Project NS0315CO

Pesticides in Honey Bee Hives in the Maritime Provinces: Residue Levels and Interactions with *Varroa* mites and *Nosema* in Colony Stress

Applicant
Nova Scotia Beekeepers Association

FINAL REPORT V2

Submitted to:

Nova Scotia Beekeepers Association
Agri-Futures Nova Scotia
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16 December 2013

1. BACKGROUND

Honey bees, *Apis mellifera,* are a critical component of global and Canadian agriculture. The Maritime Provinces have a rich history of beekeeping and beekeepers in our region make crucial contributions to pollination service in Atlantic Canada. However, beekeepers continually face high and unacceptable overwintering losses. Parasitic *Varroa* mites, the pathogen *Nosema,* and pesticides are considered key threats to honey bees. Although many beekeepers monitor for *Varroa* and *Nosema* in their hives, we know little about pesticide loads in hives in Canadian operations, or whether residues of pesticide could be acting additively/synergistically with pathogen and parasite stressors to compromise colony health. This project undertook a pan-Maritime approach, and proposed to answer the following questions:

1. **What levels of pesticides are present in hive comb (beeswax)?** Samples will be collected from representative hives in NS, NB, and PEI.
2. **Is there a relationship between in-hive pesticide loads and the presence of *Varroa* mite or *Nosema*?** During collection of samples of comb and bees, we will determine infestation levels of *Varroa* mite and *Nosema* in the same hives, permitting correlational analyses among these variables.
3. **Do effects of pesticides at sublethal concentrations act additively or synergistically with *Nosema* to compromise colony health?** Here we will expose captive bees to nothing (control), *Nosema,* pesticide, or *Nosema* + pesticide, and determine effects on longevity and learning/memory.

Logistical problems precluded us from fulfilling the third objective. See section on *Deviations From the Proposal* for an explanation. As an alternative, and based on our pesticide residue results, we conducted experiments to determine: **how do realistic doses of amitraz (Apivar) affect honey bee learning and memory retention, and octopamine levels?**

2. METHODS AND MATERIALS

2.1 Pesticide Residues in Maritime Bee Hives.

Beeswax (comb) was collected from hives in representative apiaries in NS, NB and PEI. Beeswax was collected from three randomly selected hives in each apiary and pooled to make a single sample from each apiary. Samples were collected in summer and fall. Samples were stored at minus 20° C and shipped to Dr. Roger Simonds (USDA National Science Laboratory in North Carolina) for broad spectrum pesticide screening.

2.2 In-Hive Pesticide Loads and Incidence of *Varroa* mite and *Nosema*

Bees were collected from colonies used in the pesticide residue study. Approximately 200 worker bees from the brood area of each hive were collected into jars and frozen. In the lab, 250 ml of distilled water was added to each jar, the contents were vortexed in a Labline Orbit Shaker for 5 minutes at 130 rpm, and the contents were poured through a plastic screen into a white plastic tray to separate bees from mites, which were both counted to produce an index of mite infection intensity per sample.

The same worker bees used to examine *Varroa* mite loads were examined for presence of *Nosema*. Bee abdomens from 50 individuals per sample were crushed and suspended in 50 ml of distilled water. Estimation of *Nosema* load was done with a microscope and haemocytometer at 400 x magnification.

Varroa mite counts were standardized and expressed as "Varroa per bee" to account for inconsistent amounts of bees in each sample. Differences among Varroa mite levels were assessed by ANOVA using Minitab statistical software (Minitab Inc.). Means were separated by a Tukey test ($\alpha = 0.05$).

Nosema is typically measured as an average number of spores per bee. Two species of Nosema are known to affect honey bees in this region: *Nosema apis* and *Nosema ceranae*. Both are commonly found together. Because symptoms and treatments are similar, and differentiation through microscopy alone is unreliable, both are hereafter referred to as "Nosema". Differences among infection levels were assessed by ANOVA using Minitab statistical software (Minitab Inc.). Means were separated by a Tukey test ($\alpha = 0.05$).

Correlations were conducted to determine if there was relationship between concentration of pesticide in beeswax and incidence of Varroa and *Nosema*. Correlations were done for samples taken early in the season, and later in the season. Because a certain number positive samples are required to conduct a meaningful analysis, we focused only on those pesticides that were found in more than 15% of yards, which included: 2,4 DMPF (Amitraz metabolite); coumaphos and its oxon; fluvalinate; and vinclozolin.

2.2 Effect of Amitraz on Honey Bee Learning and Memory

Amitraz (Apivar®) is used as a miticide to control Varroa mite. The pesticide residue analysis found that amitraz is commonly used in apiaries around the Maritimes study (Table 1), and as a newer product, its use is expected to increase in the coming years.

Foraging age bees were collected in a mason jar from a hive using a modified hand vacuum. Bees were cold anesthetized and topically dosed (1 µl) with amitraz dissolved in acetone at one of 5 concentrations: 0 (acetone control), 600, 6000, 60,000, and 600,000 ppb. The lowest concentration (600 ppb) is equivalent to the 95th centile amitraz dose found in all bees examined (including non-detections) in a comprehensive study of pesticide residues in honey bee colonies in the US and certain parts of Canada conducted by Mullin et al. (2010)[1]. The highest treatment we used (600,000 ppb) was equivalent to the 95th centile dose for only positive detections found in bees by Mullin et al. (2010). Bees were then held in a dark 25 °C chamber for 24 hours.

Twenty-four hours after exposure, bees were used in proboscis extension reflex (PER) bioassays to test the effects of miticide exposure on both learning and short-term memory retention. To test for learning ability, bees were cold anesthetized and individually strapped into trimmed pipette tips, exposing only their head and antennae. Bees then underwent 6 conditioning sessions at 3 min intervals. Each conditioning session consisted of a 3 sec exposure to scented airflow (rosemary oil) followed by a 4 sec stimulation of the antennae by a droplet of 30% sucrose. If the proboscis extended, the bee was allowed a 2 sec drink. The

[1] Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D and Pettis JS, High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. PLoS ONE 5:e9754 (2010).

presence of proboscis extension during scent exposure was recorded, and indicated a learning event.

Memory retention was tested both 1 and 2 hours following the conditioning sessions. No sucrose reward was presented. Bees were exposed only to the rosemary scent and the presence or absence of proboscis extension was recorded.

2.3 Effect of Amitraz on Honey Bee Hemolymph Octopamine Levels

Octopamine is often considered the insect equivalent of norepinephrine. It acts as a neuromodulator, neurotransmitter and neurohormone and is known to be involved in fight or flight responses, aggression, and most importantly, in learning and memory in honey bees and other insects. Octopamine receptors are thought to be the main target for the amitraz mode of action. For this reason, octopamine was measured as a secondary indicator of the potential of amitraz to affect honey bee learning and memory.

Bees were collected, treated and kept for 24 hours as described in section 2.2. After 24 hours, bees were cold anesthetized and 10 µL of hemolymph was collected from the heads of 5-10 bees per treatment rep using flame-stretched capillary tubes. The hemolymph was collected into 20 µL 0.1 M perchloric acid on ice. This was then brought to a 40 µL volume using chilled 0.1 M perchloric acid. These solutions were centrifuged at 13,000 rpm for 5 min using a refrigerated centrifuge. The supernatant was then collected and stored in amber colored scintillation vials at -20 °C until analysis.

Analysis was done using HPLC-UV at 224 nm using a C-18 column and a mobile phase consisting of a 7:3 v/v mixture of 0.02 M citric acid and 0.02 M sodium dihydrogen phosphate at pH 3.0. Samples underwent filtration and a four times dilution in the mobile phase before injection into a 50 µL sample loop. The mobile phase was run at a rate of 2.0 mL/min. Peak verification was done using synthetic octopamine as well as synephrine, which was used as an internal standard for accurate quantification of octopamine concentrations.

3. RESULTS

Varroa mite and *Nosema* assessments were performed on 90 of 93 samples. The remaining 3 samples were not collected because the assigned hives were dead, male-populated or missing. Of 90 samples, 43 contained Varroa mites and 56 were positive for *Nosema*. Only 22 samples were afflicted by both.

3.1 Varroa Mites

There was a significant difference in parasitism rates between summer and fall collections ($F_{1,41}$ = 8.01; P = 0.007). Summer samples were not as heavily parasitized as samples collected in fall. Only 36.7% of summer hives had Varroa mites. Of these, the mean number of mites per bee was 0.0323 (± 0.0086). Parasitism rates later increased to 63.6% with a mean of 0.1220 (± 0.0312) mites/bee.

Nova Scotia exhibited the highest level of mite infestation with 86.1% of hives contributing to a mean parasitism rate of 0.1028 (±0.0220) mites/bee ($F_{2,40}$ = 3.52; P = 0.039). This was 10-fold the amount of Varroa mites in NB and PEI. Fewer hives in PEI were infested (36.7%) and of these the mean level of parasitism was significantly less than in Nova Scotia with

just 0.0070 (±0.0016) mites/bee. Hives from New Brunswick had 0.0089 mites/bee. This was not significantly different from either NS or PEI, although it should be noted that only one hive (3.7%) from NB tested positive for Varroa. This sample contained only 2 mites.

To test differences between all apiary locations, mean numbers of mites/bee were pooled for all 3 hives samples at each apiary to give an overall representation of infection intensity within each apiary. Not every hive at each location tested positive, but each still contributed to the overall mean infection intensity index for each apiary. Infection intensity varied significantly between sites with as many as 0.2224 (±0.0892) mites/bee at one site and none at other sites ($F_{19,70} = 3.86$; $P < 0.001$).

3.2 *Nosema*

More hives were positive for *Nosema* in summer (75%) than in fall (33.3%). However, mean infection intensity levels were not significantly different between infected hives from summer and fall collections. Summer collections had a mean 2,483,333 (±457,550) spores/bee compared to 1,934,091 (±947,324) spores/bee in fall ($F_{1,54} = 0.28$; $P = 0.599$).

New Brunswick exhibited the highest level of infestation with 81.5% of hives infected and having the highest mean greatest infection intensity: 3,452,273 (±781,409) spores/bee. Nova Scotia hives had the lowest load of *Nosema* with only 44.4% of hives contributing to a mean of 1,065,625 (±298,205) spores/bee, almost significantly less than New Brunswick's infection intensity ($F_{2,53} = 3.06$; $P = 0.055$). PEI had an intermediate level of 2,223,611 (±721,029) spores/bee in 60% of hives.

As with Varroa, infection intensity was assessed by apiary location, with pooled samples at each location used to determine overall infestation per apiary. *Nosema* loads varied significantly between sites with as many as 7,920,000 (±1,650,000) spores/bee at one site and others having none ($F_{19,70} = 6.70$; $P < 0.001$).

3.3 Beeswax

Thirty-one pooled samples (hives from a single site were pooled) were screened for a comprehensive list of 174 pesticides and degradates. Seventeen pesticides residues were identified (Table 1). All samples contained at least 2 different residues and one sample had up to 8 residues. The most prevalent were the miticides: coumaphos and fluvalinate, commonly employed against Varroa, at concentrations of up to 3,390 and 328 ppb respectively. Coumaphos oxon, a metabolite of coumaphos was also found in 67.7% of samples with a maximum detection of 107 ppb. The metabolite of the miticide amitraz, 2,4 dimethylphenyl formamide (2,4-DMPF) was detected in 61.3% of samples with a maximum detection of 39,300 ppb. Vinclozolin, a fungicide, was detected in 35.5% of samples. All other residues detected were sparsely distributed amongst all other samples.

For samples taken early in the summer, there is a significant positive correlation between the concentration of fluvalinate, and the occurrence of *Nosema* in hives from those same yard (Table 2). All other correlations between pesticide concentration and Varroa mite or *Nosema* levels were not significant. There was no correlation between levels of Varroa mite and *Nosema* ($R = -0.229$; $P = 0.33$).

For samples taken in the fall, there was a positive significant correlation between concentration of 2,4 DMPF detected in hives and the levels of *Nosema* (Table 2). This

relationship was not apparent in the early sampling. There was no correlation between Varroa levels and any pesticide. As in the summer, there was no correlation between fall levels of Varroa mite and *Nosema* (R = -0.043; P = 0.88).

It is unknown why high concentrations of fluvalinate and 2,4 DMPF were correlated with levels of *Nosema* in summer and fall, respectively. High levels of these miticides possibly caused bees to be more susceptible to *Nosema* at these times of the year. However, one would expect similar correlations with the other miticides, so this seems to be an unlikely explanation.

3.4 Learning and memory

Honey bees treated with five different amitraz concentrations were evaluated for both learning and memory retention. Bee learning ranged from 92.9% for the 60,000 ppb amitraz treatment, to 97.7%, for the 6,000 ppb amitraz treatment (Fig. 1). None of the treatments were significantly different (P > 0.05).

Both 1 and 2 hours after conditioning sessions, the same bees were tested for their ability to retain what they had learned. Memory retention after 1 hour ranged from 92.9% for the control, to 97.6% for the 6000 ppb amitraz treatment (Fig. 2). After 2 hours, memory retention ranged from 91.8% for 6000 ppb to 94.6% for 600 ppb amitraz (Fig. 3). Memory retention did not change within most treatments between 1 to 2 hours post-conditioning. It only declined slightly within the 6000 and 600,000 ppb amitraz treatments. No differences in memory retention were statistically significant (P > 0.05).

Table 1. Pesticide residues (ppb) detected in beeswax from Maritime honey bee hives, 2011.

Pesticide[1]	Positive Samples	Frequency (%)	Mean	Max	SEM	LOD (ppb)
1-Naphthol	1	3.2	28	28.0	---	10
2,4 DMPF (Amitraz metabolite)	19	61.3	9196.6	39,300.0	2109.8	4
Acetamiprid	2	6.5	10.6	12.6	2.1	8
Boscalid	3	9.7	161.7	271.0	71.5	4
Carbendazim (MBC)	3	9.7	30.8	45.4	7.4	5
Chlorothalonil	1	3.2	71.3	71.3	---	1
Coumaphos	31	100.0	495.4	3390.0	135.6	1
Coumaphos oxon	21	67.7	44.8	313.0	15.2	1
Cypermethrin	1	3.2	17.8	17.8	---	4
Fluvalinate	31	100.0	113.6	328.0	14.6	1
Paradichlorobenzene	3	9.7	2660.7	7150.0	2246.0	10
Pyraclostrobin	4	12.9	30.7	46.0	6.1	15
Pyrimethanil	2	6.5	9.2	11.6	2.4	3
Tetramethrin	1	3.2	7.7	7.7	---	10
Thiabendazole	1	3.2	1.8	1.8	---	1
THPI	1	3.2	297.0	297.0	---	50
Vinclozolin	11	35.5	2.8	10.8	0.9	1

[1] 174 pesticide residues or metabolites were screened. Only those that were detected are reported.

Table 2. Pearson correlation coefficients (*R*) and P-values for correlations of pesticide concentration with Varroa and *Nosema* levels from representative hives/bee yards in NS, NB, and PEI. Significant correlations (*P*<0.05) are highlighted in bold.

Pesticide	**Jun-Jul Samples**		**Sept-Oct Samples**	
	Varroa	*Nosema*	*Varroa*	*Nosema*
2,4 DMPF	*R* = -0.129 *P* = 0.59	*R* = -0.129 *P* = 0.59	*R* = -0.292 *P* = 0.29	***R* = 0.783** ***P* = 0.001**
Coumaphos	*R* = -0.018 *P* = 0.94	*R* = -0.094 *P* = 0.70	*R* = 0.399 *P* = 0.14	*R* = -0.035 *P* = 0.90
Fluvalinate	*R* = 0.198 *P* = 0.40	***R* = 0.613** ***P* = 0.004**	*R* = 0.131 *P* = 0.64	*R* = -0.114 *P* = 0.69
Vinclozolin	*R* = -0.107 *P* = 0.65	*R* = -0.003 *P* = 0.99	*R* = -0.444 *P* = 0.10	*R* = 0.461 *P* = 0.08

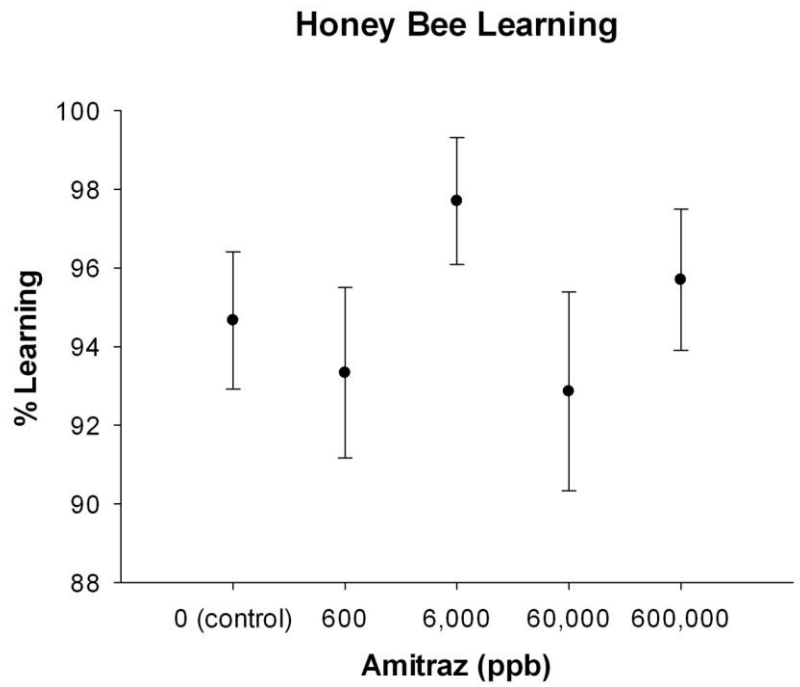

Fig. 1. Percent learning (± SEM) of amitraz-treated honey bees during proboscis extension reflex (PER) bioassays.

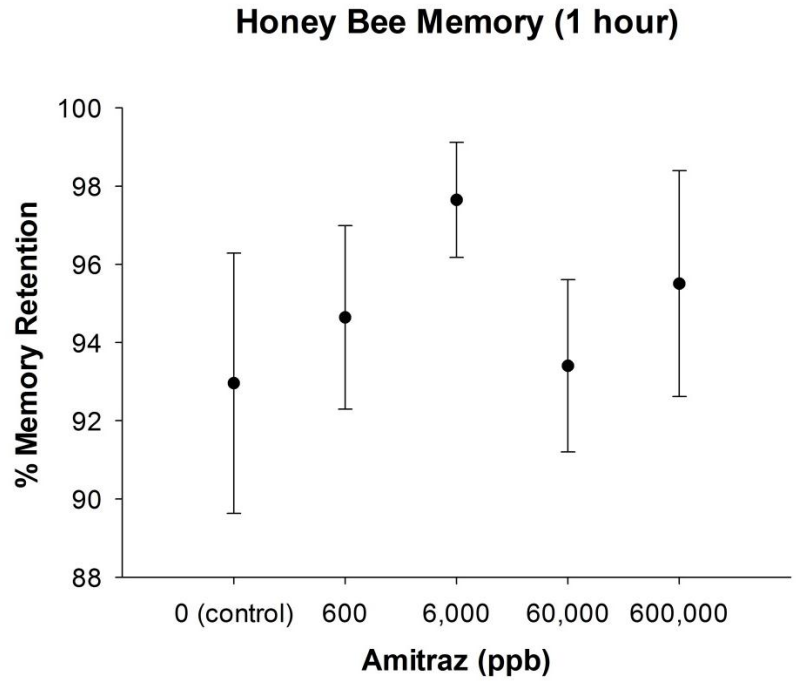

Fig. 2. Percent memory retention (± SEM) during proboscis extension reflex (PER) bioassays 1 hour after conditioning in amitraz-treated honey bees.

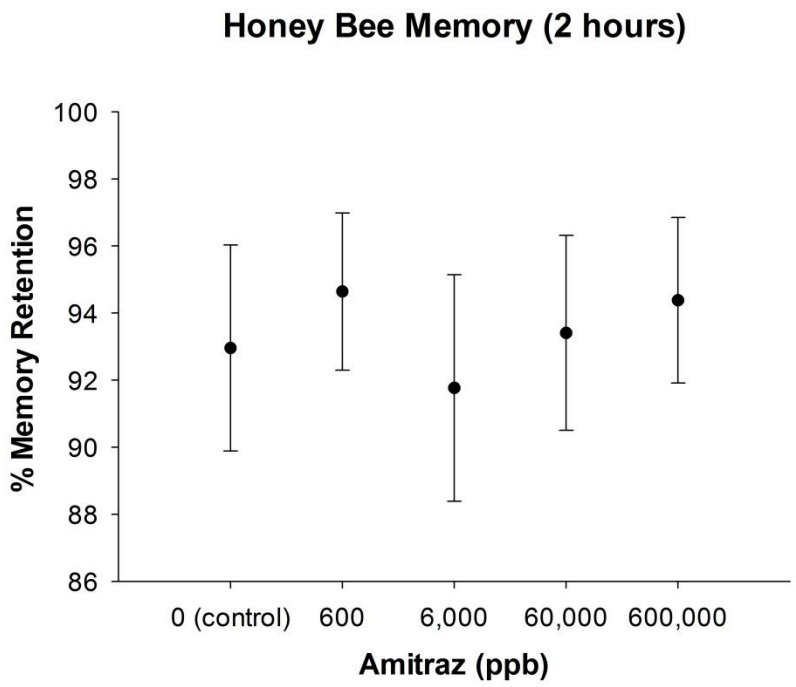

Fig. 3. Percent memory retention (± SEM) during proboscis extension reflex (PER) bioassays 2 hours after conditioning in amitraz-treated honey bees

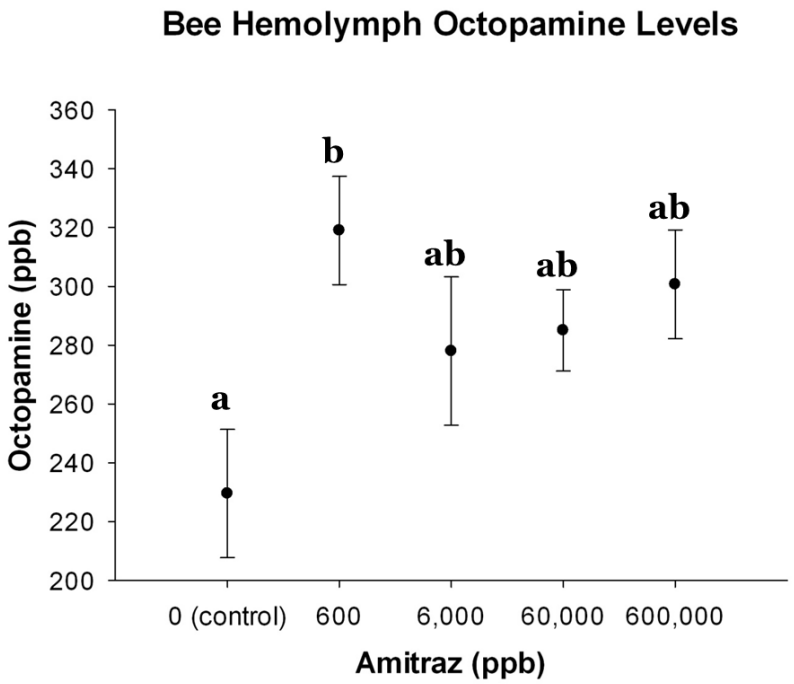

Fig. 4. Octopamine levels (± SEM) found in hemolymph of amitraz-treated honey bees.

3.5 Octopamine concentrations

Bee hemolymph from five different amitraz treatments was collected and analyzed. One-way ANOVA found that octopamine level was significantly affected by amitraz treatment ($F_{4,45}$ = 2.83; P = 0.035). A Tukey's means separation test showed that bees treated with 600 ppb amitraz had significantly higher hemolymph octopamine levels, at 319 ppb, than the control treatment, at 230 ppb. All other treatments were not significantly different (Fig. 4).

Thus, although the PER bioassay did not detect a statistically significant effect of amitraz on honey bee learning and memory retention, low concentrations of amitraz can significantly increase titres of octopamine. Additional experiments are required to determine whether or not such increases in octopamine are biologically significant.

4. DEVIATIONS FROM THE PROPOSAL

There were several deviations from the proposed research.

i. We had hoped to obtain approximately 40 samples of beeswax for residue analysis. We collected many of the samples ourselves from beehives with permission from those beekeepers. For others we sent collection kits containing instructions and all materials required to collect wax and bee samples. While many cooperators were compliant, others were not, resulting in the shortfall of samples.

ii. We had hoped to secure a MSc student for this project in May 2011. A suitable candidate was not found until May 2012. Nonetheless, much research was completed in 2011 and there were no delays in the project overall.

iii. We originally proposed to test whether *Nosema* and pesticide synergistically affect bee learning through PER or t-tube bioassays. However, several problems were encountered in conducting these experiments. It was difficult to obtain and maintain a *Nosema*-infested inoculum of high potency; this was despite having the assistance of Jason Sproule and Andony Melathopoulos our lab, who both have ample experience working with *Nosema*. In order to artificially create this population, frames of brood were removed from the hive and hatched in an incubator. Groups of bees were then removed and fed a sucrose solution mixed with a fresh *Nosema* inoculum. During the incubation period needed for *Nosema*, high mortality, accompanied by limited hive resources (new frames of young brood), precluded us from obtaining enough infected bees to maintain a fresh supply of *Nosema* inoculum. We decided that learning experiments would still be done, but they would involve a single pesticide (amitraz, which is becoming more widely used by beekeepers and has not been previously studied for potential effects on be learning), instead of a pesticide-parasite combination (see section 2.2). Additionally, since the mode of action of amitraz involves interactions with octopamine receptors, hemolymph octopamine (known to be involved in learning and memory in honey bees) octopamine was measured as a secondary indication of the potential effects of amitraz on the learning ability of honey bees.