Impacts of Insecticides and Fungicides Found in Migratory Honey Bee Colonies on Immune Function and Varroa Population Levels

Project No.: **12-POLL9-Frazier**

Project Leader: James L. Frazier
Department of Entomology
Center for Pollinator Research
Penn State University
501 ASI Building
University Park, PA 16802
814.863.7345
814.865.3048 (fax)
jfrazier@psu.edu

Project Cooperators and Personnel:
Maryann T. Frazier, Christopher Mullin, Diana Cox-Foster,
Department of Entomology, Center for Pollinator Research
Penn State University

Objectives:

Determine if the pesticide loads in pollen and wax found in migratory beekeeper colonies reduce colony fitness.

1) Assess queen egg laying, brood development, hypo-pharyngeal gland volume, and adult bee longevity in colonies treated with known levels and combinations of fungicides and insecticides.
2) Assess immune function by quantifying deformed wing virus (DWV) and Nosema levels as well as tracking down regulation of specific immune genes.
3) Assess Varroa mite levels in brood cycles before, during, and following pesticide exposure.
4) Evaluate the impacts of commercial beekeeper prepared pollen substitute to mitigate impacts of pesticide on adult longevity, hypo-pharyngeal gland size and immune function.
5) Ascertain if insecticide laden pollen, fungicide laden pollen, or pesticide laden wax has equal impacts on colony fitness or not, and if combining one or more of these gives significant additive or synergistic impacts.

Interpretive Summary:

Sub-lethal impacts of pesticides were determined through altered colony functioning during and after pesiticide dietary exposure via Megabeefeeding supplement and increasing impacts seen when dietary exposure was combined with contaminated wax comb. Partial mitigation of these impacts was observed by feeding colonies on a high protein diet supplement. The sub-lethal impacts measured during these experiments would not normally be seen by beekeepers during their routine colony level inspections. These results indicate that modifications are needed for both proper honey bee colony management as well as for improved risk assessment for honey bee safety to candidate pesticides during the registration process. Using the modifications of colony level functioning measured in our experiments as inputs to a new simulation model of honey bee colony population dynamics, model predictions (Zhu,

2013) indicated reduced size of the colony during the season of pesticide exposure, but successful survival over the winter if no other stressors were present. Such reductions in colony size carry specific costs to beekeepers and should be included in any assessment of pesticide impacts on honey bees resulting from exposure during pollination events or through unintended exposure throughout the active season.

During the first week of feeding colonies on treated diets, all diets were consumed equally, indicating that all colonies were getting an equivalent amount of pesticide treatment. Queen egg laying rate and first instar larval survival were significantly reduced more than 50% after two weeks of feeding on the fungicide contaminated diet in combination with treated wax, while only insecticide diet plus contaminated wax gave less, but significant reduction in queen egg laying. This is a major sub-lethal impact on the colony for fungicide contaminated diet, and to a lesser degree insecticide contaminated diet in combination with pesticide contaminated wax, and indicates that fungicide sprayed pollen brought back to the colony is likely to have negative impacts on the colony within two weeks of exposure. Similar potential is indicated for insecticide contaminated pollen as well.

Colony impacts from treated diets and wax combinations varied by treatment and by dates across the remainder of the field season. Pollen consumption was significantly reduced in colonies with the dimethoate insecticide treatment as a positive control, while honey collection was significantly reduced in colonies fed only insecticide diet or dimethoate diet. Insecticide diet only resulted in a loss of honey collection and colony weights throughout the season. Brood to adult ratio, and foraging frequency were unchanged for all treatments except the dimethoate positive control. Varroa levels were consistently low among all treatments throughout the season except for the insecticide diet feeding in the 4th week of the season.

Following the removal of capped brood from the treated colonies after three weeks; colonies were allowed to forage naturally and were also given supplemental feeding with a beekeeper-produced proprietary high protein diet mixture. This feeding regime produced rapid increases in brood rearing and colony weights within two weeks even for the dimethoate positive control, suggesting that increased nutrition can provide some rapid recovery to sub-lethal impacts of pesticides at the colony level. These results should be studied further with the potential to develop improved management strategies to minimize pesticide impacts following pollination events or for use during resting periods to rejuvenate colony health.

Virus levels were unusually high in our experimental colonies this season as part of a trend we have seen over the last 3-4 years. Colonies fed diets with insecticide contamination resulted in an increased titer of DWV in bees over time as compared to control or fungicide treatments. The combination of exposure routes (wax treatment plus exposure through the diet) could not be evaluated given that the treated brood had limited survival. Our plans to measure adult longevity and hypopharyangeal gland development could not be completed during this season due to high levels of disease throughout the colonies and the failure of control colonies to survive normally. The second season of treatments and corresponding longevity, disease, and hypopharyangeal gland tests are still underway at this time.

Materials and Methods:

Honey bee colonies were established from packages in April 2012 and requeened with sisterqueens **(Figure 1)**. Selection of colonies with stable queens and comparable colony dynamics reduced our test population to four colonies/diet treatment. Four colonies/treatments were fitted with pollen traps and pollen stores reduced to a minimum prior to the introduction of Megabee diet patties containing test mixtures. The test colonies were divided between two apiaries with known histories of relatively pesticide-free pollen access during most of the field season. Test diets with pesticide levels and mixtures corresponding to those measured from migratory beekeeper hives were prepared in the laboratory and fed for two weeks with renewal of the pollen patty every 5 days. Colony development was followed over two brood cycles and the resulting capped brood reared on these diets were taken to the lab and held in cages under hive temperature and humidity conditions for various tests of disease levels, immune function, adult longevity, and hypopharangeal gland development tests. Colony level measurements were taken before, during, and after the diet feeding periods. Sections of wax comb were also treated with a pesticide mix or a methanol solvent control and tested either individually or in combination with the diet mixes. The following treatments were used beginning July 14, 2012:

1. Untreated wax and Megabee Diet: Control (C)
2. Dimethoate insecticide Diet; Positive pesticide control (D)
3. Insecticide Diet (I)
4. Fungicide Diet (F)
5. Treated Wax or Control Wax (W or CW)
6. Insecticide Diet plus Treated Wax (I + W)
7. Fungicide Diet plus Treated Wax (F + W)

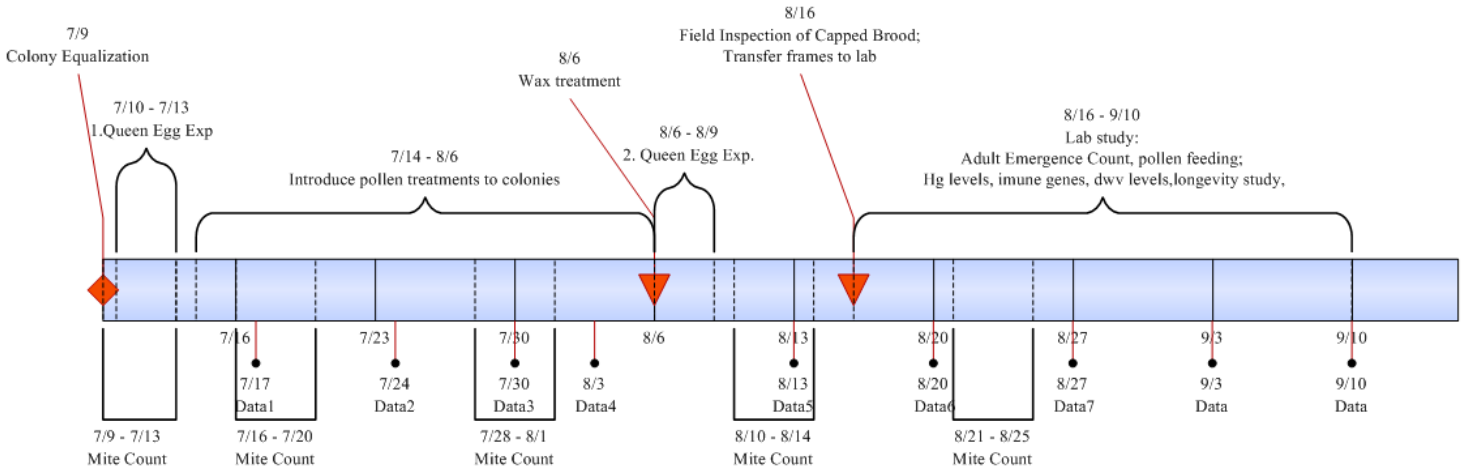


Figure 1. The experimental protocol for treated diets and wax on honey bee colonies during the 2012 field season at Penn State University.

1. PESTICIDE SOLUTION PREPARATION

   Formulated pesticides in 100% MeOH solution were made up in combination and "painted" on the wax comb using nylon bristle brushes utilizing all of the solution. Diet treatments were made with formulated pesticides made up in water. We cut out and weighed a sample of comb wax equal to the size of the test and control brood areas (11 cm x 11 cm = 6 gm) to determine the mass of wax needed to be treated to obtain the ppb levels found in the beekeeper comb analyses. A 4 gm treated sub sample of wax was analyzed for pesticide levels to confirm our treatment method dose. Another 3 gm sample of the untreated wax comb was analyzed to confirm background levels of pesticides remaining after summer usage and/or after irradiation of the comb. We sampled 3-5 combs by taking 3 gms each and combining them by freezing and grinding together to obtain a representative sample for analysis.

2. DIET PREPARATION

   We used Megabee protein supplement (Mann Lake) for bees treated with a given pesticide mix of formulated materials to give the desired levels for the test. Total protein diet needed for the test was calculated by determining the amount needed/colony (43 grams for a colony of 5000 bees) over the development period for brood x total colonies to be fed. Diet will be made up fresh and changed in the field every 5-7 days throughout the experiment. We used 239 gm diet + 203 gm sucrose + 203 ml water or 203 ml. pesticide mixture for each protein treatment. This yielded enough for treating 20 colonies with a 1 ½ inch x 10 in x 10 inch protein patty with an aluminum foil cover separating it from the top frame and the upper surface peeled back to expose the diet.

   Pesticide mixtures based on analyses of migratory colonies and those selected for each diet are indicated below (deletions due to low levels or unavailable to us):

   Insecticide (+ herbicide) diet treatment (ppb in final patty mix – Formulation used)

A.	COUMAPHOS	4 PPB	CoRAL
B.	AMITRAZ	136 PPB	Taktic
C.	CARBARYL	124 PPB	Sevin XLR Plus
D.	FLUVALENATE	103 PPB	Mavrik Aquaflow
E.	ALDICARB	8 PPB	deleted
F.	FENPROPATHRIN	1 PPB	Danitol
G.	ENDOSULFAN	2 PPB	Mionex
H.	CHLORPYRIFOS	4 PPB	Lorsban 4E
I.	METHOXYFENOZIDE	4 PPB	Intrepid 2F
J.	NORFLURAZON	5 PPB	deleted
K.	PRONAMIDE	58 PPB	deleted
	TOTAL =	388 ppb	

Fungicide Diet Treatment (ppb in final patty mix- Formulation used)

CHLOROTHALONIL12	772 PPB	Bravo Weather Stik
CAPTAN	136 PPB	Captan 80WG
AZOXYSTROBIN	7 PPB	deleted
TOTAL =	12,908 PPB	

Wax Pesticide Mixture Treatment – (ppb for wax weight- Formulation used in 100%MeOH)

A.	ALDICARB	10 PPB	deleted
B.	FLUVALENATE	3018 PPB	Mavrik Aquaflow
C.	CHLOROTHALONIL	7691 PPB	Bravo Weather Stik
D.	AMITRAZ	4213 PPB	Taktic
E.	PERMETHRIN	35 PPB	Pounce 3.2 EC
F.	FENBUCONAZOLE	3 PPB	deleted
G.	FENPROPATHRIN	3 PPB	deleted
H.	COUMAPHOS	4539 PPB	CoRAL ELI
I.	ENDOSULFAN	7 PPB	deleted
J.	ESFENVALERATE	1 PPB	deleted
K.	AZOXYSTROBIN	3 PPB	deleted
L.	CHLORPYRIFOS	2 PPB	deleted
M.	PRONAMIDE	5 PPB	deleted
N.	METHOXYFENOZIDE	81 PPB	Intrepid 2F
O.	DICOFOL	1 PPB	deleted
	TOTAL =	19,577 PPB	

A 4 gm treated diet sample was submitted for pesticide analyses to confirm our method of treatment and the exact quantities of pesticides in the mixtures. Diet was fed in an open super above the colony along with sugar water at 50% concentration for the duration of the queen egg laying test and the brood development period (15 days).

3. WAX PREPARATION

Wax from the 20 cm x 20 cm test area was removed from a frame and weighed to give the total wax mass for calculating pesticide residue levels. Pesticide solution was prepared by weighing out an appropriate amount of the formulated material, dissolving in 100% MeOH solution of appropriate volume and painting this solution onto the cells of the treatment area with a nylon bristle brush repeatedly until all solution was used and frames allowed to dry in the hood overnight. Effort was made to give an even distribution of solution over the test area. An additional sample was prepared and treated for the sub-sample for pesticide analysis.

4. DISEASE SAMPLE PREPARATION AND ANALYSIS

Samples of 50 bees per colony were collected from house bees prior to feeding the diets to the colonies (July 6, 2012) and then later from the frames containing the treated and control wax (Sept. 1, 2012). These samples were frozen at -80°C and later analyzed for viral diseases using RT-PCR using methods previously published (Singh et al. 2010). Selected samples were sequenced to confirm the identity of the viruses and their strains.

Results and Discussion:

During the first week of stable colony development prior to feeding any test diets, background measurements were taken on all colonies to confirm colony functioning including brood to adult ratio, foraging frequency, and overall colony weights reflecting the total brood, honey, and pollen stores. **Figure 2** shows these background colony measures and together with the measured queen egg laying rates shown in **Figure 3**, these measures were used to remove colonies (158,162,166,167,168,170,172,181,186, and 190) from the test pool of colonies due to low brood numbers, foraging, and queen egg laying rates.

During the first week of feeding treated diets to the colonies, Megabee patties were weighed before and after 5 days of feeding to determine if all diets were being consumed in the equal amounts. **Figure 4** shows that all treated diets were consumed in amounts equal to the control diets and to each other indicating that all colonies were acquiring equivalent amounts of treatment.

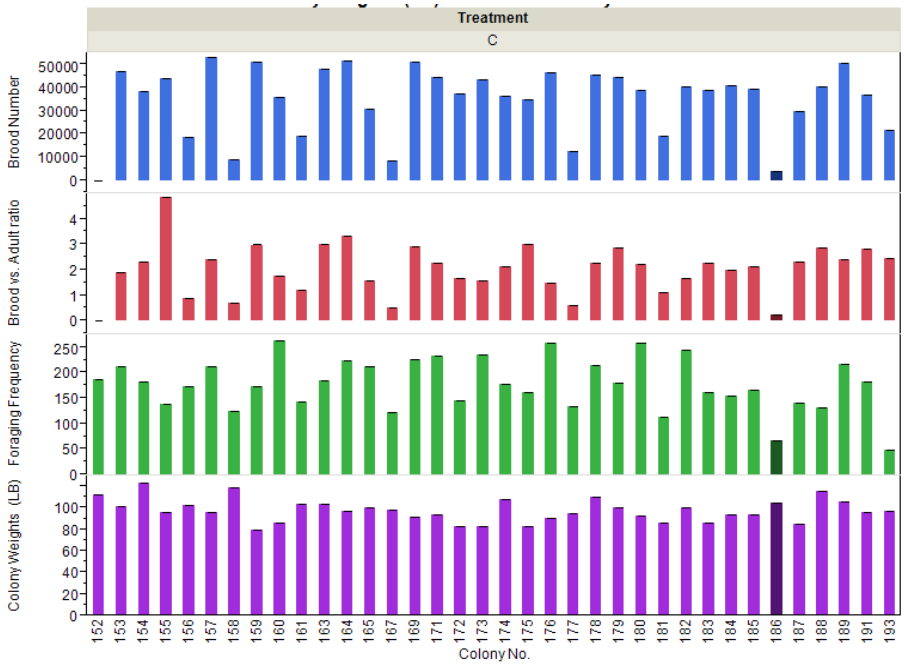


Figure 2. Colony level measurements prior to diet feeding resulted in some colony removal from the testing pool.

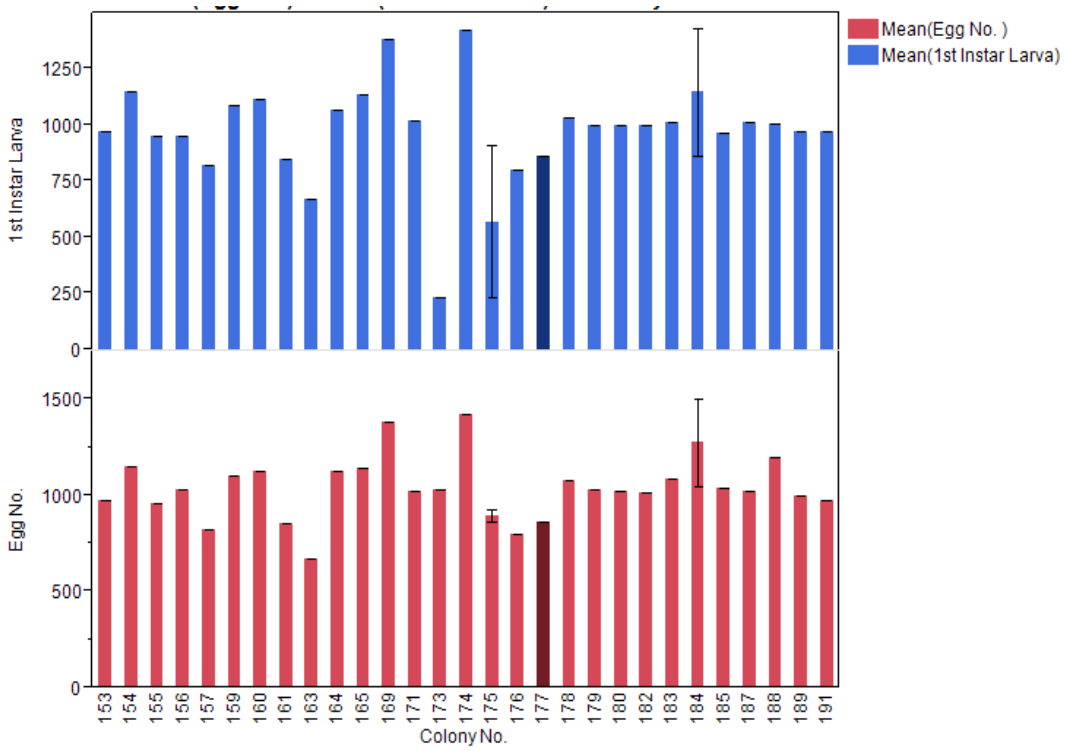
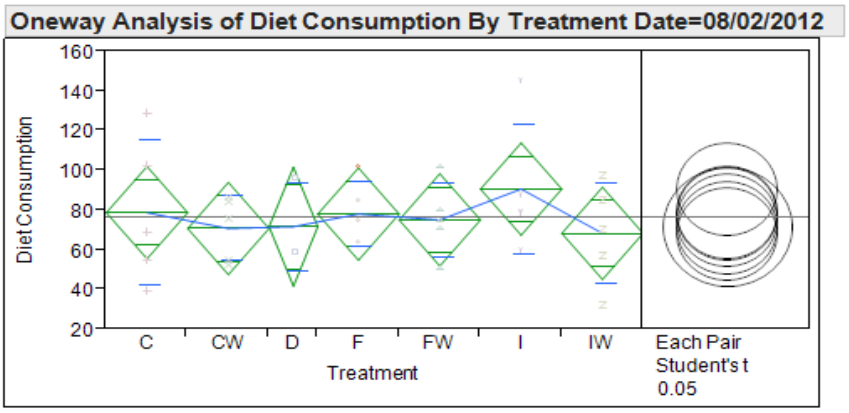


Figure 3. Queen rgg-laying rate and first instar survival prior to treatments allowed for selection of stable colonies for further testing.



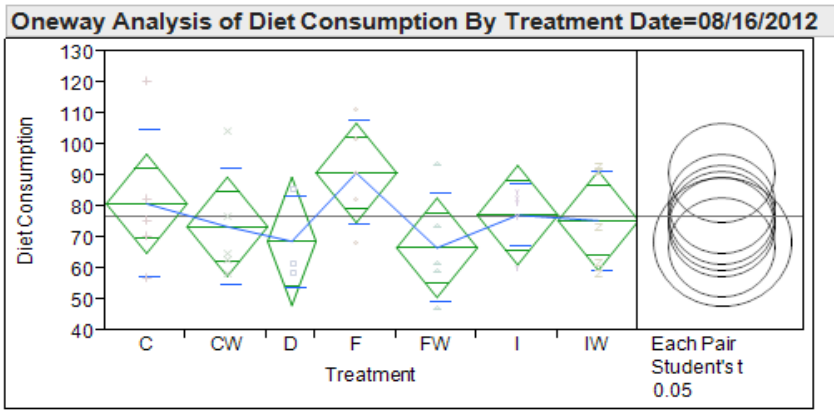
Connecting Letters Report

Level		Mean
I	A	90.886000
C	A	79.010000
F	A	78.284000
FW	A	75.364000
D	A	71.926667
CW	A	70.996000
IW	A	68.594000

Levels not connected by same letter are significantly different.

Figure 4. Diet consumption during the first week of treatment with showing no significant differences.

During the second week of diet feeding, however, feeding on some diets was reduced including a reduction in dimethoate positive control and in fungicide diets combined with treated wax (**Figure 5**). The fungicide diet alone was not reduced, indicating that fungicide levels in the diet were not the important factor for reduction, but when combined with contaminated wax it was significant. We anticipated that the dimethoate diet would potentially reduce consumption since this level of insecticide was chosen to kill some bees and potentially larvae within the test colonies. It should be pointed out that only the fungicide diet plus contaminated wax combination was significantly reduced from both the first and second week feeding levels on all other diets.



Connecting Letters Report

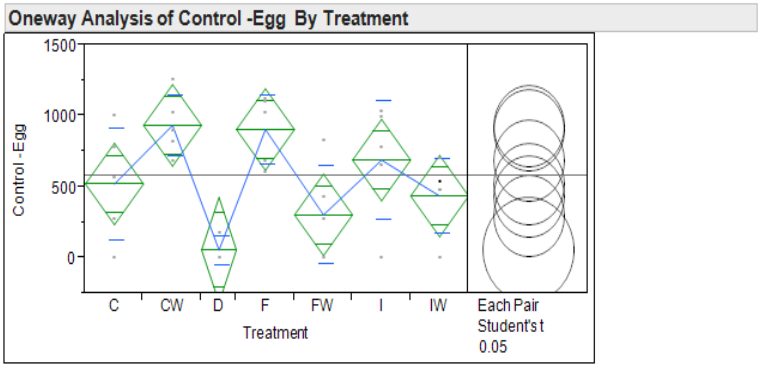
Level		Mean
F	A	91.022000
C	A B	81.068000
I	A B	77.404000
IW	A B	75.634000
CW	A B	73.682000
D	A B	68.943333
FW	B	66.874000

Levels not connected by same letter are significantly different.

Figure 5. Diet consumption during the second week of treatment showing reduction in fungicide diet combined with treated wax compared to fungicide diet only.

The most dramatic sub-lethal effects of feeding the colony on contaminated diets occurred during the second week of feeding where both queen egg laying rate and survival of first instar larvae were greatly reduced to about 50% of control values (**Figures 6, 7, and 8**). The dimethoate diet gave by far the lowest level egg laying, followed by significant reductions for Insecticide diet and then fungicide diets combined with treated wax to levels half and one third of control levels respectively. This clearly indicates that the combination of treated diets and treated wax is far more deleterious that treated diets alone, and supports a conclusion that the most deleterious sub-lethal impacts on queen egg laying and larval survival require these combinations. **Figures 7** and **8** clearly indicate the significance of the combination impacts on egg laying and first instar survival - both impacts that would be easily missed in normal beekeeper colony level evaluations. However these impacts were determined in our experimental protocols because we were interested in taking detailed measures of potential impacts on the colonies to use in verifying our mathematical simulation model under

development during the summer of 2012. These results raise important considerations for how sub-lethal impacts of pesticide contaminated pollen and nectar coming into bee colonies managed for pollination should be monitored to permit both management of healthy hives and for determining adequate risk assessment during evaluation of candidate pesticides for registration purposes.



Connecting Letters Report

Level					Mean
CW	A				937.20000
F	A	B			907.60000
I	A	B	C		692.80000
C		B	C	D	524.20000
IW			C	D	439.20000
FW			C	D	307.00000
D				D	59.33333

Levels not connected by same letter are significantly different.

Figure 6. Queen egg laying during the second week of treatment showing significant reductions for insecticide and fungicide diets when combined with treated wax.

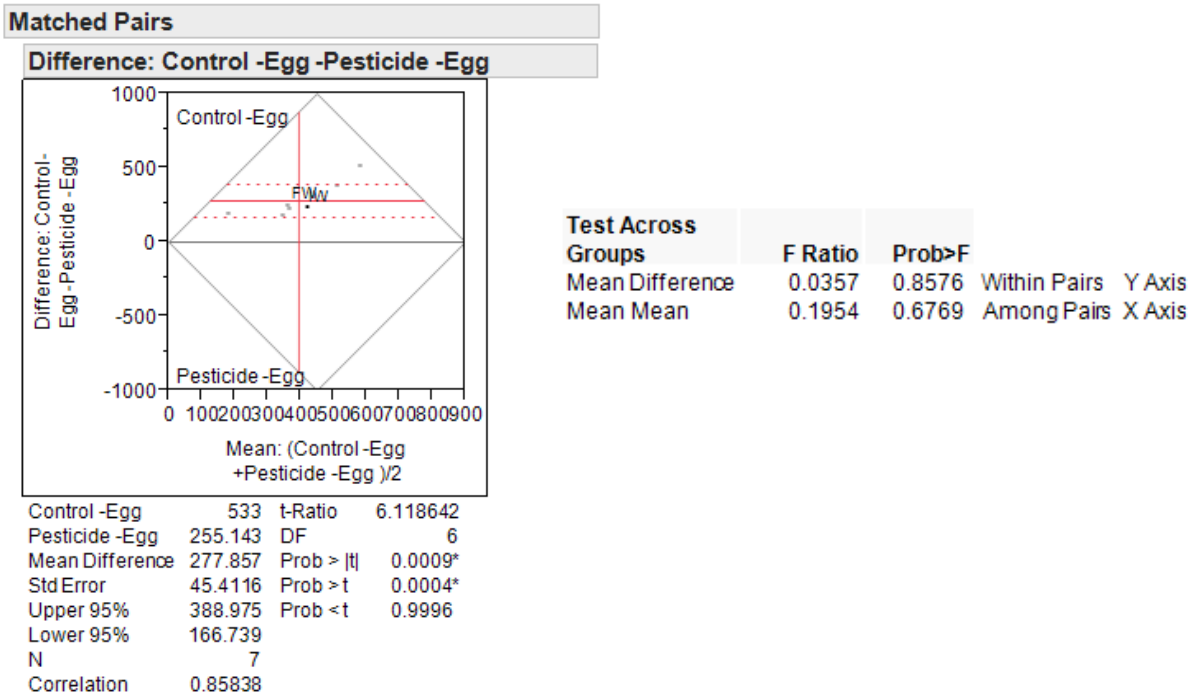


Figure 7. Paired tests of significant reduction in queen egg laying rate resulting from feeding of pesticide diets.

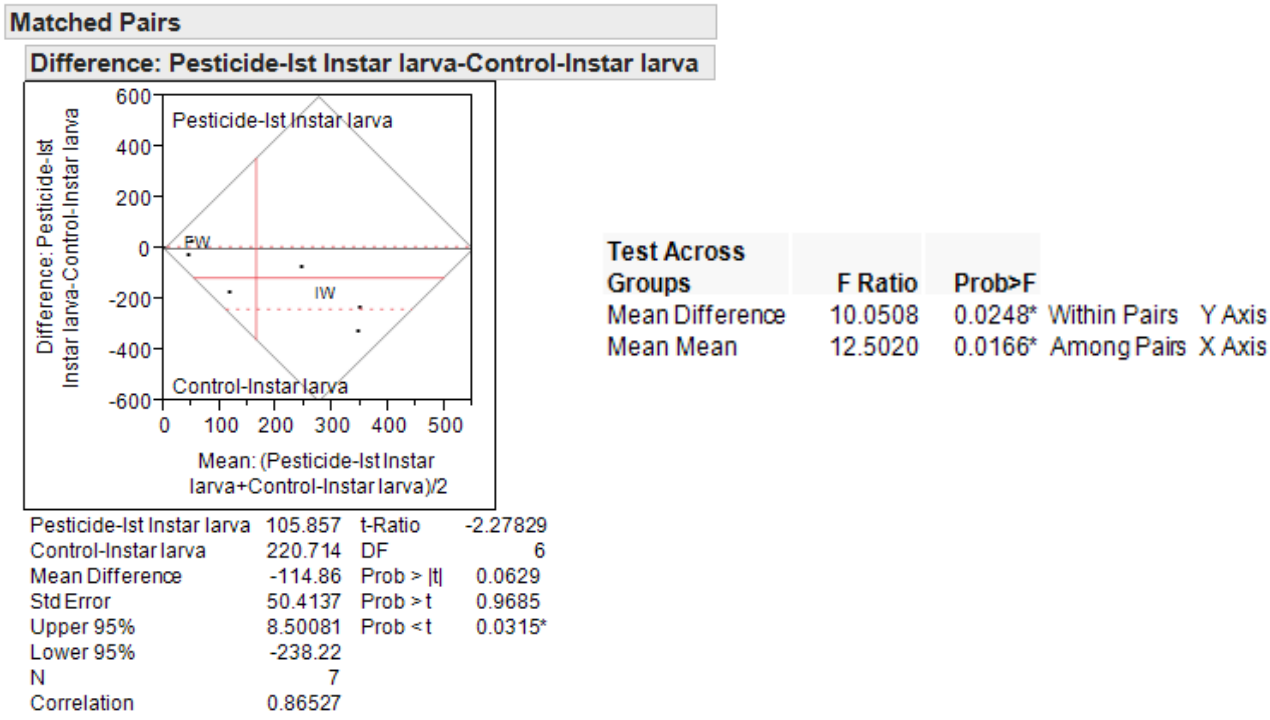


Figure 8. Paired tests of significant reduction in first instar survival resulting from feeding of pesticide diets.

After the diet feeding period of two weeks, the experimental colonies were followed for several weeks to determine additional impacts by monitoring multiple aspects of colony functioning. **Figures 9, 10**, and **11** show these impacts beginning one and a half weeks after diet feeding. For the last date (9/20/12) the impacts of feeding the proprietary beekeeper high protein supplemental diet are apparent where some rapid improvements in colony dynamics were evident.

Honey stores were significantly reduced in colonies with insecticide treated diets throughout the periods following feeding, and this was unique to this treatment alone. This measure is important given that the ability of the bees to forage for nectar and to make honey was not impeded in any way during the experiment. This impact on honey production was even greater for the insecticide treatment as compared to the dimethoate treatment suggesting that this effect is due to altered behavior of foragers more than just death of adult bees anticipated from dimethoate feeding. Indeed, the amount of pollen consumed is significantly reduced for the dimethoate feeding colonies throughout the period suggesting that dead bees and possibly brood were the cause of this reduced consumption (**Figure 9**). **Figure 10** indicates that reduced numbers of larvae, pupae, and adult bees were produced in the dimethoate treated colonies, and this too showed some recovery following the feeding of high protein diet supplement at the 9/20/12 reading (**Figure 10**).

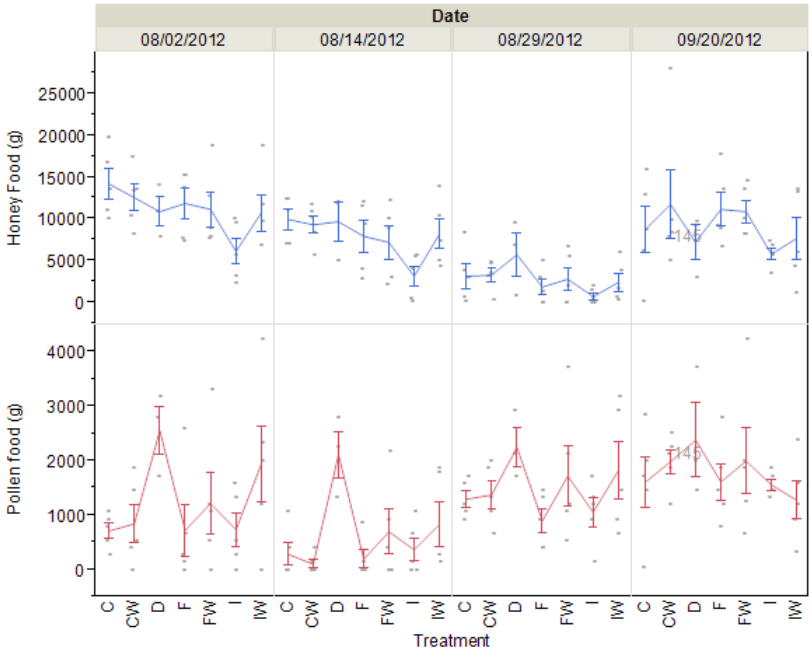


Figure 9. Colony measures of honey collection and pollen utilization during and following feeding of pesticide diets and combinations with treated wax showing reduction in honey collection for insecticide diet and reduction in pollen feeding by dimethoate positive control (higher levels of pollen food indicate that the pollen patties were not consumed as much).

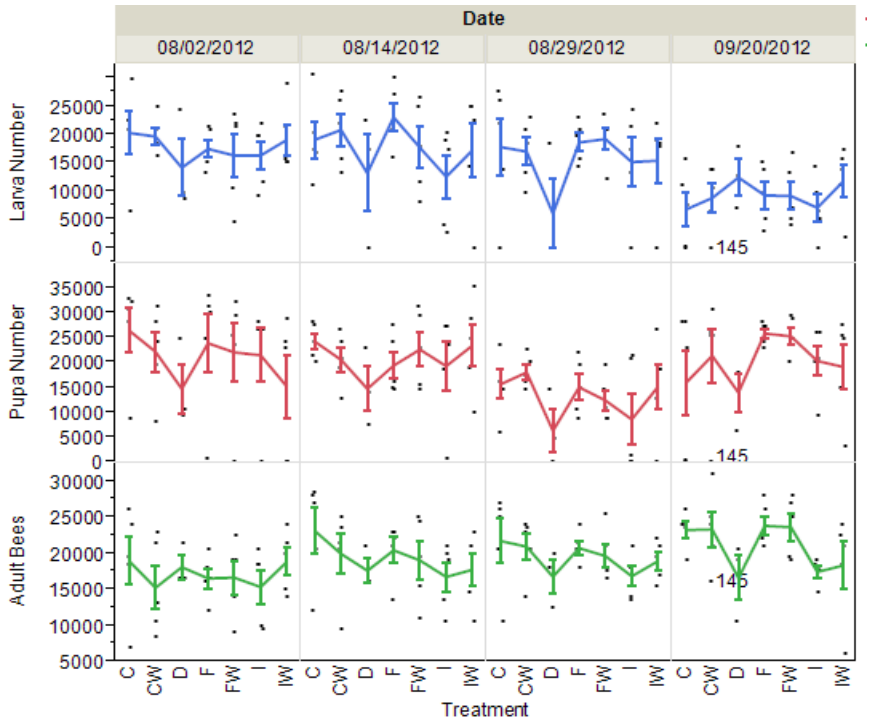


Figure 10. Colony measures of larvae, pupae, and adult bees during and following feeding of pesticide diets combined with treated wax showing reductions for dimethoate or insecticide diets and recovery in larval and pupal numbers following high protein supplemental feeding (9-20-2012).

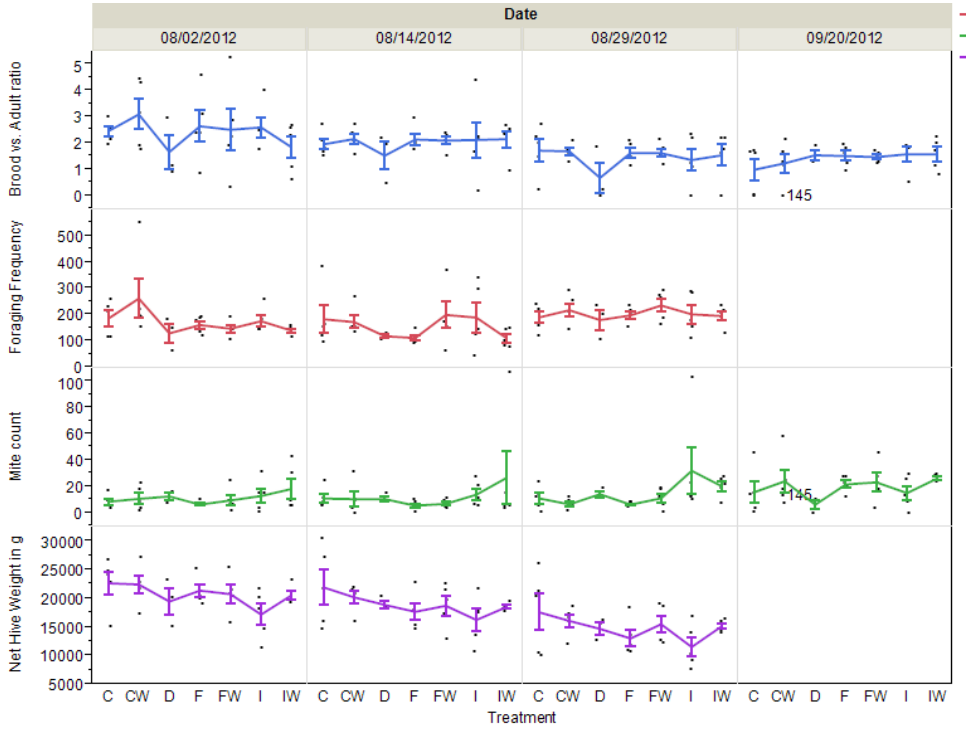


Figure 11. Colony level measures during and after feeding on contaminated diets. Significant increase for mite count for insecticide diet on 8/29 and significant reductions in hive weights for insecticide diet at 8/02, 8/14, 8/29 dates.

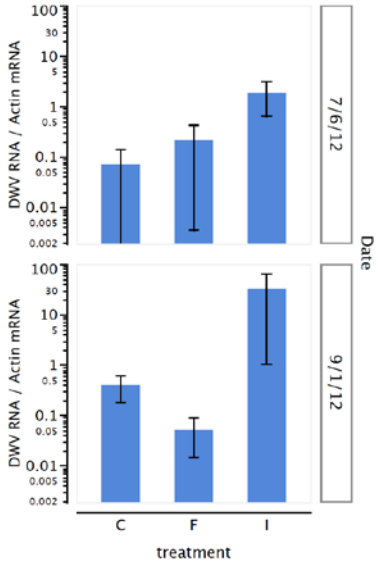


Figure 12. Colonies fed diets with insecticide contamination resulted in an increased titer of DWV in bees over time as compared to control or fungicide treatments. (3-way ANOVA, treatment/date/colony (nested in treatment), treatment p= 0.0001, 2 df, ss 38.9947, F=10.5629).

In the early samples in July 2012, indications of high disease states were present. All of the colonies had significant prevalence and titers of viral diseases at elevated levels. The early samples of the bees yielded 6 different viruses with positive detections. Deformed wing virus was present in 100% of all the colonies at a fairly high prevalence level (> 90% of bees were infected). Likewise, sacbrood virus (SBV) was present at very high prevalence in all the colonies. Black queen cell virus (BQCV) was present in most of the colonies at a more moderate level. Kashmir bee virus was present in most of the colonies and Israeli acute paralysis virus (IAPV) was found at less than 10% of the bees. Only a few samples were infected with Lake Sinai Virus-2. Chronic bee paralysis virus and acute bee paralysis virus were not detected.

The sampling date in September 2012 was marred by the lack of available bees for virus analysis. Between July and September, several of the colonies had diminished populations of bees, making it impossible to collect samples for virus analysis. In particular, only 3 colonies with treated wax comb had significant number of bees to sample from the pesticide treated comb. Problems with bee survival prevented thorough analysis of the impact of the pesticides upon the viral diseases in the bees. In the repeat of the experiment in 2013, plans were made to compensate for the high mortality of the bees by sampling on a more frequent time period. Given the cost of the analyses and the personnel time required, only select time periods will be able to analyze at the current level of resources.

Even with the problems in sampling, the pesticide exposure in the diets of the bee colonies had significant impacts. The colonies fed the mixture of insecticides had a significant increase

in the titers of the DWV virus (p= 0.05) as compared to the control colonies and the fungicide colonies (**Figure 12**). There was a significant interaction between the time and the treatment, with the level significantly increasing in the colonies fed insecticide. The levels of DWV in the control and fungicide treatments also tended to increase during the season as well. The levels of DWV infection in the bees was markedly higher than the infections observed in colonies in 2004 (Yang and Cox-Foster 2005), with the titers of the viral infections near the levels observed in varroa-parasitized bees with suppressed immune systems. The prevalence of the DWV and SBV infections also significantly increased over the season from 58% to 92% for DWV ($p < 0.0001$ Pearson test, ChiSquare) and from 54% to 63% for SBV (p= 0.0290 Pearson test, ChiSquare).

In the three colonies that could be sampled following exposure to treated wax, there was a tendency for the DWV titers to be lower in the bees exposed to the pesticide contaminated wax; however, the viral titers were still significantly elevated (**Figure 13**). The role of the viral diseases in the immature brood survival has not been adequately investigated. Given the significant impacts on queen laying, survival of early instar larvae, and decreased survival to adult stage, the role of the viruses needs to be investigated more. Potentially, the few bees that survived the exposure to the contaminated wax represented those with lower initial viral diseases, while their sisters with higher initial viral titers succumbed due to the impacts of the added pesticide exposures.

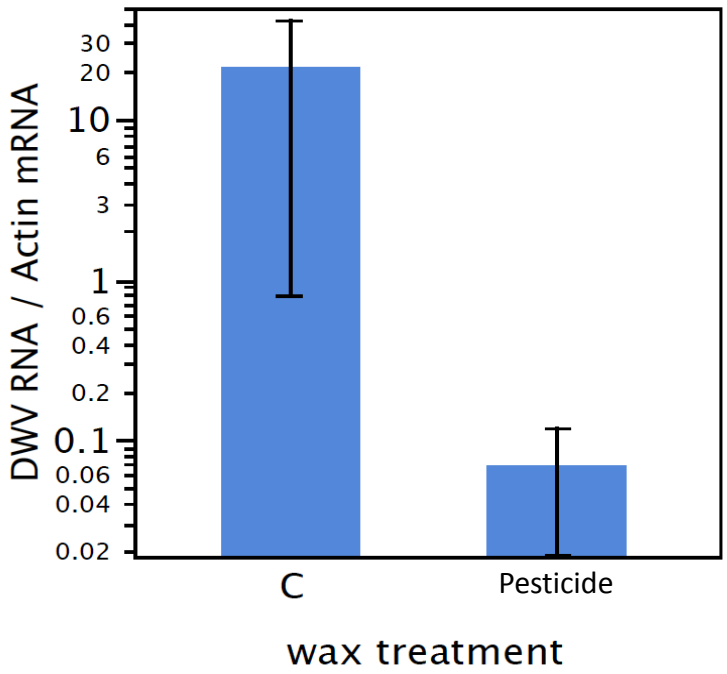


Figure 13. Bees reared on treated wax tended to have lower levels of DWV infection as compared to those reared on control wax; however, the survival rate of the bees reared in the treated wax was significantly less than those reared in the control wax.

Plans for Publication:

Results of these experiments will be combined with our migratory beekeeper pollen and wax analyses over the season and written for publication as soon as the results are presented at the Almond Board Meeting in 2013.

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