The Influence of Cover Crop on Honey Bee Nutrition and Gut Microbes and On Colony Growth and Activity

Project No.: **15-POLL14/14A-McFrederick/Meikle/Carroll**

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Objectives:

1) Develop methods to exploit data from continuous monitoring of bee hives in the field.
2) Determine the effect of strategically planted oilseed cover crops that bloom prior to, and shortly after the almond pollination, on honey bee nutrition, health, and queen quality.
3) Determine if cover crops affect the honey-bee gut microbiota when compared to bees fed high fructose corn syrup.
4) Understand the interplay between cover crops, honey bee nutrition, health, queen quality, and microbes by synthesizing the results from objectives 1-3.

Interpretive Summary:

Lack of forage is thought to be one of the major contributing stressors leading to poor honey bee colony health. Colonies experience nutritional stress in the times of dearth before and after almond pollination due to the lack of alternative forage and reliance on supplemental feeds. To determine if the availability of supplemental forage before the almond bloom increases honey bee colony health, we followed 32 colonies over the winter and spring of 2015-2016. We split these colonies among 4 sites in late December; 2 sites with readily available forage and 2 sites with limited forage. We then followed colony weight, temperature, growth metrics, nutrition, and microbiome through April 2016. We are still conducting laboratory analyses and will have a better understanding of the effects of forage availability soon. Even without these final data, however, our colony survival data allow us to recommend supplemental forage for honey bee health. Small differences in forage availability (one month of forage, with ~2.5X more pollen in the forage treatment colonies compared to the no forage colonies) led to drastic differences in post-almond bloom colony survival. 81% of the no-forage treatment colonies failed while only 19% of the forage treatment colonies failed. Importantly, these trends only became apparent after the almond bloom. These data suggest that even moderate amounts of supplemental forage made available to honey bees before the almond bloom will likely lead to greater colony survival. Almond growers may be able to secure honey bee contracts by providing pre-bloom forage as an incentive.

Materials and Methods:

In November 2015 40 honey bee colonies were identified at apiaries in southern Arizona that occupied 1-2 10-frame deep boxes and had marked queens. Of those hives, 32 were selected for monitoring while the remaining 8 hives were used as “sentinel” hives, two such hives at each treatment site. Pre-almond sites were two plots at “Red Rock” and two plots at MAC (Maricopa Agricultural Center), both north of Tucson. The Red Rock plots had ample available forage: Rapini, Brittlebush, Creosote, African Sumac, Filaree. The MAC plots had no available forage. The sentinel colonies at all four plots were equipped with pollen traps to verify availability or lack of forage. Sampling and supplemental feeding occurred as outlined in **Table 1**.

Table 1. Experimental timeline 2015-2016.	
Nov. 19 -23	Hive inventory and identification
Nov. 27	Maintenance feeding: 250 g BeePro patties for all hives
Dec. 7-10	Pre-treatment sampling and evaluations. Temperature sensors installed.
Dec. 11	Maintenance feeding: 250 g BeePro + 3 L 1:1 sugar syrup for all. Pollen traps placed on sentinel hives.
Dec. 24	Treatment sites not quite ready. 3 L sugar syrup given to all hives.
Dec. 30	All hives moved to respective treatment sites and placed on electronic scales.
Dec. 31 - Jan. 25	Hives in treatment plots. Hives inspected periodically to ascertain their health. Sentinel hives monitored on several occasions for pollen collection.
Jan. 26-29	Hive sampling and evaluations.
Feb. 2-4	Hives moved to almond orchard (Blackwell's Corner, CA) and installed on scales. Hives fed sugar syrup.
Feb. 4-29	Hives in CA almonds
Mar. 3-5	Hives sampling and evaluations. All hives moved to Keck's Corner (about 25 km away) for post almonds and placed on scales. Hives fed sugar syrup.
Mar. 6 - Apr. 4	Hives in post-almond site.
Apr. 4-8	Post-almond hive sampling and evaluations. All hive scales and associated equipment moved to Arizona.
Apr. 9	All surviving hives moved back to Arizona.

Methods Objective 1: Develop methods to exploit data from continuous monitoring of bee colonies in the field

All 32 hives were fitted with Thermochron temperature sensors. Six hives in each group were placed on electronic scales, for a total of 24 hives on scales. As hives died, other hives from the same treatment groups were placed on the scales; statistical analysis will of course take this into account. During hive evaluations, each frame was gently shaken to dislodge adult bees, then weighed, photographed using a 16.3-megapixel digital camera (Pentax K-01, Ricoh Imaging Co., Ltd.) and replaced in the hive. The area of sealed brood per frame was estimated from the photographs using ImageJ version 1.47 software (W. Rasband, National Institutes of Health, USA). The total weight of the adult bee population, or "adult bee mass," was calculated by subtracting the combined weights of

Hives at MacSouth site before pollination. Solar panel on lower right

hive components (i.e. lid, inner cover, box, bottom board, frames, entrance reducer, internal feeder) obtained at the start of the experiment from the total hive weight recorded the midnight prior to the inspection. At each inspection, 3-5 g of adult bees, wax and honey were each collected from each hive into 50 ml centrifuge tubes and stored at -20ºC. Pooled samples of adult bees, honey and wax collected will be analyzed for pesticide residues (174 compounds) by the Laboratory Approval and Testing Division, Agricultural Marketing Service, USDA, Gastonia, NC. Continuous data were divided into daily average data and within day detrended data. Sine curves were fit to the detrended data using 3-day samples taken sequentially by day; the resulting running average values and sine wave amplitudes were considered separately.

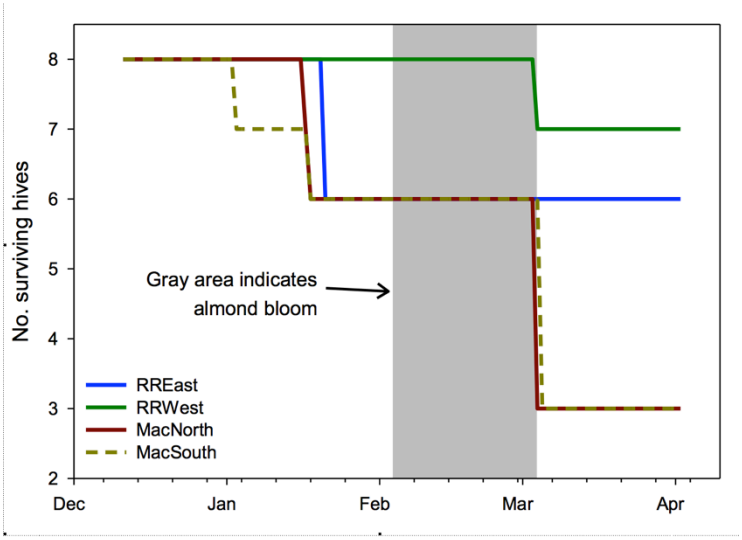

Figure 1. Survivorship of hives during the course of the study (not including sentinel hives). RREast and RRWest were considered to have "good" forage while MacNorth and MacSouth did not. In 2015-16 we lost a total of 13 of 32 hives, whereas in 2014-15 we lost a total of 3 of 16 hives (in the Meikle group).

<u>Methods Objective 2: Effect of cover crops on honey bee nutrition, health and queen quality.</u>

Changes in colony performance were linked to changes in individual bees by monitoring worker nutrition, foraging, and queen performance over time. The effects of supplemental forage on bee nutritional health were determined by analyzing the nutritional content of stored pollen and the key nutrient reserves of bees. Nutritional deficiencies can be detected as nutrient shortages or imbalances in both the insect and its food. Nutrients in both bees and their food can be analyzed at a microscale (mg or µL amounts) level using subsamples of homogenized tissues (see methods below). Some tissues were partitioned to provide subsamples for both nutritional and microbial analyses, for example queen guts were removed, and the rest of the queens are therefore available for nutritional analyses. Approximately 500 mg each of corbicular pollen and bee bread (stored pollen) was sampled from each colony when available. Corbicular pollen on bees was collected by pollen trap as previously described, while bee bread samples were obtained by coring multiple stored pollen cells with a modified pipette tip. Pollen samples were analyzed for total protein and total lipid contents as well as amino acid, fatty acid, sterol, carbohydrate, water soluble vitamin, and fat-soluble vitamin compositions. When supplemental forage was not available,

Hives at almond pollination at Blackwell's Corner, CA.

nectar and pollen substitutes were evaluated for nutrient contents. Five individual capping (5th instar) larvae, emerging adult bees, nurse bees, and incoming forager s were collected from each colony. The former two developmental stages represent key stages in the nutritional development of bees (i.e. at the end of larval feeding and at the beginning of adult feeding), while the latter two perform critical nutritional functions. In the event that brood was not present, frame bees were substituted for nurse bees. Larvae were analyzed for fresh mass, total protein content, and total lipid content. Adult bees were characterized for fresh mass, total protein content of the hypopharyngeal gland, and total lipid content of the fat body.

All nutrients were identified and quantified by comparison against known internal and external standards. To breach the exine coat, pollen samples were ruptured by osmotic shock and vibration before chemical extraction (Human and Nicolson 2003). All tissues were homogenized by BeadBeater or grinding in liquid nitrogen before chemical analyses. Lipids were extracted by Folch extraction (Folch *et al.* 1957) and total lipids quantified by a chromic acid oxidation assay (Amenta 1970). Proteins and amino acids were digested by heated acid hydrolysis and neutralized before analysis (Otter 2012). Total protein content of neutralized hydrolyzed proteins was determined by a Pierce BCA assay (Thermo-Fisher, Rockford, IL). Hydrolyzed amino acids were derivatized and characterized by EI GC-MS analysis (Kaspar *et al.* 2008). Fatty acid, sterol, and carbohydrate components were derivatized by silylation and characterized by EI GC-MS analysis (modified after (Aliferis *et al.* 2012)). Water soluble vitamins and antioxidants were extracted under acid hydrolysis and analyzed by HPLC (Lebiedzinska *et al.* 2007). Fat soluble vitamins and antioxidants were extracted, saponified, and separated by HPLC based on the methods of Wang et al. (2007) and Slavin and Yu (2012). Body masses and nutrient contents were compared across treatments and sampling occasions with a mixed-model repeated-measures ANOVA with treatment, time and their interaction as fixed effects and colony within treatment as a random effect.

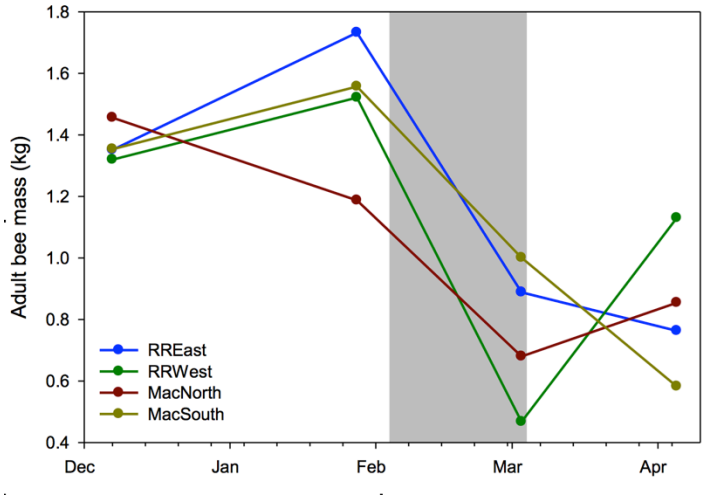

Figure 2. Adult bee masses calculated from hive evaluations. Note that the number of hives per treatment group changed drastically, and the loss of smaller colonies explains much of the "growth" in adult bee mass after almonds.

The effects of supplemental forage on queen productivity and queen retention were periodically assessed through the forage and almond pollination periods. Since queen losses are often initiated by workers, we examined queen emissions of queen mandibular pheromone (QMP), a pheromone complex that signals queen right quality to the workers (Keeling *et al.* 2003; Slessor *et al.* 2005). We have recently developed methods to estimate QMP emissions from live queens that are a marked improvement over current methods, which extract QMP contents from dissected queens (Nino *et al.* 2012). Our non-destructive methods can be used to monitor individual queens through a treatment period. QMP emissions were collected after a brief isolation of the queen and her retinue workers on brood comb and

characterized by unpublished analytical chemistry methods. Queen attractiveness to workers was quantified by recording the average size of the queen retinue during two observations (Nino *et al.* 2012).

Queens were assessed for QMP emissions and retinue formation during the pre-forage bloom, the pre-almond bloom, the end of almond bloom, and the end of the post-bloom forage crop. After the last assessment of live queens, queens were removed and sacrificed to assess fecundity metrics and gut microbes. Ovariole development and spermatheca contents will be quantified in dissected queens (Niño et al. 2012). To allow comparisons with previous studies, we will also perform conventional estimates of mandibular gland QMP contents (Niño et al. 2012).

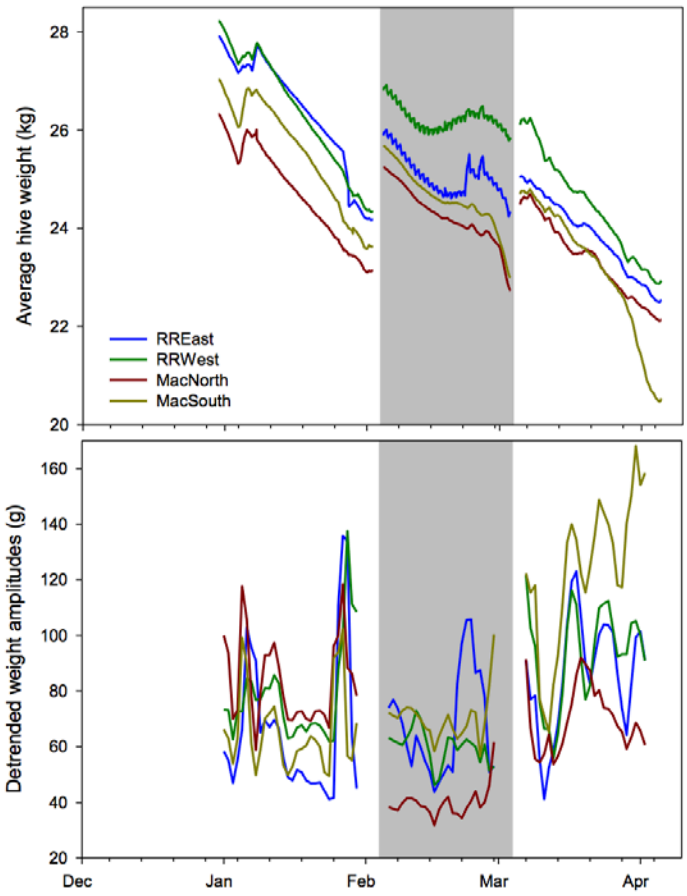

Figure 3. Upper figure shows average total hive weight from the start of the study (Dec. 30) until the end. Increases in hive weight in February and March were due to 1) feeding hives sugar syrup; and 2) the loss of smaller hives (which raised the average hive weights). Note that the strongest colonies grew during almonds, but not the weak groups. Lower figure shows detrended weight amplitudes, which correspond to foraging activity. Note the active foraging while hives were at the treatment sites, particularly by hives with little natural forage in the vicinity. After almonds, the heavy loss of colonies by the MacNorth and MacSouth groups contributed to high variance within those groups.

Retinue sizes were compared across treatments and sampling occasions with a mixed-model repeated-measures ANOVA with treatment, time and their interaction as fixed effects and colony within treatment as a random effect. QMP emissions sampled sequentially from individual queens were normalized against the initial baseline value, arcsine transformed, and compared across treatments and sampling occasions with mixed-model repeated-measures ANOVA. All other queen metrics sampled on a single occasion were compared across treatments by a one-way ANOVA. QMP emissions and retinue size were compared separately against brood production (estimated from frame photographs as described earlier) to determine if pheromone emissions or queen attractiveness are correlated with queen/worker reproductive productivity (brood production).

<u>Methods Objective 3: Effects of cover crops on gut microbiota.</u>

To test the hypothesis that artificial diet versus forage will influence the composition of the honey bee gut microbiome, we are characterizing the gut microbiome from our experimental colonies. We collected five worker bees (when available) from each colony at four different time points. We surface sterilized each sample by rinsing each sample in a 10%

bleach solution, followed by three rinses in sterilized, deionized water. We then dissected the gut from each sample, using sterilized dissection trays and tools.

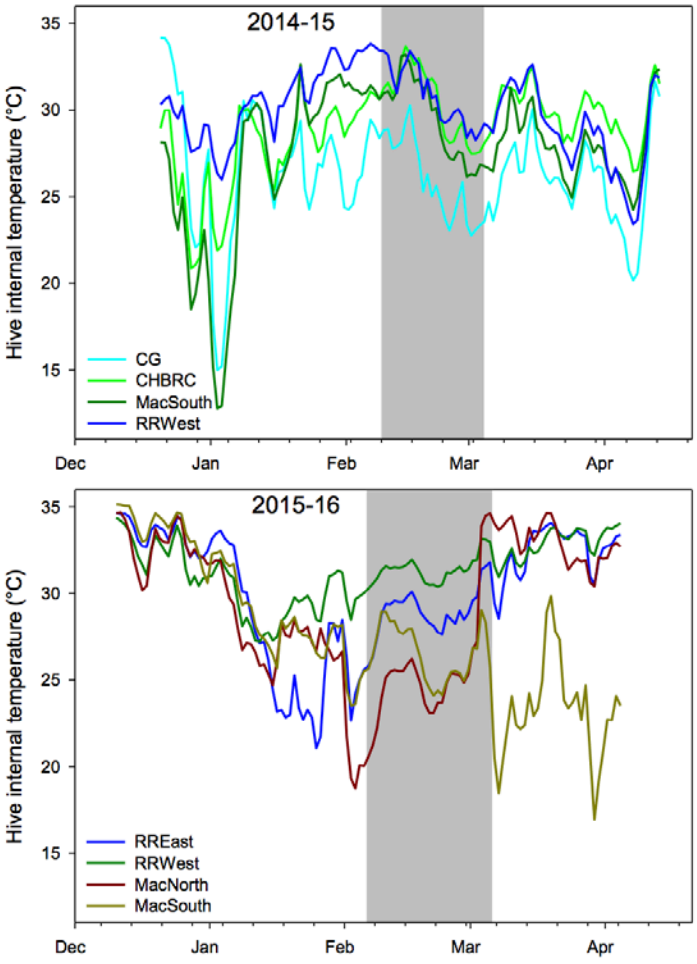

Figure 4. Average daily hive internal temperatures, comparing data from 2014-15 (upper graph) with that of 2015-16 (lower graph). Note the differences in the patterns of changes, largely due to external weather. We have found internal temperature to be associated with hive vigor - healthy hives tend to have higher temperatures during cold periods than weaker hives. Hives in 2014-15 were all offered pollen patty during the treatment period, but only hives in the CG and RRWest treatment groups were offered natural pollen. In the 2015-16 experiment, both the Red Rock treatment groups showed higher temperatures during pollination. Hives from MacNorth, perhaps the least favorable of all the treatment groups, showed increased average temperatures after pollination, owing to the loss of the weaker colonies. The MacSouth group also lost many weak hives at that same time, but even the strong colony in that group showed a large decrease in internal temperature just after the end of almond pollination for unknown reasons.

To quantify bacterial communities found in the workers and the queens, we used next-generation sequencing. We have extensive experience with these methods (McFrederick *et al.* 2012; USDA 2012; McFrederick *et al.* 2013; 2014), and recently co-authored a paper creating standard methods for analyses of the honey bee gut microbiota as part of the COLOSS BEEBOOK (Kwong & Moran 2013; Engel *et al.* 2013a). To identify gut bacteria and determine their relative abundances, we sequenced a gene found in all bacteria, using modern sequencing technology that allows us to generate thousands of DNA sequences from a single sample (Illumina DNA sequencing). We then compared the sequences that we obtained to sequences from a publically available database (NCBI's GenBank). This allows us to identify the different bacterial DNA sequences, and therefore, the bacteria from the honey bee gut samples.

We analyzed the next generation sequencing data using standardized methods (Engel et al. 2013). We used the QIIME pipeline (Martinson et al. 2011; Kuczynski et al. 2011; Moran et al. 2012) and UniFrac analyses (Hamady & Lozupone 2009; Martinson *et al.* 2012; Engel *et al.* 2012) to create community dissimilartity matrices. We tested for differences by forage plot, timepoint, and whether the colonies had been placed on forage or no-forage plots in the winter. To determine if the microbiomes of these groups significantly differed, we used Adonis analysis, a type of permutational MANOVA (Oksanen *et al.*). We visualized these differences using non-metric multi-dimensional scaling (Ramette 2007), a non-parametric ordination analysis.

<u>Objective 4: Understand the interplay between cover crops, honey bee nutrition, health, queen quality, and gut microbes.</u>

Through the proposed collaboration of researchers from different fields, we will be able to build a complete picture of how cover crops in almond orchards affect honey bee nutrition, health, queen quality, and gut microbes. In objectives 1-3 we will use cutting-edge technology to measure the response of these individual variables to cover crop treatment, and in objective 4 we will synthesize the results from objectives 1-3. Using individual correlation analyses and correcting for multiple tests, we will determine if variation in honey bee nutrition, health, queen quality and colony-level growth and activity significantly correlates with variation in gut communities. To use the gut microbial community data in these analyses, we will use principal coordinates analysis to summarize microbial community composition into a single continuous variable (Ramette 2007). All bees that we sample for gut microbes will also be used for nutrient analyses, and we will therefore be able to determine if nutrient levels correlate with gut microbial community composition. We will also quantify and correlate gut microbial communities and queen mandibular pheromone production from the queen samples. Finally, we will use running average and detrended colony weight data from the period coinciding with our sampling of bees for gut microbial analysis to determine if colony growth and forager activity correlates with gut microbial community composition. As this synthesis is a novel approach, we will explore further analysis options as well. While the ability to determine if cover crop affects honey bee nutrition, health, queen quality, and gut microbes by themselves is a powerful approach, this synthesis will allow us to further determine if gut microbes underlie significant effects of cover crop treatments. If so, gut microbes may be the mechanism by which cover crops affect bee health. We will therefore be able to make recommendations of cover crop plantings that will maximize the beneficial properties of the honey bee gut microbiota. The proposed research may help secure low cost pollination services for future almond crops.

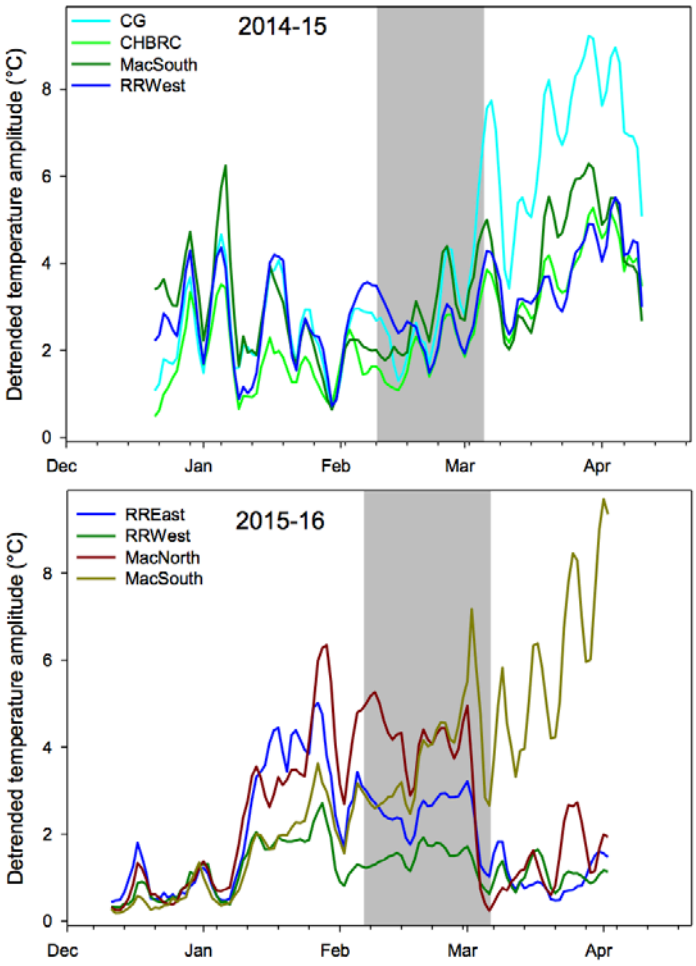

Figure 5. Temperature amplitudes refer to the within-day variability in internal hive temperature, and thus has been found inversely proportional to the presence of brood (high amplitudes: little or no brood; low amplitudes: usually brood present) and to the size of the adult bee mass in winter. The low amplitudes in the 2015-16 data in December and the beginning of January correspond well with the observed higher hive internal temperatures; both those measures indicate the presence of some brood. This was not the case in 2014-15. However, little brood was produced just before or during almond pollination. After pollination, brood production increased (and weaker hives died), particularly in the Red Rock treatment groups.

Results and Discussion:

The presence or absence of mid-winter (January) forage had an unexpectedly severe, but delayed, impact on colony survivorship **(Figures 1-4)**. Significantly fewer no-forage

colonies than forage colonies survived to the post-almond evaluation timepoint (April 4-8th, 2016, **Figure 1**). Among colonies pooled by treatment, 3 no-forage colonies survived and 13 no-forage colonies failed while 13 forage colonies survived and 3 forage colonies failed (X2=10.125, Chi-square analysis with Yates correction, p=0.001). Poor overwintering forage impacted colony survivorship in a delayed fashion; notably, no-forage colonies failed during and after almond pollination (February, March, and early April) rather than immediately after the overwintering forage period (end of January).

A relatively small difference in forage availability during the winter therefore had a large effect on colony survivorship. That is, the treatment effect was severe despite the modest duration (1 month) and differences (approximately 2.5x in sentinel colony) in incoming corbicular pollen. Critically, the difference in incoming corbicular pollen was not absolute due to the presence of additional winter weeds around the fields (forage treatment colonies 106.6 ± 13.7 SE g, no-forage treatment colonies 41.8 ± 12.4 SE g corbicular pollen trapped in sentinel colonies, mostly mustards and composites) nor was the amount of pollen collected in the pollen traps sufficient to completely feed a spring colony. Apparently, the difference was enough to impact colony survivorship and may point at the likelihood that colonies only need a small amount of pollen to enhance production in the early spring.

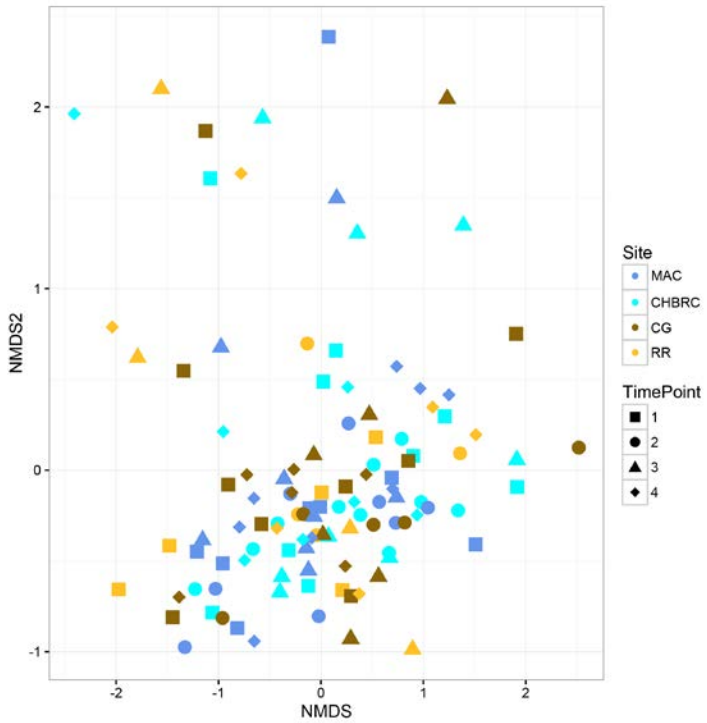

Figure 6. NMDS ordination of honey bee worker gut microbial communities (2014-2015). There is no readily apparent clustering by site, indicating that Rapini treatment and geographic variation in overwintering sites did not affect microbiome structure. Microbiome structure does differ by time point ($Adonis_{df=1,110}$, P = 0.046), although clustering in the NMDS plots are not completely obvious. Time point 2 samples, which are from the almond pollination time point, have lower values on NMDS2, likely driving the significant differences.

<u>Objective 1: Develop methods to exploit data from continuous monitoring of bee colonies in the field</u>

For statistical analysis, data points every 5 days were used (amplitude values were calculated on 72-hour datasets so data 5 days apart are independent). Prior to almond pollination, “plot” (i.e. RREast, RRWest, MacNorth or MacSouth) had a significant effect on amplitude ($F_{3,355}$=2.75, P=0.0425) but no post hoc contrasts were significant. During pollination “plot” had a much stronger effect ($F_{3,144}$=12.99, P<0.0001) and post hoc contrasts showed that RRWest was different from MacNorth and MacSouth (P<0.0001 for both) as well as RREast (P=0.0032). After almond pollination, “plot” was again significant ($F_{3,86}$=19.73, P<0.0001) and all pairwise comparisons were significant, except between RREast and MacNorth.

<u>Objective 2: Effect of cover crops on honey bee nutrition, health and queen quality.</u> We are currently processing workers and food materials to quantify nutrient reserves in

bees and the nutrient quality of food materials. We will characterize queen pheromone emissions from queens once the dissection of queens is completed for Objective 3. We will complete approximately 4,200 chemical analyses of the workers, queens, queen retinues, and food materials as well as frame analysis of colony productivity by October 31, 2016.

Objective 3: Effects of cover crops on gut microbiota

We have dissected and extracted DNA from all bees from 2015-16, but we are still in the process of library preparation. We expect to have all analyses done on the 2015-2016 samples by December 2016, in time for the Almond Conference.

We have finished all analyses for the 2014-2015 data. During this first field season, the forage treatments unfortunately failed to work, as the colonies placed on the no-forage treatment plots had lots of pollen and nectar coming in from an unidentified source. The results are shown in the top panel of **Figure 5, Figure 6** and last year's report. The contaminant forage also resulted in only subtle differences in the microbiomes of the different colonies (**Figures 6,7**). Unsurprisingly, Rapini treatment did not affect microbiome structure ($Adonis_{df=1,110}$, P = 0.64). The location at which each colony was overwintered was also not statistically significant ($Adonis_{df=3,110}$, P = 0.74). Time point, however, was significant ($Adonis_{df=1,110}$, P = 0.046). While the effect of time point was not large, there were detectable patterns in the abundances of specific bacteria during different timepoints (**Figure 7**). For example, *Bartonella apis* - one of the core gut microbes, was particularly abundant in 9 colonies during the almond pollination, but not at opther time points. *Bartonella apis* ferments glucose, arabinose, and xylose (Kešnerová *et al.* 2016), and may be important in carbohydrate utilization in the honey bee gut. Two different Acetobacteraceae bacteria (*Parasaccharibacter apis* and alpha 2.1) were also abundant in 6 colonies during the first winter sampling point. *Parasaccharibacter apis* has been shown to increase honey bee larval survival (Corby-Harris *et al.* 2014), and may be important in spring build up for almond pollination.

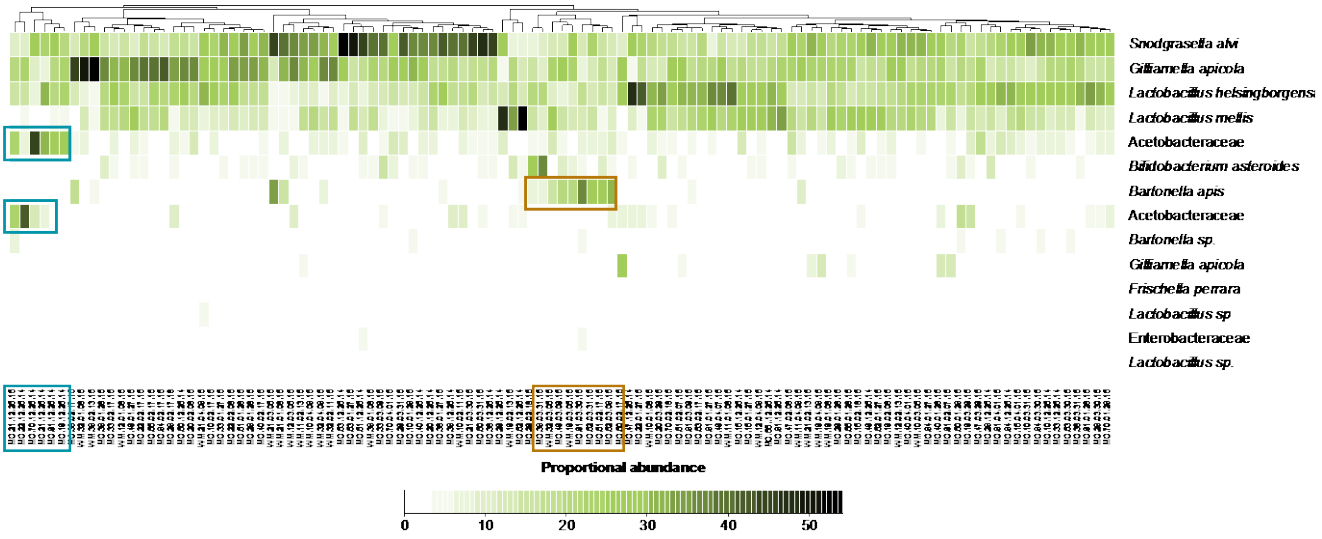

Figure 7. Heatmap of all 2014-2015 honey bee gut microbiome samples. Each column represents a colony, and each row represents one of the gut bacteria. Shade of the cell represents proportional abundance as indicated in the legend. The samples outlined in blue are enriched in *Parasaccharibacter apium* and Alpha 2.1 during the winter. The samples in brown are enriched in *Bartonella apis* during the almond pollination.

Research Effort Recent Publications:

None yet to report.

References Cited:

Aliferis KA, Copley T, Jabaji S (2012) Gas chromatography-mass spectrometry metabolite profiling of worker honey bee (*Apis mellifera* L.) hemolymph for the study of Nosema ceranae infection. *Journal of Insect Physiology*, **58**, 1349–1359.

Amenta JS (1970) A rapid extraction and quantification of total lipids and lipid fractions in blood and feces. *Clinical Chemistry*, **16**, 339–346.

Corby-Harris V, Snyder LA, Schwan MR *et al.* (2014) Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov. *Applied and Environmental Microbiology*, **80**, 7460–7472.

Crailsheim K, SCHNEIDER L, HRASSNIGG N *et al.* (1992) Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*) - Dependence on individual age and function. *Journal of Insect Physiology*, **38**, 409–419.

Engel P, James RR, Koga R *et al.* (2013a) Standard methods for research on *Apis mellifera* gut symbionts. *Journal of Apicultural Research*, **52**, 1–24.

Engel P, Kwong WK, Moran NA (2013b) *Frischella perrara* gen. nov., sp. nov., a gammaproteobacterium isolated from the gut of the honey bee, *Apis mellifera*. *International Journal of Systematic and Evolutionary Microbiology*, **63**, 3646–3651.

Engel P, Martinson VG, Moran NA (2012) Functional diversity within the simple gut microbiota of the honey bee. *Proceedings Of The National Academy Of Sciences*, **109**, 11002–11007.

Folch J, Lees M, Stanley G (1957) A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, **226**, 497–509.

Forsgren E, Olofsson TC, Vásquez A, Fries I (2010) Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. *Apidologie*, **41**, 99–108.

Hamady M, Lozupone C (2009) Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *The ISME Journal*, **4**, 17–27.

Johnson RM, Mao W, Pollock HS *et al.* (2012) Ecologically Appropriate Xenobiotics Induce Cytochrome P450s in Apis mellifera. *PLoS ONE*, **7**, e31051–e31051.

Kaspar H, Dettmer K, Gronwald W, Oefner PJ (2008) Automated GC-MS analysis of free amino acids in biological fluids. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, **870**, 222–232.

Keeling CI, Slessor KN, Higo HA, Winston ML (2003) New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. *Proceedings Of The National Academy Of Sciences*, **100**, 4486–4491.

Keller I, Fluri P, Imdorf A (2005) Pollen nutrition and colony development in honey bees: part I. *Bee World*, **86**, 3–10.

Kešnerová L, Moritz R, Engel P (2016) *Bartonella apis* sp. nov., a honey bee gut symbiont of the class Alphaproteobacteria. *International Journal of Systematic and Evolutionary Microbiology*, **66**, 414–421.

Koch H, Schmid-Hempel P (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings Of The National Academy Of Sciences*, **108**, 19288–19292.

Koch H, Schmid-Hempel P (2012) Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host-parasite system. *Ecology Letters*, **15**, 1095–1103.
Kostarelou-Damianidou M, Thrasyvoulou A, Tselios D, Bladenopoulos K (2015) Brood and honey production of honey bee colonies requeened at various frequencies. *Journal of Apicultural Research*, **34**, 9–14.
Kuczynski JJ, Stombaugh JJ, Walters WAW *et al.* (2011) Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Current Protocols in Bioinformatics*, **Chapter 10**, Unit10.7.
Kwong WK, Moran NA (2013) Cultivation and characterization of the gut symbionts of honey bees and bumble bees: *Snodgrassella alvi* gen. nov., sp. nov., a member of the Neisseriaceae family of the Betaproteobacteria; and *Gilliamella apicola* gen. nov., sp. nov., a member of Orbaceae fam. nov., Orbales ord. nov., a sister taxon to the Enterobacteriales order of the Gammaproteobacteria. *International Journal of Systematic and Evolutionary Microbiology*, **63**, 2008–2018.
Lebiedzinska A, Marszall ML, Kuta J, Szefer P (2007) Reversed-phase high-performance liquid chromatography method with coulometric electrochemical and ultraviolet detection for the quantification of vitamins B-1 (thiamine), B-6 (pyridoxamine, pyridoxal and pyridoxine) and B-12 in animal and plant foods. *Journal of Chromatography A*, **1173**, 71–80.
Mao W, Schuler MA, Berenbaum MR (2013) Honey constituents up-regulate detoxification and immunity genes in the western honey bee Apis mellifera. *Proceedings Of The National Academy Of Sciences*, **110**, 8842–8846.
Martinson VG, Danforth BN, Minckley RL *et al.* (2011) A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology*, **20**, 619–628.
Martinson VG, Moy J, Moran NA (2012) Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and Environmental Microbiology*, **78**, 2830–2840.
McFrederick QS, Cannone JJ, Gutell RR *et al.* (2013) Specificity between lactobacilli and hymenopteran hosts is the exception rather than the rule. *Applied and Environmental Microbiology*, **79**, 1803–1812.
McFrederick QS, Wcislo WT, Taylor DR *et al.* (2012) Environment or kin: whence do bees obtain acidophilic bacteria? *Molecular Ecology*, **21**, 1754–1768.
McFrederick Q, Mueller U, James RR (2014) Interactions between fungi and bacteria influence microbial community structure in the *Megachile rotundata* larval gut. *Proceedings Of The Royal Society B-Biological Sciences*, **In Press**.
Moran NA, Hansen AK, Powell JE, Sabree ZL (2012) Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS ONE*, **7**, e36393.
Nino EL, Malka O, Hefetz A *et al.* (2012) Effects of honey bee (*Apis mellifera* L.) queen insemination volume on worker behavior and physiology. *Journal of Insect Physiology*, **58**, 1082–1089.
Oksanen J, Blanchet FG, Kindt R *et al.* vegan: community ecology package.
Oldroyd BP (2007) What's Killing American Honey Bees? *PLoS Biology*, **5**, e168.
Oliver R (2012) 2012 Almond Pollination Update. *American Bee Journal*, **152**, 315–322.
Olofsson TC, Alsterfjord M, Nilson B, Butler É, Vásquez A (2014) *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov., *Lactobacillus melliventris* sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kullabergensis* sp. nov., isolated from the honey stomach of the honeybee

Apis mellifera. *International Journal of Systematic and Evolutionary Microbiology*, **64**, 3109–3119.
Otter DE (2012) Standardised methods for amino acid analysis of food. *British Journal of Nutrition*, **108**, S230–S237.
Ramette A (2007) Multivariate analyses in microbial ecology. *FEMS Microbiology Ecology*, **62**, 142–160.
Sagili RR, Pankiw T (2007) Effects of protein-constrained brood food on honey bee (*Apis mellifera* L.) pollen foraging and colony growth. *Behavioral Ecology And Sociobiology*, **61**, 1471–1478.
Sheesley B, Poduska B (1968) Supplemental feeding of honey bees. *California Agriculture*, **22**, 2–4.
Sheesley B, Poduska B (1970) Strong honeybee colonies prove value in almond pollination. *California Agriculture*, **24**, 4–&.
Slavin M, Yu LL (2012) A single extraction and HPLC procedure for simultaneous analysis of phytosterols, tocopherols and lutein in soybeans. *Food chemistry*, **135**, 2789–2795.
Slessor KN, Winston ML, Le Conte Y (2005) Pheromone communication in the honeybee (*Apis mellifera* L.). *Journal of Chemical Ecology*, **31**, 2731–2745.
Turnbaugh PJ, Ridaura VK, Faith JJ *et al.* (2009) The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science translational medicine*, **1**, 6ra14.
Turnbaugh P, Hamady M, Yatsunenko T (2008) A core gut microbiome in obese and lean twins. *Nature*.
USDA (2012) *Report on the National Stakeholders Conference on Honey Bee Health*. USDA.
USDA (2013) *Noncitrus Fruits and Nuts*. USDA.
Vásquez A, Forsgren E, Fries I *et al.* (2012) Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. *PLoS ONE*, **7**, e33188.
Wheeler MM, Robinson GE (2014) Diet-dependent gene expression in honey bees: honey vs. sucrose or high fructose corn syrup. *Scientific Reports*, **4**.