The Effect of Oxalic Acid Treatments on Drone Semen Viability and Queen Survival, Egg Laying Rate, Egg Viability and Brood Production

Project No.: **08-POLL5-Ellis**

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Objectives:

Examine the sub lethal effects of OA on reproductive members of the colony, drones and queens. Examine queen survival and productivity and drone sperm viability in OA treated queens and drones. The response variables measured for drones will be sperm number and viability. For queens, queen longevity, egg laying rate, egg viability and brood production will be examined.

Background and Justification:

In the spring of 2007, some beekeepers in United States experienced severe unexplained colony losses. A team of scientists (Cox-Foster et al. 2007) identified the chemical burden of both in-hive and out-of-hive toxic chemicals as a potential contributing factor to colony stress, and they recommended further investigation of the role of toxins in the hive as a factor in predisposing colonies to collapse.

Honey bees are highly sensitive to most pesticides compared to other insects. This may be due to their habit of foraging for pollen and nectar, foods that contains few plant allelochemicals. Despite their sensitivity to pesticides, miticide treatments were the first line of defense when *Varroa* mites were found in the United States (Ellis 2001). Currently eight miticides are registered for *Varroa* suppression in North America. While these miticides have been evaluated for worker bee toxicity, most of them have not

been examined for toxicity to reproductive members of the colony. Likewise sub lethal effects on drones and queens have not been investigated for most compounds (Desneux et al. 2007, Belzunces et al. 1993).

Since 1987, beekeepers have used a variety of miticides to suppress *Varroa* mite populations in their colonies. Some of the miticides used in beehives leave residues in hive products (principally beeswax) that could potentially interact with subsequent treatments synergistically to shorten the life span of worker bees, reduce sperm viability in drones, or to increase the frequency of supersedure in queens.

- Some beekeepers are treating colonies with miticides as many as 3-4 times per year.
- Prior to 1985 the only agricultural chemicals used in beehives were antibiotics.
- Some miticides have been found to persist as residues in hive products (especially beeswax).
- Since beekeepers began putting miticides in their colonies, many have reported dwindling colonies, poor matings and high rates of queen supersedure.
- While the above effects may be due to biotic stress, such as *Varroa* mites, *Nosema ceranae* or bee viruses, they may also be due to chemical residues in hives or chemical interactions.

The scientists conducting this study have previously investigated the toxological properties of *oxalic* acid (OA) applied to worker honey bees and their *Varroa* mite parasite. This research extends previous work on OA to determine if it affects queen survival and drone sperm viability. OA is low in cost, and it is a highly effective varroacide when applied to broodless colonies. An investigation of reproductive effects will be an important contribution to OA registration, and it will provide scientists and beekeepers research-based knowledge about the bee safety of a promising varroacide.

Interpretive Summary:

Drone semen number and viability were not affected by OA exposure.

Once queens were mated and began laying, no queens were lost or superseded during the experiment. The only variable that exhibited a significant response was egg viability with the high dose queens having reduced egg viability when compared with low dose and untreated colonies.

Queen exposure to a dose of OA that is higher than queens would normally be exposed to, did not reduce egg laying or sealed brood production. However, it did result in a significant drop in egg viability. Since there were no differences in sealed brood production 8 weeks post treatment, it appears that the drop in egg viability may be a temporary condition that disappears over time. Treatment with a lower dose of OA that more closely resembles what a queen could be exposed to during colony treatment did not result in any reduction in egg laying, egg viability or sealed brood production. Neither the high dose nor the low dose reduced queen survival.

This study supports the recommendations that that beekeepers should be careful not to exceed the recommended dose and that they should treat colonies when brood is not present in their colonies. In this study, excessive dosing and treating when brood was present reduced egg viability.

Material and Methods:

Drone Sperm Number and Viability Studies

- Drone combs were inserted in test colonies in May 2008. After queens had oviposited in them, they were moved above a queen excluder to emerge.
- In June 2008 we captured 800 drones from above the excluder.
- We then treated cohorts of 200 drones with 2 concentrations of OA (100 and 200 µg in .5 µl acetone).
- Another 200 drones were treated with acetone only and served as controls.
- All drones were then marked with enamel paint on their thoraces using a separate color for each treatment and returned to their colony.
- We then removed the queen excluders.
- We recaptured the marked drones 14 days later and determined their sperm counts and sperm viability by dual florescent staining (Collins 2000, Garcia-González, and Simmons 2005).
- Drone sperm viability and quantity were examined in a completely randomized design using a t-test to compare outcomes. Data were analyzed using SAS.

Detailed procedures: We went through the target colony, frame by frame, early in the morning when all drones are present. We placed drones in a queen excluder box. The box was then either taken back to the lab for immediate semen collection, or placed inside a queen bank for storage. Drones were not to be kept in the box for more than one hour outside a colony. Wearing gloves, we fully everted the drones by crushing the thorax between the thumb and index finger on the right hand and squeezing the abdomen with the left hand.

We looked for the tan colored semen, careful not to contaminate semen by touching it to the drone itself. We rinsed the semen off of the drone into a 200 microliter tube using 150 microliters of modified Kiev diluent in a 1000 microliter pipette. We recollected the diluent and repeated rinse if necessary. We then stained sperm using LIVE/DEAD® Sperm Viability Kit (L-7011) (Molecular Probes, Inc; Eugene, OR) with staining protocol for insect sperm modified from (Garcia-Gonzalez and Simmons, 2005).

Next we transferred 12 microliters of diluted semen to a new 200 microliter tube and added 1 microliter of diluted SYBR14 (0.1mM) and mixed with a pipette. We then incubated at room temperature in the dark for 10 min. We added 2 microliters of propidium iodide (2.4mM), mixing with the pipette. Next we incubated the samples at room temperature in the dark for an additional 10 min. To record sperm viability under the microscope, we transferred 12 microliters of dyed semen to a clean microscope slide and cover with a cover slip. We took pictures of 5 fields of view (at 100x) using both red and green filter. We counted live and dead sperm using EBImage/R.

Mated Queen Survival, Egg Laying Rate, Egg Viability and Brood Production in a Colony

- We established 45 mated queens in 3 frame nuclei.
- We divided colonies into 3 groups and treated 2 groups with 2 concentrations of OA. The third group received acetone only (Aliano et al, 2006).
- We recorded queen survival, egg production, egg viability and brood production.
- Queen experiments followed a completely randomized design. The least square means procedure in SAS was used to analyze results.

Detailed procedures: We placed mature queen cells in 45 three-frame nuclei. We divided the nuclei into 3 groups: untreated (1 μl of acetone), low dose (18 μl OA per μl); high dose (180 μl OA per μl). The high dose was equal to the LD_{10} for worker bees, and the low dose was 10 fold lower than the LD_{10}. When queens began laying, they were narcotized with CO_2 and one of the 3 treatments was applied. Queens were then returned to their colonies and monitored for 8 weeks. Four response variables were measured for all colonies: queen survival, egg production, egg viability and sealed brood production.

Results:

Drone Sperm Number and Viability Studies

Table 1. We found no evidence that oxalic acid treatment negatively impacted sperm viability or number. No significant differences were detected in the 3 treatment groups (P = 0.19)

Proportion of viable sperm in drones treated with OA

Oxalic acid (μg)	Proportion alive	std deviation
0	.622	.026
100	.559	.061
200	.617	.058

Mated queens study

Table 2. There were no significant differences in the number of eggs laid during a 24 hour period. Means were compared using the least square means procedure.

Eggs Laid in 24 Hours

Treatment	Mean No. Eggs Laid(±SEM)	Mean Comparisons	*P* - Value
Control	315.7 ± 68.8	Control vs. High	0.350
High Dose	400.3 ± 58.7	Control vs. Low	0.420
Low Dose	387.6 ± 56.2	High vs. Low	0.870

Table 3. There were significant differences in the egg viability of high and low dose queens and of high dose and untreated queens. Means were compared using the least square means procedure.

Egg Viability

Treatment	Percent Egg Viability(±SEM)	Mean Comparisons	*P* - Value
Control	.835 ± .092	Control vs. High	0.004
High Dose	.467 ± .073	Control vs. Low	0.970
Low Dose	.841 ± .073	High vs. Low	0.001

Table 4. There were no significant differences in the square inches of brood 8 weeks after treatment. Means were compared using the least square means procedure.

Square Inches of Brood

Treatment	Mean No. Eggs Laid(±SEM)	Mean Comparisons	*P* - Value
Control	131.2 ± 75.9	Control vs. High	0.200
High Dose	92.3 ± 54.8	Control vs. Low	0.580
Low Dose	115.2 ± 67.25	High vs. Low	0.380

Queen Survival

Table 5. Queen survival 8 weeks post treatment with 2 doses of OA. Neither the high dose nor the low dose reduced queen survival.

Treatment	Percent of Queens Surviving
Control	100%
High Dose	100%
Low Dose	100%

Discussion:

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Once queens were mated and began laying, no queens were lost or superseded during the experiment. The only variable that exhibited a significant response was egg viability with the high dose queens having reduced egg viability when compared with low dose and untreated colonies.

Queen exposure to a dose of OA that is higher than queens would normally be exposed to, did not reduce egg laying or sealed brood production. However, it did result in a significant drop in egg viability. Since there were no differences in sealed brood production 8 weeks post treatment, it appears that the drop in egg viability may be a temporary condition that disappears over time. Treatment with a lower dose of OA that more closely resembles what a queen could be exposed to during colony treatment did not result in any reduction in egg laying, egg viability or sealed brood production. Neither the high dose nor the low dose reduced queen survival.

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