Antioxidants in Wax Cappings of Honey Bee Brood

Project No.: **08-POLL3-Sammataro**

Project Leader: Diana Sammataro, Ph.D.
Research Entomologist
Carl Hayden Honey Bee Research Center
2000 East Allen Road
Tucson, AZ 85719
(520) 670-6380 ext. 121
Fax: (520) 670-6493
e-mail: diana.sammataro@.ars.usda.gov

Project Cooperators and Personnel:
B. LeBlanc, J. Finely

Objectives:

1) Determine antioxidant levels in the wax cappings of honey bee brood and compare cappings from *Varroa*-infested and uninfested brood.
2) Test antioxidants found in wax cappings for miticidal activity against *Varroa* mite using the mite vial assay.

Interpretive Summary:

Antioxidants and antioxidant enzymes make up a large portion of the honey bee immune system. We investigated if there was a difference in the antioxidant level of wax cappings from infested vs. non-infested brood in workers and drones from several bee lines. The results confirm that there is a difference in antioxidant activity in cappings of bee brood between *Varroa*- infested vs. non-infested cells. This would indicate that the bees are responding to mite invasion and activity while in the pupal cell. These results demonstrate that antioxidant activity is dynamic in a bee colony and may shed some light on the nutritional requirements and even supplements that could benefit bees.

The next phase of this work is to feed bees supplementary antioxidants in their food to help boost their immune system and perhaps mitigate the harmful effects of mite predation of bee larvae. We will also look at if particular antioxidants are lethal to mites in laboratory assays.

Materials and Methods:

Two types of samples were collected; cappings from *Varroa*-infested vs. uninfested worker and drone brood and cappings from certain queen lines. Colonies headed by

specific lines of queen honey bees included Russian (R), African (A), SMR (S), Hygienic (H) and Italians from California (CA); all were located at the Carl Hayden Bee Research Center (Tucson AZ). Five colonies per line were all installed on new beehive equipment and foundation and maintained for several years in the same hive furniture. Cappings were collected from known-aged brood, obtained by caging the queen for 24 hours (Finely and Sammataro 2008). Cappings that were from infested cells were stored separately from uninfested cells. All cappings were put in Eppendorf tubes and stored in the freezer (0°F -19.4C) until needed. Cappings were collected between June of 2006 and August of 2008 from at least 3 colonies of each queenline (workers) and 2 CA lines (Drones).

Five cappings from infested vs. uninfested brood were weighed --these were the small samples. In the second experiment, we pooled all the cappings from all four African bee colonies (A), all days (738.45mg) and all three Russian colonies (B), all days (834.4mg). The rest of the mite cappings (C), all from the Russian colonies were also pooled (59.4mg).

To dissolve the wax in the small samples, we added 100uL of methanol and put each tube in the ultrasound for 10 min; after which they were placed in the -70C freezer overnight. The next day, after they defrosted 200uL more of methanol was added and again placed in the ultrasound for 10 min.

Determination of Antioxidant Activity Using the DPPH Radical Scavenging Method.
The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay is a robust, basic antioxidant assay. We used a modification of the assay conditions reported by Hatano et al. (1988). Ethyl alcohol (280 uL) is mixed with the solvent extract (72 uL) in a scintillation vial and DPPH solution (242.5 uL of a 51 mg DPPH/100 mL methanol) added; the vial is capped and vortexed. The more active samples turn color, from purple to light yellow. Immediately following, 160 uL is transferred to each of the 96 wells of the microplate and the samples are run in triplicate. The micro-plate is sealed (Thermal Seal, Excel Scientific, Wrightwood, CA) and incubated in the dark for 1 hour at room temperature. After a programmed 30 second shake cycle, the absorbance is recorded at 517 nm. Extraction solvents (72 uL) volumes is used as controls for each solvent extract. The percent activity is calculated by the following equation by substituting the mean of the absorbance values:

$$\% \text{ scavenging activity} = 100 \times \frac{[\text{control-sample}]}{\text{control}}$$

Determination of Antioxidant Activity Using the FRAP Assay.
The ferric reducing – antioxidant power (FRAP) assay was performed according to conditions reported by Benzie & Strain (18). Water (350 uL; 18 M-Ohm; Barnstead) was mixed with 100 uL of FRAP solution. The FRAP solution was freshly prepared by mixing: 10 mM 2,4,6-tripyridyl-S-triazine (TPZ) in 40 mM HCl, 20 mM $FeCl_3$ solution and 0.3 M NaOAc/HOAc buffer at pH=3.6 at (1:1: 10) parts per volume respectively.

$$[Fe^{2+}]_{mM} = 0.235 \times (\text{Abs@593 nm}) + 0.0054;\ R^2=0.9992$$

Quercetin, a flavanol, was selected because it is a polyphenolic that is found in bee collected pollen, fruits and legumes. By performing the FRAP and DPPH assay under identical conditions as the extracts and by varying the concentrations of quercetin, we could determine the antioxidant activity of the extracts in terms of quercetin equivalents.

Results and Discussion:

In our preliminary investigations, we did find some significant differences in the activity of the cappings between infested and non-infested workers and drones. The results are summarized in the graphs.

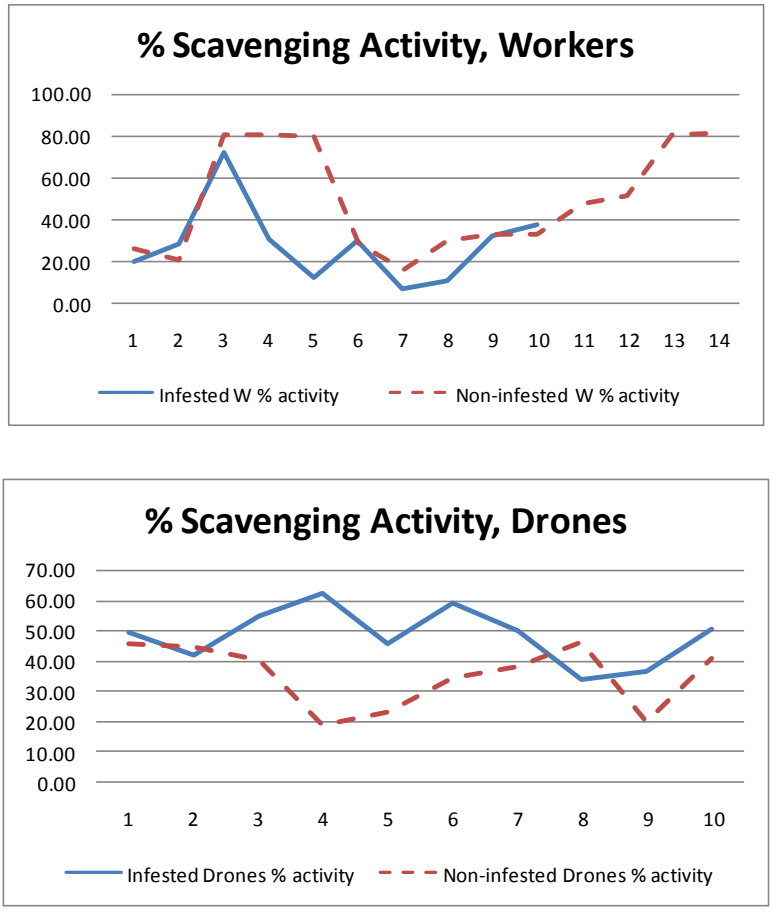

Figure 1. DPPH analysis summary: Infested drones had significantly higher scavenging activity than infested workers, and non-infested drones had lower activity than non-infested workers (F=9.3, df=1, P=0.04).

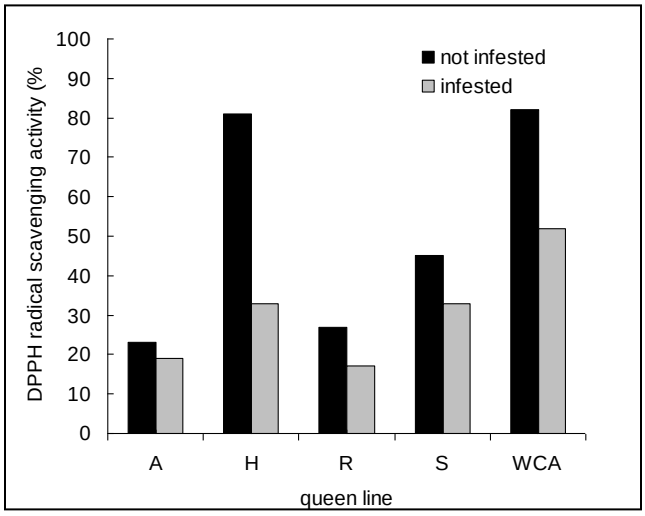

Figure 2. FRAP results showing Quercetin equivalents, of worker queenlines. African (A) differed from Hygienic (H) and Italians (WCA); Hygienic (h) differed from Russian (R) and African (A), Russian (R) was different from all except African (A); SMR (S) was different from Russian (R) and Italian (WCA) was different from African (A) and Russian (R). The intercept ($F=258.3$, $df=1$, $P=0.00$) and differences were between infested vs. non-infested ($F=8.1$, $df=1$, $P<0.01$).

The results confirm that there is a difference in antioxidant activity in cappings of bee brood between *Varroa*- infested vs. non-infested cells. This would indicate that the bees are responding to mite invasion and activity while in the pupal cell. These results demonstrate that antioxidant activity is dynamic in a bee colony and may shed some light on the nutritional requirements and even supplements that could benefit bees.

We also looked at selected antioxidants to determine if they are lethal to mites in laboratory assays. Some antioxidants were administered in a vial assay and while all caused some mite mortality, further testing needs to be done on dosage and application in bee colonies (**Figure 3)**

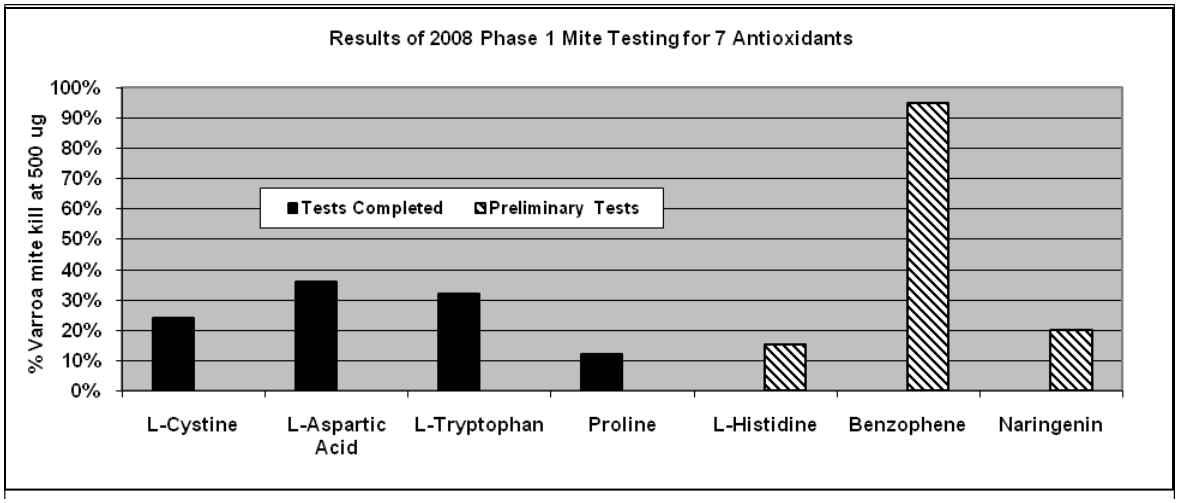

Figure 3. Seven antioxidant compounds in mite-assay trial. Benzophene worked the best (highest mite kill) but is the most hazardous to use.

We will continue evaluating these compounds during the next year. The next phase of this work is to feed bees supplementary antioxidants in their food to help boost their immune system and perhaps mitigate the harmful effects of mite predation on bee larvae.

References cited:

Benzie, I. F. F., and J. J. Strain. 1996. Ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Anal Biochem* 239:70-76.

Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. 1988. Two new flavinoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chemical and Pharmaceutical Bulletin,* 36: 1090-2097.

Recent Publications:

This work is currently being finalized for publication.