Original article

Honey bee colonies provided with natural forage have lower pathogen loads and higher overwinter survival than those fed protein supplements

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Abstract – 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.

pollen nutrition / digestion / amino acids / *Nosema* / viruses

1. INTRODUCTION

The health and longevity of individuals and the growth of populations depend on the availability and nutritional value of food. The nutritional needs of honey bees are met with nectar and pollen. Nectar provides carbohydrates, and pollen supplies protein and all other nutrients (Brodschneider and Crailsheim 2010). Pollen is essential for colony growth, but when it is unavailable, managed hives are fed protein supplements (PS) (Nabors 2000; Mattila and Otis 2006; DeGrandi-Hoffman et al. 2008). Colonies usually are fed PS in the spring when pollen stores are low, and flowering plants and foraging weather are inadequate to support vigorous brood rearing.

However, feeding PS in fall and through the winter has become increasingly common in the US especially for hives that pollinate early crops such as almonds.

Recently, land managers have been encouraged to plant bee forage to provide pollen for colonies used in almond pollination (http://www.almonds.com/newsletters/outlook/fall-time-plant-bee-forage). Do these plantings improve colony health and survival to greater degree than those receiving adequate amounts of PS? Studies comparing PS with pollen fed as patties indicate that they are equivalent in palatability (DeGrandi-Hoffman et al. 2008), hypopharyngeal gland development in nurses (DeGrandi-Hoffman et al. 2010; Peng et al. 2012), hemolymph protein titers (DeJong et al. 2009; Morais et al. 2013), brood production, and colony growth (Saffari et al. 2010). However, viral loads are lower in bees fed pollen than PS (DeGrandi-Hoffman et al. 2010) perhaps because pollen feeding affects the

expression of genes associated with metabolic and immune function (Alaux et al. 2010, 2011).

Previous studies comparing PS and pollen were conducted in cages or in colonies for no more than a month. This might not be long enough for differences between pollen and PS to manifest. We compared the effects of feeding colonies PS with providing natural forage (*Brassica rapa*—rapini) from November to February. This is when colonies are in holding yards prior to almond pollination. We selected rapini because it blooms profusely in the southwestern USA during the fall and winter and is in Brassicacae, a family with highly nutritious pollen (Singh and Singh 1996). We compared the protein and amino acid composition of PS with rapini pollen, estimated protein digestion, and examined the effects of food source on immunity by measuring *Nosema* and virus titers. We also recorded queen losses and colony growth and survival. We found that PS might not provide all the nutrients needed to maintain low levels of pathogens and prevent queen loss overwinter.

2. MATERIALS AND METHODS

Rapini seed (Rapini broccoli Raab var. Quarantina, My Eco Gardens, Colville, WA, USA) was planted at the University of Arizona West Campus Agricultural Facility in Tucson, Arizona, USA (site 1), and the Red Rock Agricultural Facility in Red Rock, Arizona, USA (site 2), during the first week of October, 2013. Fields were seeded with 0.3 kg per hectare. Bloom began in November.

Colony establishment and measurements 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 13 km away at the Carl Hayden Bee Research Center, Tucson, AZ, USA. 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.

Colony sizes were measured at the start (November 12) and end (February 11) of the study. Areas on both sides of each frame with brood (all stages) were measured using a grid with 5 cm×5 cm squares that covered the entire side of a comb. Values from the frame measurements were summed to obtain brood and adult bee estimates for the colony (DeGrandi-Hoffman et al. 2008). Colonies were inspected monthly for the marked queen. 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.

Diet consumption Diet 1 was fed as premade patties. Diet 2 patties were made by mixing granulated sugar (sucrose) with water (2:1) and adding MegaBee® powder at a 1:1 weight ratio. The mixture was covered for 12 h so the powder could absorb the syrup before making patties. All patties were weighed (113 g) and placed on the top bars of frames. Unconsumed patties were removed weekly throughout the study, weighed to estimate consumption, and replaced with a new patty.

Hemolymph collection and soluble protein analysis We measured hemolymph protein concentrations in five nurse bees (adult workers feeding larvae) from all colonies when the hives were established (baseline protein levels) and at 3-week intervals from November to February using methods described in DeGrandi-Hoffman et al. (2010, 2013). Hemolymph also was sampled from day 5 larvae (five per colony). Larvae were rinsed in phosphate-buffered saline (PBS) to remove any brood food prior to sampling (Chan and Foster 2008). Hemolymph from five workers or larvae was pooled, and a 1-μL sample was added to 99 μL of PBS containing 1 % EDTA-free Halt Protease Inhibitor Cocktail (HPIC) (#78437, Thermo Scientific, Rockford, IL). Samples were stored at −80 °C until analyzed using bicinchoninic acid (BCA) protein assay (#23225, Thermo Scientific). Absorbance was measured at 562 nm with a Synergy HT spectrophotometer (BioTek Instruments, Inc., Winooski, Vermont). Protein quantitation was estimated using a standard curve generated from serial dilutions of bovine serum albumin.

Soluble protein in pollen, diets, and hindgut Soluble protein in pollen was measured using corbicular

loads from bees foraging on rapini. We also sampled stored pollen (i.e., beebread) from colonies at both sites. Pollen and beebread samples were pooled and divided into three subsamples for analysis. Soluble protein concentrations in the PS were measured using three random samples from patties of each diet. In all cases, 1 mg of the food source was added to 999 μL of PBS with 1 % EDTA-free HPIC and analyzed for total soluble protein as described above using BCA.

As a measure of digestion, soluble protein concentrations in the hindgut contents (ileum and rectum) of nurse bees were measured and compared with concentrations in the PS and pollen. Nurse bees were used because they consume and digest more pollen than older bees (Crailsheim et al. 1992). Hindguts were removed from the bees, and a 1-μL sample of the contents was added to 99 μL of PBS and 1 % EDTA-free HPIC. Samples from the five bees per colony were combined, and the pooled sample was added to 900 μL of PBS with 1 % EDTA-free HPIC. Samples were stored at −80 °C until analyzed as above. Digestion was estimated by: soluble protein concentration in hindgut contents/soluble protein concentration in food source.

AA analysis Random samples of pollen, beebread, and PS were taken as described above for soluble protein analysis. Free amino acids (AAs) were quantified by mixing 10-mg portions with 200 μL 0.03 M HCl to solubilize the free AA, filtering them through a Gilson 200-μL filtered pipette tip and centrifuging the sample. The supernatant (100 μL) was analyzed with GC/MS using an EZ:faast kit for free physiological AA (Phenomenex Inc., Torrance, CA, USA).

All analyses were conducted on an Agilent model 7890 GC (Agilent, Palo Alto, CA, USA) coupled to a 5975 quadrupole Mass Selective Detector (MSD) operated in Selected Ion Monitoring mode. A 1.0-μL derivatized sample was injected in split mode (Agilent 7693 Autosampler Injection System) and separated on a Zebron ZB-50 capillary column (30-m length, 0.25-mm ID, and 0.25-μm film thickness). A deactivated borosilicate single taper glass liner (SGE Focus Liners) was used to minimize sample loss. Injector temperature was 250 °C, with helium as the carrier gas at a flow rate of 1.2 mL/min. Initial oven temperature (110 °C) was held for 1 min, then ramped up at 30°/min to 320 °C, and held for 5 min. AAs were identified and quantified by comparing peaks against known retention times and characteristic mass fragments (m/z) of derivatized AA standards. We detected and quantified nine essential (essential amino acid (EAA)), six conditional (essential in times of stress), and five nonessential AAs (Nelson and Cox 2012).

Nosema levels At the start and end of the study, 25 bees per colony were collected from honey frames and stored in alcohol (Smart and Sheppard 2012; Travers et al. 2012). The samples were divided into groups of five bees. Abdomens were removed, placed in a mortar with 5 μL of ultra pure water, and crushed with a pestle. A 1-μL sample was pipetted into a hemocytometer and covered with a glass slip. *Nosema* spores (presumably *Nosema ceranae* spores as *Nosema apis* is not readily detectable in apiaries) were counted at ×40 magnification and converted to total spores per bee using methods described in Fries et al. (2013). The process was repeated for each group of five bees. The percentage of groups with spores and the total number counted in all groups were used as estimates for the colony.

Virus titers Colonies were sampled (five nurse bees per colony) after foraging on rapini or fed PS for four brood cycles. All samples were snap frozen in liquid nitrogen and stored at −80 °C until analyzed for *black queen cell virus* (BQCV), *deformed wing virus* (DWV), *Israeli acute paralysis virus* (IAPV), *Kashmir bee virus* (KBV), and *Sac brood virus* (SBV) using methods described in Chen et al. (2005). Virus levels were quantified using the comparative Ct method (ΔΔCt method) that is based on the number of cycles needed to generate a fluorescent signal above a predefined threshold. The average Ct value (ΔCt) of each virus was normalized using the Ct value for housekeeping gene β-actin (ΔCt=(Average $Ct_{target\ x}$)−(Average $Ct_{\beta\text{-actin}}$)). The group with the lowest ΔCt 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 treatment group was calculated using the formula $2^{-(\Delta\Delta Ct)}$ and expressed as an *n*-fold difference relative to the calibrator.

Statistical analysis All data were evaluated for normality and homogeneity of variance prior to analysis. Protein and individual AA concentrations were compared among food sources with one-way analysis of variance (AOV). Repeated measures AOV was used to compare PS consumption and soluble protein

concentrations in the hemolymph and hindguts. Initial *Nosema* spore counts were compared among treatments using log transformation followed by AOV. *Nosema* counts were converted to proportional change (final spore count/initial spore count) and compared among treatments using AOV. *Nosema* infection can interfere with digestion (Malone and Gatehouse 1998; Dussaubat et al. 2012), so we tested for a correlation between final spore count in a colony and proportion of undigested protein in the hindgut of bees from that colony. Colony size comparisons were made using AOV with the proportional population change (ending/starting population size) as the test statistic. The proportions of queens and colonies lost during the study were compared using z-tests. Statistical analyses were conducted using Minitab (Minitab Inc, State College, PA, USA) and SPSS (Field 2005).

3. RESULTS

Protein and AA analyses 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 pollen or beebread ($F_{2,26}$=7.08, P=0.004) (Figure 1).

We detected 20 AAs, with proline having 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 I). Histidine, lysine, and
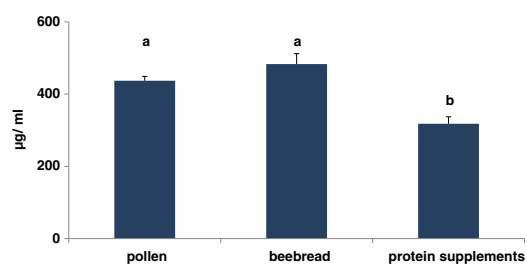
Figure 1. Average concentration (±SE) of soluble protein in rapini pollen, beebread, and two protein supplements extracts. Protein concentrations of the supplements did not differ, so data were combined. Averages with the *same letter* are not significantly different as determined by an analysis of variance followed by a Tukey's multiple comparison test ($F_{2,26}$=7.08, P=0.004).

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

Consumption There was no difference between diet 1 and 2 consumptions ($F_{1,15}$=0.184, P= 0.67). Colonies consumed an average of 66.4± 4.6 g of diet 1 and 63.6±3.5 g of diet 2 per week.

Soluble protein concentrations in bees Baseline hemolymph protein concentrations did not differ among the treatment groups ($F_{3,36}$=1.24, P= 0.3). The concentrations in nurse bees ($F_{3,30}$= 0.196, P=0.898) and larvae also did not differ among treatments ($F_{3,31}$=1.28, P=0.3) after feeding on PS or pollen for four brood cycles.

Soluble protein concentration of hindgut contents was significantly higher in bees feeding on PS compared with pollen or beebread ($F_{3,29}$= 90.6, P<0.0001) (Figure 3). The contents contained about 65 % of the soluble protein in the PS compared with 30 % of the pollen or beebread.

Nosema spore counts 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). 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).

Virus titers Of the viruses we screened for, DWV and BQCV were detected at the highest titers and with the greatest frequency (Figure 5). BQCV titers had the greatest fold increases in colonies fed PS and lowest in those foraging on rapini.
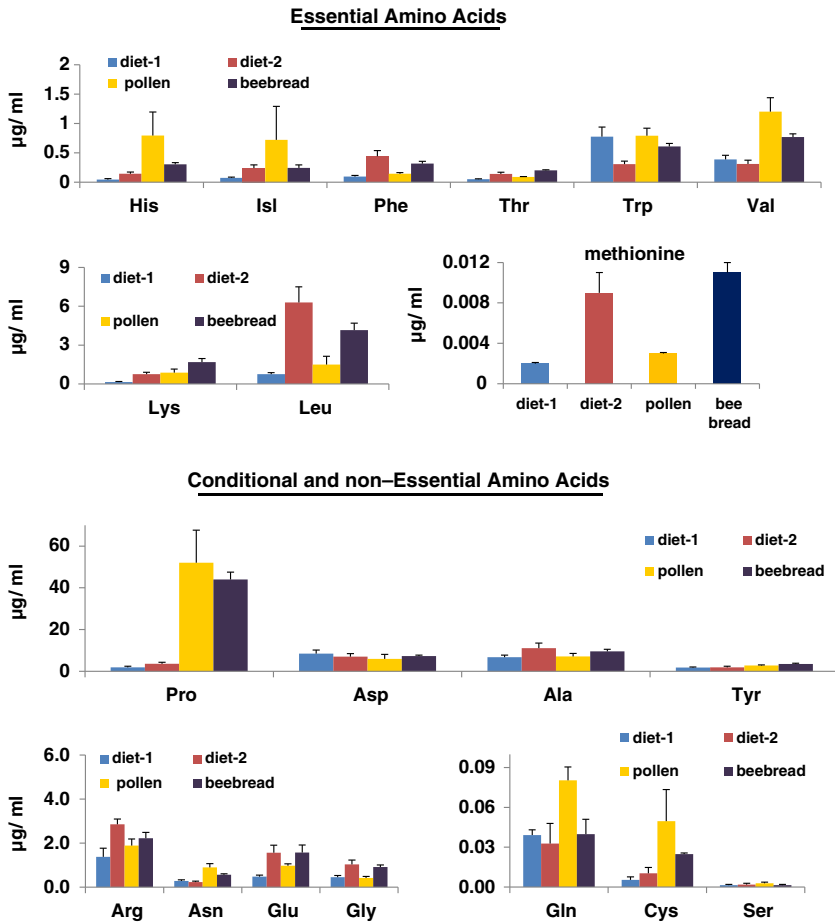
Figure 2. Average amounts (±SE) of free amino acids per milligram of two different protein supplements (diet 1 and diet 2), rapini pollen, and beebread extracts.

There were no clear effects of diet on DWV levels.

Colony growth 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 4.0 ± 0.3 frames of bees (approx. $10,024\pm250$ adults) and 1.8 ± 0.1 frames of brood. At the end of the study, populations sizes of surviving colonies were similar to when they began and did not differ significantly among treatment groups (adult bees: $F_{3,27}=0.86$, $P=0.47$, frames of brood: $F_{3,27}=0.85$, $P=0.48$).

Queen and colony survival Queen losses were highest among colonies fed diet 1 (z-score=1.84,

$P=0.03$) (Figure 6). Colony mortality also was higher in those fed PS than foraging on rapini, but the differences were not significant (z-score=1.4, $P=0.07$).

4. DISCUSSION

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

Table I. Amino acid concentrations found in two protein supplements (diets 1 and 2), rapini pollen and beebread extracts.

Amino acid	Diet 1	Diet 2	Pollen	Beebread	$F_{3,8}$, P value
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	0.002 b	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 a	1.88 b	1.88 b	4.48, 0.04
Cysteine	0.006 c	0.01 c	0.07 a	0.02 b	46.18, <0.0001
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
Nonessential					
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

Means followed by the same letter are not significantly different at the 0.05 p level

nutritional differences might have contributed to the higher pathogen levels in colonies fed PS and greater queen and colony losses especially for diet 1.
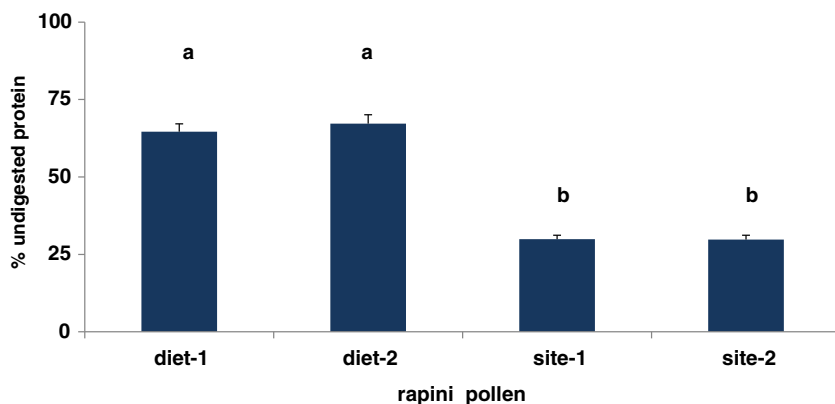

Figure 3. Percentage of undigested soluble protein (±SE) in the hindgut of bees fed protein supplements (diets 1 and 2) or rapini pollen at two different sites (sites 1 and 2). Means followed by the *same letter* are not significantly different as determined by analysis of variance ($F_{3,29}$=90.6, P<0.0001) followed by a Tukey's W procedure.
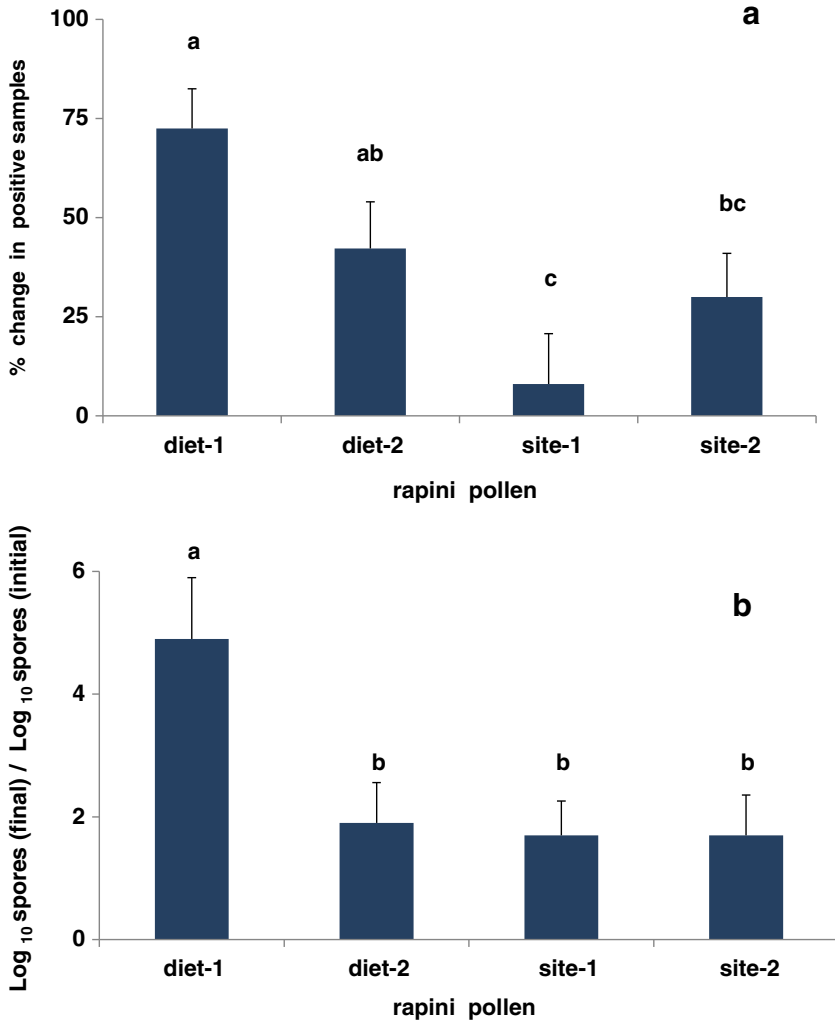
Figure 4. Average changes (±SE) from the start to the end of the study in the percentage of samples with *Nosema* (**a**) from colonies fed protein supplements (diet 1 or 2) or rapini pollen at two different sites (sites 1 or 2). Average changes in the number of spores per colony (±SE) are shown in **b**. Means followed by the *same letter* are not significantly different as determined by a one-way analysis of variance followed by a Fisher's test (changes in the percentage of bees testing positive for *Nosema*: $F_{3,33}=5.19$, $P=0.005$, changes in spore numbers: $F_{3,34}=4.10$, $P=0.014$).

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 predigestion 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 (Roulston and Cane 2000; Di Pasquale et al. 2013).
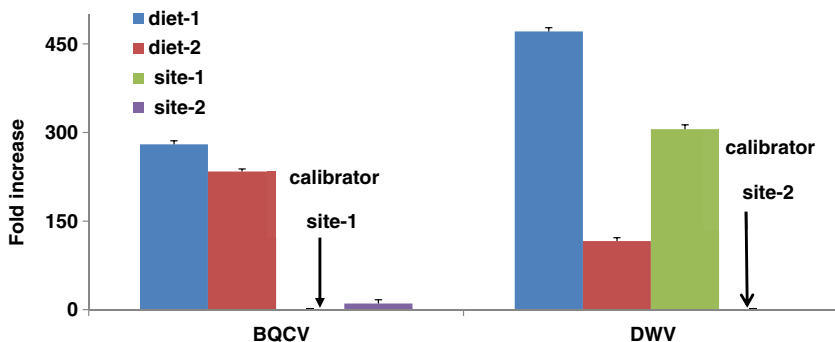
Figure 5. Fold increases (±SE) in black queen cell virus (BQCV) and deformed wing virus (DWV) titers estimated by normalizing the titers (ΔCt=(virus titer for sample)−(β actin)) for the treatment. ΔCt for site 1 was the calibrator for BQCV and site 2 for DWV. $\Delta\Delta$Ct is the difference between ΔCt for the treatment and the calibrator. The fold increase was calculated by $2^{-(\Delta\Delta Ct)}$.
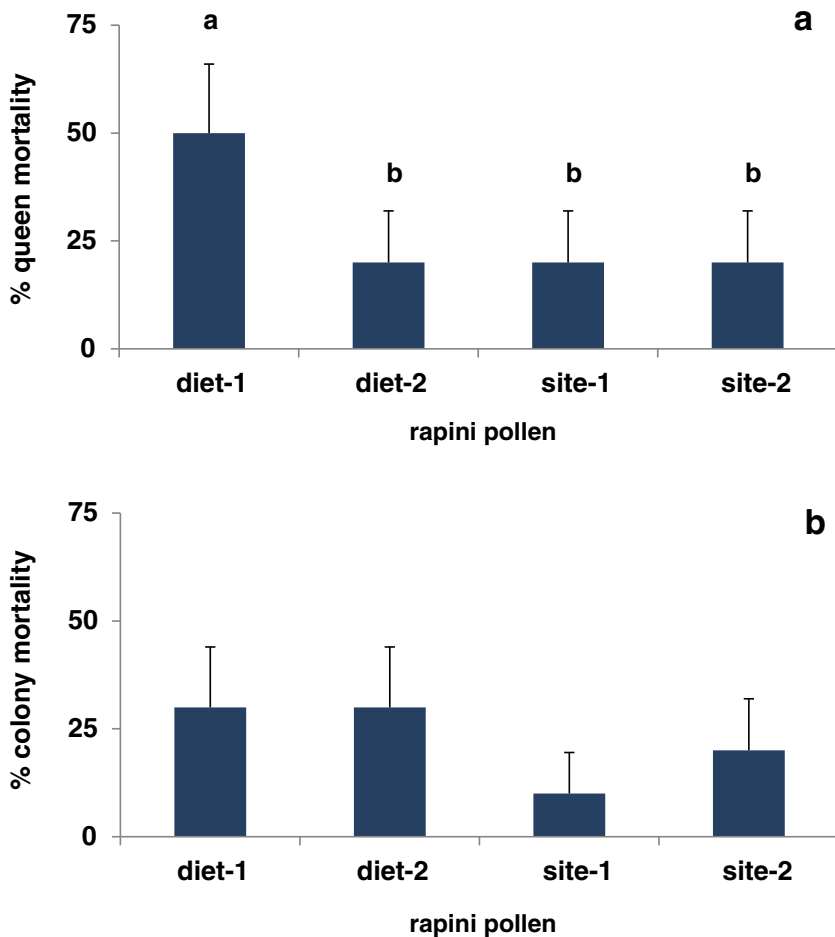
Figure 6. Percentages of queens (**a**) and colonies (**b**) (±SE) that died during the study period in colonies fed protein supplements (diet 1 or 2) or rapini pollen at two different sites (site 1 or 2). Queen losses were significantly higher in colonies fed diet 1 than those fed diet 2 or rapini (z-score=1.84, P=0.03). Colony losses were not significantly different (z-score=1.4, P=0.07).

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 *B. 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 and 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 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 and 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 and 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 that we measured. All EAAs 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 AAs 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 remains 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 USA have been between 22 and 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 USA. 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.

Les colonies d'abeilles disposant d'un approvisionnement naturel ont moins de pathogènes et présentent une survie hivernale plus élevée que celles nourries de compléments protéiniques

alimentation en pollen / digestion / acides aminés / *Nosema* / virus

Honigbienenvölker mit einer Nahrungsversorgung durch natürliche Sammeltätigkeit haben eine geringere Belastung mit Pathogenen und höhere Überwinterungsraten als Völker , die mit Proteinzusätzen gefüttert werden

Pollenernährung / Verdauung / Aminosäuren / *Nosema* / Bienenviren

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