ALMOND BOARD PROJECT PLAN/RESEARCH GRANT PROPOSAL

Project Number: 05-GD-02

Project Year: 2005 Anticipated Duration of Project: 12 months

Project Leaders: Allen C. Cohen, University of Arizona, Department of Entomology
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Project Title: Improving vigor of honey bees in almond pollination systems to increase resistance to mite and microbial infestations.

Keywords: honey bee vigor, resistance to mites and microbes, improvement of almond pollination

Commodity(s): Honey bees, almonds

I. Problem and Its Significance

Honey bee pollination is essential to production of almonds. The vigor of the colonies placed in almond orchards for pollination is directly proportional to nut set. However, because of the mixed feeding habits of honey bees (polyphagy) and their complex nutritional requirements, it is difficult for bees using a single nectar and pollen source. Furthermore, as a result of several factors inherent in the demands of almond pollination, honey bees are already highly stressed by the time they are introduced into the almond orchards to do their job as prolific pollinators. The fact that almond blossoms appear early in the year means that the bees have just come through the stresses of winter with the inherent population declines that take place at this time. They have been subsisting on stored products and supplements that beekeepers provided (artificial or semi-artificial diets that are inherently less suitable than fresh pollens and nectars that they can find in the large variety of plants that are blooming in mid- to late spring and summer). The already stressed bees are further stressed by the process of transporting them sometimes for long distances.

Several sources of stress reduce population numbers and vigor of the honey bees that are used in the orchards, and these stresses and the reduction in vigor decrease the effectiveness of the bees in pollinating the almond blossoms. This adverse effect is further compounded as the season progresses, and the bees in more southern orchards are transported to the northern orchards a little later in the season. Furthermore, it is becoming increasingly evident that the use of high fructose corn syrup, despite the intentions of this practice to reduce nutritional pressures on bees, is one of the factors that increases nutritional stress and decreases natural resistance to Varroa and disease. The overall effect of this stress and the resulting decline in colony vigor is that nut set is reduced and honey bee colonies are harmed so that both the almond growers and the bee keepers are negatively impacted in economic terms.

The goal of this proposal is to develop supplements for bees prior to and during their transport to almond orchards so that the vigor and disease resistance of bee populations is improved and greater numbers of bees are available to increase pollination efficiency of transit colonies.

Table 1. Stress factors involved in the reduction of honey bee populations and vigor resulting in losses in pollination efficiency in almond orchards.

Stress Factor	Effects	Causes
1) Malnutrition	Reduction of longevity, reduced brood production, Susceptibility to other stresses	Deficiencies of vitamins, specific minerals, antioxidants, protein components
2) Diseases	Reduction of longevity, reduced brood production, Susceptibility to other stresses, Death	Viruses, Bacteria, Protozoa, Fungi—loss of natural defenses against microbes
3) Parasites	Depletion of resistance, loss of body fluids and metabolic components	Varroa mites, tracheal mites, other mites, small hive beetles, raiding predators, and helminthes
4) Environmental (lack of appropriate & diverse plants)	Poor quality of propolis, increased susceptibility to other stresses (especially microbes and mites)	Lack of available resin components from plants (key flavonoids, phenolics, and terpenes missing)
5) Management (moving bees)	Malnutrition, reduction in longevity and brood production, loss of foraging vigor	Prevention or truncation of foraging, depletion of storage resources, temperature extremes, water depletion, introduction of parasites and diseases
6) Management (Over-harvesting)	Population reduction, depletion of energy stores and other nutrients	Excessive removal of honey, pollen, and propolis

II. Objectives & Plans and Procedures (Modified January, 2006)

Objective I: Determination of mite feeding characteristics of mites. Mite digestive systems will be examined under a dissecting microscope and a compound microscope to determine the relationship of gut structure and gut contents to the nature of the target diet that the mites are extracting from their bee hosts. Also, the hemolymph of bees will be extracted and analyzed in bees representing a wide range of developmental ages. The hematocrit of the bee hemolymph will be used to help assess the amount of lipid, cellular, and liquid (plasma) present in the mites' feeding target. Our hypothesis is that mites require higher concentrations of macronutrients (lipids, proteins, and complex carbohydrates) than can be obtained in a simple clear liquid such as adult bee hemolymph or particulate-free plasma.

Objective II: Laboratory trials of nutritional supplements to determine feeding preferences, uptake of minerals, and optimization techniques to improve absorption and transport of nutrients in the bees' bodies. The trial mixtures will be unique mineral formulations with the organic form of each mineral presented in sugar solutions both as individual minerals and combinations.

Objective III: Measurements of hemolymph antioxidant and antimicrobial potential in relationship to mineral supplements. Hemolymph from bee pupae from hives with and without the mineral/antioxidant supplements will be removed and tested for antioxidant capacity and ability to inhibit growth of *Bacillus subtilis* or other species not pathogenic to be and shown to be good models for determination of defense mechanisms.

Observations of gut structure of mites and hemolymph of bees: The gut (digestive system) structure of Varroa mites will be observed with dissecting microscopes after dissection to reveal gross anatomy. These observations will be extended further to higher magnifications with compound microscopes and imaging systems, using mites that have been cleared with lactic acid, glycerol, water (1:1:1).

Total antioxidant power (FRAP) assay. The method of Benzie and Strain (1999) as modified by Cohen and Crittenden (2004) will be used to determine the total antioxidant power as indicated by the ferric-reducing antioxidant power (FRAP). Aliquots of 5 :l of extract or standard will be added to 95 :l of ferric-TPTZ reagent (300mM acetate pH 3.6 buffer, 2,4,6-tripyridyl-s-triazine in 40 mM HCl, and 20 mM $FeCl_3$ in a 10:1:1 ratio). This mixture will be placed in the well of a cuvette and measured at 593 nm. As a standard, $FeSO_4$ will be used and antioxidant power will be expressed as µmolg $^{-1}$FRAP. Extract C (the ascorbate-free and lipophilic-free extract) will be used to estimate the phenolic component of FRAP activity. Reagents will be obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) or Spectrum Chemical Company (Gardena, CA). The FRAP assays and DPPH free radical scavenging activities will be measured in hemolymph of bees that have been provided with mineral supplements and those that have not.

Determination of Phenoloxidase (monophenol, L-dopa: oxygen oxidoreductase, EC 1.14.18.1) is a multi-copper oxidase, which plays an key role in melanin synthesis, for defense against intruding microorganisms and parasites, wound heating and cuticle pigmentation. A phenoloxidase from the hemolymph of honey bee pupae has a reported molecular mass of 70 kDa,. Optimal pH and temperature were reported to be 6.5 and 20^o C, respectively. Honey bee phenoloxidase is activated by trypsin and inhibited by protease inhibitors and phenylthiourea

Assay for PO activity

PO assays will be carried out in typical reaction mixtures containing 25 µl of pupal hemolymph, 0.01 M sodium cacodylate, pH 7.0, and 0.2 mM L-dopa or 0.1 mM dopamine in a final volume of 700 µl. Changes in absorbance will be measured at 490 nm. Measurements will be made at time intervals where the velocity of reaction will be proportional to the enzyme concentration. A PO unit (U) was defined as the amount of enzyme that increases 0.1 U of absorbance at 490 nm. Specific activity will be expressed as U/mg protein. For some tests, 1.4 U trypsin will be added to reaction mixtures prepared and assayed as described above. In this case, 5 mM $CaCl_2$, will be used as trypsin cofactor.

Ascorbic Acid Determination: Fraction A (0.9 ml total aqueous extract) will be mixed with 0.1 ml of 25 U/ml ascorbate oxidase and incubated at 25°C for 2 h. After incubation, 50 :l of sample will be mixed with 0.95 ml of ferric-TPTZ reagent, and measured at 593 nm. A blank, to which 0.1 ml water will be added rather than the ascorbate oxidase, will be prepared in the same way. The difference between the sample and the blank is the value for ascorbate. L-Ascorbic acid will be used as the standard.

Acceptance of mineral and antioxidant supplements: All supplements, dissolved in 50% sucrose syrup in concentrations ranging from 1% to 0.03%, will be offered to bees in Petri dishes placed within hives. The minerals to be tested will include organic salts of sodium, potassium, magnesium, manganese, calcium, iron, and zinc. Antioxidants to be tested are carotenoids, tocopherols, and ascorbates. The design of the acceptance experiments is to compare weight losses of the syrup/additive mixtures, with only concentrations of the same additive in each comparison. In other words, for example, only calcium salts of various concentrations will be made rather than comparison of calcium acceptance with magnesium. This procedure should allow us to determine the most acceptable concentrations of each additive individually so that the final mixture of all individual additives will be the optimal, most palatable combination.

Statistical Tests: All grouped data will be tested with a Fisher's PLSD test (SAS 1999). Also, optimization of the various diet formulations will be assessed by Pareto analysis as specified in the Statview Program for testing relative contributions of various components in quality control systems (SAS 1999).

RESULTS:

Objective 1: Varroa mite digestive systems were shown to be simple, nearly straight tubes, with very slight branching into diverticula. The gross anatomy of the Varroa mites' digestive system is that of an organism that uses food that is highly concentrated with nutrients such as proteins and lipids. Liquid-feeders have digestive systems that are highly convoluted with the special length and complex twists and turns being adaptations for counter-current flow of digestive system contents and extensive readsorption of excess water that must be removed from the system. The relatively simple system of the Varroa mites can function adequately only if foods of highly concentrated nature are ingested. This is substantiated by determination of the hematocrit of hemolymph (more properly "hemocoel fluids") from larvae and early pupae (2-4 day old pupae) where the lipid content ranges from 12-17% of the hemolymph volume; the cellular materials (most dense fraction) is between 18-24%; and the plasma (clear) fraction ranges from 59-70%. The preliminary conclusion that is indicated by these measurements is that the main feeding target of Varroa mites is a slurry consistency rather than a clear liquid. These findings raise questions about the mechanisms of food selection and ingestion by Varroa mites during their period of most rapid growth. One further observation that supports the consistency of these observations is that direct visualization of the gut contents of actively feeding Varroa reveals solid-looking materials filling the mites' gut lumen.

The observation of tyrosine nodules (Figure 1 and as discussed by Erickson et al 1997) and comparisons of the differential cuticular darkening reactions in frozen pupae indicate mite-induced failure of the phenoloxidase system. The planned tests on mineral supplementation (especially copper salt treatment) could not yet be conducted, but will be completed in the fall of this year.

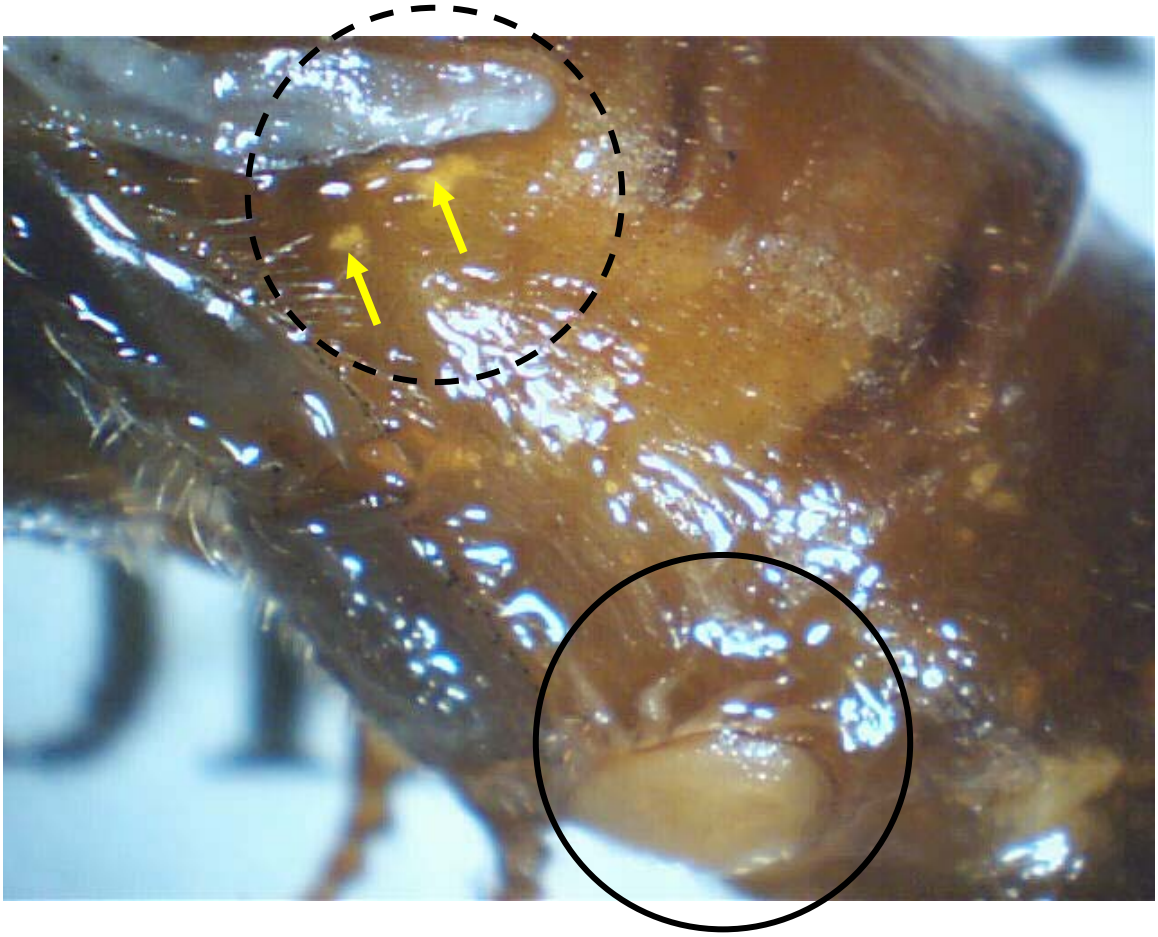

Figure 1. Varroa-infested bee (mite is circled with a solid line), showing crumpled wing and tyrosine nodules pointed out with yellow arrows. Visualization of the tyrosine nodules was made possible by soaking the bee in 70% ethanol.

Objective 2:

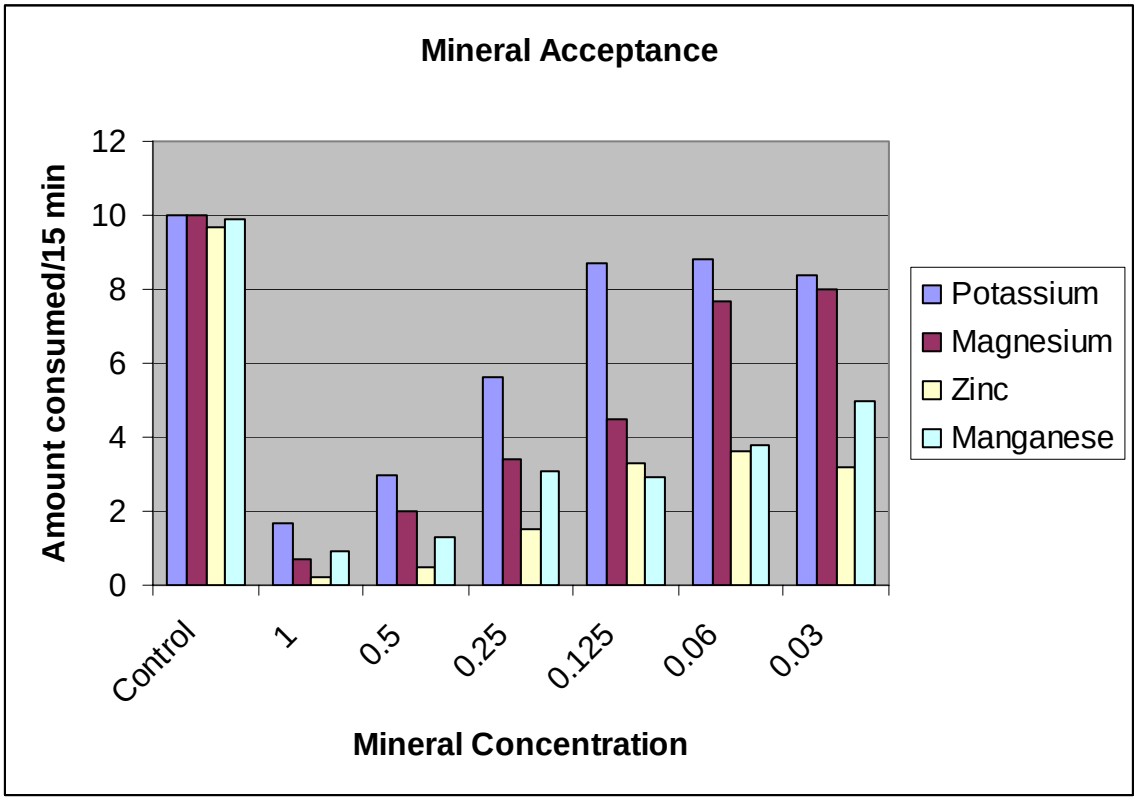

Figure 2. Amounts of syrups containing potassium, magnesium, zinc, and manganese consumed per 15 minute period by honey bees. Syrups were placed in colonies in open Petri dishes, and the control was syrup alone with no additives.

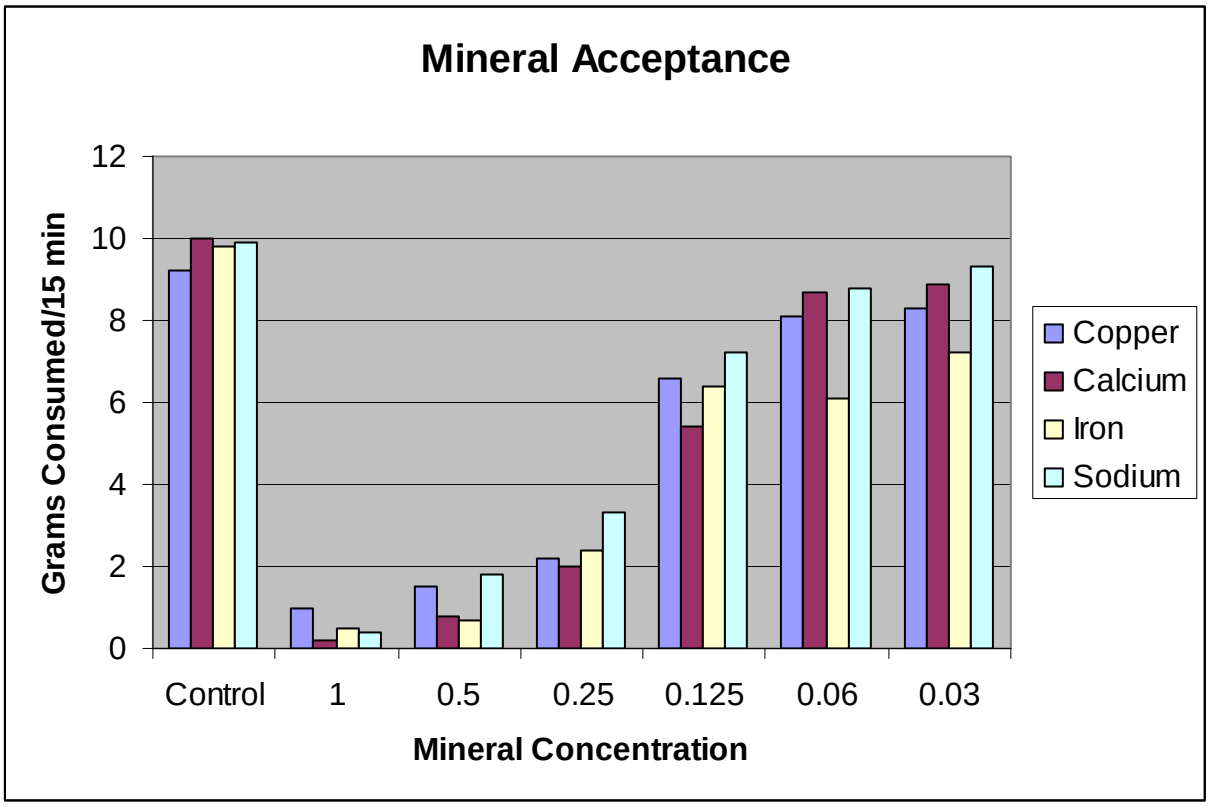

Figure 3. Amounts of syrups containing copper, calcium, iron, and sodium consumed per 15 minute period by honey bees. Syrups were placed in colonies in open Petri dishes, and the control was syrup alone with no additives.

As indicated by Figures 2 and 3, the controls were consistently most acceptable to the bees. Also, in general, the lowest concentrations of each mineral were most acceptable. Also evident in these figures, certain minerals were less acceptable than others with iron compounds being less palatable than sodium or potassium.

Objective 3:

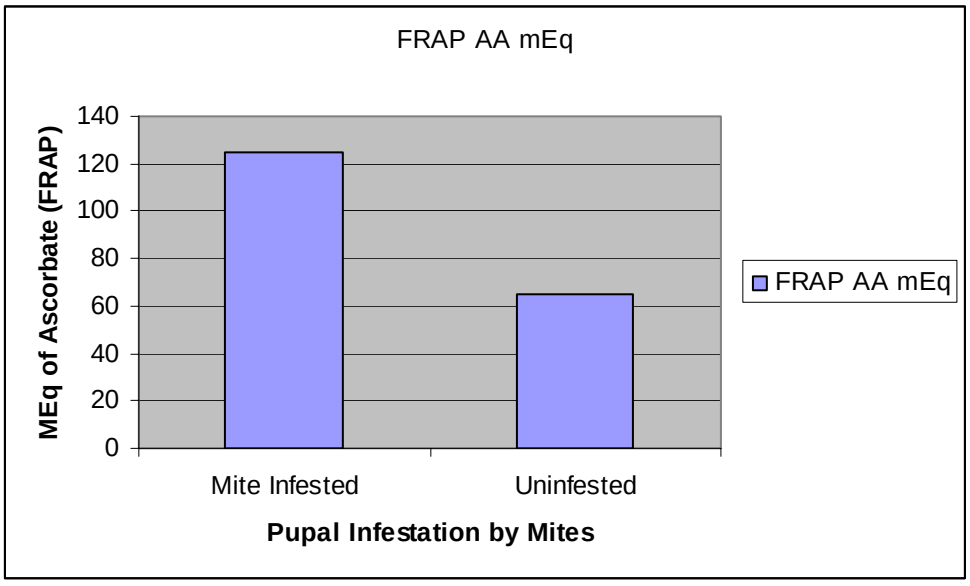

Figure 4. Ferric reducing antioxidant potential (FRAP) activity in hemolymph of pupal honey bees with and without mite infestations in cells.

Figure 4 demonstrates that bees that are infested with mites have a higher antioxidant (FRAP) potential than bees that are uninfested. We interpret this unexpected result as an indication that

the infested bees are stressed by the Varroa attack and that they are responding to the stress by producing some kind of endogenous antioxidant such as uric acid or glutathione. Atmowidjojo et al. (1999) showed that water and temperature stressed honey bees had elevations in hemolymph uric acid content, and Cohen and Patana (1982) and Cohen (1983) showed that other insects responded to physiological stresses with increased uric acid, and this purine and related purines can act as excellent antioxidants and free radical scavengers.

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