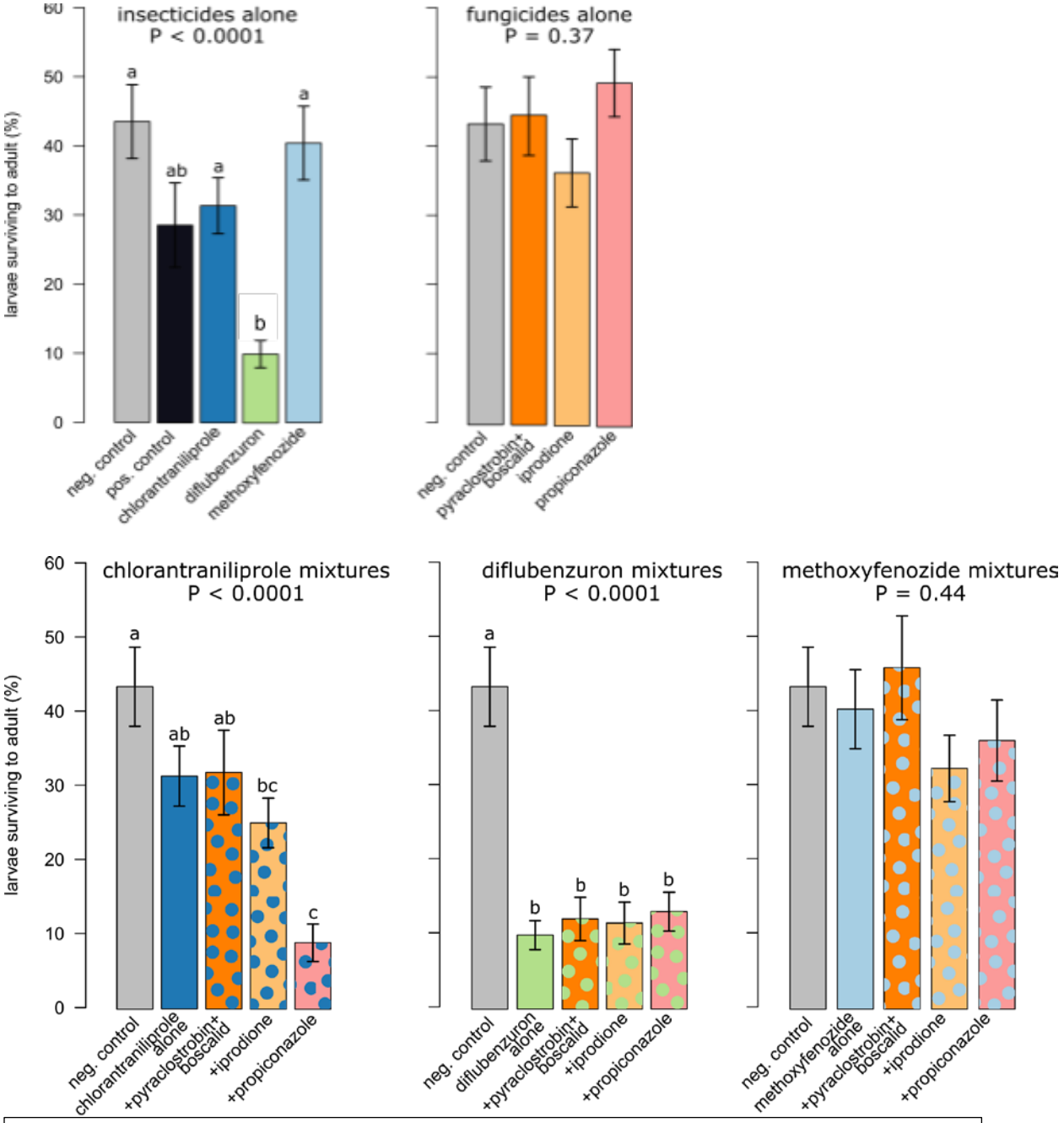

Figure 4. Survival (mean ± standard error) of larvae treated once with insecticides alone, fungicides alone or insecticide-fungicide combinations . Bars with different letters (a or b) are significantly different from each other. Each treatment group was replicated 12 times (N = 12).

Objective 3. Queen development and health

The proportion of grafted larvae that developed into adult queens differed significantly among treatments, as larvae reared on pollen treated with Altacor and Dimilin 2L had lower emergence success (**Table 3)**. Fewer queens reared by workers fed pollen treated with Dimilin 2L survived more than 7 days after emergence, although the difference was not statistically significant. Although virgin and mated queens in the Altacor and Altacor-Tilt combination treatment groups showed lower survival probabilities compared to the control group, the difference was not statistically significant.

Table 3. Percentages of larvae developed into fully capped queen cells, cells with adult queen emergence, emerged adults that survived 7 days as virgin queens, and queen success in mating colonies. One-way ANOVA and Chi-squared tests (χ^2) were performed to compare the percentages across treatments. Statistically significant difference among treatments are indicated by a P value < 0.05.

Queen development						
	Control	Tilt	Altacor	Altacor &Tilt	Dimilin 2L	Statistics
Cells capped, % (mean ± SE)	80.1 ± 7.0 N = 7	85.3 ± 7.5 N =6	75.8 ± 7.0 N = 7	91.1 ± 7.0 N = 7	62.2 ± 7.0 N = 7	F(4, 29) = 1.54 P = 0.22
Adult emergence, % (mean ± SE)	86.7 ± 7.0 N = 7	99.1 ± 7.4 N = 6	86.4 ± 6.9 N = 7	91.5 ± 6.9 N = 7	65.3 ± 6.9 N = 7	F(4, 29) = 3.18 **P = 0.028**
Adult survival (7 day), % (mean ± SE)	70.1 ± 10.6 N = 7	64.7 ± 11.4 N = 6	62.4 ± 10.6 N = 7	57.3 ± 10.6 N = 7	30 ± 10.6 N = 7	F(4, 29) = 2.17 P = 0.097
Queen mating and colony survival						
	Control	Tilt	Altacor	Altacor &Tilt	Dimilin 2L	Statistics
Mated & survived, wk 2	82.8% N = 29	58.3% N = 24	70.0% N = 30	63.3% N = 30	56.7% N = 30	χ^2 = 6.1 P = 0.19
Survived, wk 6	62.1% N = 29	58.3% N =24	53.3% N = 30	56.7% N = 30	30.0% N = 30	χ^2 = 8.8 P = 0.10

Pesticide residue analysis

Pesticide residue sampling from closed swarm box experiments show that concentrations of all pesticides decrease as nurse bees consume pollen and then secrete royal jelly. However, diflubenzuron is present at greater concentration in royal jelly, relative to the original pollen concentration, than chlorantraniliprole (**Table 4**).

Table 4. Final concentrations of the active ingredients (in ppm) in pollen treated with the formulated pesticides and levels of the active ingredients detected in nurse bees and royal jelly in queen cells, compared to pollen that was not treated. The values are displayed as mean ± one standard error around the mean, with range (min - max) and sample size (N) shown in parentheses.

active ingredient	treatments	pollen	nurse bees	royal jelly
fungicide propiconazole (target 90 ppm)	with Tilt	72.8 ± 8.67 (42 - 120, N = 9)	1.7 ± 0.42 (0.46 - 3.4, N = 8)	0.14 ± 0.03 (0.027 - 0.29, N = 9)
	no Tilt	0.09 ± 0.03 (0 - 0.41, N = 15)	0.030 ± 0.015 (0 - 0.17, N = 15)	0.0005 ± 0.0004 (0 - 0.005, N = 13)
insecticide chlorantraniliprole (target 40 ppm)	with Altacor	29.5 ± 2.81 (15 - 42, N = 10)	0.36 ± 0.09 (0.05 - 0.73, N = 8)	0.05 ± 0.009 (0.02 - 0.10, N = 10)
	no Altacor	0.06 ± 0.04 (0 - 0.56, N = 14)	0.001 ± 0.001 (0 - 0.01), N = 12	0 (0, N = 12)
insecticide diflubenzuron (target 100 ppm)	with Dimilin	84.8 ± 23.95 (51 - 180, N = 5)	9.28 ± 1.43 (6.5 - 13, N = 4)	0.49 ± 0.21 (0.2 - 1.1, N = 4)
	no Dimilin	0.12 ± 0.05 (0 - 0.86, N =19)	0.003 ± 0.002 (0 - 0.03, N = 16)	0 (0, N = 18)

Research Effort Recent Publications:

Wade, A., Lin, C.-H., Regan, E., Johnson, R. Combined toxicity of insecticides and fungicides applied to California almond orchards to honey bee larvae. Insects. *In revision.*

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Mullin, C. A., M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. vanEngelsdorp, and J. S. Pettis. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PloS one 5:e9754.

Schmehl, D. R., H. V. V. Tomé, A. N. Mortensen, G. F. Martins, and J. D. Ellis. 2016. Protocol for the in vitro rearing of honey bee (Apis mellifera L.) workers. Journal of apicultural research.

Spivak, M., and G. Reuter. 1994. Successful queen rearing: Short course. Minnesota Extension Service, University of Minnesota.