Project Report – January 2018
Understanding the effects of nutrition and juvenile hormone on reproductive output in alkali bees (Nomia melanderi)
and
Characterizing microbial associates of alkali bees

Principal Investigator
Karen M. Kapheim, Department of Biology, Utah State University, Logan, UT

Introduction
Health and reproduction are critical aspects of native pollinator biology, because the number of pollinators available each year is limited by the survival and number of offspring produced the previous year. Furthermore, pollination by bees is mostly the result of female bees collecting nectar and pollen to provision their developing offspring. Understanding the factors that influence bee health and reproductive output, as well as the dietary needs of female bees during different stages of their reproductive cycle is thus a crucial element of maintaining pollinator populations.

Alkali bees (Nomia melanderi) are important native pollinators of alfalfa (Medicago sativa), and thus are a valuable resource for alfalfa seed growers. I have proposed experiments to investigate the reproductive biology of female alkali bees, and to survey the bacterial and fungal associates of alkali bees throughout their life cycle. I completed two field seasons in Touchet Valley, WA in 2015 and 2016. I have shared some of the results from these field seasons in previous years’ reports. This 2018 report includes only new results stemming from continued analysis of these data. In the case where these results are published, I have sent the publication to the WASGA to keep on file.

Research Objectives
1. Identify hormonal and nutritional factors that influence variation in reproductive potential in female alkali bees

   Introduction
   Variation in nutrition and hormone cycling in the early stages of adulthood influence reproductive activity in other bees, but how these factors influence reproductive maturity in alkali bees is unknown (Kapheim, 2017). We investigated the nutritional and endocrine requirements for reproductive maturation in young adult female bees by experimentally manipulating each of these factors in the lab and observing their effects on reproductive physiology.

   Methods
   We tested the effects of juvenile hormone (JH) and nutrition on reproductive development among newly emerged alkali bee females. JH has many functions in insects, including development of reproductive organs (i.e., testes, ovaries) and oogenesis (i.e., egg development) (Nijhout, 1994). We focused newly emerged females for two reasons. First, this is an especially sensitive time for reproductive development among bees. Females of a bee species related to alkali bees emerge with undeveloped ovaries, and protein consumption is required for their eggs to develop (Kapheim, 2017, Kapheim et al., 2012). Other bee species emerge from development with very low levels of endogenous JH, which then increase during the course of ovary development (Bloch et al., 1996, Robinson et al., 1991, Smith et al., 2013). Second, this is a practical way to standardize age among experimental bees. It is also a phase of the life cycle that can be easily recognized by seed growers, should practical applications arise from this research.
Newly emerged females were collected from emergence traps that had been placed over undisturbed surfaces by gently coaxing them into 15 ml conical tubes, and the tube was placed in a cooler with an ice pack for transport to the laboratory. Upon returning to the lab, we randomly assigned each bee to a treatment group. We conducted two different experiments. The first experiment tested the nutritional needs of female reproductive maturation and the second tested the effects of JH and influences from other bees on reproductive maturation.

**Experiment 1 treatment groups:**

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Age at collection (days)</th>
<th>Diet in the lab</th>
<th>Field or lab?</th>
</tr>
</thead>
<tbody>
<tr>
<td>New females</td>
<td>1</td>
<td>n/a</td>
<td>Field</td>
</tr>
<tr>
<td>Reproductive, nesting females</td>
<td>Unknown</td>
<td>n/a</td>
<td>Field</td>
</tr>
<tr>
<td>Sugar only</td>
<td>10</td>
<td>Carbohydrates only</td>
<td>Lab</td>
</tr>
<tr>
<td>Sugar + pollen</td>
<td>10</td>
<td>Carbohydrates + protein</td>
<td>Lab</td>
</tr>
<tr>
<td>Sugar + pollen + fresh alfalfa flowers</td>
<td>10</td>
<td>Carbohydrates + protein + environmental vitamins/minerals</td>
<td>Lab</td>
</tr>
</tbody>
</table>

**Experiment 2 treatment groups:**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Rearing environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μg JH-III</td>
<td>Alone</td>
</tr>
<tr>
<td>Solvent control</td>
<td>Alone</td>
</tr>
<tr>
<td>Handling control</td>
<td>Alone</td>
</tr>
<tr>
<td>50 μg JH-III</td>
<td>With an older, reproductive female</td>
</tr>
<tr>
<td>Solvent control</td>
<td>With an older, reproductive female</td>
</tr>
<tr>
<td>Handling control</td>
<td>With an older, reproductive female</td>
</tr>
</tbody>
</table>

All lab-reared bees were housed in cylindrical cages for 10 days at the Forage Genetics facility in Touchet, WA. Cages were kept at 22-28°C, 40-85% RH, and 13 h light:11 h dark with full spectrum lighting. Bees in all groups except ‘sugar water only’ were fed a mixture of sterilized sucrose and pollen: 30 ml of 35% (w/v) sucrose mixed with 2.5 g of finely ground honey bee pollen (Betterbee, Greenwich, NY, USA), and food was replaced daily. Bees in the ‘sugar water only’ group received sterile 35% (w/v) sugar solution. When bees reached 10 d of age, they were chilled at 4°C for 5 min, placed in individually-labeled tubes, and flash-frozen in liquid nitrogen. Samples were stored in liquid nitrogen until return to Utah State University, where they were transferred to a -80°C freezer. The 10 day period provides enough time for ovary development, as alkali bees typically lay their first egg on the third day after emergence (Bohart & Cross, 1955).

For JH treatments, JH-III (product E589400, Toronto Research Chemicals, Inc., Toronto, ON, Canada) was dissolved in dimethylformamide (DMF) (Fisher Scientific, Fair Lawn, NJ, USA) at a concentration of 50 μg μl⁻¹. This dose was chosen based on our previous research with alkali bees (Kapheim & Johnson, 2017b). We applied the treatments with a pipette tip to the thorax of
each bee while they were secured in their harnesses. Bees in the JH and solvent groups received 1 μl of JH-III dissolved in DMF or DMF only, respectively. Bees in the all other groups were touched lightly with a clean pipette tip. The treatment procedure was repeated 5 d later.

We also collected newly emerged females and actively nesting females to compare reproductive development in the lab to reproductive development when females emerge from over-wintering and when they are reproductively mature. All bees were collected at the same time of day. These samples were flash-frozen immediately upon collection.

Upon returning to USU, we performed abdominal dissections of each bee to measure oocyte maturation and Dufour’s gland size as a function of nutrition and hormone treatments. One of the primary functions of the Dufour’s gland in alkali bees is to secrete chemicals (Batra, 1972). It is connected to the sting, and thus plays a lubricating role in egg-laying. Most importantly, ground-nesting bees line the inside of their cells with secretions from this gland, and it thus serves a prominent reproductive role.

For additional methodological detail, please see Kapheim & Johnson (2017a).

**Results and Discussion**

Adult female alkali bees can rapidly develop eggs upon emergence, even without mating. Most newly emerged bees had very small oocytes, at early stages of development (stages I-II), but a few had mature oocytes (stage V) in both ovaries, similar to those of actively nesting females (Fig. 1A). All of the actively nesting females we collected had oocytes at the later stages of maturation (stages III-V), and most (70%) had at least one resorbing oocyte. Egg resorption occurs when mature oocytes degenerate if there is no opportunity to oviposit available. This may mean that alkali bee reproduction is more limited by the time and energy required for nesting building and brood provisioning (e.g., floral resource availability, foraging effort) than by the time and energy required for completion of oogenesis.

![Fig. 1. Effects of diet on oocyte maturation in alkali bees. Newly emerged females had oocytes at significantly earlier stages of maturation than females in the other treatment groups (ordinal logistic regression: Z=−4.61, P=3.99×10^-6; number of individual bees: newly emerged n=26, sugar n=11, sugar+pollen n=20, sugar+pollen+alfalfa n=10, reproductive n=7). Shading within bars indicates the proportion of maximum viable terminal oocytes in each stage of maturation, with stage I and II as pre-vitellogenic, III and IV as vitellogenic, and V as mature.](image)

The initial stages of egg development in adult female alkali bees does not require a protein source. Females reared in the lab for 10 days had significantly longer oocytes and Dufour’s glands than newly emerged females, but there were no significant differences in these metrics among females reared on different diets (Fig. 2). However, actively nesting females had
significantly more developed anatomy than either newly emerged or lab-reared females (Fig. 2). All but one of the lab-reared females and actively nesting females had oocytes in the later stages of maturation (stages III-V), and there were no significant differences in the egg stage among these groups (Fig. 1). However, none of the lab-reared bees in experiment 1 had a completely mature oocyte (stage V) (Fig. 1). Approximately half of the lab-reared females had at least one resorbing oocyte, and these were mostly in stage IV. Together, these results indicate that alkali bees do not require a source of protein for the initial stages of egg production. They likely use nutritional reserves carried over from development as an energy source for oogenesis. However, the completion of reproductive maturation may be hastened by ecological cues, such as nesting substrate or access to mates.

Fig. 2. Effects of diet on reproductive maturation in alkali bees. (A) Maximum viable terminal oocyte length and (B) Dufour’s gland length were significantly different between newly emerged females, 10 day old lab-reared females and nesting females (oocytes: $F_{4,89}=30.68$, $r^2=0.58$, $P<4.79\times10^{-16}$; Dufour’s gland: $F_{4,101}=45.80$, $r^2=0.64$, $P<2.20\times10^{-16}$; number of individual bees: newly emerged $n=36$, sugar $n=14$, sugar+pollen $n=22$, sugar+pollen+alfalfa $n=14$, reproductive $n=20$). Diet did not have a significant effect on reproductive development when newly emerged females were reared in the lab for 10 days. Boxes represent the interquartile range, with the line as the median. Whiskers extend to 1.5 times the interquartile range. Circles are outliers. Letters indicate significant differences ($P<0.001$ in Tukey post hoc tests).

JH has a positive effect on reproductive physiology in young, non-reproductive female alkali bees. Females treated with JH were the only lab-reared females that developed completely mature oocytes (stage V) (Fig. 3). Females treated with JH also had significantly longer oocytes and Dufour’s glands than the control females (Fig. 4). However, reproductive maturation was not affected by whether females were reared in the same cage as another bee or not (Fig. 4).
Fig. 3. Effects of JH on oocyte maturation in alkali bees. There were significant differences in stage of oocyte maturation among JH treatment groups ($\chi^2=23.20$, $P=7.31\times10^{-4}$, number of individual bees: sham n=16, DMF n=17, JH n=7). Shading within bars indicates the proportion of maximum viable terminal oocytes in each stage of maturation, with stage I and II as previtellogenic, III and IV as vitellogenic, and V as mature.

Together, these results demonstrate that alkali bees do not require dietary protein during the initial stages of reproductive maturation, but that JH enhances this process. If ecological cues, such as nesting or mating opportunities, impose reproductive limitations on alkali bees, this can apparently be overridden by JH treatment. In this way, alkali bees seem to differ from another important alfalfa pollinator, *Megachile rotundata*, which required dietary protein to become reproductively active (Richards, 1994). This disparity may reflect differences in nutritional stores leftover from development, as *M. rotundata* overwinter as adults and *N. melanderi* overwinter as pre-pupae.

Fig. 4. Effects of endocrine and social treatments on reproductive development in alkali bees. (A) Maximum terminal oocyte length and (B) Dufour’s gland length were significantly different between lab-reared females treated with juvenile hormone (JH) versus controls, but variation in the social environment did not significantly affect reproductive development (oocytes: $F_{5,41}=6.68$, $r^2=0.45$, $P=1.23\times10^{-4}$; Dufour’s gland: $F_{6,57}=8.77$, $r^2=0.48$, $P=8.97\times10^{-7}$); number of individual bees: sham+solitary n=12, sham+social n=12, dimethylformamide (DMF)+solitary n=12; DMF+social n=10; JH+solitary n=10; JH+social n=8). Boxes represent the interquartile range, with the line as the median. Whiskers extend to 1.5 times the interquartile range. Circles are outliers. Letters indicate significant differences ($P<0.05$ in Tukey post hoc tests). Gray bars, social treatment; white bars, solitary treatment.
Unlike for some other bees (Kapheim, 2017), cues from the social environment do not affect the physiological response to JH. This means that high-density nesting is unlikely to negatively impact reproductive maturation, though it could have consequences for the rate of nest building, which seems to be a limiting factor in egg-laying rate.

2. Characterize microbial associates of alkali bees

Introduction

Alkali bees have many microbial enemies which can lead to disease or spoil pollen provisions in the nest (Batra et al., 1973, Batra & Bohart, 1969, Johansen et al., 1982), but bees also associate with many beneficial microorganisms (Vasquez et al., 2012). Some bacteria found in bee nests function to prevent food spoilage, including pollen balls provided to larvae (Anderson et al., 2014, Mcfrederick et al., 2012). Other bacteria found in the guts of honey bees and bumble bees have been shown to decrease infection rates when exposed to pathogens or parasites (Forsgren et al., 2010, Koch & Schmid-Hempel, 2011, Vasquez et al., 2012).

Advances in DNA sequencing technology have provided a means for studying the entire community of bacterial and fungal microorganisms in a given environment, without the need for culturing-based techniques. This technology was first used to characterize the microbial community associated with honey bees (Cox-Foster et al., 2007, Martinson et al., 2011), and was a major breakthrough in understanding the role of microorganisms in bee health. A potential outcome of characterizing the microbial communities associated with bees is the development of probiotic treatments to improve the health of commercially managed species. However, administering probiotics that are not endogenously associated with the bee can cause weakened immune systems and increase mortality (Ptaszyńska et al., 2016). A first step toward developing probiotic treatments is thus identifying the microbes that naturally associate with each species. Nesting in the ground may expose alkali bees to a unique set of microbes that have not been previously characterized in other bees (none of whom are ground-nesting), and this may be an important source of microbial acquisition and function.

I characterized the diversity and relative abundance of bacterial associates of alkali bees throughout their life cycle to determine the how these microbes are acquired and maintained. This work will establish a foundation upon which future studies of beneficial and harmful microbes can be manipulated to investigate impact on alkali bee health.

Methods

We collected samples for this project from four bee beds in Touchet, WA in June 2016. Nest samples included brood cell walls from nests, pollen balls, eggs, small larvae, and pre-pupae. I also used the guts from the bees in experiment 1 of the study above (i.e., newly emerged females, actively nesting females, lab-reared females on sterile diets of sugar water or sugar water and pollen). All samples were flash frozen upon collection.

We isolated bacterial DNA from each sample using MBio Powersoil DNA Isolation kits. DNA was sent to the University of Illinois Roy J. Carver Biotechnology Center for PCR amplification of the V4 hypervariable region of the 16S rRNA gene, using established primers (Caporaso et al., 2012). Amplicons were then sequenced on an Illumina MiSeq V2 to obtain paired-end, 250 bp reads. Sequence differences in this gene can be used to distinguish operational taxonomic units (OTUs), which are pragmatic proxies for microbial species.

After quality filtering, we identified OTUs based on 97% similarity and aligned them to the SILVA v128 database, using the ‘pick_open_reference_otus.py’ script within the QIIME v1.9.1 environment (Caporaso et al., 2010). We excluded OTUs with only a single read, OTUs
identified as mitochondria or chloroplast, and OTUs which were present in our control samples (i.e., no template included in the DNA isolation and PCR steps). We further filtered the data to exclude OTUs that were not observed more than 10 times in at least 2% of the samples. We also excluded samples with fewer than 100 reads. We then used the frequency of each OTU in each sample for statistical comparisons across sample types and bee beds.

Results and Discussion
After filtering, we detected 2,071 taxa in 61 samples. There were no OTUs unique to the lab-reared females, which suggests that foreign bacteria were not introduced into bee guts as a function of lab-rearing.

There are significant differences in the microbial communities associated with alkali bees at different life stages. A PCoA ordination plot of the log-transformed OTU frequencies reveals that small, feeding larvae and pollen balls have more similar bacterial communities to each other than to brood cell walls and pre-pupae, which are also similar to one another (Fig. 5). The gut bacteria of newly-emerged females is more similar to that of lab-reared females and cell walls/pre-pupae than that of actively nesting females (Fig. 5). Multivariate statistical testing using the function adonis within the R package vegan revealed that both sample type and bee bed of origin were significant factors of variance in the community composition of bacteria associated with each sample (sample type: F = 4.57, p = 0.0001; bed: F = 2.40, p = 0.0001). Pairwise comparisons between sample types revealed highly significant differences between each bee bed and sample type, except lab-reared and newly-emerged females. This finding could be due, in part, to differences in variance within sample types or bee beds. To test this, we used the function betadisper and found significant differences among sample types (F = 2.50, p = 0.03) and bee beds (F = 2.86, p = 0.05) overall. Pairwise tests revealed marginally significant differences in variance between pollen balls and guts of lab-reared females (p = 0.05) and between bee beds A & D (p = 0.02). It is likely that the significant differences found among bee beds reflects the uneven sampling that occurred in each bee bed. For example, we did not collect any adult females from bed D. The dispersion estimates thus reveal that differences in variance are not the primary driver of differences observed in the microbiome profile of each sample type.

Together, these results suggest that major shifts of the microbiome occur with each life stage of alkali bees. Alkali bees, like other holometabolous insects, undergo a complete metamorphosis after the larval feeding stage is complete. This involves a clearing of the gut, which could also eliminate bacteria. Thus, the bee microbiome is similar to its food source during the developmental feeding stage (pollen balls and small larvae), but is more similar to that of its surrounding after feeding is complete and the gut has been cleared (cell walls and pre-pupae).

These results also suggest that adult alkali bees acquire a significant portion of their gut microbiomes from the environment. Females reared in the lab for 10 days on a sterile diet (sugar water or sugar water + pollen) had highly similar gut microbiomes to newly emerged bees, but both were significantly different from those of actively nesting females (posthoc, pairwise comparisons to nesting females: new - p = 0.015, lab - p = 0.002).
Alkali bee life stages are also accompanied by changes in the diversity of their associated microbial communities. The cell walls of brood cells have the highest diversity of all sample types, as measured by the Shannon index (Fig. 6). Pre-pupae and adult female guts have the lowest diversity of all life stages (Fig. 6). Nesting females had similar diversity levels to that of pollen balls and small larvae (Fig. 6). Bacteria that contribute to the increased diversity in cell walls belong to phyla that make up less than 2% of the phyla in bee samples (e.g., Gemmatimonadetes, Chloroflexi, Planctomycetes) (Fig. 7). We did not detect significant differences in alpha diversity among bee beds ($F = 2.20, \ p = 0.10$).
Fig. 6. Alpha diversity among sample types. Shannon index measures taxonomic diversity as a function of species richness and evenness (Anova: F = 0.45, p = 5.05x10^{-12}). Letters indicate significant differences (P<0.05 in Tukey post hoc tests). Boxes represent the interquartile range, with the line as the median. Whiskers extend to 1.5 times the interquartile range.

Some of the bacteria in the adult female guts are acquired from the environment. Groups of bacteria that increase in abundance the most after emergence, and which are not acquired when reared in the lab, include members of the families Weeksellaceae, Xanthomonadaceae, and Lactobacillaceae (Fig. 8). Xanthomonadaceae is a family of insect-vectored phytopathogens, including species known to infect alfalfa (Chatterjee et al., 2008). Lactobacillaceae are common in the guts of other bee species, and are typically acquired from the environment (McFrederick et al., 2013, McFrederick et al., 2012). Compared to nesting females, newly emerged and lab-reared females have significantly higher abundance of bacteria in the families Alcanivoracaceae, Enterobacteriaceae, Sphingobacteriaceae, Mycobacteriaceae, Rhodobacteraceae, Rhodospirillaceae, Dietziaceae, Moraxellaceae, and Pseudomonadaceae (Fig. 8). Bacteria from these families are likely outcompeted by environmentally acquired bacteria in the guts of reproductive females.
Fig. 7. Relative abundance of major phyla in each sample type. Only phyla comprising > 2% of the total microbiome are included.
Fig. 8. Differences in abundance of gut bacteria among actively newly emerged (left) or lab-reared females (right) and actively nesting females. Log2 fold change represents the relative abundance of each taxa in newly emerged or lab-reared females, as compared to nesting females. Points on the left side of the y-axis (<0) are more abundant in nesting females; points on the right side of the y-axis (>0) are more abundant in newly emerged or lab-reared females.

References


