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 2014 to present.

Interpretive Summary:

Malnutrition is a major cause of colony losses. In managed hives, bees are fed protein supplements (PS) during pollen shortages. If bees were provided with natural forage instead of PS, would they have lower pathogen levels and higher queen and colony survival? We addressed this question by either providing colonies with forage (*Brassica rapa* – rapini) or feeding them PS from November to February. Soluble protein concentrations in the PS were lower than the rapini pollen as were levels of most amino acids. Nurse bees digested less of the protein in PS than the pollen. Hemolymph protein titers in nurse bees and colony growth did not differ between those fed PS or foraging on rapini. However, colonies fed PS had higher levels of *Black Queen Cell Virus* and *Nosema* and greater queen losses indicating that natural forage might improve overwintering survival.

In summary, the study indicates that planting rapini or other forage 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. European honey bee (*Apis mellifera ligustica*) colonies were established with adult bees, brood, a marked laying queen (Pendell Apiaries, Stonyford, CA, USA), and no pollen stores. The colonies had feeders with 30% sucrose solution during the entire study. Ten hives were established within 0.4 km of each planting when bloom began. Colonies fed PS were 13km away at the Carl Hayden Bee Research Center, Tucson, AZ. Ten colonies each were fed either BeePro® (Mann Lake, Hackensack, MN, USA –diet-1) or MegaBee® (Dadant, Chico, CA, USA- diet-2). The hives had pollen traps (Front porch pollen traps, Brushy Mountain Bee Farm, Moravian Falls, NC, USA) that were checked every 2-3 days. Trapped pollen was removed as were dead bees and debris behind the trap.

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 (AA) 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 samples were 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 derivatization 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.

AA 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. AA standards were used to determine two characteristic mass

fragments (m/z) and retention times of individual derivatized AA. We detected and quantified nine essential (EAA), six conditional (essential in times of stress), and five non-essential AA (Nelson & Cox 2012).

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 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 new patties were given to the colonies. 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. We replaced lost queens with marked mated queens from the same stock. Colonies with less than one frame covered with bees were counted as a colony loss.

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). Counts were converted to total spores per bee using methods described in Fries et al. (2013).

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.

Soluble protein concentrations did not differ between sites or diets so values for each food source were combined. PS had significantly lower soluble protein levels than rapini pollen or beebread ($F_{2, 26} = 7.08$, $p = 0.004$) (**Figure 1**).

Of the 20 AA we detected, proline had the highest concentration (pollen and beebread) and methionine and serine the lowest (**Figure 2**). Individual EAA concentrations differed significantly among the food sources in all cases except isoleucine and valine (**Table 1**). Histidine, lysine and threonine concentrations were higher in pollen or beebread than PS. Leucine, lysine, methionine, and threonine concentrations were higher in beebread than pollen. In general, diet-1 had the lowest concentrations of EAA.

Of the 7 conditional AA, cysteine and proline concentrations were significantly higher in pollen and beebread than in either PS. Non-essential AA concentration did not differ among the food sources except for asparagine, which was highest in pollen and beebread and lowest in diet-2.

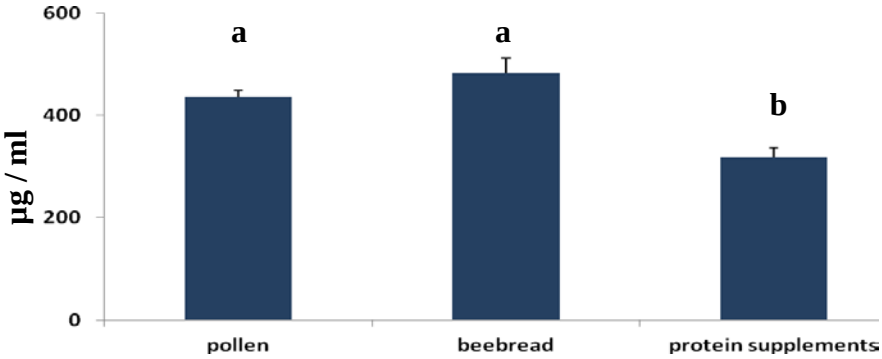


Figure 1. Average concentration (± SE) of soluble protein in rapini pollen, beebread and two protein supplements. Protein concentrations of the supplements did not differ so data were combined. Averages with the same letter are not significantly different as determined by an analysis of variance followed by a Tukey's multiple comparison test ($F_{2, 26} = 7.08$, $p = 0.004$).

Table 1. Amino acid concentrations found in two protein supplements (Diet-1 and 2), rapini pollen and beebread. Means followed by the same letter are not significantly different at the 0.05 p-level.

Amino Acid	Diet-1	Diet-2	Pollen	Beebread	$F_{3,8}$, p
Essential					
histidine	0.04 c	0.14 b	0.4 a	0.3 a	28.5, < 0.0001
isoleucine	0.07	0.24	0.72	0.72	0.94 , 0.45
leucine	0.76 b	6.31 a	1.53 b	4.15 a	11.52, 0.003
lysine	0.14 b	0.75 b	0.87 b	2.66 a	19.32, 0.001
methionine	0.02	0.009 a	0.003 b	0.011 a	22.7, <0.0001
phenyalanine	0.01 c	0.44 a	0.14 bc	0.32 ab	8.29, 0.008
threonine	0.05 c	0.14 b	0.09 bc	0.23 a	17.5, 0.001
tryptophan	0.78 a	0.31 b	0.79 a	0.61 ab	4.19, 0.047
valine	0.14	0.57	0.4	0.8	2.46, 0.137
Conditional					
arginine	1.34 b	2.86	1.88 b	1.88 b	4.48, 0.04
cysteine	0.006 c	0.01 c	0.07 a	0.02 b	46.2, < 0.001
glutamine	0.04	0.04	0.08	0.04	3.67, 0.063
glycine	0.45 b	1.04 a	0.41 b	0.91 a	6.97, 0.013
proline	1.89 b	3.56 b	52.3 a	44.1 a	11.02, 0.003
tyrosine	1.8 b	1.9 b	2.8 ab	3.5 a	4.32, 0.044
serine	0.002	0.002	0.003	0.001	0.41, 0.75
Non-Essential					
alanine	6.8	11.1	9.6	7.1	1.67, 0.249
asparagine	0.29 bc	0.23 c	0.89 a	0.56 ab	9.76, 0.005
aspartic acid	8.5	6.9	5.9	7.3	0.46, 0.717
glutamic acid	0.48	1.56	0.97	1.57	3.88, 0.064

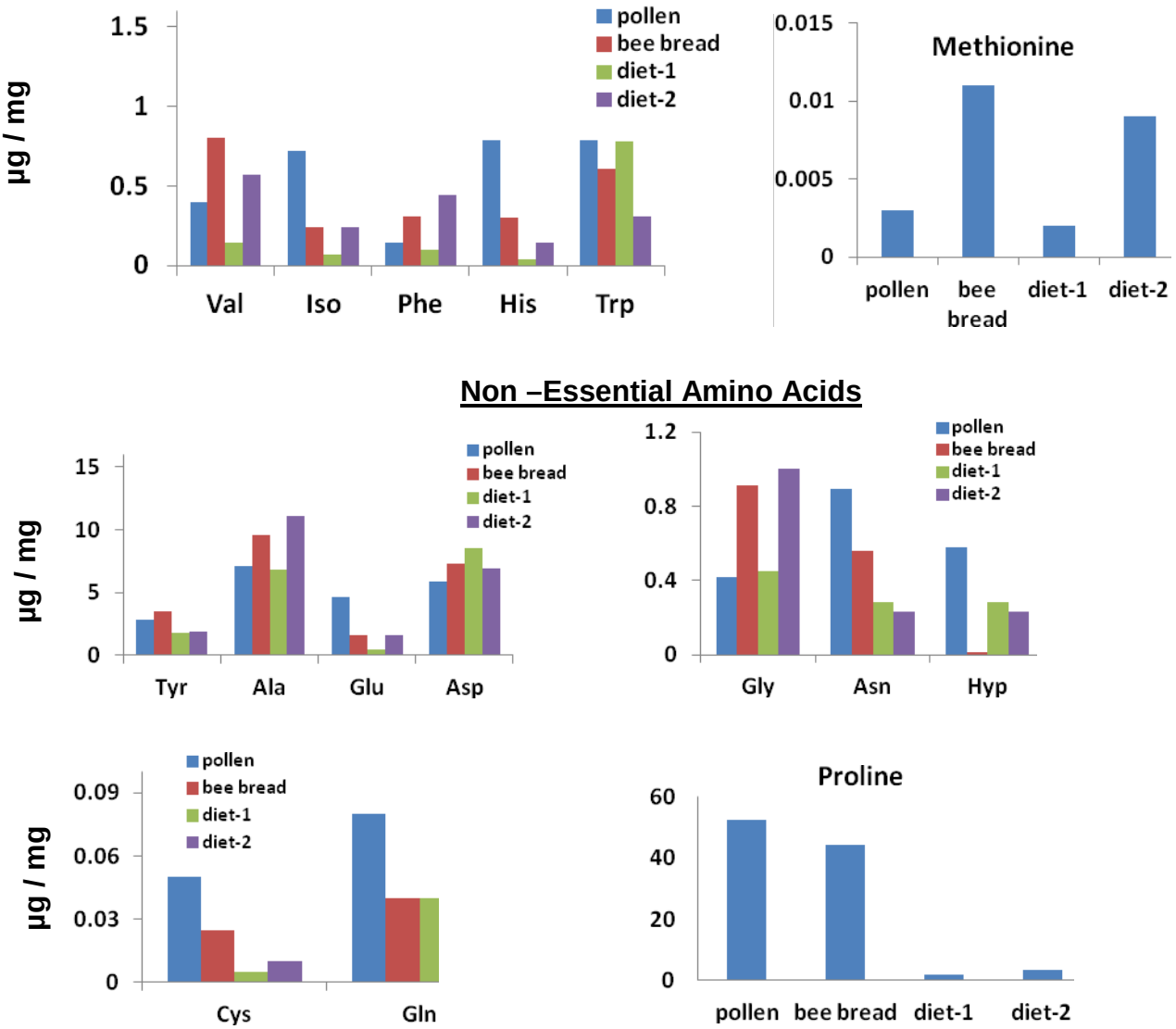


Figure 2. Essential and non-essential amino acids in two protein supplement diets (diet-1 and diet-2) and in rapini (*Brassica rapa*) pollen and bee bread made from it.

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.

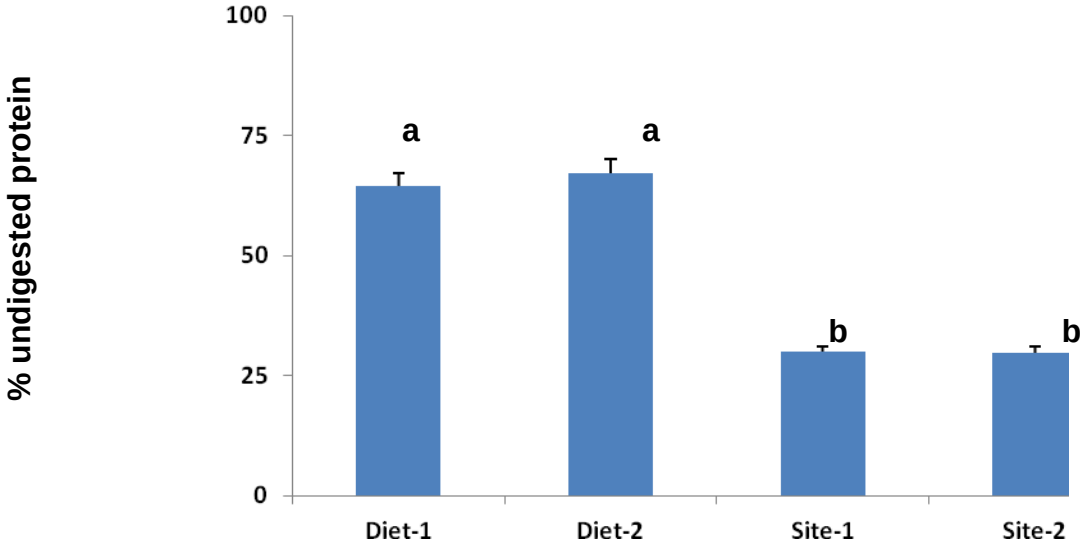


Figure 3. Percentage of protein in diets fed to colonies that were recovered in the hindgut of nurse bees. Means followed by the same letter are not significantly different at p < 0.05 as determined by F-test ($F_{3,29}$ = 90.6, p < 0.0001)and Tukey's W procedure.

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 30% of the colonies fed either PS. Colony losses for those foraging on pollen at sites-1 and 2 were 10 and 20% respectively (**Figure 4)**. 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 or foraging on rapini at either site lost queens. 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).

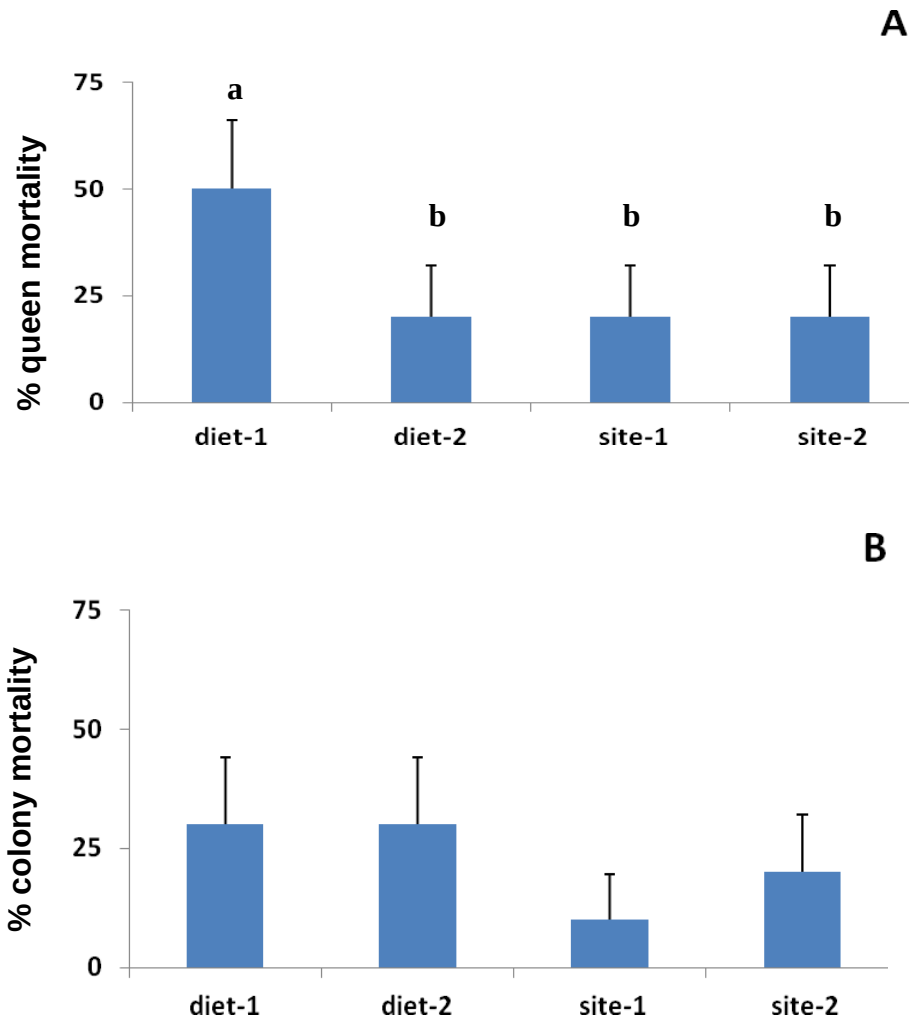


Figure 4. Percentages of queens (A) and colonies (B) that died during the study period in colonies fed protein supplement diets (diet-1 or 2) or foraging on rapini at two different planting sites (site-1 or 2). Queen losses were significantly higher in colonies fed diet-1 than those fed diet-2 or foraging on rapini (z- score = 1.84, p = 0.03). Colony losses were not significantly different among treatment groups (z-score = 1.4, p = 0.07).

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 (**Figure 5**).The greatest fold increases in DWV titers occurred in colonies fed diet-1.

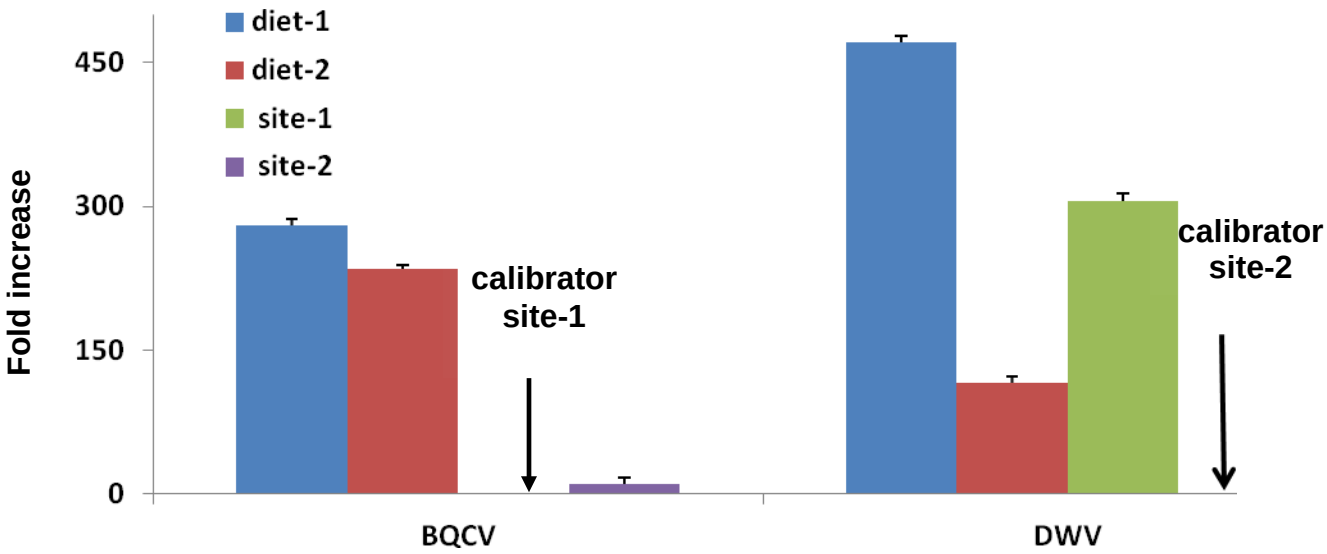
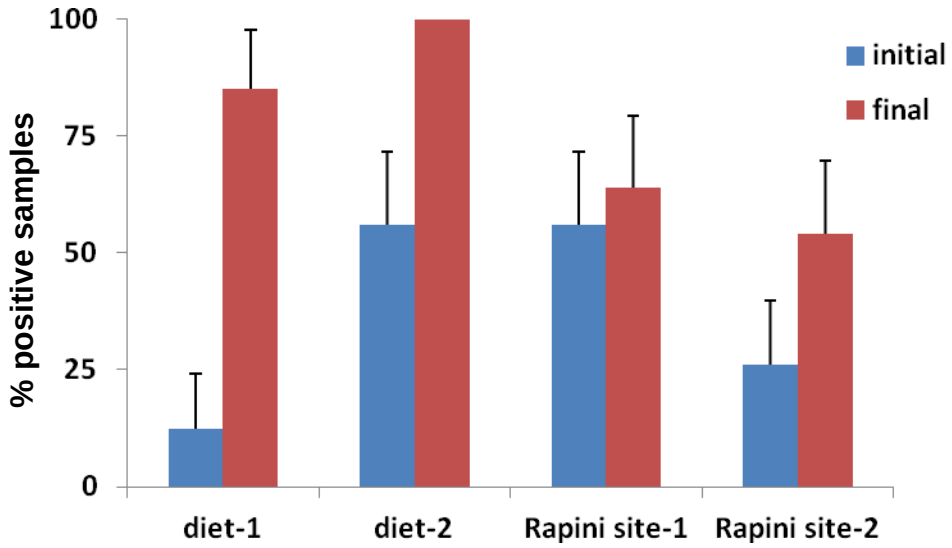


Figure 5. Fold increases in Black Queen Cell Virus (BQCV) and Deformed Wing Virus (DWV) titers estimated by calculating normalized virus titers (Δ Ct = virus titer for sample - beta actin) for treatment and control groups. ΔCt for site-1 was used as the calibrator for BQCV and site-2 for DWV. ΔΔCt was calculated as the difference between ΔCt for the treatment group and the calibrator. The fold increase was calculated by: $2^{-(\Delta\Delta Ct)}$.

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 6**). Colonies fed diet-2 and those foraging on rapini at site-1 had similar percentages of infected bees when the study began. At the end of the study, colonies fed PS, particularly diet-1, had the greatest increases in samples testing positive for Nosema ($F_{3,34}$ = 5.19, p = 0.005) and the highest average number of spores per colony ($F_{3,34}$ =4.10, p = 0.014) (**Figure 4**). Spore counts in colonies fed diet-1 were more than 4x higher than initial counts. There was a significant correlation between final spore counts for the colony and the concentration of undigested protein in the hindgut of bees from those colonies (Pearson correlation = 0.445, n = 35, p = 0.007).



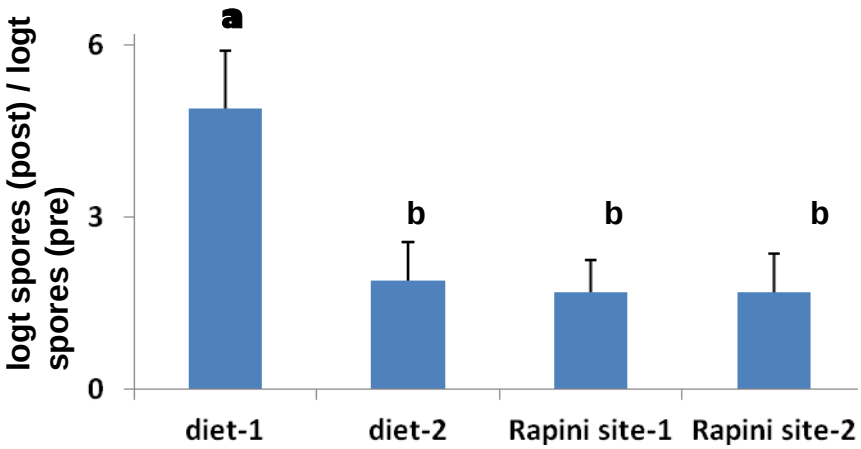


Figure 6. Percentage of worker bee samples testing positive for nosema spores before (initial) and after (final) feeding on protein supplement diets (diet-1 or 2) or foraging on rapini at two different sites for 9 weeks (A). The proportional increase in nosema spores (log transformed) between initial 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:

This study compared the nutritional value of two PS with rapini pollen, and measured the effects on pathogen loads and colony survival. Both PS had less soluble protein than pollen and beebread and less was digested. Many AA concentrations also were lower in the PS. Despite the nutritional differences, hemolymph protein titers and population sizes of surviving colonies did not differ among the treatments. However, the nutritional differences might have contributed to the higher pathogen levels in colonies fed PS and greater queen and colony losses especially for diet-1.

Pollen and beebread had similar soluble protein levels, but differed in concentrations of certain AA. The beebread might have had pollen from sources other than rapini and caused the

differences. However, similar results from controlled feeding studies have been reported (Human and Nicolson 2006; DeGrandi-Hoffman et al. 2013). Possible reasons include the activity of proteolytic enzymes added to pollen during storage, and metabolism by microbes in stored pollen (Gilliam et al. 1989; Metges 2000). However, a recent study suggests that bacteria in stored pollen contribute to its preservation rather than pre-digestion or nutrient conversion (Anderson et al. 2014). Though the reasons for nutritional differences between pollen and beebread are not clear, both should be included in nutritional analyses of a pollen source.

Bees digested less of the protein in the PS than the pollen. Our estimates were similar to Wang et al. (2014) who reported digestion of *Brassica rapa* pollen to be about 67%. In contrast, bees digested only about 35% of the protein in the PS. The differences might be that PS are difficult to digest because they contain soy or barley flour and eggs as protein sources, none of which are part of a bee's natural diet.

We found a significant relationship between *Nosema* levels and the amount of protein in the hindgut. Nosema replicates within midgut tissue (Higes et al. 2007; Dussaubat et al 2012) and likely impairs digestion and nutrient absorption. Nutritional stress is exacerbated when there is Nosema infection because this Microsporidia relies on the host to furnish energy for growth and reproduction (Mayack & Naug 2009; Martín-Hernández et al. 2011; Holt et al. 2013). Queen loss might also be more common because infected workers are less likely to feed nestmates (Naug and Gibbs 2009). In our study, colonies fed diet-1 had the greatest incidence of Nosema and the highest queen and colony losses.

Though protein digestion was lower for PS than rapini pollen, hemolymph protein titers did not differ. Colonies fed PS had pollen traps on the entrance, but some pollen might have entered the hive since traps capture only a portion of what is collected. Though we are not certain that the bees fed exclusively on the PS, it probably was the primary protein source. Others have reported no effects of protein stress on soluble protein titers, but stressed colonies produced less brood (Schmickl & Crailsheim 2001; Rueppell et al. 2008; Willard et al 2011). In our study, brood production did not differ between colonies fed PS or pollen possibly because it occurred in the winter when brood rearing rates are low. In a study comparing PS with pollen in summer, brood rearing was lower in those fed PS (Hocherl et al. 2012).

Colony survival was similar between those fed PS and foraging on rapini. PS patties were replaced weekly, so the colonies were opened more often than those foraging on rapini. This difference did not seem to affect survival. However, we may have underestimated colony losses particularly in those fed diet-1 because we replaced lost queens. Queen loss in winter can be fatal to a colony. Even if a queen is reared, drones usually are not available for mating. Queen failure is a leading cause of overwinter colony loss (Brodschneider & Crailsheim 2010; vanEngelsdorp et al 2010, 2013). Those losses might be associated with poor nutrition and higher pathogen loads that together compromise the care and health of queens.

Differences in AA concentrations between the PS and pollen may have contributed to the pathogen levels we measured. All EAA were present in the PS and pollen, but in some cases, concentrations were lower in the PS. The conditional AA cysteine and proline were much lower in both PS than in pollen. These AA constitute large portions of certain antimicrobial peptides

such as the cysteine-rich roylasin (Fujiwara et al. 1990) and the *Apis*-specific apidaecin that contains almost 30% proline (Casteel et al. 1990). Immune components respond in a nutrient-specific manner (Cotter et al. 2010). Whether the responses are driven by the nutritional demands of the immune traits, direct effects on immune gene expression, or indirect effects from diet-based changes to microbial communities in the gut remain to be determined. Relevant to this final point is that gut microbial communities might differ considerably between bees feeding on PS and pollen (Pernice et al. 2014) and influence immunity and other measures of fitness.

For the past 7 years, winter colony losses in the U.S. have been between 22-36% (Steinhauer et al. 2014). The losses are due to many factors several of which are related to the nutritional state of the colony. Feeding colonies PS in their current formulations does not appear to remedy problems of poor nutrition or reduce colony losses. In our study, we lost 30% of the colonies fed PS, which is within the range of annual winter losses in the U.S. Though PS can help colony populations to build in the spring, our study indicates that PS alone might not sustain the health and survival of colonies over prolonged periods in the winter especially when colonies remain active. Under these conditions, planting forage for bees could be part of the solution for reducing colony losses.

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.”

Research was presented in a Symposium - Nutrition and the Health and Behavior of Wild and Managed Bees - at the National Meetings of the Entomological Society of America, Portland, OR November, 2014. Title of talk: “Comparing nutrient acquisition from natural forage vs. protein supplements and measuring the effects on colony health”.

DeGrandi-Hoffman G, Chen Y, Rivera R, Carroll M, Chambers M, Hidalgo G, Watkins DeJong E. 2015.Honey bee colonies provided with natural forage have lower pathogen loads and higher overwinter survival than those fed protein supplements. Apidologie DOI: 10.1007/s13592-015-0386-6

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