Can Application Time Limit Fungicide Exposure to Honeybees in Almonds?

Project No.: **16-POLL16-Johnson/Pettis**

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Objectives:

Objective1: To ascertain the concentrations of each fungicide inside and outside of the spray area in an almond orchard when sprayed with the fungicides, cyprodinil and propiconazole. Objective 2: To ascertain if honeybee foraging behavior was differently affected when subjected to an evening fungicide spray event compared to a morning fungicide spray event.

Interpretive Summary:

The original intent was to study iprodione and propiconazole (prop) but constraints necessitated our approving a fungicide of interest other than iprodione. Approval by the Almond Board was given for the fungicide cyprodinil (cyp), as a replacement for iprodione because cyp 1) is a fungicide with a different mode of action from prop, 2) is routinely applied to almond trees, 3) can be analyzed by the National Science Laboratory in Gastonia, NC, and 4) has the same limit of detection (LOD of 2 parts per billion [ppb])) as prop by LC-MSMS (liquid chromatography tandem mass spectrometry). Prop's mode of action is to disrupt membrane rigidity (1) and cyp's mode of action is to disrupt protein biosynthesis (2).

Key Findings: Concentrations of the two fungicides were greatest in the spray areas and decreased as distance from the spray area increased, as expected. Returning foragers to the hives and pollen bearing returning foragers to the hive were consistently present on all three days of the study (control day, post PM spray, and post AM spray) and showed no significant differences based on spray events. However, foragers visiting treated trees decreased after the PM spray and was significantly decreased after the AM spray compared to control foragers. Pollen collected at the hives located at the edge of the spray areas showed the greatest detections of fungicides, containing 8.5 % cyp and 0.75 % prop after the PM spray and 2.5 % cyp and 7.3 % prop after the AM spray relative to the concentrations in anther pollen from trees located within the spray areas. Fungicides applied to the orchard appear to have different rates of degradation within a 24 hr period. In anther pollen, cyp concentrations

dropped 60.8% and prop concentrations dropped 46.1% from the post PM spray day (Day 2) to the post AM spray day (Day 3) in the PM treatment areas.

Materials and Methods:

Location and Spray Parameters: The almond ranch under study was located near Shafter, Kern County, California. The rectangular ranch consisted of 300 acres containing about 33,950 almond trees with each tree 18 ft. apart in rows 22-24 ft. apart. The ranch was equally divided into four quadrants by two bisecting roads (N/S and E/W). Each of the four treatment areas of the study consisted of 5.5 acres and occupied one of the four corners of the orchard. Tilt (CA) (100-617-ZG) containing the active ingredient prop (41.80%) and Vanguard WG (CA) (100-828-ZB) containing the active ingredient cyp (75.00%) were sprayed at the maximal allowable rate for almonds. These fungicides must be monitored if sprayed at a wind speed of 7 mph and are not allowed to be sprayed at wind speeds over 10 mph due to hazards associated with drift. According to the manufacturer's Safety Data Sheet, both fungicides are toxic to aquatic organisms. Both fungicides were applied by air rig spray using an R-11® Spreader-Activator (2935-50142) spreader with an 8000 Ga tank and a nozzle size 16. The spray rate of the spreader was 2.5 mph.

General Parameters: The bisecting roads (N/S and E/W) of the orchard were populated with 600 beehives, provided by a commercial beekeeper from the north central US. To prepare for the study, each quadrant of the orchard was described by an acronym containing three parameters: (1) the quadrant location was designated by a two-letter code containing cardinal directions, (2) a "P" for prop or a "C" for cyp, and (3) AM or PM to designate morning or evening spray times, respectively. As an example, "SW PPM", designates the orchard's southwest corner as the location where prop was applied as an evening spray. A control location in the center of the orchard was equidistant from each of the four spray locations (**Figure 1**). The study took place over three days from February 10–February 12, 2018, designated hereafter as Days 1-3. Data were collected throughout each day on all study days. On Day 1 which preceded spray events, control data was collected. The evening spray of cyp and prop was complete at 7pm on Day 1. On Day 2, post-PM spray data was collected. The morning spray occurred on Day 3 at 6 AM and the data collected was considered post-AM spray data. Personal protective equipment was required to enter the orchard on the morning of day 3. Data was collected according to the following three metrics:

1) Bloom density: An area of 1 m^3 was marked on trees (n=4) in each bloom assay location to count the number of blossoms daily as an assessment of bloom progress.
2) Anther collection on almond trees

Anthers, the pollen producing structure of the blossom, were collected once daily for the three days of the study. Samples of almond blossom anthers were collected in four treatment areas and one control area of the orchard preceding the spray event (control)
and following the morning and evening sprays. Using scissors, each observer cut anthers into a sample vial from several trees for a 10-minute period.

Pollen Collection from Hives: Pollen traps were installed at the entrances of three hives in each location to capture corbicular pollen from foragers. Any alternative entrances at the hive were

closed with duct tape. The representative 5 g vial from each area was a three-part composite of a third of pollen collected from each of the three pollen traps mixed together. Samples were collected preceding the spray event and following the morning and evening spray.

Weather and Prevailing Winds: The hourly weather was downloaded from the Bakersfield Airport, CA weather station, the NCDC-NOAA weather station nearest to Shafter, CA through a NOAA site (www.ncdc.noaa.gov). The weather data categories of interest for each day were temperature (temp), relative humidity (RH), cloud cover, precipitation, atmospheric pressure (atm press), prevailing wind directions, wind speed, and times for sunrise and sunset. This data was collected to address potential issues of fungicide drift after spray events.

Fungicide Residue Analysis: Anther and pollen samples were analyzed for prop and cyp by LC-MSMS at the USDA-AMS National Science Laboratory in Gastonia, North Carolina. The limit of detection for both fungicides was 2 parts per billion (ppb).

Results:

Average bloom density increased throughout the three study days as shown in (**Figure 1**).

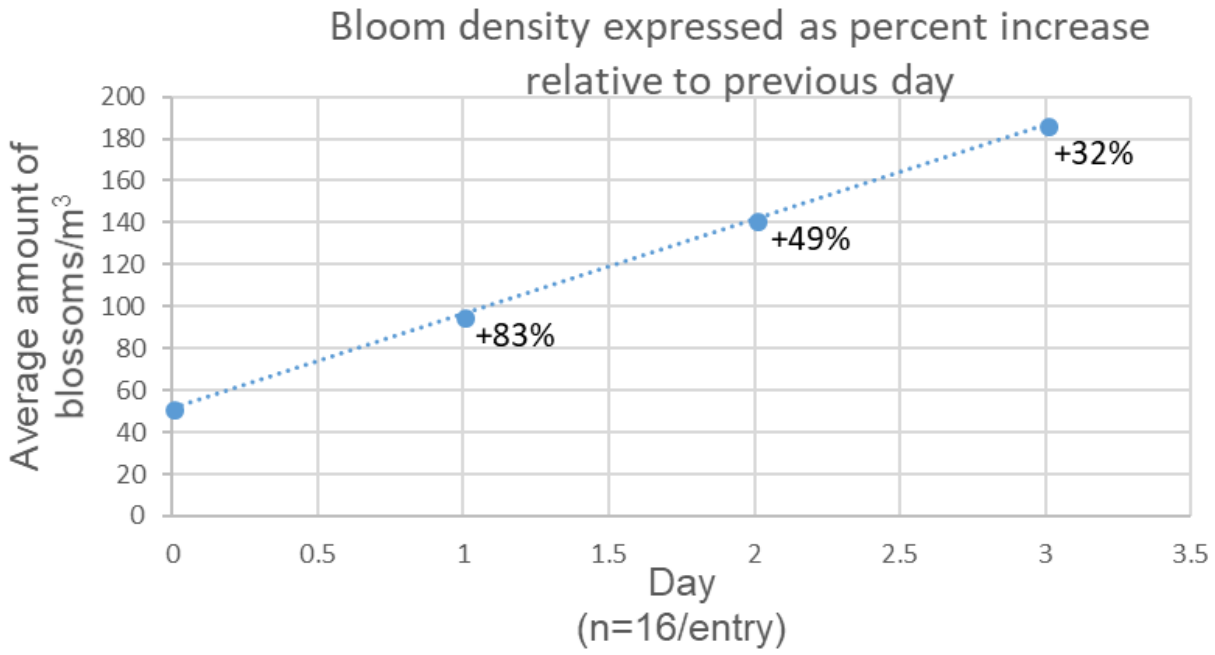


Figure 1: Bloom density. “Day 0” data was collected as a pre-control day, but the data was not used except in this one chart and only for reason of relative relationship. There were sufficient blooms to study throughout the three days.

Fungicide residue results: Concentration levels of both fungicides at various locations across the orchard are illustrated for each day in (**Figures 2-4).**

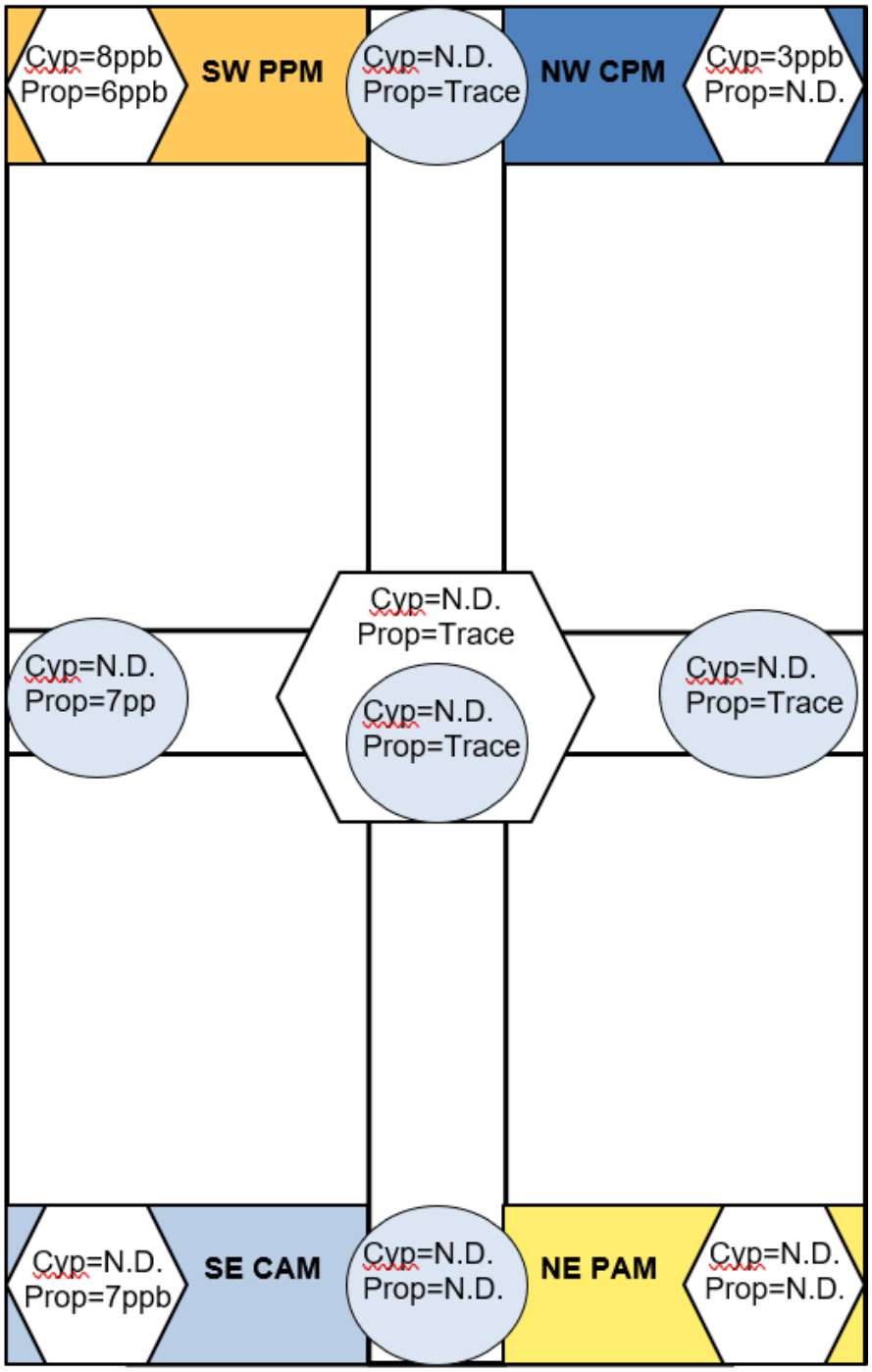


Figure 2: Day 1, Control Day. Concentration levels are reflective of cyp and prop background levels before the spray's events were initiated. Spray areas are designated by cardinal direction descriptions as the first two letters, the pesticide that will be sprayed (P= Prop, C= Cyp), and either PM or AM to describe future spray event times. Data within hexagons reflect background concentration in anther pollen on trees (that will be in the future spray areas). Data within the ovals represent background concentration in pollen collected at the hives before future spray events.

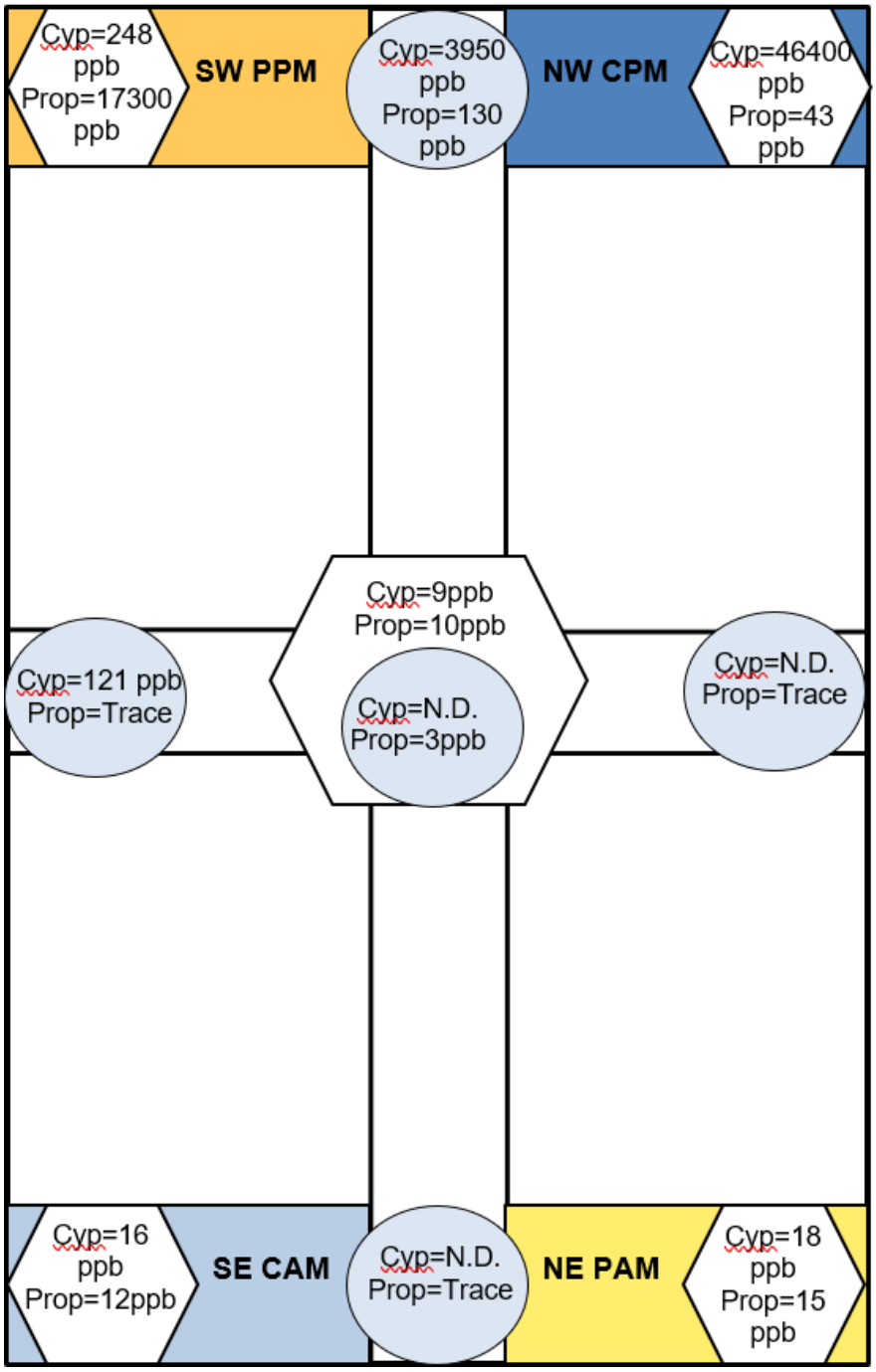


Figure 3: Day 2, post-PM spray event. All shapes and letter codes are the same as **Figure 2**. Detection levels are reflective of concentration of the Cyp spray event that took place in 5.5 acres in the northwest section and of the Prop spray event that took place in 5.5 acres in the southwest section of the orchard at 7pm the previous evening on Day 1. Samples were collected on Day 2. Small amounts of cross detection of one fungicide into the spray area of the other fungicide, presumably due to spray drift, can be seen within the PM spray areas.

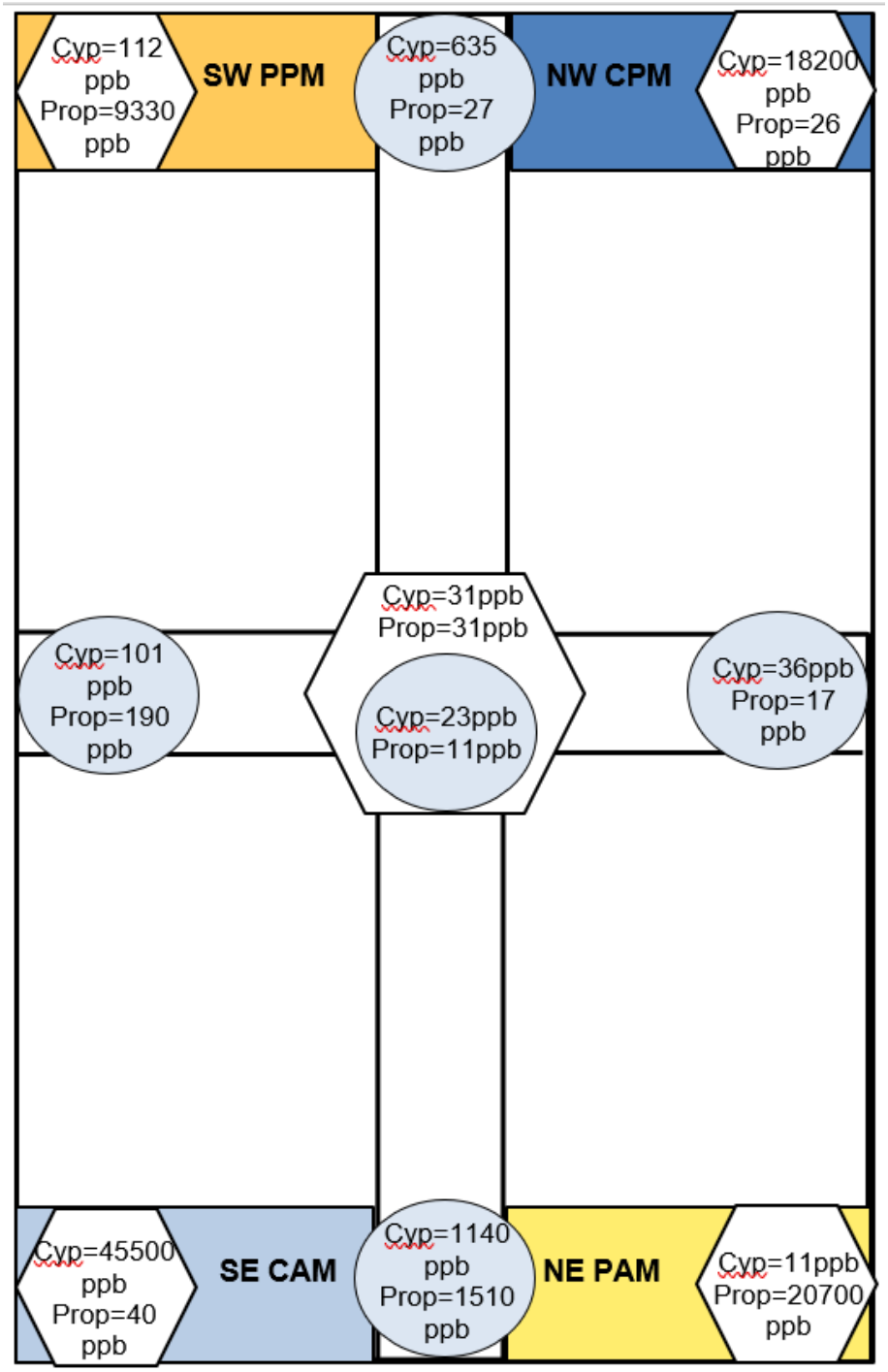


Figure 4: Day 3, post-AM spray event. All shapes and letter codes are the same as **Figure 2**. Concentration levels are reflective of the Cyp spray event that took place in 5.5 acres in the southeast section and of the Prop spray event that took place in 5.5 acres in the northeast section of the orchard at 6am on Day 3. Residual concentration by fungicides from the PM spray event is still present but diminished (Cyp= 39.2%, Prop= 53.9% of original concentration levels) on the west side of the orchard, presumably from degradation over time by physical effects. Samples were collected on Day 3. Spray drift may account for the cross detection of small amounts of one fungicide into the AM spray area of the other fungicide.

Anther pollen results: Explanation of distances: On Day 1 there was no spray event but to compare results, 10000 ft was chosen to represent Control data. Distance determinations from spray events are shown in (**Figure 5**).

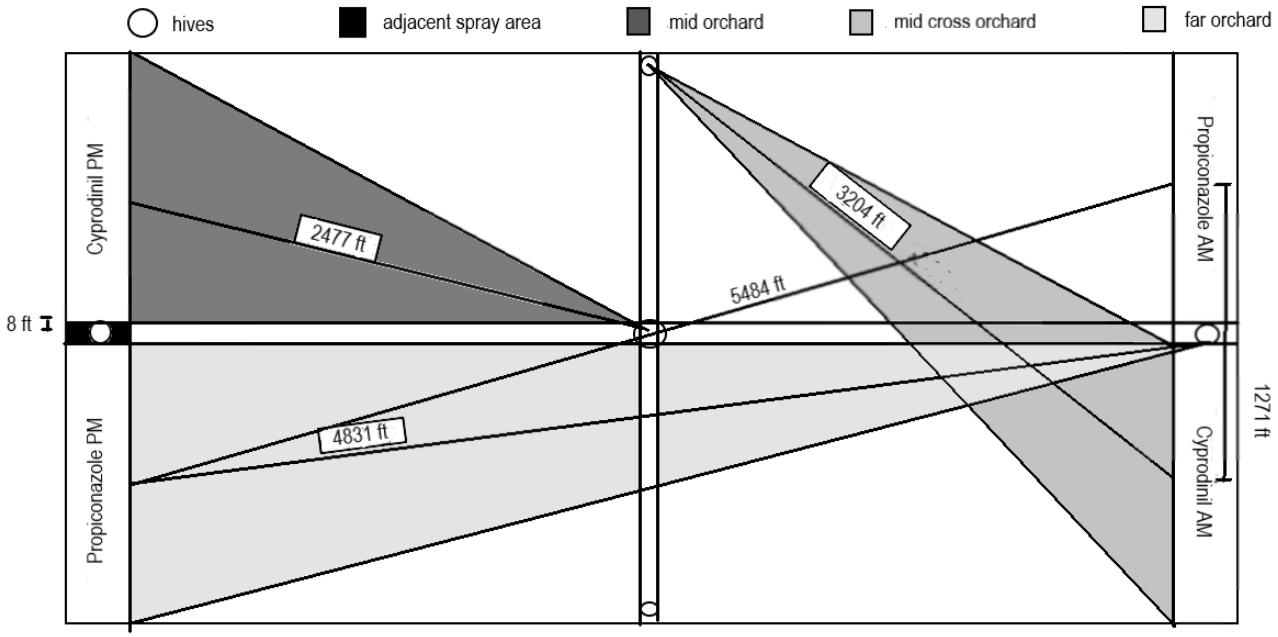


Figure 5: Orchard distances were calculated by a simple distance from the halfway point of one area to another halfway point (8ft, 1271ft, 5484ft) or by calculating the length of a line from the angle of a right triangle to the halfway point of its opposite side (2477 ft, 3204 ft, 4831 ft) using the Pythagorean Theorem (shaded triangles).

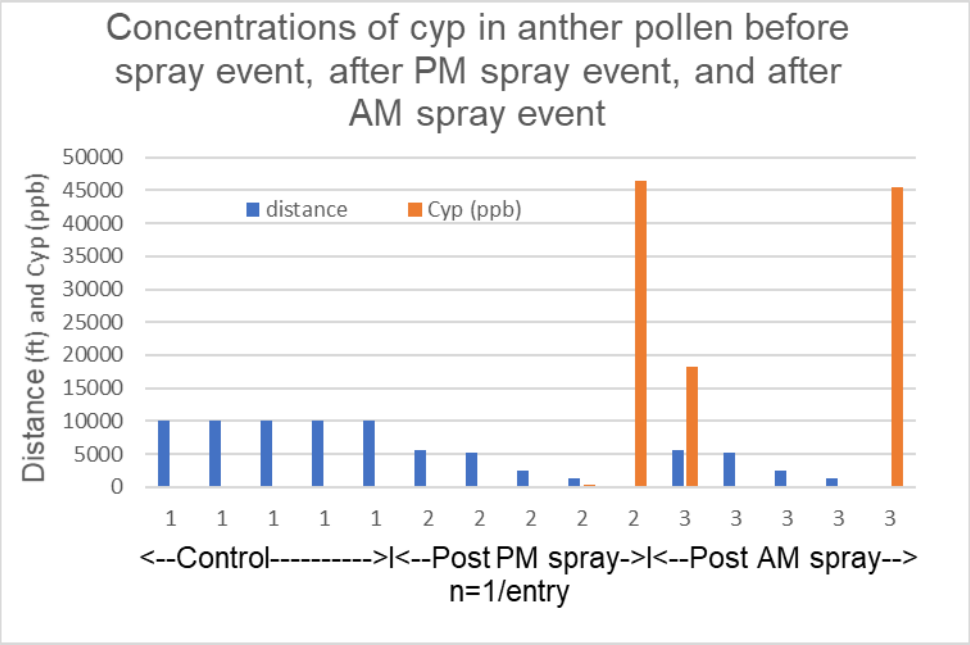


Figure 6: Cyp concentrations in anther pollen for both spray events is greatest in the spray areas (distance =zero). The relatively high peak of cyp at the greatest distance on Day 3 is residual cyp from the PM spray the day before and reflects degradation of cyp over 24 hrs.

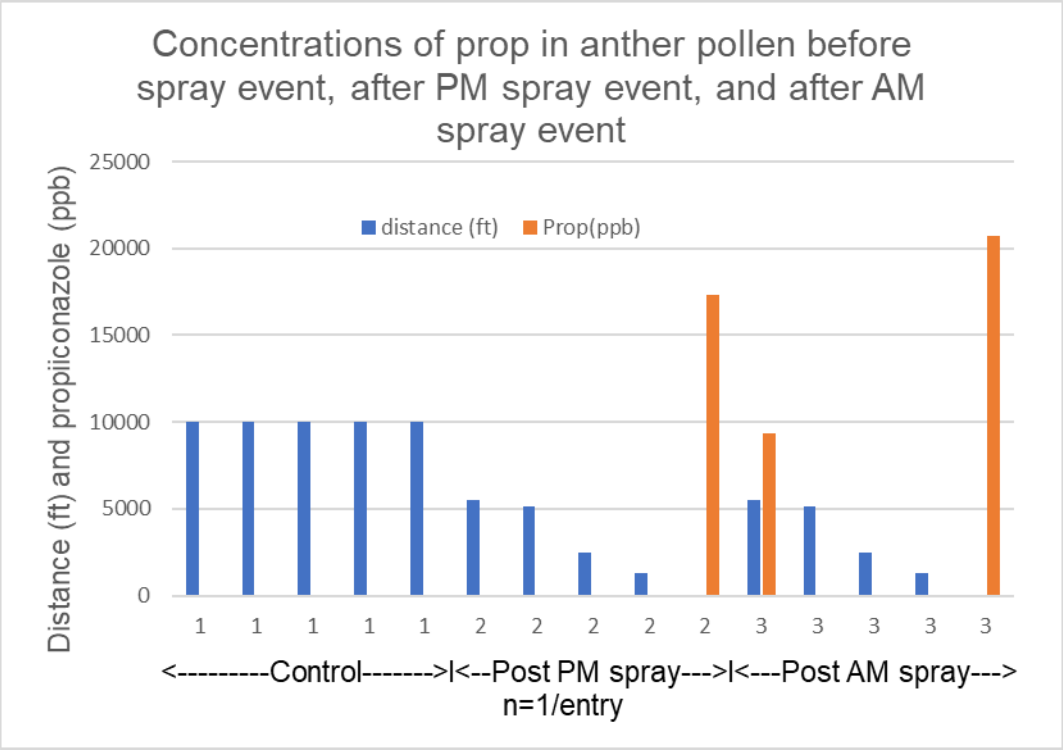


Figure 7: Prop concentrations were greatest within the spray areas for both spray events (distance=zero). The high detection level at the greatest distance from the spray area on Day 3 is residual prop from the PM spray the day before and reflects degradation of prop over 24 hours.

Hive pollen results:

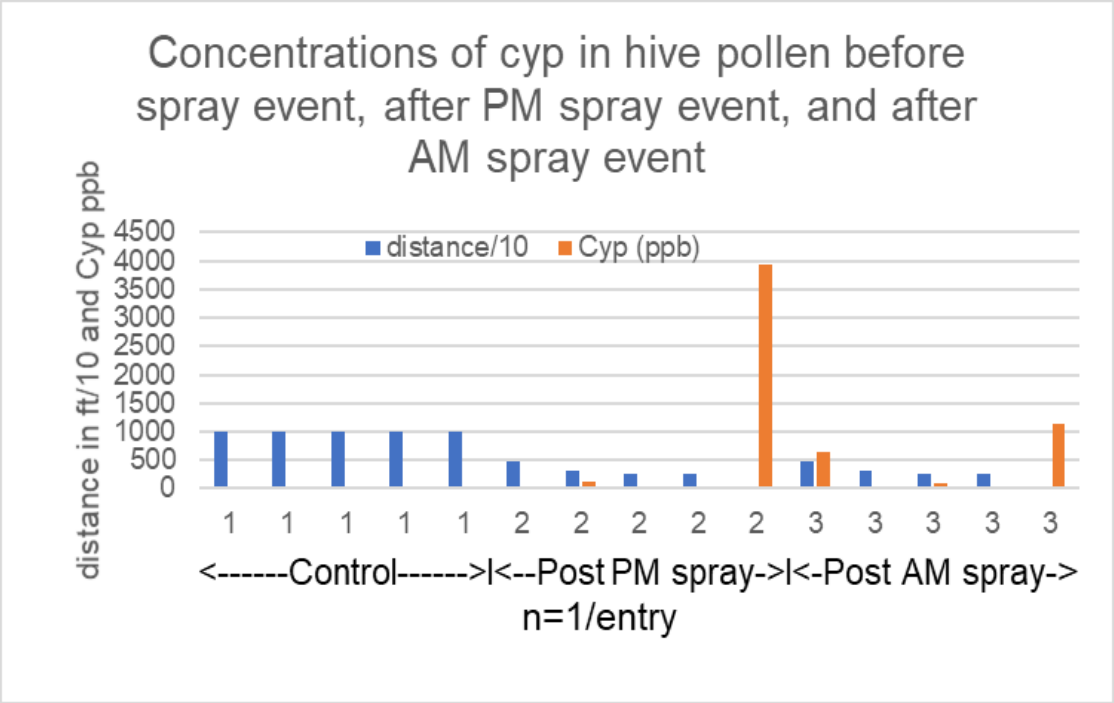


Figure 8: Hive pollen closest to the spray areas had the greatest detection level of cyp. The high cyp concentration level at the greatest distance from the spray area on Day 3 is likely to reflect cyp-exposed pollen collected from the PM spray event over 24 hrs. Decreased collection of the contaminated pollen in the PM spray area on Day 3 and the low collection of pollen nearest to the AM spray area on Day 3 cannot be attributed to differences in spray event concentrations because the post AM cyp concentration and the post PM cyp concentration in anther pollen at the trees were almost equal (**Figure 6**). Behavior modification by foragers visiting treated blossoms may be a factor but determining the actual behavior change would require extensive research.

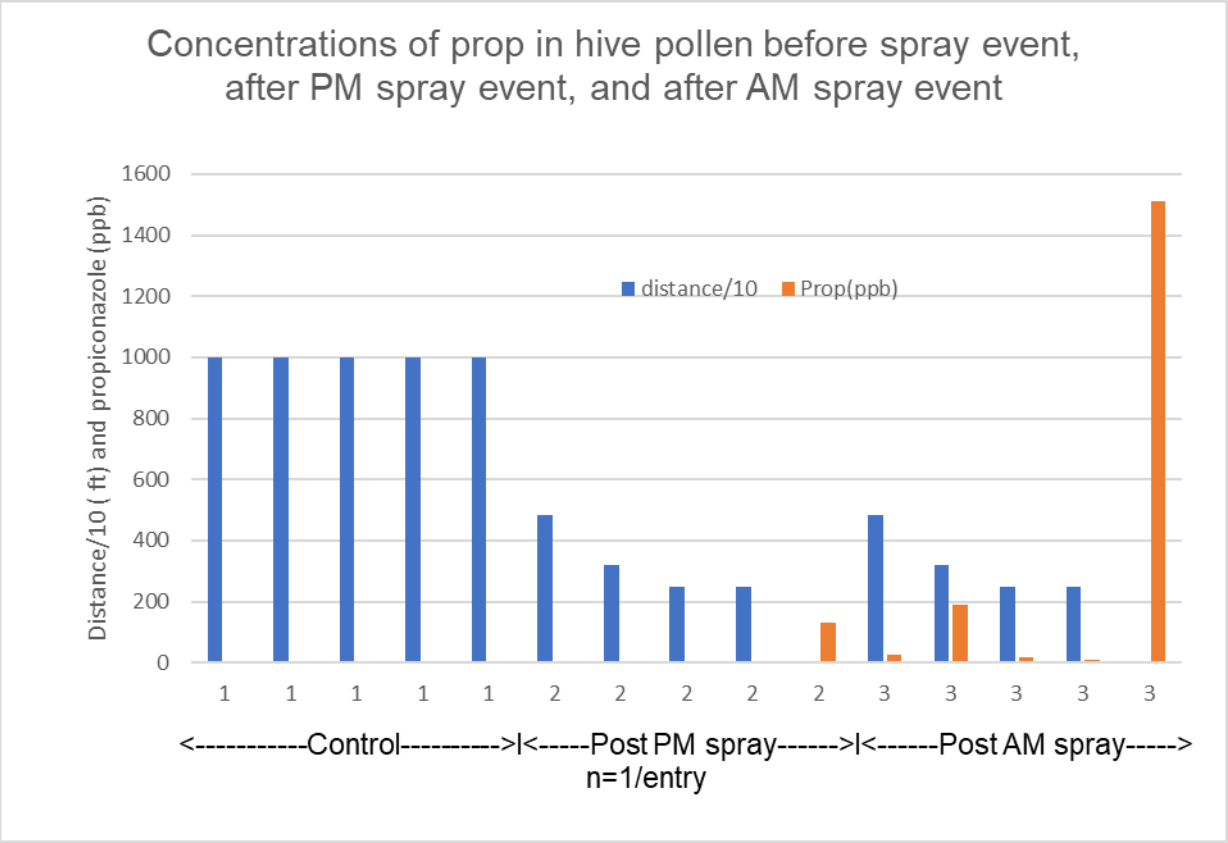


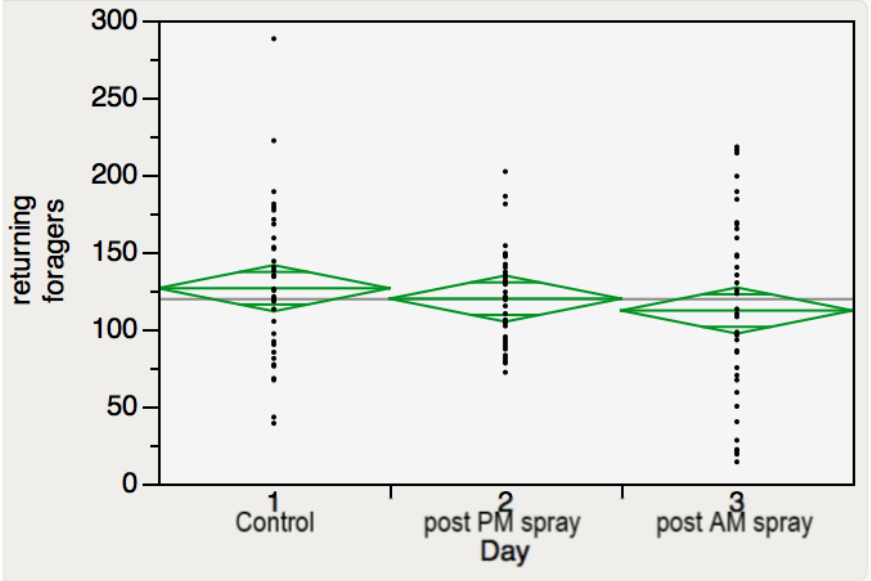
Figure 9: The highest concentration levels of prop in hive pollen occurred closest to the spray sites. Hive pollen values after the PM spray are much lower than hive pollen values after the AM spray. This result represents the best data presented herein that may reflect the difference between the PM and AM spray. Prop may have degraded overnight after the 7PM spray (see **Figure 6**) and/or the foragers underwent a behavior modification which resulted in less collection of pollen in the prop spray area. After the AM spray, relatively larger amounts of prop-exposed pollen were collected and returned to the hive, possibly because prop did not have as much time to degrade due to less time passage from the spray event, or the foragers had less time to initiate behavior modification to diminish the collection of prop-exposed pollen. It is unclear why there is a relatively large peak (190ppb) at the hive located 3204 ft from the spray event on Day 3.

Forager results: There were three categories of foragers under study:

1) Total number of foragers returning to the hives in all locations over the three days,
2) Number of foragers bearing pollen returning to the hives in all locations over the three days
3) Number of foragers that were counted on the blooms on the trees in the sprayed areas over the three days.

These three data sets were analyzed by a OneWay Anova program using JMP®, version 10.0.0 (64-bit edition), SAS Institute, Inc 2012

Oneway Analysis of returning foragers By Day



Missing Rows 120

Oneway Anova

Summary of Fit

Rsquare	0.015492
Adj Rsquare	-0.00134
Root Mean Square Error	47.66069
Mean of Response	119.3833
Observations (or Sum Wgts)	120

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	2	4182.07	2091.03	0.9205	0.4012
Error	117	265770.30	2271.54		
C. Total	119	269952.37			

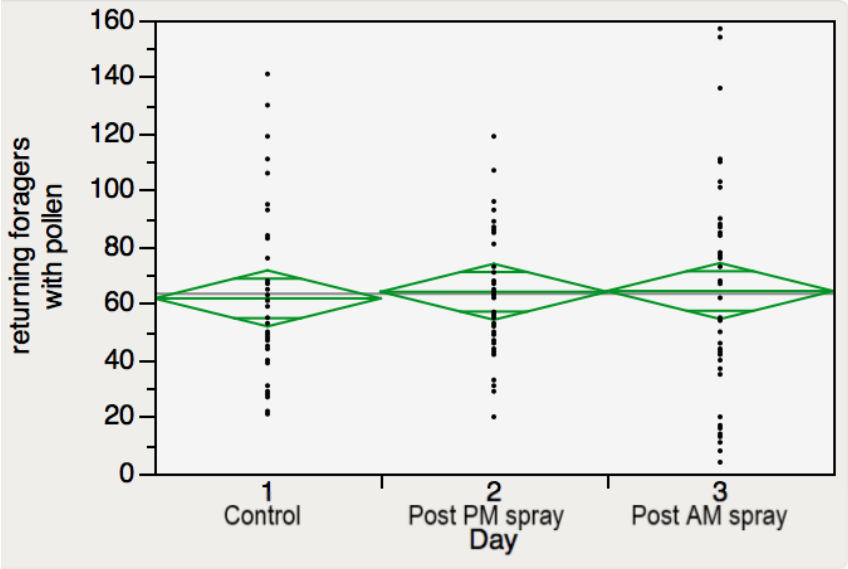
Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	40	126.450	7.5358	111.53	141.37
2	40	119.700	7.5358	104.78	134.62
3	40	112.000	7.5358	97.08	126.92

Std Error uses a pooled estimate of error variance

Figure 10: Total number of foragers returning to the hive in all locations per day of study. From the results in (**Figure 10)**, total returning foragers show no significant differences by day.

Oneway Analysis of returning foragers with pollen By Day



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Oneway Anova

Summary of Fit

Rsquare	0.0014
Adj Rsquare	-0.01567
Root Mean Square Error	31.54381
Mean of Response	63.44167
Observations (or Sum Wgts)	120

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	2	163.22	81.608	0.0820	0.9213
Error	117	116416.38	995.012		
C. Total	119	116579.59			

Means for Oneway Anova

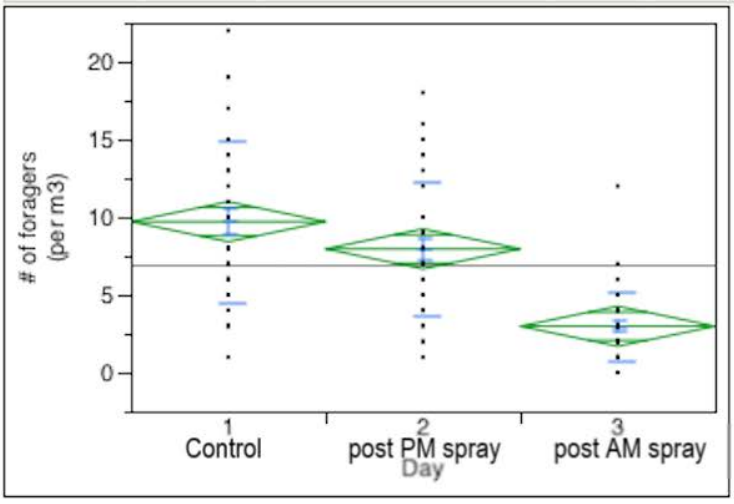
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	40	61.8000	4.9875	51.922	71.678
2	40	64.1250	4.9875	54.247	74.003
3	40	64.4000	4.9875	54.522	74.278

Std Error uses a pooled estimate of error variance

Figure 11: Number of foragers bearing pollen returning to the hives in all locations over the three days

From the results in (**Figure 11**), returning foragers bearing pollen showed no significant differences by day

Oneway Analysis of # of foragers (per m3) By Day



Missing Rows 120

Oneway Anova

Summary of Fit

Rsquare	0.329101
Adj Rsquare	0.317632
Root Mean Square Error	4.119243
Mean of Response	6.875
Observations (or Sum Wgts)	120

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	2	973.8500	486.925	28.6964	<.0001*
Error	117	1985.2750	16.968		
C. Total	119	2959.1250			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	40	9.70000	0.65131	8.4101	10.990
2	40	7.95000	0.65131	6.6601	9.240
3	40	2.97500	0.65131	1.6851	4.265

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
1	40	9.70000	5.22420	0.82602	8.0292	11.371
2	40	7.95000	4.32020	0.68308	6.5683	9.332
3	40	2.97500	2.22443	0.35171	2.2636	3.686

Figure 12: Number of foragers that were counted on the blooms of the trees in the sprayed areas of the orchard over the three days

The only category of forager data that showed significant differences was 3) the foragers that visited the sprayed trees (**Figure 12**). This effect may be due to spray time or to accumulation of exposure over time or both. Day 3 counts of foragers visiting sprayed trees were significantly lower than the Day 1 and Day 2 counterparts suggesting that exposure to fungicides on the blooms caused a diminishment of forager visits at the trees without influencing overall foraging behavior at the hives. At all hive locations, total foragers continued to return at the same rates, and pollen bearing foragers returned at the same rates. The decrease of visiting foragers at the sprayed trees occurring over time but becoming significant by Day 3 may be attributable to several factors. The bees may be avoiding the sprayed trees for physical reasons; the fungicide concentration may cause the blossoms to become more difficult landing surfaces by making the blossoms more slippery or sticky (as examples). The blossoms may be unattractive to honeybees for chemical reasons; the fungicides may bear an unattractive odor, mask the natural odor of almonds, or may irritate the feet of the bees (as examples). The blossoms may be avoided for biological reasons: the fungicides may disorient, or even kill the foragers (as examples) thereby decreasing the number of returning foragers who would waggle dance enthusiasm for a given direction or distance (3) causing progressively fewer bees to visit fungicide-sprayed trees (4,5). These hypothetical reasons to explain forager avoidance of the trees would require extensive research to elucidate the actual cause(s).

Weather issues: Sunrise on day 1 occurred at 6:48am and rose one minute earlier for each subsequent day. Sunset occurred at 17:33 pm and set one minute later for each subsequent day. Wind directions were variable for all three days and no precipitation fell throughout the study period. (**Table 1**) summarizes key meteorological measures for the three days.

Table 1: Weather conditions for Shafter, CA for Feb10-Feb12, 2018

Day	Highest temp °C /time	Lowest temp °C/time	RH High/ low	Avg. wind speed mph	Highest wind speed mph	% clear sky	Atm Press High/ low
1	23.9/13:54	10/23:54	69/32	6	14	100	29.43/29.30
2	22.2/14:54	5.6/6:54	64/17	4.9	16	100	29.49/29.21
3	15.6/14:54	5.6/3.54	73/33	5.3	11	54	29.44/29.29

The PM sprays took place at a wind speed of 6 mph with winds from the NNW and the AM spray took place at 7 mph with winds from the ESE, in accordance with the label instructions. Generally, weather was calm and consistent for three days. Spray events occurred on time because wind speeds were calmer than or at the label recommendation of 7 mph at the times of the sprays. The afternoon of Day 3 was lightly overcast compared to the clarity of the two previous days. Weather can be a confounding factor in field studies because pesticide spray drift can create unwanted zones of exposure, precipitation and high windspeeds can diminish forager counts (6), and heavy dews could potentially wash the fungicides from the tree surfaces. The weather pattern for this study was basically ideal for diminishing confounding factors**.**

Discussion:

Concentration levels of both fungicides were highest on the tree blossoms within the spray area as seen by the anther pollen data. Concentration levels orchard wide generally followed an inverse relationship with distance: the higher the fungicide concentration, the closer the distance to the spray area.

Foragers that returned to the hive and foragers bearing pollen that returned to the hive were not significantly different across the three-day study. However, foragers that were counted in the tree blossoms of the spray areas showed a significant drop by the third day of the study, illustrating a behavior difference possibly related to the timing of the spray events and/or sequential spray events. Another behavior change that may be directly impacted by the AM versus the PM spray times is the concentration of prop in hive pollen. The longer lapse of time before foragers had access to the prop-sprayed blossoms may have allowed prop to degrade and caused less prop to be found in hive pollen after the PM spray than after the AM spray. It is reassuring to note that the largest concentrations of cyp and prop were in the spray areas (drift was a minor factor) and that the levels of concentration were similar for both spray events, which confirms that the air rig application method provided consistent delivery. Both cyp and prop had small quantifiable background levels of concentration (>2ppb LOD) on the Control Day (Day 1) but the background contribution was small or non-existent relative to spray event concentrations. Background levels might be contributed by previous spray events or drift from recently sprayed neighboring orchards, the history of which might be difficult to find.

One confounding factor may be unavoidable. When spray events took place, the number of blooms was evolving. It is impossible to control the opening of the blooms so within spray areas, some blooms will open and will not be as contaminated as blooms which were fully open at the time of the spray. This orchard was populated with Monterey and non-Pareil tree varieties which may bloom on slightly different schedules. Averaging the results should lessen the specific differences in blossoms in the spray areas.

The research objectives were to establish a method to analyze fungicide exposure levels to honeybees in the field and to ascertain if one could perceive behavior aberrations in foragers after timed sprayed events. This research successfully supported both objectives suggesting that the research design is worthy for further studies. Future research might include:

- determining the behavioral cause for the significant decrease of foragers on sprayed blooms over the three-day study
- testing four pesticides in an orchard (one at each corner) to ascertain if synergistic relationships can be measured on honeybees in the field.
- testing if degradation rates of different compounds in the field occur at different rates
- testing if different application methods have differing impacts on bee behavior and
- testing if the passage of a longer time (a week+?) causes behavior modifications based on an accumulated load of xenochemicals and if so, do the behavior modifications eventually lessen over time (several weeks?).

Special thanks go to Dr. Steven Cook for advice on analysis.

Research Effort Recent Publications:

If permission is granted by the Almond Board, this research will be orally presented at an American Chemical Society lecture at Drexel University, Washington DC in Sept 2018, the York County Beekeepers Assoc at the Penn State York Campus, PA in Sept 2018, and at the Chesapeake Bay Watershed Project at Reisterstown, MD in Oct 2018. This research will be submitted for potential publication to the Journal of Economic Entomology or to the Journal of Environmental Entomology in Fall 2018.

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