Comparing the Effects of Protein Supplements vs. Natural Forage on Colony Health

Project No.: **13-POLL1-DeGrandi-Hoffman**

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Objectives:

1. Determine nutritional quality of pollen from rapini (*Brassica rapa*) flowers and the bee bread made from it and compare this with the protein supplement diet.
2. Compare hemolymph protein concentrations of nurse bees from colonies foraging on rapini and those feeding on a protein supplement diet.
3. Compare brood production between colonies foraging on rapini and those feeding on a protein supplement diet.
4. Compare virus titers of nurse bees and larvae between colonies fed protein supplement and those foraging on rapini

This is a progress report for research conducted in November 2013 to August 2014.

Interpretive Summary:

Beekeepers that bring colonies to California in the fall feed their bees' protein supplements until almond bloom because pollen is not available. If plantings of fall and winter forage were available, would this improve colony health, growth and survival? To address this question, we compared the nutritional composition of two commercially available protein supplements (Diet-1 or Diet-2) with pollen from a fall mustard (Brassicaceae) called rapini (*Brassica rapa*). After 9 weeks of either feeding on the protein supplements or foraging on rapini, we compared the health, growth and survival among the groups of colonies. The protein supplements we chose had similar concentrations of soluble protein and colonies consumed similar amounts of each. However, both protein supplements had significantly less protein than the rapini pollen and bee bread and in some cases, lower levels of essential amino acids. Bees digested only about 35% of the protein in the supplements compared with 70% in the pollen. On average, 35% of the colonies fed protein supplements died during the study compared with 15% foraging on rapini. On average, 35% of the colonies fed protein supplements died during the study compared with 15% foraging on rapini. On average 35% of the colonies fed diet lost queens during the study compared with 20% foraging on rapini. By the end of the study, colonies fed protein supplements had the greatest fold increases in Black queen cell virus and highest percentages

of bees testing positive for Nosema. Those fed Diet-1 also had the greatest fold increases in Deformed wing virus and and Nosema. The study indicates that planting rapini could decrease queen and colony losses and provide healthier colonies for almond pollination. The results from this study indicate that colonies foraging on rapini prior to almond bloom have greater survival and lower disease titers than those fed protein supplements, supporting the investment in forage plantings rather than supplements for colonies used in almond pollination.

Materials and Methods:

Rapini planting. Rapini was planted from seed at the University of Arizona West Campus Agricultural Facility in Tucson, AZ USA (Site-1) and at the University of Arizona Red Rock Agricultural Facility in Red Rock, AZ USA (site-2) during the first week of October, 2013. Fields were seeded at a rate of 1.5lbs per acre at site-1 and 2lbs per acre at site-2.

Colony establishment. Ten colonies of European honey bees were established within 0.25mi of each rapini plantings when bloom began in November, 2013. The colonies contained adult bees, brood and a marked laying queen. A second group of colonies were located at the Carl Hayden Bee Research Center, Tucson, AZ USA and fed protein supplements (PS) only. Pollen traps were put on these colonies to prevent pollen from entering. These hives were more than 5 mi away from the rapini plantings. Two different commercially available protein supplements (PS, hereafter referred to as Diet-1 and Diet-2) were fed to the colonies. Ten colonies were fed Diet-1 and 10 fed Diet-2.

Objective 1. Protein and amino acid analysis in diets and pollen. Total soluble protein concentrations in the pollen, bee bread and both protein supplements were estimated using a bicinchoninic acid (BCA) protein assay (#23225, Thermo Scientific). Absorbance was measured at 562 nm using a Synergy HT spectrophotometer (BioTek Instruments, Inc., Winooski, Vermont). Protein concentration was estimated using a standard curve generated from serial dilutions of bovine serum albumin (BSA) (details of methods in DeGrandi-Hoffman et al. 2010, 2013).

Free amino acid analysis was conducted using 10 mg portions of Diet-1 and 2 patties (commercial preparations), corbicular pollen and bee bread. The sample was mixed with 200 µl 0.03M HCl to solublize the free amino acids. The mixture was filtered through a Gilson 200µl filtered pipette tip. 100 µl clear sample was taken for analysis using the protocol based on the EZ:faast kit for free physiological amino acids by GC/MS (Phenomenex Inc., Torrence, CA, USA). This protocol employs an ion-exchange cleanup and a derivitzation of the amino acids for GC/MS analysis. The polar nature of amino acids requires derivatization to make amino acids volatile and less reactive prior to GC/MS analysis.

Amino acid analyses were conducted on an Agilent model 7890 Gas Chromatograph/Mass Spectrometer (GC/MS), (Agilent, Palo Alto, CA, USA) equipped with model 5975 quadrupole Mass Selective Detector (MSD) operated in Selected Ion Monitoring (SIM) mode. The GC/MS system is equipped with the Agilent 7693 Auto sampler injection system. The separation of the derivatized samples was on a Zebron ZB-50 capillary column, 30m length, 0.25mm Id and 0.25µm film thickness. Instrument conditions were: Oven Temperature was set at 110°C, held for 1 minute, then ramped up at 30°/min to 320°C and held for 5 min. The total run time is 13

minutes. The injector temperature is 250°C, Helium is the carrier gas, flow is 1.2 ml/min., septum purge flow 3 ml/min., split ratio 10:1, source temperature is at 230°C, MS Quad at 150°C. A 1.0 µl aliquot of derivatized sample was injected into GC/MS for separation. The glass liner was deactivated borosilicate single taper gooseneck (SGE Focus Liners). The transfer line to the MSD was kept at 310°C. The MSD was operated in scan mode (50–420 *m*/*z*) and in SIM mode. Amino acid standards were used to determine two characteristic mass fragments (m/z) and retention times of individual derivatized amino acids.

Eighteen amino acids were detected and quantified. These were: tyrosine, glycine, alanine, tryptophan, serine, phenylalanine, proline, asparagine, valine, glutamic acid, leucine, isoleucine, trans-4-hydroxyproline, glutamine, methionine, asparagine, cysteine and histadine.

Objective 2. Hemolymph collection and total soluble protein analysis. Both PS were fed to bees in patty form. Diet-1 was packaged as pre-formed patties. Diet-2 was sold in powder form and patties were made according to the manufacturer's instructions. Diet-1 and 2 patties were weighed prior to putting them on the top-bars of colonies. The patties were removed and weighed (if any diet remained) weekly and a new patty was given to the colony. The difference between initial patty weight and weight 7 days later was used as the estimate of diet consumption for the colony.

Measurements of hemolymph protein concentrations between colonies foraging on rapini and those fed PS was used as a proxy to compare worker nutritional status in a manner similar to other studies (e.g. Bitondi and Simoes 1996; Cremonez et al. 1998; Cappelari et al. 2009; DeGrandi-Hoffman et al. 2010, 2013). Adult bees actively feeding larvae (i.e., nurse bees) were sampled from all colonies (5 nurses per colony) every 3 weeks. Hemolymph was extracted and analyzed for soluble protein concentrations using methods described in DeGrandi-Hoffman et al. (2010, 2013).

As a measure of digestion, soluble protein concentrations in the hindgut of nurse bees were measured and compared with the protein concentration in the protein supplements and rapini pollen and bee bread. Soluble protein concentrations in the hindgut were determined by removing the abdomen of nurse bees and opening it to reveal the digestive tract. The bees were the same as those used to measure hemolymph protein concentrations. Hindgut contents were collected by making an incision into the hindgut using dissection scissors, and inserting a micropipette; 1µl of the hindgut contents were collected from each bee. The contents were transferred to a 2µl microcentrifuge tube containing 99µl of phosphate buffered saline (PBS) with 1% EDTA-free Halt Protease Inhibitor Cocktail (#78437, Thermo Scientific, Rockford, IL). The hindguts from 5 bees per colony were sampled and pooled; 100µl of each pooled sample was combined with 900µl of the PBS and Halt solution in a 2ml centrifuge tube. Samples were stored at -80°C until analyzed.

Total soluble protein concentrations of the hindgut contents were estimated using a BCA protein assay (#23225, Thermo Scientific). Absorbance was measured at 562 nm using a Synergy HT spectrophotometer (see above). Protein concentrations in hindgut samples were estimated using a standard curve generated from serial dilutions of bovine serum albumin (BSA).

Objective 3. Colony measurements. Colony sizes at the start and end of the study were measured using techniques described in DeGrandi-Hoffman et al (2008). Briefly, the areas on each frame with brood and bees were measured on both sides of each frame and then summed for the colony. The presence of the marked queen also was recorded at 3 week intervals. If queens were lost, they were replaced with mated queens from the same queen line.

Objective 4. Virus titers. Five bees actively nursing larvae were sampled per colony. All samples were placed in liquid N and stored at -80 °C until analysis for viruses using methods described in Chen et al. (2005) and DeGrandi-Hoffman et al. (2010). We tested for the presence of the following viruses: Black queen cell virus (BQCV), Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV) and Sac brood virus (SBV).

The output of qRT-PCR assays of each virus was interpreted by using the comparative Ct method (ΔΔCt Method). The virus level was quantified based on the value of the cycle threshold (Ct) which represents the number of cycles needed to generate a fluorescent signal above a predefined threshold and therefore is inversely proportional to the concentration of the initial target that has been amplified. For each detected virus, the average Ct value (ΔCt) of a virus was normalized using the Ct value for the corresponding β-actin following the formula: $\Delta Ct = (\text{Average } Ct_{\text{virus x}}) - (\text{Average } Ct_{\beta\text{-actin}})$. The group with the lowest level of the minimal virus level was chosen as a calibrator. The ΔCt value of each group was subtracted from the ΔCt of the calibrator to generate ΔΔCt. The virus concentration in each group was calculated using the formula $2^{-(\Delta\Delta Ct)}$ and expressed as an n-fold difference relative to the calibrator.

Nosema levels also were measured at the start (prior to feeding PS) and end of the study to determine the effects of nutrition on the titers of this pathogen.To estimate Nosema levels, 25 bees were sampled from each colony and stored in alcohol. Samples from each colony were split into groups containing five bees that were analyzed together for Nosema spore levels. The abdomens were removed, placed in a mortar with 5 µl of ultra pure H_2O (1 µl per bee) and crushed using a pestle. The supernatant (1 µl) was pipetted into a hemocytometer chamber (0.100 mm deep) and covered with a glass slip. Spores were counted at 40x magnification in the four large corner squares and the large center square of the hemocytometer grid (each large square contains 16 smaller squares). The total number of spores per bee was calculated using the George E. Cantwell equation (American Bee Journal, June 1970):

$$\frac{\text{Total spores}}{\text{number of squares (80)}} \bullet 4{,}000{,}000 = \text{total number of spores per bee}$$

This process was repeated for each group of five bees to generate an average from five estimates of nosema levels per colony.

Results and Discussion:

Objective 1. Protein and amino acid analyses of diets. Rapini pollen and bee bread did not differ in protein concentrations at the two sites, so values from each site were combined. Soluble protein in bee bread was significantly higher than in pollen (**Figure 1**). Both protein

supplements had similar protein concentrations. These concentrations were lower than that of rapini pollen ($F_{2,9} = 11.33$, $p = 0.003$).

We could detect 7 of the 10 essential amino acids in pollen, bee bread and the protein supplements (**Figure 2**). Derivatives of arginine, threonine, and lysine were not stable enough for quantification. Amino acid levels differed between pollen and bee bread; valine, phenyalanine and methionine levels were higher in bee bread than pollen while isoleucine and histidine were lower. We found differences in amino acid concentrations between pollen and bee bread in our previous studies (DeGrandi-Hoffman et al. 2013). The changes in amino acid concentrations could be due to enzymatic activity cleaving amino acids from proteins at particular sites (increasing concentrations) or possible conversion of amino acids into energy through microbial metabolism (decreasing concentrations).

The concentrations of essential amino acids were lower in the diets than in the rapini pollen. Diet-1 had lower concentrations than in pollen for all essential amino acids except for tryptophan. For Diet-2, isoleucine, histidine and tryptophan concentrations were lower than in pollen.

We detected 10 non-essential amino acids in pollen, bee bread and the protein supplements. Levels of tyrosine, aspartate and proline were similar between pollen and bee bread. Alanine and glycine levels were higher in bee bread compared with pollen and glutamine, asparagine, cysteine, glutamate and hydroxyproline were lower. The protein supplements had similar amounts of all non-essential amino acids with the exception of alanine and glycine which were higher in Diet-2. Levels of glutamine, asparagine, hydroxproline, cysteine, and glutamate in both diets were lower than in pollen and glycine levels in Diet-2 was higher but similar to levels in bee bread. The greatest difference in amino acid concentrations between the diets and rapini pollen was in proline where concentrations were more than 10x higher in the pollen than the protein supplements. Insects can detect proline in their food and use it as an energy substrate to fuel flight and other high energy activities (Brosemer and Veerabhadrappa 1965; Crabtree and Newsholme 1970; Carter et al.2006). Proline also is important in insect immunity. There is a family of proline-rich, inducible, antibacterial peptides that are 18–34 amino acid residues in length present in a number of insect species. This includes abaecin from *Apis mellifera* in which proline makes up greater than 25% of the amino acid composition of this peptide (Casteel et al. 1990; Gillespie et al 1997). In combination with lower protein concentrations and reduced rates of protein digestion, bees feeding on protein supplements might have reduced immune responses compared with bees feeding on pollen.

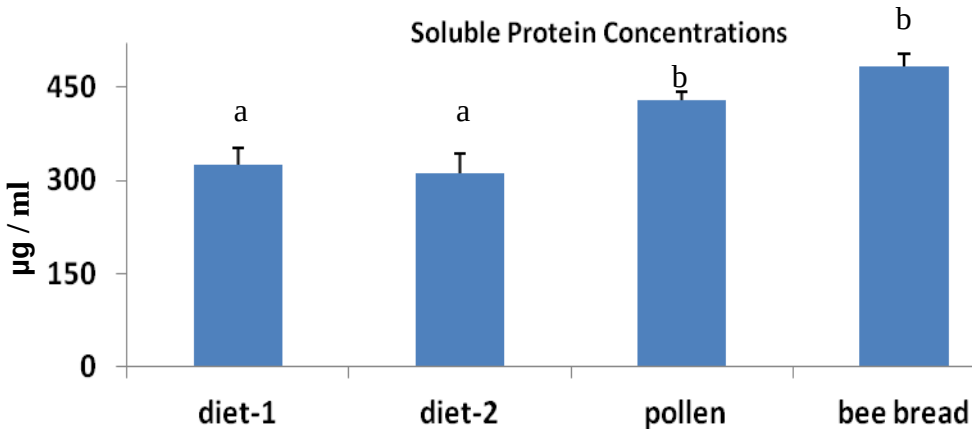

Figure 1. Soluble protein concentrations in two protein supplement diets (Diet-1 and 2) and from pollen and bee bread collected by honey bee colonies foraging on rapini (*Brassica rapa*). Means followed by the same letter are not significantly different at the 0.05 level as determined by Tukey's W method. A separate analysis comparing protein concentrations between pollen and bee bread, indicated significant differences between them ($t_{38} = 2.33$, $p = 0.026$).

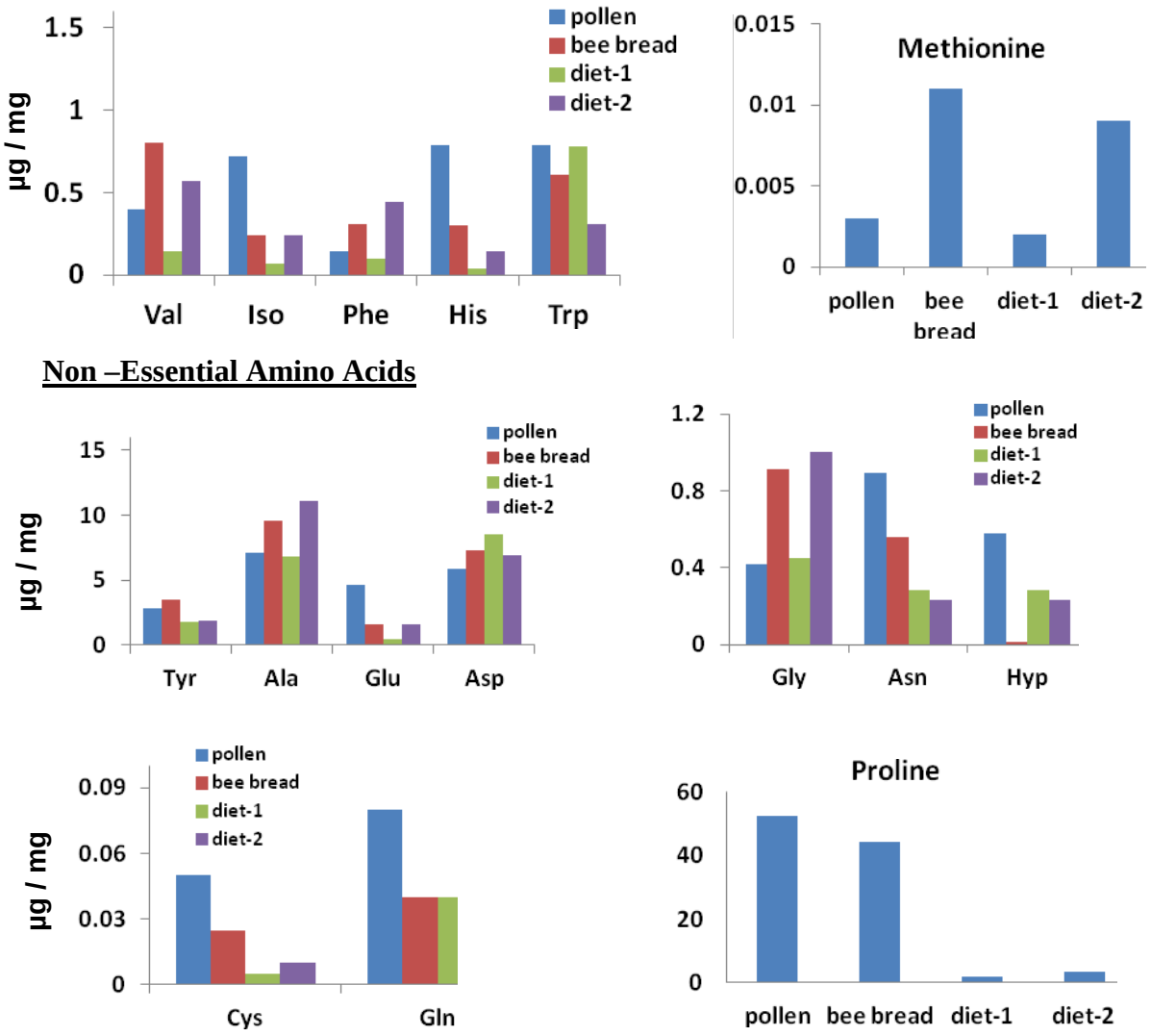

Figure 2. Essential and non-essential amino acids in two protein supplement diets (Diet-1 and Diet-2) and in rapini (*Brassica rapa*) pollen and bee bread made from it.

Objective 2. Hemolymph protein concentrations in bees. Both diets were equally palatable to the bees; there was no difference in consumption between them during the 9 week study ($F_{1,15} = 0.184$, $p = 0.67$). The colonies consumed an average of 66.4 ± 4.6g of Diet-1 and 63.6 ± 3.5g of Diet-2 per week.

Protein concentrations in nurse bees at the start of the study did not differ among the treatment groups ($F_{3,36} = 1.24$, $p = 0.3$). After three brood cycles, hemolymph protein concentrations did not differ among nurse bees from colonies feeding on different protein supplements or foraging on rapini ($F_{3,30} = 0.196$, $p = 0.898$). The hemolymph protein concentrations in larvae also did not differ among diet treatments ($F_{3,31} = 1.28$, $p = 0.3$).

The proportion of protein in the diets that was detected in the hindgut of nurse bees was significantly higher for those feeding on protein supplement diets compared with rapini pollen ($F_{3,29}$ = 90.6, p < 0.0001) (**Figure 3**). About 65% of the protein in either Diet-1 or 2 was in the hindgut. Undigested protein values for the pollen averaged 30% for both sites.

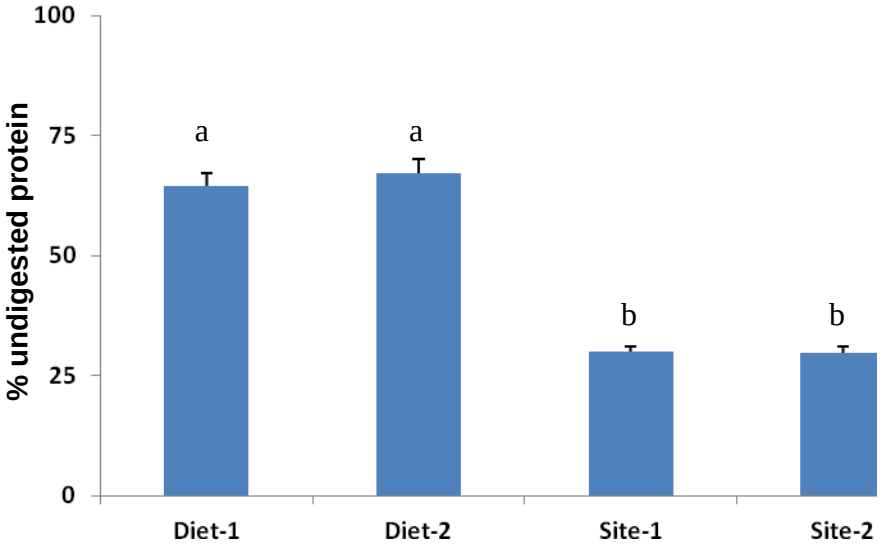

Figure 3. Percentage of protein in diets fed to colonies that were recovered in the hindgut of nurse bees. Means followed by the same letter are not significantly different at p < 0.05 as determined by F-test and Tukey's W procedure.

Objective 3. Colony growth and queen survival. Colony sizes at the start of the study did not differ among treatment groups (adult populations: $F_{3,36}$ = 0.13, p = 0.94; frames of brood: $F_{3,36}$ = 0.42, p =0.74). Colonies averaged 4.1 ± 0.3 frames of bees and 1.8 ± 0.1 frames of brood. By the end of the study, we lost 40% of the colonies fed Diet-1 and 30% of those fed Diet-2. Colony losses for those foraging on pollen at sites-1 and 2 were 10 and 20% respectively. Queens also were lost during the study period. The highest queen losses occurred in colonies fed Diet-1 (50%). Twenty percent of the colonies fed Diet-2 and 20% of the colonies foraging on rapini at either site lost bees during the study. There were no differences in sizes of the colonies that survived until the end of the study among the treatment groups (frames of brood: $F_{3,28}$ = 1.78, p = 0.17, adult bees: $F_{3,28}$ = 0.79, p = 0.51).

Objective 4. Virus Titers. Of the 5 viruses we screened for in the nurse bee samples, Deformed wing virus (DWV) and Black queen cell virus (BQCV) were detected at the highest titers and with the greatest frequency. BQCV titers were highest (greatest fold increases) in colonies fed either Diet-1 or Diet-2 and lowest in colonies foraging on rapini.The greatest fold increases in DWV titers occurred in colonies fed Diet-1.

Nosema titers. Initial assessments of Nosema indicated that the colonies fed Diet-1 or foraging rapini at site-2 had the lowest percentages of infected bees at the start of the study (**Figure 4**). Colonies fed Diet-2 and those foraging on rapini at site-1 had similar percentages of infected bees. Colonies fed Diet-1 had significantly lower titers of Nosema spores at the

start of the study compared with the other treatments which did not differ ($F_{3,34}$ = 3.97, p = 0.016). By the end of the study though, colonies fed either Diet-1 or 2 had the highest percentages of infected bees. Nosema spores were detected in all bees from colonies fed Diet-2, and in 85% of those fed Diet-1. Spore counts were significantly greater in colonies fed Diet-1 compared with the other treatments which did not differ. Spore counts in colonies fed Diet-1 were more than 4x higher than initial counts.

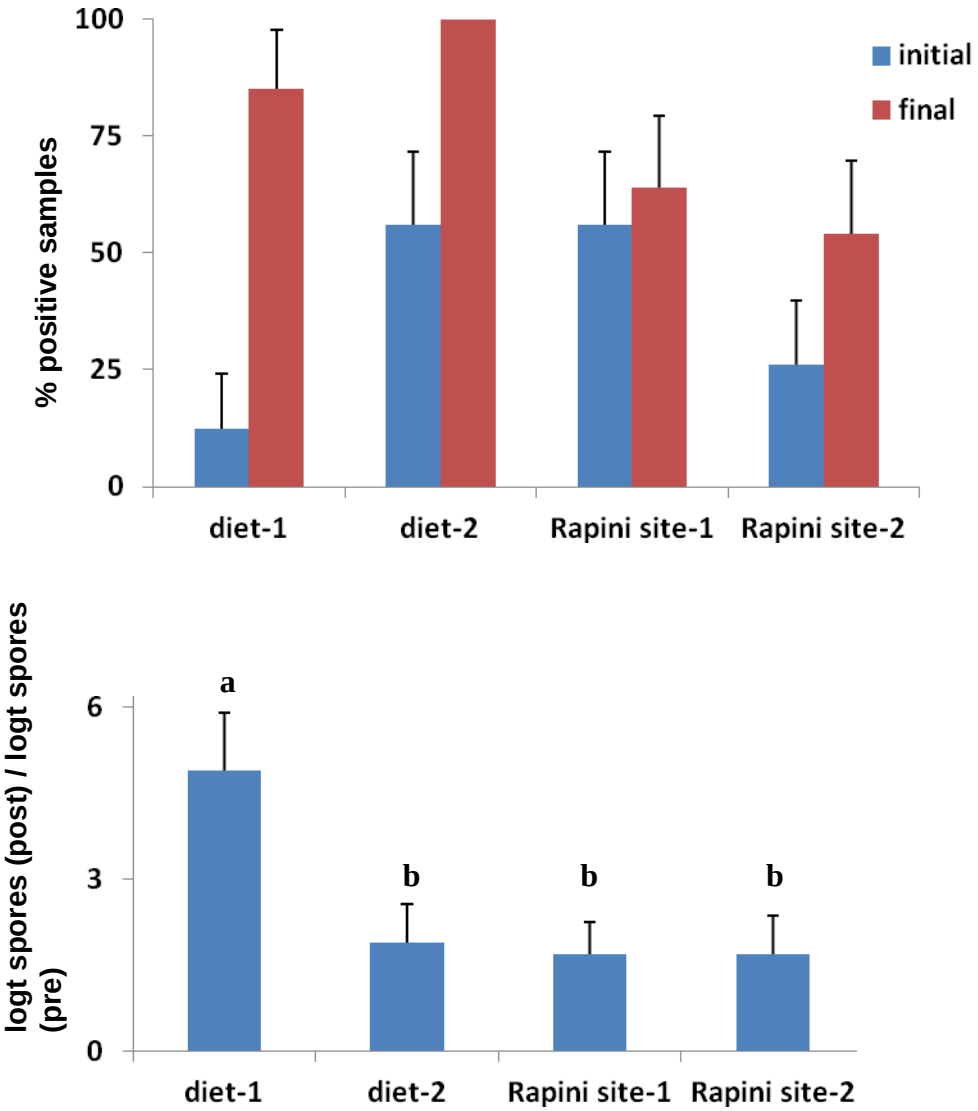

Figure 4. Percentage of worker bee samples testing positive for Nosema spores before (initial) and after (final) feeding on protein supplement diets (Diet-1 or 2) or foraging on rapini at two different sites for 9 weeks (A). The proportional increase in Nosema spores (log transformed) between initial numbers (logt spores pre) and 9 weeks later (logt spores post). Means followed by the same letter are not significantly different as determind by one-way analysis of variance followed by Tukey's W muliple comparison test.

Conclusions:

The protein supplements used in this study are commercially available and commonly used by beekeepers. Both diets differed from rapini pollen in protein and amino acid concentrations; rapini pollen and bee bread has about 30% more soluble protein than the protein supplements and higher levels of several essential amino acids. Bees were able to digest and acquire more protein from pollen and bee bread than the protein supplements. Though hemolymph protein levels in nurse bees did not differ among the colonies fed protein supplements or foraging on rapini, lower protein and amino acid levels in combination with reduced protein acquisition with protein supplements might have contributed to higher disease titers and greater queen and colony losses. For almond growers, higher colony losses when bees are fed protein supplements would reduce almond yields since they are dependent on colony number per acre. The results from this study indicate that colonies foraging on rapini prior to almond bloom have greater survival and lower disease titers than those fed protein supplements, supporting the investment in forage plantings rather than supplements for colonies used in almond pollination.

Research Effort Recent Publications:

Research findings were presented at the Pacific Branch Meetings of the Entomological Society of America Meetings, April, 2014. Title of talk: “Comparing nutrient acquisition from natural forage vs. protein supplements and measuring the effects on honey bee colony growth”.

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