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For The

2018 Winter Seed School Conference

January 28 - 30, 2018

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WESTERN ALFALFA SEED GROWERS
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PROGRAM
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Sunday, January 28
5:00 pm – 7:00 pm - Get Acquainted Reception – *Sponsored by – International Pollination Systems*

Monday, January 29
7:00 – **APRI Proposal Review Meeting**
7:00 am – Registration Desk Opens – Trade Show Ongoing
Session 1
8:30 – **Conference Kickoff –** Shane Johnson, Western Alfalfa Seed Growers Association
8:40 – **NAFA Update** – Beth Nelson, President, National Alfalfa and Forage Alliance (NAFA)
9:10 – **Seed Industry Export Hoops and Hurdles** – Chet Boruff, CEO, Association of Official Seed Certifying Agencies (AOSCA)
9:50 – **Break – Raffle Drawing** – *Coffee and Breaks Sponsored by – Northstar Seed, Leafcutters*

Session 2
10:10 – **Unmanned Aerial System (UAS)-Guided Releases of Predatory Mites for Management of Spider Mites in Strawberry** - Elvira Simone de Lange, University of California - Davis
10:50 – **DowDupont: How the Merger will affect the Seed Industry** – Ron Cornish, General Manager Alforex Seeds
11:20 – **Alfalfa Seed Production Survey** (US Growers Only)
12:20 – **Lunch & Raffle Drawing –** *Sponsored by – Alforex Seeds*

Session 3 – Research Session
1:40 – **Microbes & Alfalfa Leafcutting Bee Health** – Dr. Quinn McFrederick, University of California, Riverside
2:00 – **Enhancing and Protecting Populations of Alfalfa Seed Pollinators** – Dr. Walsh, Washington State University, Prosser, WA
2:20 – **Use of Traps to Monitor & Control Hymenopteran Pests of Megachile rotundata During Storage, & Incubation** – Dr. Pitts-Singer, USDA-ARS. Logan, UT
2:40 – **Revisiting Bomber Flies Control & Exploring Provision – Soil Moisture Relations** – Dr. Cane, USDA-ARS, Logan, UT
3:00 – **Factors Affecting Alkali Bee Reproduction and Microbial Associates** - Dr. Karen Kapheim, Utah State University, Logan, UT
3:20 – **Raffle Drawing/Announcements**
3:30 – **Presenter's Meet and Greet Social & Researchers Poster Panel –** *Sponsored by JWM Leafcutters*
5:00 – **WASGA Board of Directors Meeting**

Tuesday, January 30
Session 4
8:00 – **Welcome** – Shane Johnson, Western Alfalfa Seed Growers Association
8:10 – **Alfalfa Seed Production in Idaho – Video Presentation**
8:20 – Diana Cox-Foster, USDA-ARS, Logan, UT
8:40 **– Linking Pollinator Behavior to Gene Flow to Improve Coexistence –** Johanne Brunet, USDA-ARS VCRU, Madison, WI
9:00 – **MP3 Update and the Pesticide Registration Process** – Erik Johansen, Washington State Department of Agriculture
9:30 – **State of the Industry Report (USA & Canada)** – Robin Newell, S&W Seed
10:10 – **Break – Raffle Drawing** – *Coffee and Breaks Sponsored by – Northstar Seed, Leafcutters*
10:30 –**Succession Planning Workshop** – Corey Brock, Brock Law Firm and Todd King, Leffel Otis Warwick PS
12:00 – **Raffle Drawing/Announcements**
Conference adjournment

Optional Tour (pre-registration required)
1:30 – **Rebecca Creek Distillery Tour (Meet in Hotel Lobby to board Bus)**

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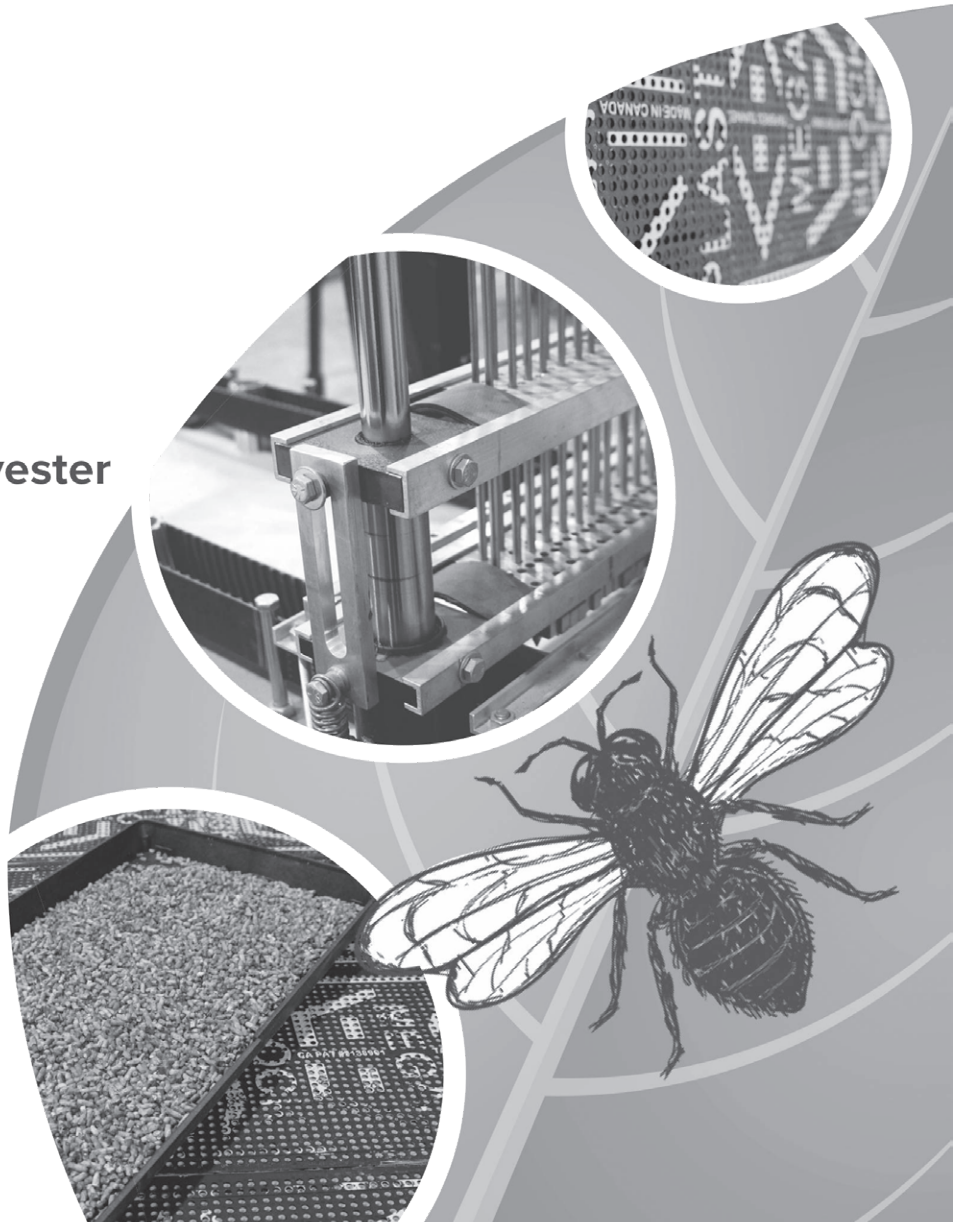

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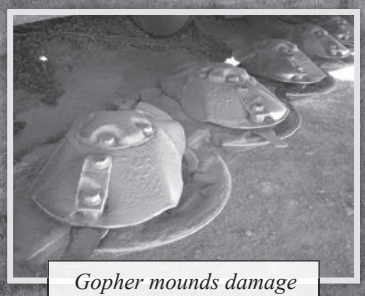

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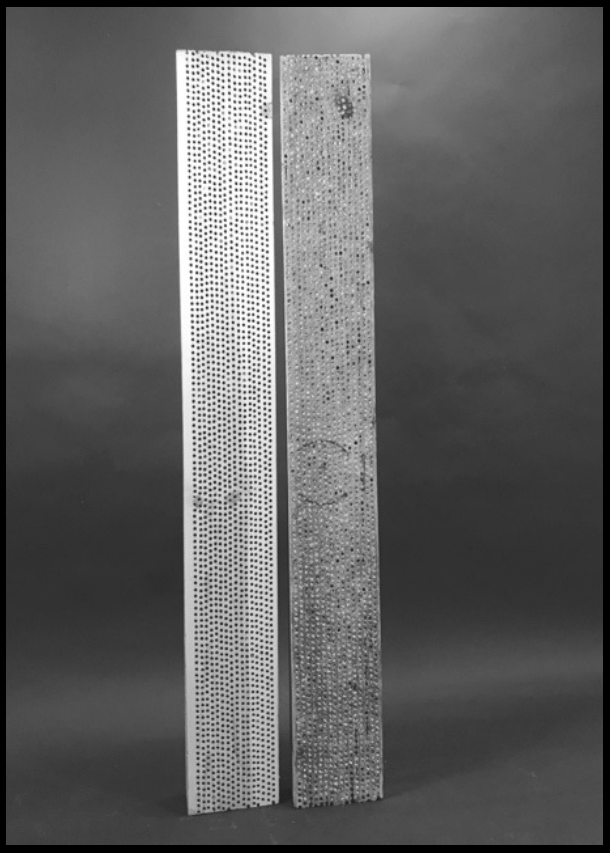

Project Title: Microbes and Alfalfa Leafcutting Bee Health
Lead Investigator / Affiliation: Quinn McFrederick, University of California, Riverside
Collaborating Investigator(s) / Affiliation(s): Hoang Vuong, UC Riverside; Theresa Pitts-Singer and Ellen Klinger, Logan Bee Lab
Hyperlink to research website and/or curriculum vitae: http://melittology.ucr.edu

Introduction and Justification:

Alfalfa Leafcutting Bees (ALCBs) are known to associate with various microbes (McFrederick et al. 2014). For example, we have found bacteria that are abundant in pollen provisions and the larval gut (McFrederick et al. 2017) and appear to be commensal, at least in regards to ALCB nutrition. In previous APRI funded research, we tested a strain of one of these commensal bacteria, which we then identified as *Lactobacillus kunkeei*, for inhibitory activity against *Ascosphaera aggregata*, the causative agent of the brood disease chalkbrood. We did not find any inhibitory activity of this bacterium, either on its own or when co-inoculated with other wild bacteria. Since this project, however, we have discovered that this bacterium is related to *L. kunkeei*, but is actually part of a complex of three closely related species that are all distinct from *L. kunkeei* (Fig. 1). We refer to these bacteria as the *L. micheneri* group, which includes *L. micheneri*, *L. timberlakei,* and *L. quenuiae*. Based on the contents of their genomes, these bacteria have very different possible functions. We therefore tested the functional capabilities of multiple strains of these bacteria.

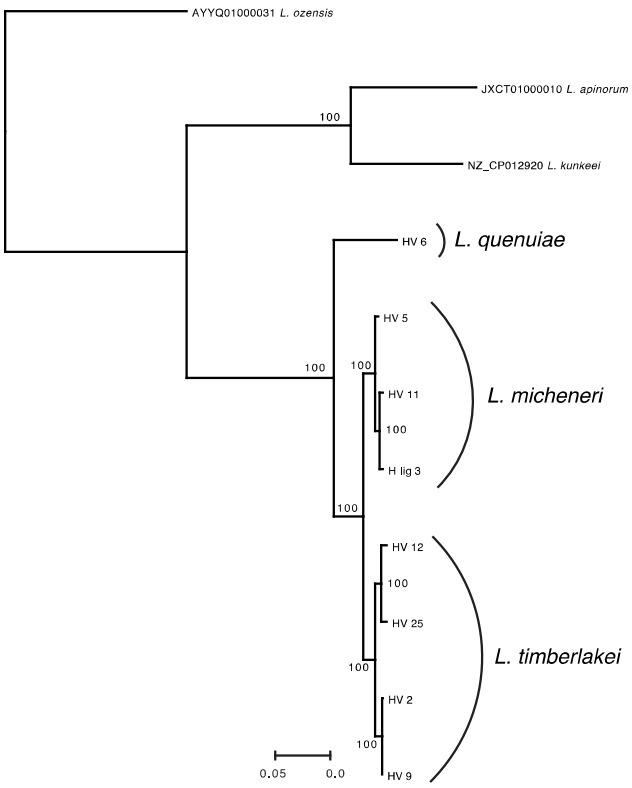

Fig. 1. Phylogenetic tree based on six protein coding, single copy genes. We have discovered 3 new species of bacteria that associate with megachilid bees including the ALCB.

Our goal here was to learn more about the possible function of these bacteria so that we can eventually leverage them to increase ALCB health. We used functional assays to determine if these bacteria exhibit antifungal properties that may either protect the pollen provision from spoilage or inhibit the growth of *Ascosphaera* pathogens found in the pollen provisions and larvae. The unexpected evolutionary diversity of these bacteria suggests that they may also have unexpected functional diversity.

Objective:

1) Assay various *Lactobacillus* species for fungal inhibitory properties, which could prevent food spoilage and/or protect the developing larvae inside the nest.

Methods:

Isolation of bacteria and fungi

To further explore the function of various *Lactobacillus* strains, we performed culture plate assays. To obtain pure cultures for these assays, we used 30 strains of lactobacilli that we had isolated from various bees (including ALCBs) using de Mann, Rogosa, and Sharp agar plates supplemented with 20% fructose (MRS + 20% F, (De Man et al. 1960; Endo et al. 2012). These bacteria are not host specific (McFrederick et al. 2017), so bacteria isolated from other bees are potential probiotics for the ALCB. To identify bacteria, we sequenced the 16S rRNA gene using universal bacterial primers (Lane 1991).

We isolated fungi from ALCB nests by plating pollen provisions onto potato dextrose agar and V8 agar plates. We tested 13 unique fungal isolates as well as *Ascosphaera* spores at two concentrations from 5 ALCB larval cadavers that had succumbed to chalkbrood disease. To identify fungi, we extracted DNA and sequenced the internal transcribed spacer using universal fungal primers (White et al. 1990).

Preparation of *Lactobacillus*-conditioned media and agar plug for challenge assays by mycelial plugs or fungal spores

We grew *Lactobacillus* in liquid MRS+2F (de Man, Rogosa, and Sharpe media with 2% fructose supplement) media for two days prior to plating or filter sterilization. We recorded an OD_{600} between 0.3 and 0.4 of the liquid cultures of the 30 strains of *L. micheneri* group bacteria. we filtered 700uL of the liquid culture for well diffusion assays and plated 100uL of the liquid culture on MRS+2F agar plates. We stored the culture filtrate at -20C for no longer than 3 weeks. To obtain plugs of MRS agar covered in *Lactobacillus* cells, we grew *Lactobacillus* on MRS+2F agar plates until a lawn formed. Once the agar plates were ready, we stored the plates in a 4 °C refrigerator for less than 3 weeks. We cultivated myceliated fungi for a week before boreing fungi. We only bored agar containing mycelia from the edges of the growth on the agar plate. We cultivated fungi on agar plates until sporulation and used a metal loop to transfer at least 200,000 spores to 2.0mL of PBS. To test each bacteria's ability to inhibit *Ascosphaera* growth, we spread a minimum of 600,000 spores to 2.0mL of PBS on V8 plates.

Mycelial plug application vs *Lactobacillus* inhibition assay (calculated in distance/day)

We filled wells in agar plates with conditioned media (100uL of MRS+2F in which we grew *Lactobacillus* but filter sterilized the media before using it in the experiment) and plugs (agar plugs of diameter 0.5 cm from plates after two days of growth) approximately 1 cm from the center fungal plug (n=9). We measured the distance from the center of the plate to each plug. We then recorded days until fungi overtook the well or plug. Using this distance and the number of days, we calculated fungal growth rate towards the bacterial plug or well of conditioned media. To calculate the efficacy of a bacterial strain's ability to inhibit fungal growth, we calculated the difference between fungal growth toward a treated plug or well and fungal growth toward a control (no bacteria) plug or well on the same plate.

Spore inoculation vs Lactobacillus inhibition assay (calculated in days)

To test whether lactobacilli strains can inhibit *Ascopsphaera* growth, we plated *Ascosphaera* spores on V8 plates and subsequently added either conditioned media or agar plugs containing live lactobacilli cultures to the plates. We spread 10,000 spores (n= 9) or 20,000 spores (n=5) in 100uL of PBS across the plate. We then recorded the diameter of the clearing halo as well as the number of days until the spores take over the plug or well. We then calculated *Ascosphaera* growth by taking the difference between the number of days until the *Lactobacillus* plug or well was overgrown by spores and the number of days until the control plug or well is taken over by the fungi spores.

Results

Mycelial plug application vs *Lactobacillus* inhibition assay

We found several strains that were able to inhibit the growth of various fungal mycelia. The strain with the broadest inhibitory ability was *Lactobacillus timberlakeei* strain HV12, which inhibited mycelial growth of 3 strains of two species of fungi: *Sordaria fimicola* strain 1 and *Fusarium solani* strains s2 and s5. Seven other strains inhibited a single fungal strain. These *L. micheneri* group species strains were HV95, HV10, HV97, HV25, HV28, HV66, and EH 38. Based on the average fungi inhibition in cm/day, we found that *Sordaria fimicola* (-0.103 cm /day) was the most inhibited fungi and *Ascosphaera proliperda* strain 1 (+0.009 cm/day) was the least inhibited fungi. Conditioned media which *Lactobacillus* had grown in but had been sterilized inhibited mycelia less relative to their respective live *Lactobacillus* plugs (Mean: -.006 cm/day and -.03 cm/day [p-value .021762] n=9, Fig. 3).

Spore inoculation vs Lactobacillus inhibition assay

Similar to the mycelia plug assays, *Lactobacillus timberlakeei* strain HV12 inhibited the most fungi out of all 30 strains in 3 spore application assays (*Aspergillus niger* strains s1, s2, and s3) and inhibited the most fungi on average across all assays. *Lactobacillus micheneri* strains HV21 and HV59 2 strains each. HV21 inhibited the growth of *A. niger* strain s3 and *Ascosphaera* spores from cadaver 1 while HV59 inhibited *Ascosphaera* spores from cadavers 4 and 5. Eight other strains inhibited single fungal strains. These *L. micheneri* group species strains were HV14, HV97, HV25, HV29, HV35, HV63, HV83, and EH 38. Based on the average number of days it took for the fungi to overtake a *Lactobacillus* agar plug, *Aspergillus niger* strain 2 on V8 modified media was the most inhibited fungi while *Aspergillus niger* strain 2 on Rose Bengal Dichloran media was the least inhibited fungi. When only comparing inhibition on fungi assayed on V8 modified media the treatment of 10,000 spores from chalkbrood cadaver 4 was the least inhibited fungi.

Lactobacilli did not significantly differ in their ability to inhibit 10,000 or 20,000 spores of fungi from chalkbrood cadavers (Mean: 2.75 days and 2.46 days, n = 4, P = 0.77). Similar to the mycelial plug assays, conditioned media did poorly in fungi spore inhibition assays compared to live lactobacilli (Mean: 3.183865 days and 0.57963 days. N = 9, P = 0.00204). This case is more significant when comparing plates inoculated with 10,000 spores with wells containing *Lactobacillus*-conditioned media and plugs of *Lactobacillus* media (Mean -0.0519 days and 2.392756 days [p-value .00058] n=4).

We also recorded the diameter of the fungi inhibition zone of each *Lactobacillus* strain at the time the fungi overtook the strain's corresponding control agar plug in each

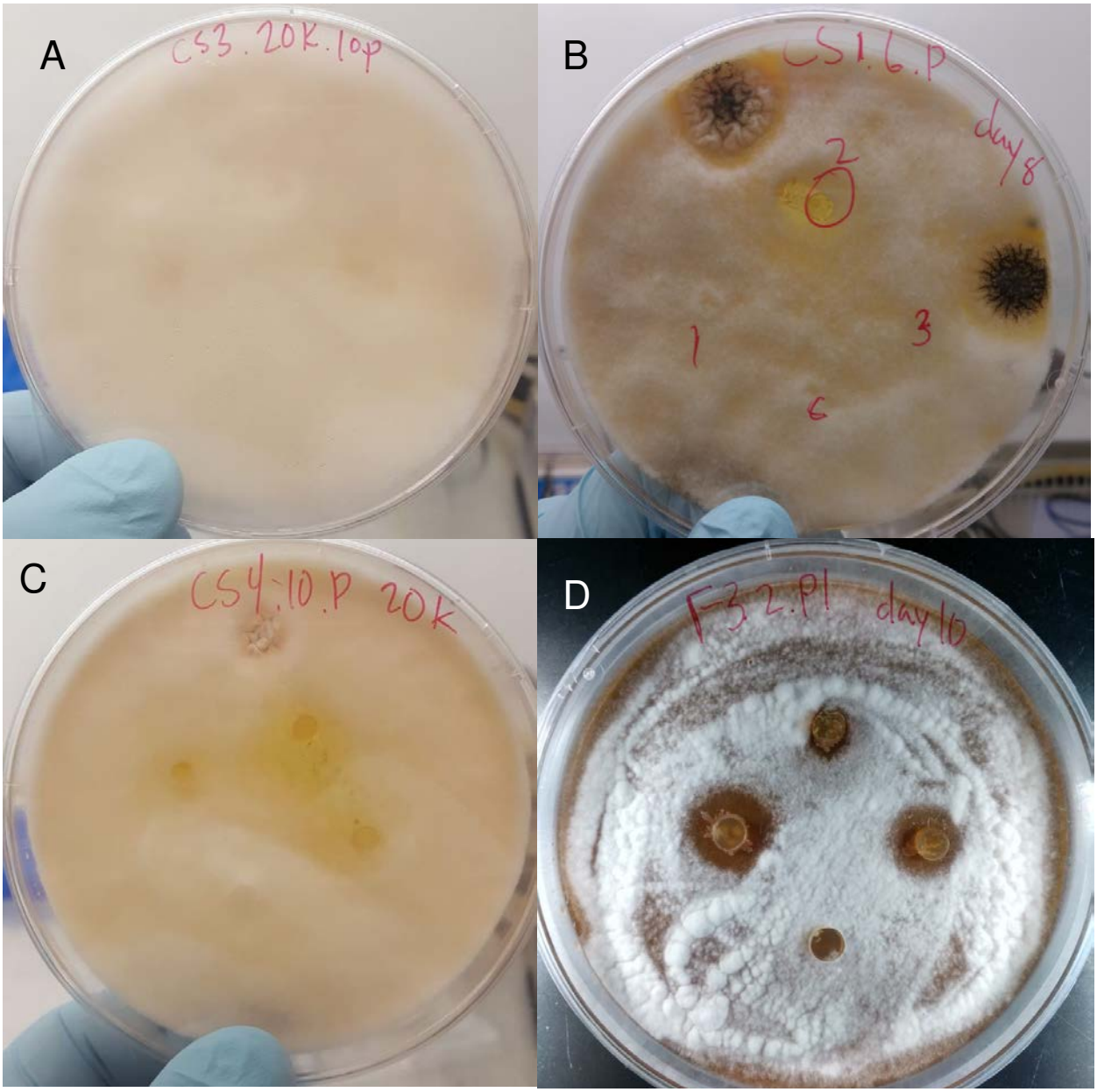

Fig. 2. Examples of no inhibition (A) versus inhibition of *Ascosphaera* by strains HV28 (B, circled plug), EH38 (C, top plug), and inhibition of *Fusarium solani* by strains HV4 (D, left).

assay. Like the mycelia plug assays, *Lactobacillus timberlakei* strain HV12 produced the largest fungi inhibition zone out of all 30 strains in 3 spore application assays and produced the largest fungi inhibition zone on average across all assays. *Lactobacillus micheneri* strains Hlig3 and HV59 and *Lactobacillus timberlakeei* strain HV4 produced the largest fungi inhibition zone in 2 assays each. Seven other strains inhibited single fungal strains. These *L. micheneri* group strains were HV14, HV95, HV11, HV3, HV21, HV83, and EH 38. Based on the average diameter, *Aspergillus niger* strain 2 on V8 modified

media was the most inhibited fungi while *Aspergillus niger* strain 2 on Rose Bengal Dichloran media was the least inhibited fungi. When only comparing inhibition on fungi assayed on V8 modified media the treatment of 20,000 spores from chalkbrood cadaver 5 was the least inhibited fungi.

Discussion

Nearly half (14) of the 30 *Lactobacillus micheneri* group strains that we studied inhibited the growth of one or more fungal strains. Interestingly, all of the 4 fungal genera that we studied were inhibited by one or more bacterial strains, but not every strain or species was inhibited by any strain. A couple of fungal strains were inhibited by 2 or more fungal strains, but most fungi were inhibited by only one bacterial strain. There is therefore variance in the efficacy of strains in inhibiting fungi as well as variance in the resistance of fungal strains to bacteria. The unexpected diversity of these bee-associated bacteria appears to result in functional diversity as well. The inhibitory strains may be useful 'probiotic' bacteria, i.e. bacteria that can be provided to the bees to improve the bees' health. As no one bacteria inhibited all fungi, however, cocktails of probiotic bacteria may be necessary to effectively inhibit fungi.

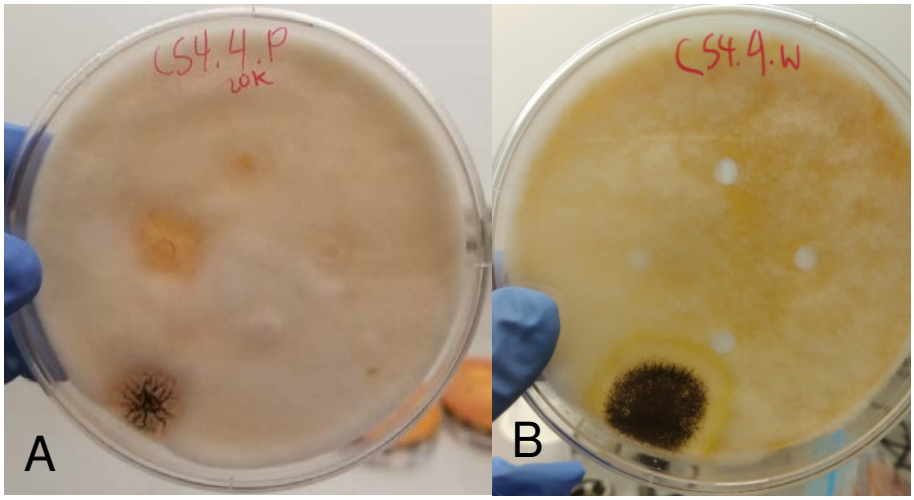

Fig. 3. Inhibition assays with live bacteria on agar plugs (A) versus conditioned media (B) which the same bacteria were grown in but was then sterilized. HV12 (left plug) inhibited *Ascosphaera* growth as live plugs (A) but not as conditioned media (B).

For several *Aspergillus niger* strains we performed inhibition assays with both mycelia and spores. Interestingly, only one bacterial strain could inhibit the growth of *A. niger* mycelia. Furthermore, the strain that inhibited mycelial growth (HV28), did not inhibit the growth of spores. Three separate strains were able to inhibit spore growth. This indicates that certain strains may be better at inhibiting the establishment of spores while others may be able to inhibit actively growing fungi. These different strains may serve different roles depending on the type and life stage of fungi present.

Of the three bacterial species we studied (*L. micheneri*, *L. timberlakei*, and *L. queneiae*), all of the inhibitory strains belonged to either *L. micheneri* or *L. timberlakei*. We have only found one strain of *L. quenuiae* so far, so inhibitory *L. quenuiae* strains may exist but remain undiscovered. As there was no difference in the ability of *L. micheneri* or *L. timberlakei* to inhibit fungi, the variation in this trait appears to lie at the strain and not species level.

We found that conditioned media (media that we grew lactobacilli in then filter sterilized) was not efficient at inhibiting fungi. Fungal inhibition occurred only in the presence of live bacteria. Two possible explanations for this phenomenon exist. First, it is possible that the presence of fungi induces secretion of a defensive compound by the bacteria. To make the conditioned media we grew the bacteria in the absence of fungi, so inducible defenses would be lacking from this conditioned media. The second possibility is that any defense compounds that the bacteria secrete are rapidly degraded, meaning that live bacteria need to be present to continually produce the inhibitory compounds.

The next step in this research is to test these inhibitory strains in bee pollen provisions. The agar plate is an artificial environment, and interactions may differ in a more natural environment. For example, the resources in pollen provisions are readily used by both fungi and bacteria, which was not the case with the agar media we used in our studies. We were unable to culture both fungi and bacteria on the same media, so all of the assays were done by placing small plugs of bacteria media on top of the fungal media. The fungi therefore had more available resources compared to the bacteria, but some of the bacteria were nonetheless able to inhibit the growth of the fungi. In the brood cell the bacteria may have greater inhibitory capabilities.

These lactobacilli are all easily culturable, and several possible routes toward application exist. One possibility would be to dehydrate these bacteria and apply them to alfalfa flowers as a dust. Lactobacilli are easily freeze-dried and revived, but such applications would take some development. Another possible application would be to inoculate artificial feeders and nest surfaces with live bacteria that the bees could acquire and incorporate into their pollen provisions. Before we explore these or other options, however, we need to test whether the antifungal properties translate to the field and verify that the bacteria are beneficial and not harmful to the bees.

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Pest and Pollinator Management on Alfalfa Produced for Seed 2017

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Activities Detailed in this report:

1. **Insecticide efficacy trials in alfalfa produced for seed.**
2. **Alfalfa Leafcutting Bee (ALCB) overwintering success following large-scale field treatment with beleaf, sivanto, and transform Insecticides**
3. **Impact of Beleaf, Transform, and Sivanto Insecticides on ALCB Foraging Behavior**
4. **Monitoring the population of alkali bees in the Touchet Valley**

Part 1: Insecticide efficacy trials in alfalfa produced for seed.

Insecticides were screened for their ability to control the major pests of alfalfa produced for seed and for their potential negative impact on beneficial arthropods in late spring 2015. Field plots were established at the irrigated Agriculture Research and Extension Center near Prosser, WA. Established plots were 360 ft^2 (18 ft. wide and 20 ft. long) and all treatments were replicated 4 times in a randomized complete block design. Insecticides were applied to mimic grower timing at the pre-bloom period of production.

Treatments were applied on June 5, 2015 using a CO_2-powered backpack sprayer equipped with a four-nozzle boom using 19.8 gallons of water per acre as a carrier. Five 180° sweeps per plot were used pre-application and post-application on June 4, 9, 11, and 15 as a means to sample Lygus and other arthropod abundance including aphids, spiders, big-eyed bugs, minute pirate bugs, assassin bugs, lace wings, lady bird beetles and weevils. Weevil abundance was substantially lower in these plots then they had been in prior years. Abundance counts for the other arthropods can be provided on request.

Analysis of variance (ANOVA) was conducted on insect abundance counts for each pest on each respective sample date. The insect abundance counts for each pest by insect treatment was then compared to the untreated control population means in pair wise *t*-tests. All of the insecticides tested significantly reduced adult Lygus populations at 10 days after treatment, but in small plots these data are difficult to interpret, given the high mobility of Lygus adults. Most of the candidate insecticides

except Rimon and Sivanto tested provided highly significant (p<0.05) knockdown of Lygus nymph populations at 4 days after treatment and some provided residual suppression of Lygus nymphs 6 and 10 days following treatment. However the Lygus nymph population dropped to very low levels in all the plots including the untreated plots at 10 days after treatment. This coincided with the onset of a heatwave where temperatures exceded 100° F for several days. Most of the insecticides tested except for Rimon and Beleaf provided significant (p<0.05) control of cowpea aphids. Rimon has no effect on aphids and is not registered for control of aphids so these comparisons are irrelevant for Rimon. Our results with Beleaf are surprising given that Beleaf typically provides effective suppression of aphid populations. The candidate insecticides Transform and Sivanto provided equivalent levels of residual control of aphids and Lygus nymps when compared to the industry-standard prebloom insecticides Bifenture, Dimethoate, and Cobalt.

Pre bloom Insecticide trials WSU Prosser. Applications were made on June 5, 2015 in 19.8 gallons water per acre

Arthropod counts are counts are average number of individual in 5 sweeps per plot.

Each treatment was replicated 4 times on 18 by 20 foot plots (360 sq feet)

6/4/2015 Pretreatment		**Days after**	**Lygus**	**Lygus**	**Cowpea**		**Big eyed**	**Minute**	**Alfalfa**	**Ladybird**
Treatment	**rate/oz/acre**	**treatment**	**adults**	**Nymph**	**aphids**	**Spiders**	**bugs**	**pirate bugs**	**weevil**	**beetles**
Beleaf 50 SG	2.8	Pre treatment	17.5	22.25	27.75	0	0	0.25	0	0.5
Beleaf 50 SG /Steward EC	2.8/11.3	Pre treatment	32.5	18.25	41.5	0.5	0	0	0	1
Bifenture EC	6.4	Pre treatment	18.25	19	42.5	0	0	0	0	0.75
Cobalt Advanced	16	Pre treatment	17	18.5	26.5	0.25	0	0	0	0
Dimethoate 4 ECX	16	Pre treatment	17.75	23.75	53.75	0	3	0	0	0
Rimon .83 EC	12	Pre treatment	14.25	21.75	49	0	0	0	0	0.5
Rimon 0.83 EC`/Steward EC	12/11.3	Pre treatment	25.75	18.5	39.25	0	0	0	0	0
*Sivanto 200 SL**	14	Pre treatment	17.75	15.75	39.75	0	0	0	0	0.75
Sivanto 200 SL/Steward EC*	14/11.3	Pre treatment	22.75	23.75	30.25	0	0	0.25	0	0
Transform WG	2.25	Pre treatment	30.5	21.75	48.75	0.5	1	0	0	0.25
Transform WG /Steward EC	2.25/11.3	Pre treatment	18.5	13.25	38.75	0.25	0	0	0	0.75
Untreated			18.75	13	30	0	0	0	0	0
6/9/2015 5 Days after treatemt										
Beleaf 50 SG	2.8	4 days	11.75*	1.5	0**	0	0.25	0	0.75	0.25
Beleaf 50 SG /Steward EC	2.8/11.3	4 days	12*	0.75	0**	0.5	0	0	0.5	0.5
Bifenture EC	6.4	4 days	2**	1.25	0**	0	0.25	0	1	0
Cobalt Advanced	16	4 days	5**	0.25	0**	0.25	0.25	0	0	0.25
Dimethoate 4 ECX	16	4 days	5**	1	0**	0	0	0	1	0
Rimon .83 EC	12	4 days	3**	4.25	1.75	0	0	0	0.5	0.25
Rimon 0.83 EC`/Steward EC	12/11.3	4 days	4**	0.75	3.25	0	0	0	0	0.25
*Sivanto 200 SL**	14	4 days	11.5*	3.75	9.5	0	0	0	0.25	0.25
Sivanto 200 SL/Steward EC*	14/11.3	4 days	6.25**	1.25	0.5**	0	0	0	0.5	0.25
Transform WG	2.25	4 days	8.25*	1	0**	0.5	0	0	0.25	0
Transform WG /Steward EC	2.25/11.3	4 days	5.25**	1	0**	0.25	0	0	0	0
Untreated			18	1.25	2.5	0	0.5	0	0.25	0
		Days after	**Lygus**	**Lygus**	**Cowpea**		**Big eyed**	**Minute**	**Alfalfa**	**Ladybird**
6/11/2015 7 Days after treat	**rate/oz/acre**	**treatment**	**adults**	**Nymph**	**aphids**	**Spiders**	**bugs**	**pirate bugs**	**weevil**	**beetles**
Beleaf 50 SG	2.8	6 days	15.25	5.25	11.5	0	0.75	0	0	1.5
Beleaf 50 SG /Steward EC	2.8/11.3	6 days	11.25	3.25	4.25*	0	0.25	0	0	0
Bifenture EC	6.4	6 days	14	2.5*	3**	0	0	0.25	0	0
Cobalt Advanced	16	6 days	18.5	0.75**	3**	0	0	0.5	0	0.25
Dimethoate 4 ECX	16	6 days	7.5*	0**	0**	0	0	0	0	0.75
Rimon .83 EC	12	6 days	11.5	2.75*	14.5	0	0.25	0	0	1.75
Rimon 0.83 EC`/Steward EC	12/11.3	6 days	8.75*	0**	3.75*	0	0.25	0	0	1
*Sivanto 200 SL**	14	6 days	10.5*	2.5**	1**	0	0.25	0.5	0	0.25
Sivanto 200 SL/Steward EC*	14/11.3	6 days	3.5**	1**	1**	0	0	0	0	0.5
Transform WG	2.25	6 days	6.75**	0**	1.5**	0	0	0	0	0.25
Transform WG /Steward EC	2.25/11.3	6 days	17.75	1**	3.75*	0	0.25	0	0	0
Untreated			17.5	6.5	10.5	0	0.5	0	0	1
6/15/2015 10 Days after treatment										
Beleaf 50 SG	2.8	10 days	6*	0	3.5	0	0.25	0.5	0.25	0
Beleaf 50 SG /Steward EC	2.8/11.3	10 days	4.75*	0	7.5	0	0.25	1	0.25	0.25
Bifenture EC	6.4	10 days	4*	0	0**	0	0	0.25	0.25	0
Cobalt Advanced	16	10 days	9.25	1	1.75	0	0	0	2.25	0.25
Dimethoate 4 ECX	16	10 days	3.25**	0	0**	0	0	0.5	0.75	0.25
Rimon .83 EC	12	10 days	4.5*	0	0.5**	0	1	0.75	2.75	0
Rimon 0.83 EC`/Steward EC	12/11.3	10 days	5*	0	2*	0.25	0.75	0	0	0
*Sivanto 200 SL**	14	10 days	7.75*	0.5	2*	0	0	0.75	0.25	0
Sivanto 200 SL/Steward EC*	14/11.3	10 days	5.5*	0	0**	0.75	0	0.25	0	0
Transform WG	2.25	10 days	1**	0	0.75**	0	0.25	0	0.5	0.25
Transform WG /Steward EC	2.25/11.3	10 days	7*	0	0.75**	0	2.5	4.5	0	0.25
Untreated			17.25	0.75	4.25	0	9	0	0.75	0.25

Insecticides in italics are not registered for use on alfalfa produced for seed

**/ Populations means are significantly lower then the untreated control in pairwize t-tests at p<0.01

*/ Populations means are significantly lower then the untreated control in pairwize t-tests at p<0.05

Insecticide efficacy in large field plot 2016.
At sunset on June 20, 2016 the field that was detailed for 2016 in part 1 of this report was treated with either Transform at 1.5 oz/acre or with BeLeaf at 2.8 oz/acre. The application of Transform was made at sunset to 40 acres between 9:00 and 9:30 PM. Subsequently the application of BeLeaf to 40 acres was made between 9:45 and 10:15 PM. A 40 acre control field where no insecticide was sprayed was immediately north of the treated field. Pretreatment sweepnet samples were taken from the 80 acre field that was to be treated with Transform and BeLeaf and from the Control field (unsprayed) on June 17, 2017. Sweep net samples consisted ten sweeps per field. A single "sweep" consist of five 180° sweeps and then counting the insect captured. Similar counts were taken post treatment on June 23, 2017, three days post insecticide treatment. These treatments are pseudo replicated so statistical values are not being displayed. That said, the Transform application numerically reduced the abundance pest Lygus compared to the Beleaf treatment and the control plots.

Table 2. Pest insect abundance per sweep pre and post insecticide application.

June 17, 2016 Pre treatment	Lygus Adults	Small nymphs	Large nymphs	Aphids
Experimental field	16.16	1.63	1.42	19.10
Control field	17.6	1.35	0.85	11.2

June 23, 2016 Post treatment	Lygus Adults	Small nymphs	Large nymphs	Aphids
Control	19.56	3.76	0.60	12.00
Beleaf	18.56	1.36	0.40	2.52
Transform	3.36	0.45	0.00	0.50

Table 3. Beneficial arthropod abundance per sweep pre and post insecticide application.

June 17, 2016 Pre treatment	BEB[1]	Spiders	LW[2] larva	LBB[3]	MPB[4]
Experimental field	1.07	0.20	0.50	0.36	0.82
Control field	0.40	0.33	0.2	0.35	0.67

June 23, 2016 Post treatment	BEB[1]	Spiders	LW[2] larva	LBB[3]	MPB[4]
Control	0.54	0.33	0.20	0.62	0.67
Beleaf	0.60	0.50	0.27	0.40	0.60
Transform	0.75	0.00	0.00	0.33	0.00

[1]/ Big eyed bugs, [2]/ Lacewing, [3]/ Ladybird beetles, [4]/ Minute pirate bugs

Insecticide Efficacy 2017

Insecticides were screened for their ability to control the major pests of alfalfa produced for seed and for their potential negative impact on beneficial arthropods in late spring 2017. Field plots were established at the irrigated Agriculture Research and Extension Center near Prosser, WA. Established plots were 360 ft^2 (18 ft. wide and 20 ft. long) and all treatments were replicated 4 times in a randomized complete block design. Insecticides were applied to mimic grower timing at the pre-bloom period of production.

Treatments were applied on July 20, 2017 using a CO_2-powered backpack sprayer equipped with a four-nozzle boom using 19.8 gallons of water per acre as a carrier. Five 180° sweeps per plot were used pre-application on July 19 and post-application on July 21, 25, and July 28 as a means to sample Lygus and other arthropod abundance including aphids, spiders, big-eyed bugs, minute pirate bugs, assassin bugs, lace wings, lady bird beetles and weevils. Abundance counts for the other arthropods can be provided on request.

Analysis of variance (ANOVA) was conducted on insect abundance counts for each pest on each respective sample date. The insect abundance counts for each pest by insect treatment was then compared to the untreated control population means in pair wise *t*-tests. All of the insecticides tested significantly reduced adult Lygus populations at 1 day after treatment, but in small plots these data are difficult to interpret, given the high mobility of Lygus adults. However the Lygus nymph population increased rapidly in all the plots except the Transform plots and Transform was the only insecticide that was effective after 5 days. All treatment effects were done by 10 days after treatment. It should be noted that these plots were established on a 1 acre block of poorly managed alfalfa hay on the WSU IAREC Roza unit. A standout on the 1 day after treatment was the efficacy provided by Biological Soil and Foliar. Typically Lygus are fairly resilient to being killed by biological-based insecticides. We plan on completing additional larger scale trials with this product in 2018. It is exempt from tolerance and would be very easy to register on alfalfa produced for seed.

				Adults		Nymphs	
MS df=8				932*		5019**	
error df=27				379		932	
Product	**Rate/acre**		Days after Trt	Mean	SE	Mean	SE
Beleaf 50 SG	2.8	oz/acre	1	34.5	11.45	68.25	26.28
Biological Soil & Foliar	96	Fl oz/acre	1	11.25	3.42	14*	8.22
Biological Soil & Foliar	128	fl oz/acre	1	37.25	17.95	53*	14.90
Grandevo	48	oz/acre	1	21.5	9.32	51.25*	24.26
Naled	16	Fl oz/acre	1	32.25	3.90	52*	10.35
Sivanto*	14	Fl oz/acre	1	30.75	13.86	34.25*	18.98
Transform	1.5	oz/acre	1	4.25*	0.95	1.5*	0.50
Transform	2.25	oz/acre	1	5*	1.83	5.5*	0.65
Control			1	47.5	9.51	114.5	7.80

				Adults		Nymphs	
MS df=8				49.4		1797*	
error df=27				35.2		618	
Product	**Rate/acre**		Days after Trt	Mean	SE	Mean	SE
Beleaf 50 SG	2.8	oz/acre	5	14	2.97	26.25	8.37
Biological Soil & Foliar	96	Fl oz/acre	5	11.25	4.11	58.25	23.68
Biological Soil & Foliar	128	fl oz/acre	5	11.25	1.93	49.5	16.07
Grandevo	48	oz/acre	5	10.75	2.39	51.25	9.39
Naled	16	Fl oz/acre	5	11.5	3.77	38	5.05
Sivanto*	14	Fl oz/acre	5	14.5	2.90	54.5	17.70
Transform	1.5	oz/acre	5	4	1.22	3.75*	2.17
Transform	2.25	oz/acre	5	7.25	3.42	7*	1.78
Control			5	14.75	2.87	56.25	8.18

				Adults		Nymphs	
MS df=8				33.9		223	
error df=27				93.7		155	
Product	**Rate/acre**		Days after Trt	Mean	SE	Mean	SE
Beleaf 50 SG	2.8	oz/acre	8	14.5	4.29	22.25	7.73
Biological Soil & Foliar	96	Fl oz/acre	8	19.5	5.04	29.25	5.74
Biological Soil & Foliar	128	fl oz/acre	8	18.25	7.04	17.75	6.24
Grandevo	48	oz/acre	8	19.5	4.99	22.25	8.79
Naled	16	Fl oz/acre	8	22.75	5.14	32	5.20
Sivanto*	14	Fl oz/acre	8	15.75	4.61	27.5	8.46
Transform	1.5	oz/acre	8	15	2.94	7.5	3.66
Transform	2.25	oz/acre	8	19.25	4.33	20.5	2.40
Control			8	14.25	4.19	28.5	4.86

				Adults		Nymphs	
MS df=8				66.4		133	
error df=27				58.7		72	
Product	**Rate/acre**		Days after Trt	Mean	SE	Mean	SE
Beleaf 50 SG	2.8	oz/acre	10	20	4.18	17	2.12
Biological Soil & Foliar	96	Fl oz/acre	10	10.25	1.49	16.25	6.42
Biological Soil & Foliar	128	fl oz/acre	10	18.75	7.03	19.75	6.82
Grandevo	48	oz/acre	10	19.25	2.10	18.75	2.46
Naled	16	Fl oz/acre	10	22.25	3.77	22.75	4.25
Sivanto*	14	Fl oz/acre	10	17.75	2.29	18.25	2.43
Transform	1.5	oz/acre	10	24	2.38	23	2.16
Transform	2.25	oz/acre	10	20.75	4.87	35.25	4.13
Control			10	23.25	3.12	19	4.14

Part 2. Alfalfa Leafcutting Bee (ALCB) ALCB Overwintering Success Following Large-Scale Field Treatment with Beleaf, Sivanto, and Transform Insecticides

Experimental Design: In the summer of 2015, binder boards were placed in domiciles for three weeks following spray treatment with Beleaf and either Transform or Sivanto. After three weeks, the binder boards were collected, x-rayed, and stored in a grower cooler for the winter. Binder boards were then incubated and emerged bees were counted and weighed. In the summer of 2016, a similar

procedure was carried out, with the exception that binder boards remained in the field for the duration of the season.

2015 Results:

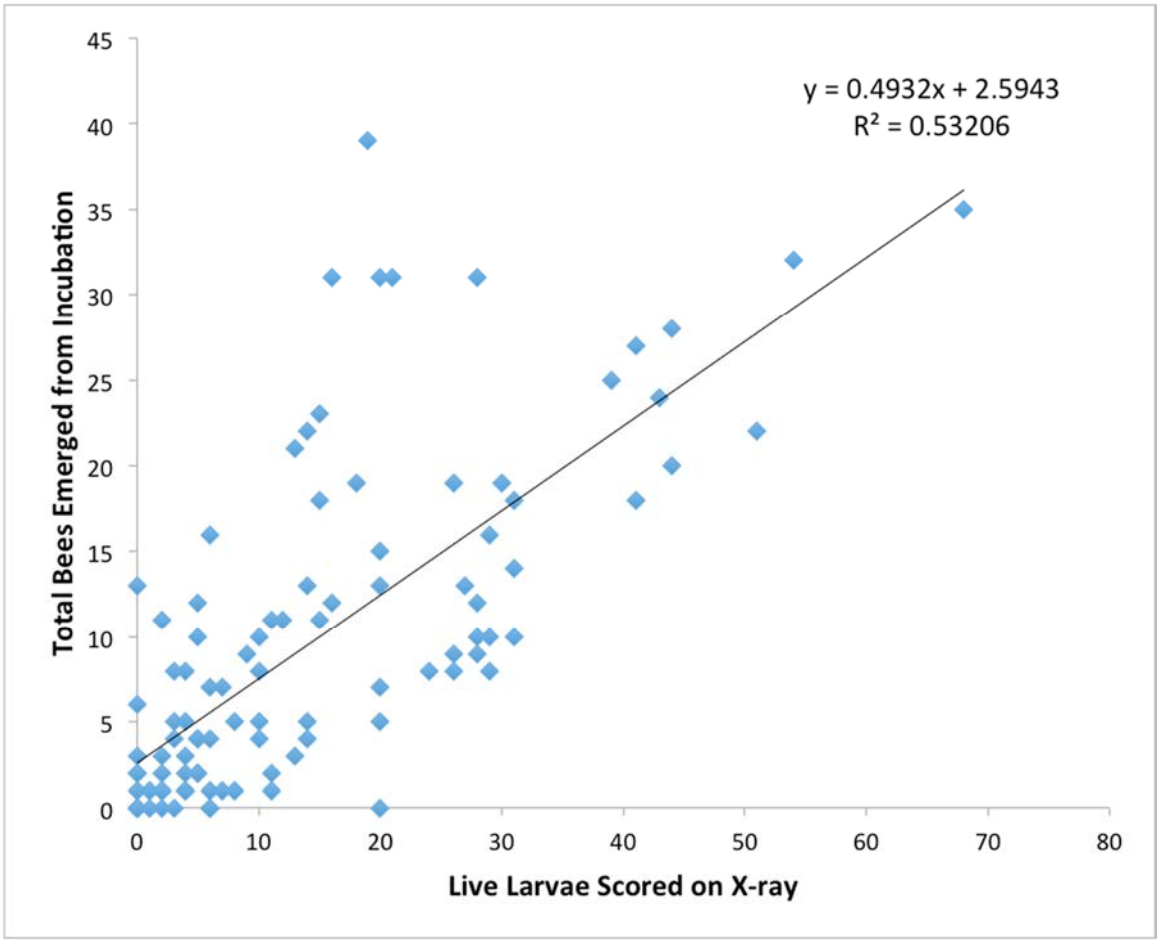

Figure 1: Correlation of the number of bees emerged following incubation in the spring of 2016 with the number of live larvae determined by scoring x-rays of the nests.

Figure 1 represents the correlation between the scoring of x-rays of paper tubes from binder boards for live larvae and the number of bees that emerged following incubation. There is a fairly strong correlation, which allows us to make predictions of the number of bees that will emerge following incubation of the tube nests in spring.

The first trial compared ALCB exposed to Beleaf with those exposed to Sivanto. Significantly more males emerged from binder boards in the Beleaf (mean = 13 males, SE = 1.27) treatment than in the Sivanto treatment (mean = 5 males, SE = 1.34) (t-test, p = 0.0266). In contrast, the number of females emerged did not differ

significantly between treatments (p = 0.0558). Results of scored x-rays found that the total number of cells provisioned was significantly higher in the Beleaf treatment (mean = 14 cells, SE = 6.41) than in the Sivanto treatment (mean = 5 cells, SE = 6.33) (p = 0.0333).

The second trial compared ALCB exposed to Beleaf with those exposed to Transform. In the second trial, there were no significant differences in males or females emerged between treatments. There was also no significant difference in the number of cells provisioned between treatments. However, this trial had a lower sample number, which makes it more difficult to detect differences between treatments.

2016 Results:

During the summer of 2016, one large-scale field trial was conducted comparing reproductive success of bees exposed to Beleaf and Transform. One field was blocked into 25-acre plots each containing four domiciles, and 8 binder boards were incubated from each treatment. The number of live larvae scored from the x-rays was again well correlated with the total number of bees that emerged from incubation (Fig. 2). In total, 157 bees emerged from the Beleaf incubation, with a male-to-female sex ratio of 1.34:1 (Fig. 3). 280 bees emerged from the Transform incubation, with a male-to-female sex ratio of 0.97:1 (Fig. 3).

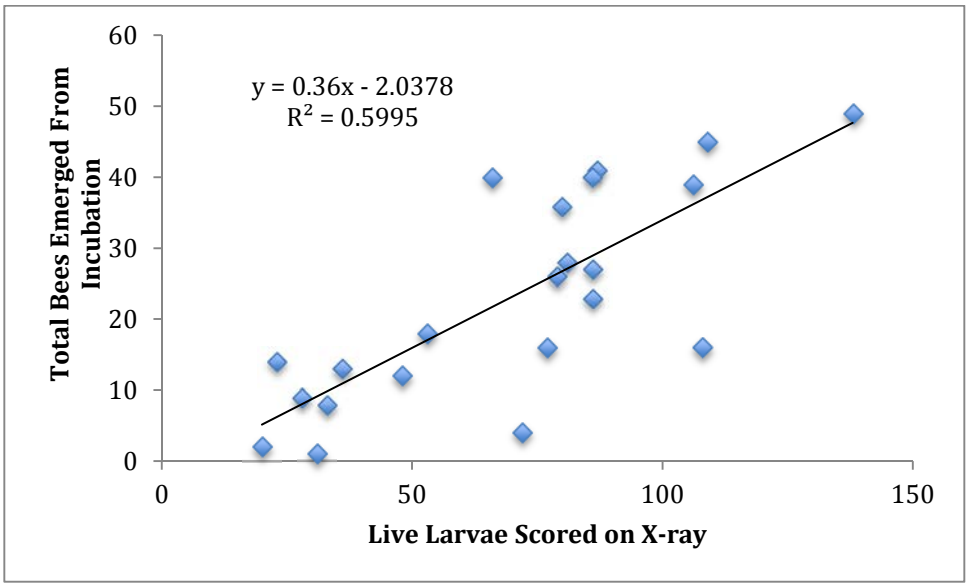

Figure 2: Correlation of the number of bees emerged following incubation in the spring of 2016 with the number of live larvae determined by scoring x-rays of the nests.

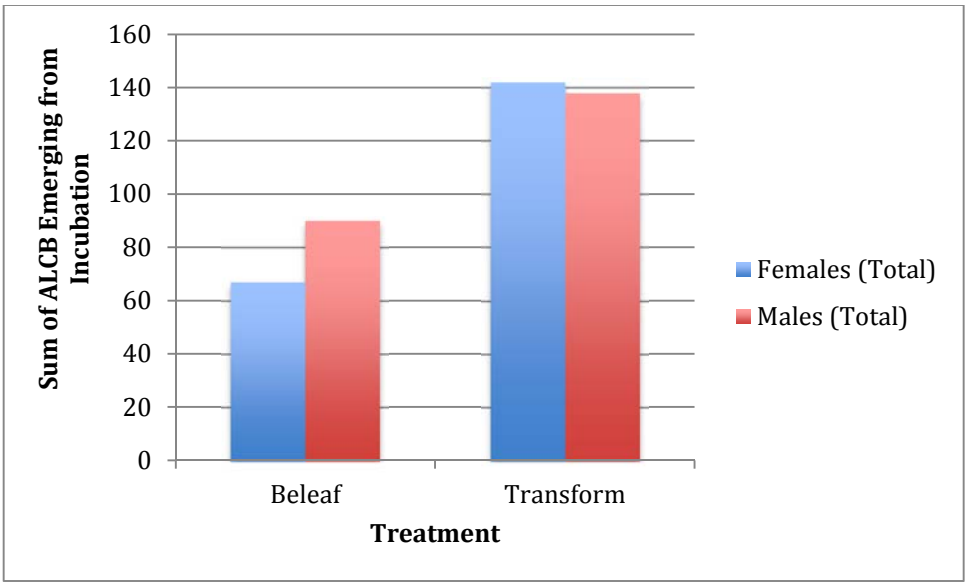

Figure 3: The total number of male and female ALCB emerging from incubation in binder boards collected from Beleaf and Transform plots.

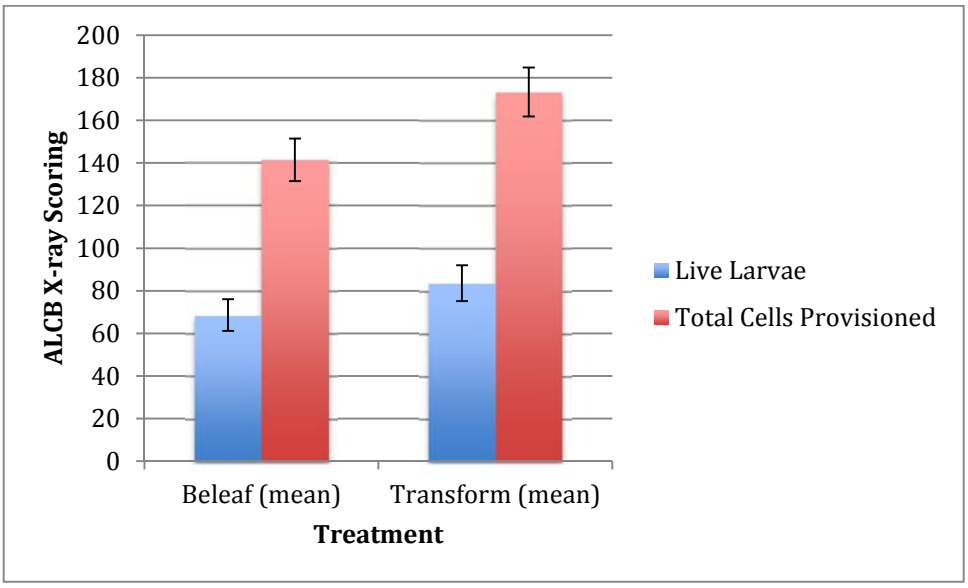

Figure 4: Error bars represent one standard error from the mean.

Part 3: Impact of Beleaf, Transform, and Sivanto Insecticides on ALCB Foraging Behavior

Experimental Design: In the summer of 2015, cameras were placed in domiciles for an hour the morning before, one day after, and three days following spray by Beleaf, Sivanto and/ or Transform. Videos were watched during the winter and duration of pollen and leaf foraging trips were recorded.

2015 Results:

	Duration (Minutes)		
Foraging Event	**Mean**	**Standard Error**	**N**
In Nest (Leaf)	3.64	0.27	39
In Nest (Pollen)	2.54	0.18	370
Leaf Trip	6.96	1.15	38
Pollen Trip	17.35	1.01	206

Duration of foraging trips. In Nest (Pollen): time spent in nest following pollen trip; In Nest (Leaf): time spent in nest following leaf trip ; Pollen Trip: time away from nest until returning with pollen ;Leaf Trip: time away from nest until returning with leaf.

Trials: Summer of 2015	**Mean Duration of Pollen Trip (min)**	**Standard Error**	**N**
Wagoner June 15th	15.63	1.14	112
Buckley June 24th	21.20	1.94	80
Wagoner July 7th	9.11	2.55	14

The average amount of time a bee spent foraging for pollen varied substantially between trials, suggesting that the conditions were very different between trials. All of the trials found the mean pollen trip duration to be greater than 14.9 minutes, the trip duration estimated by Klostermeyer and Gerber in 1969.

Bees in the field sprayed with Sivanto spent significantly longer foraging for pollen (mean pollen trip = 27.97, SE = 3.53) than in the control field (mean pollen trip = 18.95, SE = 2.24) (Wilcoxon Rank Sums, Z = 2.23, p = 0.0259). The mean duration of a pollen trip in both the unsprayed field and the Sivanto treatment was significantly longer in the Mike Buckley trial than in the other two trials.

2016 Results:
In 2016, there were no significant differences in the duration of pollen and leaf trips between Beleaf and Transform treatments prior to the spray on 20 June 2016 or after the spray on 21 June 2016 or 23 June 2016.

In 2016, nest “activity” was measured. Nest activity was defined as the number of unique events in which a bee enters one of 20 nest holes over the course of 10 minutes. Whether bees were carrying pollen, a leaf, or had no visible provision was recorded. There was not a significant difference in the number of nest visits between treatments on any date.

Part 4: Monitoring the Population of Alkali Bees in the Touchet Valley

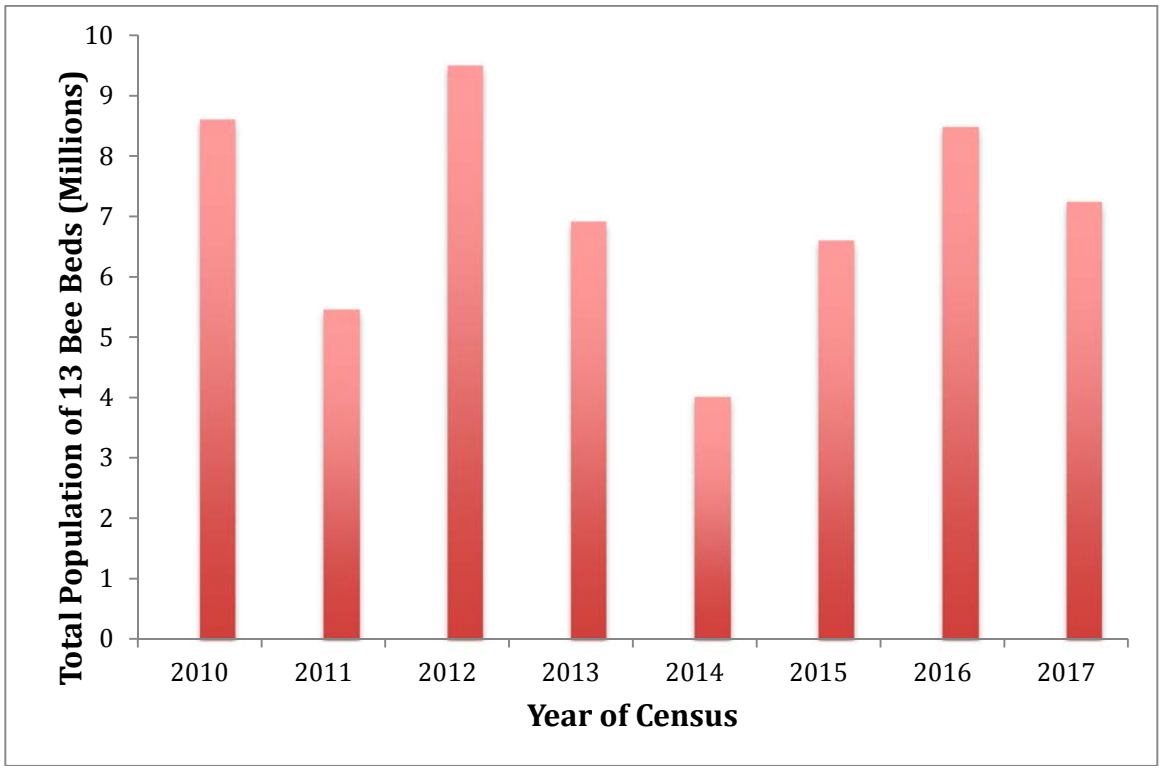

Figure 5. The estimated alkali bee population in the Touchet Valley from 1999 to 2017.

Understanding *Melittobia* biology in the context of commercial management of *Megachile rotundata*

Theresa Pitts-Singer[1] and Alan Anderson[2]
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[2]Biology Department, Utah State University, Logan, Utah

Introduction & Objectives

The alfalfa leafcutting bee (ALCB), *Megachile rotundata*, is a managed solitary species used for the pollination of alfalfa. These managed bees are susceptible to parasitism when placed en masse in alfalfa fields. Parasites and parasitoids include a variety of wasps. For example, *Sapyga pumila* is a univoltine kleptoparasite that kills the bee egg, thus using the cell's pollen-nectar mass for its own offspring development. *Pteromalus venustus*, *Monodontomerus obscurus*, and *Tetrastichus* sp. are multivoltine parasitoids that oviposit through the ALCB cell and on or through the prepupal skin; the wasps each the ALCB prepupa. The tiny females of *Melittobia* spp. chew into bee cells to parasitize by ovipositing on the outside of the prepupa, and then wasp larvae consume the prepupa.

In the past couple of years, some bee managers have encountered egregious attacks of their ALCB stocks by *Melittobia* (species yet to be determined). With the potential to be devastating to managed ALCB populations, we planned to perform trials to determine what chemical odors and style of pest trap might attract this (and other) hymenoptera parasitoids to ALCB larvae and cocoons. However, after further consideration of an approach to solve this parasitoid problem, I felt it better to first gain a clearer understanding of how this pest is able to persist in ALCB populations through winter and then during and after incubation. Therefore, Utah State University student Alan Anderson was tasked with executing experiments to fulfill the following objectives, which began in summer 2016 and continued through fall 2017:

1) Describe the reproduction and development of Utah-collected *Melittobia* using ALCB prepupae as hosts.
2) Determine at what life stages *Melittobia* can survive winter storage. Also, determine the life stage at which *Melittobia* are detectable on X-ray.
3) Determine what ALCB life stages can be parasitized by *Melittobia* sp.

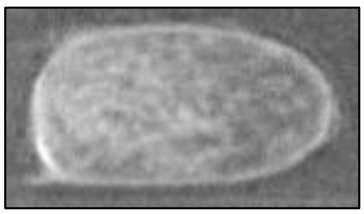
Using the results from trials in 2016 and 2017, we will be better able to proceed on developing tools for detection and control for this particular ALCB pest.

1) Methods and Results for *Melittobia* Life Cycle (2016 & 2017)

All rearing of *Melittobia* was performed in a workshop where we set up an incubator for this purpose. The workshop was isolated from any of our bee stocks, which easily could have become infected by this prolific pest.

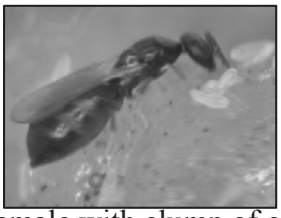
1**) 2015 & 2016 Methods and Results for Rearing and Observations**

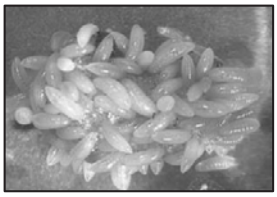
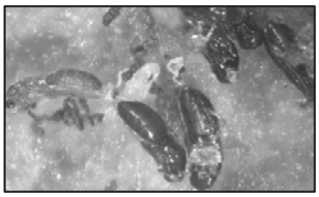
- ALCB cells parasitized with *Melittobia* were selected from ALCB cells in cold storage using X-ray detection.
- Day 0, *Melittobia* adults were introduced to ALCB cells by transferring them with a damp paint brush.
- Day 1, female wasps inspected ALCB cells and selected a location to chew through the cell.

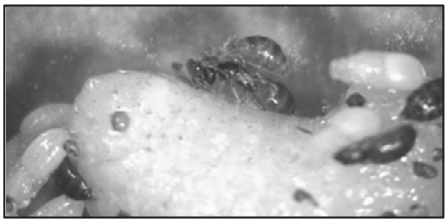


Melittobia in cell as seen on x-ray.

- Day 2, females entered the cells and began to lay eggs clumps.
- Days 4 and 5, feeding larvae lightened in color and increased 3-4 times in size.
- Day 6, larvae ceased to feed and grow; they began to turn gray.
- Days 7 and 8, larvae defecated and returned to a light yellow body color (having voided their guts). Larvae became segmented and began to pupate.
- Day 8 (end), all wasps have reached pupal stage.
- The pupal stage lasted through Days 8 - 16 for males and females. Pupal eyes became dark red as the rest of the body darkened. Males developed faster than females.
- Males emerged as adults from Days 14-22. They fought for access to females. Most died within 8 days post-emergence (never leaving the host cell).
- Females emerged as adults from Days 16-25. Most died 7-10 days post-emergence.
- Females were represented by short-winged, non-disperser and long-winged, disperser forms. There were only a few short-winged females, and although they laid eggs on the remains of the bee prepupa, there was not enough bee material left to raise a new clutch of eggs. The long-winged forms chewed out of the cell and dispersed to lay eggs in new prepupal cells.


Female with clump of eggs.



Larvae (left) and pupae (right).


Mating on host inside cell.



Adult male (left) and female (right).

2) Methods and Results for *Melittobia* Overwintering Life Stages and X-Ray Detection (2017)

- In a vial, 5-10 *Melittobia* were added to 4 *M. rotundata* cells. One Utah and two Canadian ALCB stocks were used.
- Cells were exposed to wasps for 1, 2, 3, 4, 5, 6, 7, & 8 days.
- The 4 ALCB cells were then separated into different groups: Incubator, Overwinter, X-ray, and Cut.
- Three replications per stock were set up for each exposure time, plus a 4th replicate of the 2 day exposure.

The Incubator group was used to propagate *Melittobia* for other experiments and remained at 29°C. Of the ALCB cells exposed to wasps, 51% were parasitized.

The Overwinter group was to determine at what stage *Melittobia* survive wintering. After each day of exposure, cells were placed into cold storage. Although 46.9 % of cells were infested with wasps, no life stage of *Melittobia* survived winter storage after spring incubation (and neither did bee prepupae in those parasitized cells).

The X-Ray group was monitored for the ability to detect *Melittobia* using x-rays. 43.8% of ALCB cells were parasitized, and the *Melittobia* larvae could be detected starting on Day 4 after the cells were exposed to adult female wasps.

The Cut group was used to monitor development over time and to photo-document all *Melittobia* life stages (and to confirm data collected in 2016). Results included above for Obj. 1.

3) Methods and Results for *Melittobia* Oviposition on ALCB Larvae, Pupae, and Adults (2017)

- Expose ALCB to *Melittobia* females starting when ALCB were eggs; allow ALCB to grow until they were parasitized.
- Add adult *Melittobia* to ALCB bees at the pupal stage, allowing bees to continue developing to adult stage, if possible.

Melittobia females only oviposited on the ALCB 5th instar. Usually, the wasp waited until the cocoon was complete before stinging the mature larva within. The female wasp also laid eggs on the ALCB pupa and adult, but neither wasps nor bee survived.

It was also shown in the laboratory that the *Melittobia* reared from ALCBs can parasitize and survive on the paper wasp species (*Polistes dominulus*) that commonly were found in wooden ALCB shelters in Utah alfalfa seed fields.

Discussion

Rearing *Melittobia* in the laboratory in both 2016 and 2017 yielded consistent data and provided valuable information about their life cycle and reproductive capabilities using ALCB as a host. We now understand that a prepupae in the incubator can only support one *Melittobia* generation, that the first bees emerge in just over 2 weeks, and that dispersing females could easily emerge to find new prepupal cells on which they lay could eggs. The progeny of the dispersed females could hatch as adults starting after 16 more days. Therefore, it is very important to know the *Melittobia* life stage that can successfully overwinter with the ALCB prepupae. Our trial to discover this life stage was not informative in 2017, perhaps because there is a need for some sort of prewintering phase that requires gradual cooling prior to cold storage, or something related to timing and temperature that our experiment failed to capture in our protocol.

We did find that *Melittobia* larvae are detectable on X-ray by the 4th day after presumably eggs were laid. This means that the *Melittobia* we detected on X-ray and collected from Utah wintering bee stocks were at least at the feeding larva stage at this time. Once incubated in the spring, the *Melittobia* could be emerging as adults as soon as day 7-10, overlapping with the emergence of *Pteromalus*. While *Pteromalus* is being controlled in incubators with dichlorvos and black lights, the *Melittobia* also could feasibly be controlled. But if not controlled, then the dispersing females could be entering new ALCB cells and then hatching out of cells as a new adult generation just after incubation trays are placed in the field shelters. If this timeline for adult emergence is possible and accurate, then we need to test whether *Melittobia* adults are susceptible to the levels of dichlorovos in incubators in the same manner as *Pteromalus*.

If a grower only discovers the presence of *Melittobia* when new females hatch in the shelters, it may be possible for the *Melittobia* to wait inside nesting material or even inside provisioned cells for ALCB larvae to develop to the prepupal stage. Otherwise, *Melittobia* may spend a generation in nearby paper wasp nests, which is supported by the finding that paper wasps can serve as a host of this *Melittobia* species and implies that wasps in shelters may be an alternative *Melittobia* host that serves as a source of contamination for commercial ALCB populations. After one generation in the paper wasp nests, the dispersing *Melittobia* females can return to bee nesting material where diapausing ALCB prepupae are abundant.

The information gathered in 2016 and 2017 has been very informative in understanding possible avenues of *Melittobia* infestation. With further work to determine the overwintering stages of *Melittobia*, and with new efforts to find viable methods of trapping and killing this pest, we aim to develop new management techniques for this ALCB pest as well as others.

Project Title: Revisiting bomber fly control & exploring provision-soil moisture relations
Year(s) of Study: 2017
Lead Investigator / Affiliation: James Cane, USDA-ARS PIRU, Logan
Hyperlink to research website: http://www.ars.usda.gov/Research/docs.htm?docid=18333

Objective for 2017: I) Revisit the bomber fly study with active flies, testing attraction to toxic floral bait stations (using potted lantana) and selective trapping using plastic perforated row tunnels **II)** Experiment with alkali bee provisions and their daily uptake of water vapor from nest bed soils. Duplicate with ALCB provisions in the context of their intranest atmosphere.

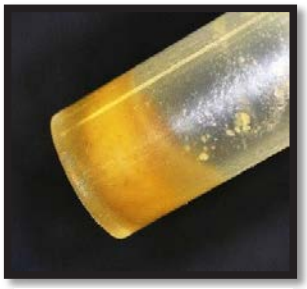
Results from 2017: We bought 2 flats of lantana starts from a Logan nursery (after cuttings from Apopka FL failed to root), potted them up and grew them in our greenhouse. We then drove them to Touchet in late June. Bees were actively nesting but bomber flies were very sparse. Scouting about, the old Byerly river bed had the most, so pots were deployed, each with a time lapse camera. The day became windy, but footage showed bee flies that occasionally flew by, or landed on the pots to sun, but never visited the flowers. Lantana likes hot summers and can be a weed in other, more humid places, but is not practical or effective for this purpose where alfalfa seed is grown.


Nest cell closure, viewed from inside nest cell

We continued our studies of nest cell water relations, again including a day's visit from Dr. Galen Campbell of Decagon Instruments. His company manufactured a rotary multiport thermocouple psychrometer for the task. Water activity of the freshly made pollen provision was much less than the surrounding salty soil, low enough to retard microbial spoilage. Water activity of the aging provision gradually increased apace larval growth, finally matching that of the surrounding soil. That added moisture came from the surrounding soil, passing through the water-permeable cap (which quickly absorbed applied water droplets). In contrast, droplets beaded up on the waterproof lining. Thus, the bee's nest cell design provides a grdual, controlled rate of provision hydration, thereby solving two problems, preventing spoilage while providing needed dietary water to the developing larva.


Tube for exposing provisions to soil humidity

Where does the water vapor go after passing the cell cap? We dug out intact, clean provision masses,, and eggs and larvae on their provisions. We measured both their water potentials and experimented with hydration. We also collected fresh alfalfa pollen from flowers. Placed in a humid atmosphere for 48h, alfalfa pollen doubled in weight, the grains visibly ballooning. Hence, alfalfa pollen itself works like a sponge, absorbing some of the soil water vapor. Fresh *Nomia* pollen balls averaged 100 mg pollen and 55 mg water. By maturity, larvae had gained 182 mg in water weight, 55 gm from nectar. The rest, 127 mg, must have come from the soil atmosphere (plus some metabolic water). How did larvae acquire that added water from the soil?


Liquefied provisions after 7 days of hydrating in mesh-bottomed tube

We again used our method to expose individual provisions to the moist atmosphere of a dampened nest bed soil. Intact provisions without a feeding larva gradually absorbed condensed soil water vapor, adding 75-90 mg of water during 7 days over fresh nesting soil (or 51-67 mg over dry nesting soil). Without a feeding larva, the provision gradually liquefied but did not mold (unlike similarly treated honey bee pollen pellets, which always molded). In contrast, half-eaten provisions from the field never looked liquefied. Hence, developing larvae must be drinking the resulting liquid as it leaches from the provision mass.

Young Nomia larva feeding on pollen provision

We conclude that a developing alkali bee larva gains most of its body water from the soil, not from the nectar in its provision. This water enters the cell as vapor passing through the unlined earthen cell cap. The provision, not the larva, absorbs this water vapor. The larva apparently drinks away this accumulating liquid, as provisions with developing larvae do not visibly liquefy, unlike provisions without a larva. The imbibed broth certainly contains nectar sugars and possibly amino acids that are leached from the pollen. The youngest larvae were tiny, just 8-11 mg, so their diet must be mostly liquid, given that an intact provision daily gained 10 mg of water that was absorbed from the soil atmosphere. Through this controlled hydration, larvae gradually gain needed dietary water without risking drowning or provision spoilage.

Mature Nomia larva finishing pollen provision

We thank Sarah Clark and Emily Hamada of the Biology Dept. at Whitman College for arranging our daily access to their lab's desiccation oven and microbalance, and John Dodd, director of the Forage Genetics research unit in Touchet, for providing us with generous lab space for our sometimes dirty bee studies.

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:

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

Experiment 2 treatment groups:

Treatment group	Rearing environment
50 µg JH-III	Alone
Solvent control	Alone
Handling control	Alone
50 µg JH-III	With an older, reproductive female
Solvent control	With an older, reproductive female
Handling control	With an older, reproductive female

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^{o}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^{o}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^{o}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 resorbtion 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.

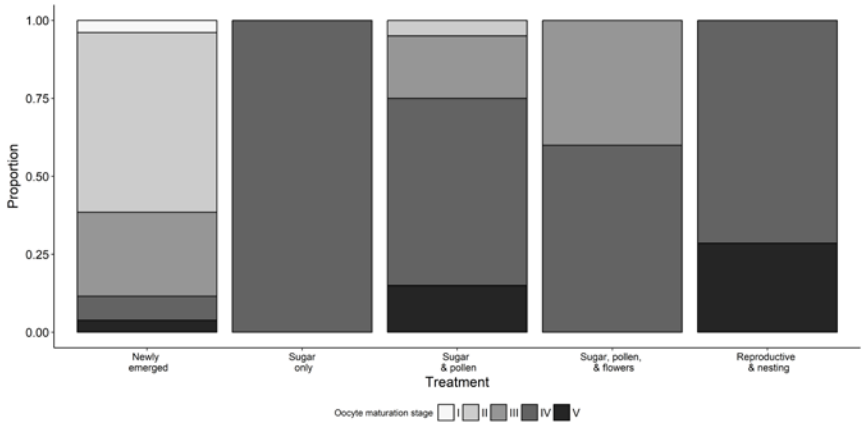


Fig. 1. Effects of diet on oocyte maturation in alkali bees. Newly emerged females had oocytes at significantly earlier stages of maturation than females in the other treatment groups (ordinal logistic regression: $Z=-4.61$, $P=3.99\times10^{-6}$; number of individual bees: newly emerged n=26, sugar 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.

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.

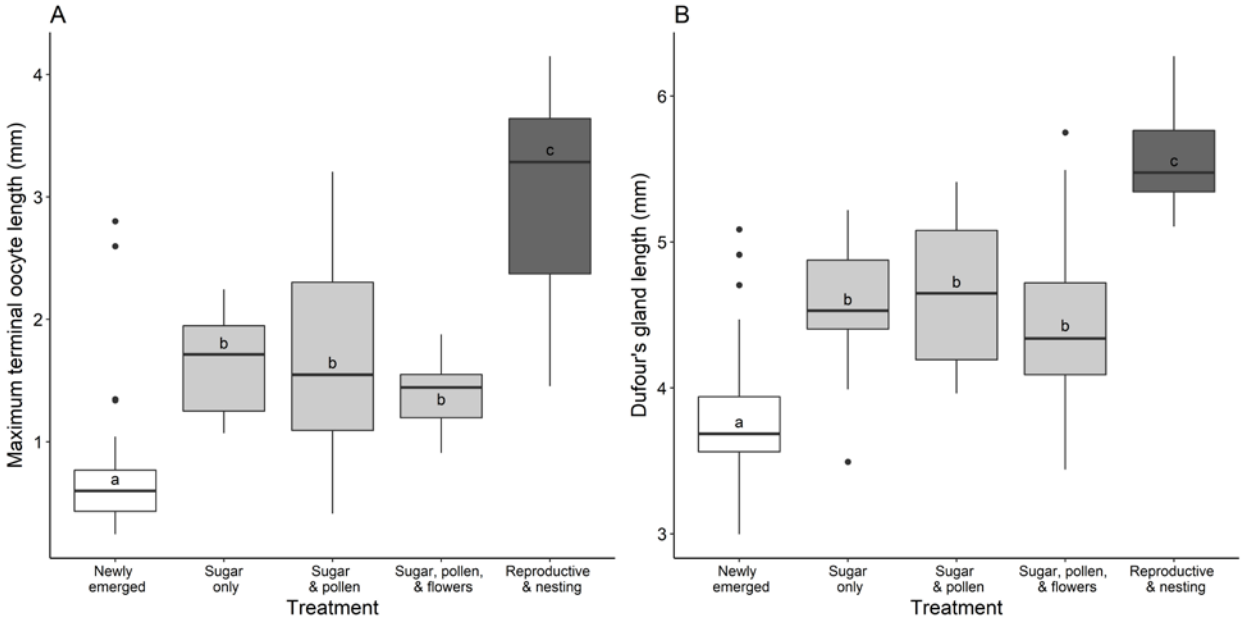


Fig. 2. Effects of diet on reproductive maturation in alkali bees. (A) Maximum viable terminal oocyte length and (B) Dufour's *gland* length were significantly different between newly emerged females, 10 day old lab-reared females and nesting females (oocytes: $F_{4,89}=30.68$, $r^2=0.58$, $P<4.79\times10^{-16}$; 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).

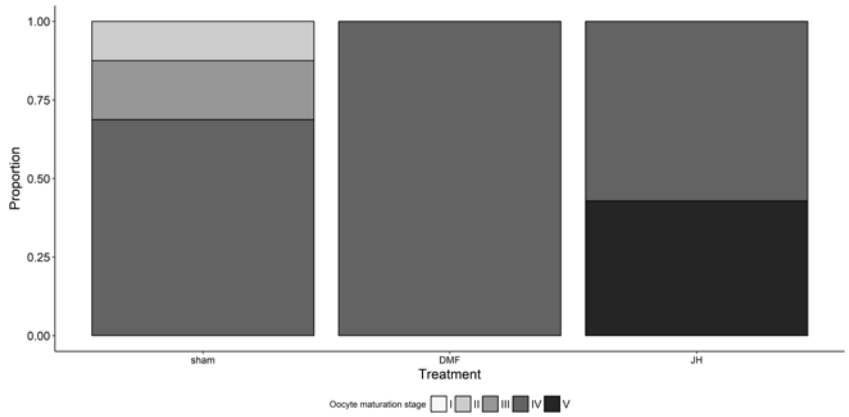


Fig. 3. Effects of JH on oocyte maturation in alkali bees. There were significant differences in stage of oocyte maturation among JH treatment groups (χ^2=23.20, P=7.31×10^{-4}, number of individual bees: sham n=16, DMF n=17, JH n=7). Shading within bars indicates the proportion of maximum viable terminal 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.

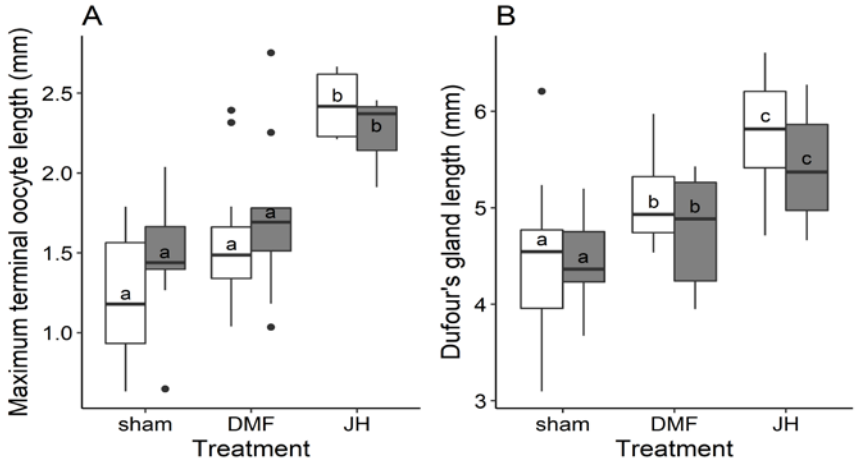


Fig. 4. Effects of endocrine and social treatments on reproductive development in alkali bees. (A) Maximum terminal oocyte length and (B) Dufour's gland length were significantly different between lab-reared females treated with juvenile hormone (JH) versus controls, but variation in the social environment did not significantly affect reproductive development (oocytes: $F_{5,41}$=6.68, r^2=0.45, P=1.23×10^{-4}; Dufour's gland: $F_{6,57}$=8.77, r^2=0.48, P=8.97×10^{-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 - $p = 0.002$).

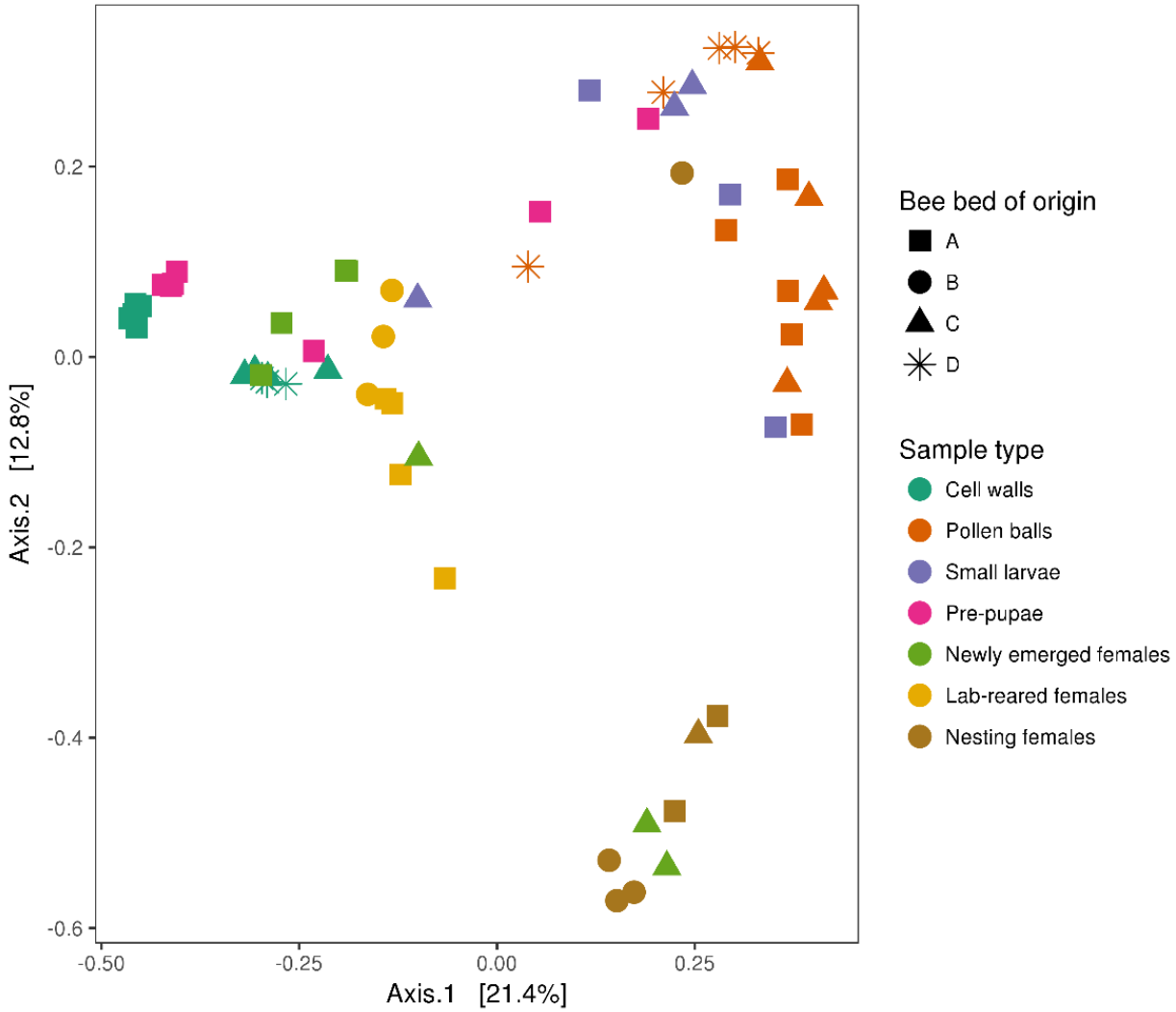


Fig. 5. PCoA ordination plot of log-transformed OTU frequencies across sample types and bee bed of origin. n = 61.

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 ($F = 2.20$, $p = 0.10$).

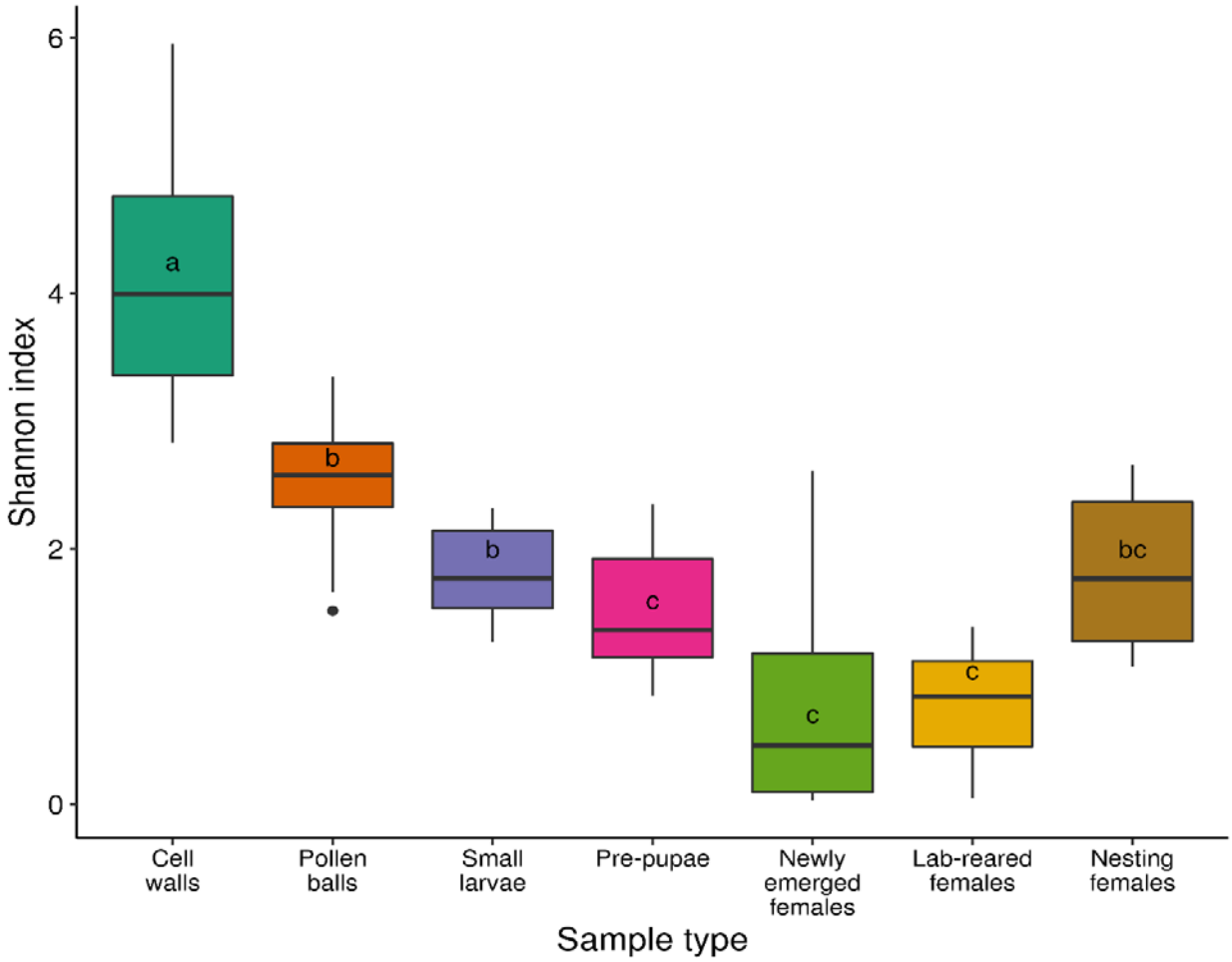


Fig. 6. Alpha diversity among sample types. Shannon index measures taxonomic diversity as a function of species richness and evenness (Anova: F= 0.45, $p=5.05x10^{-12}$. Letters indicate significant differences ($P<0.05$ in Tukey post hoc tests). Boxes represent the interquartile range, with the line 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.

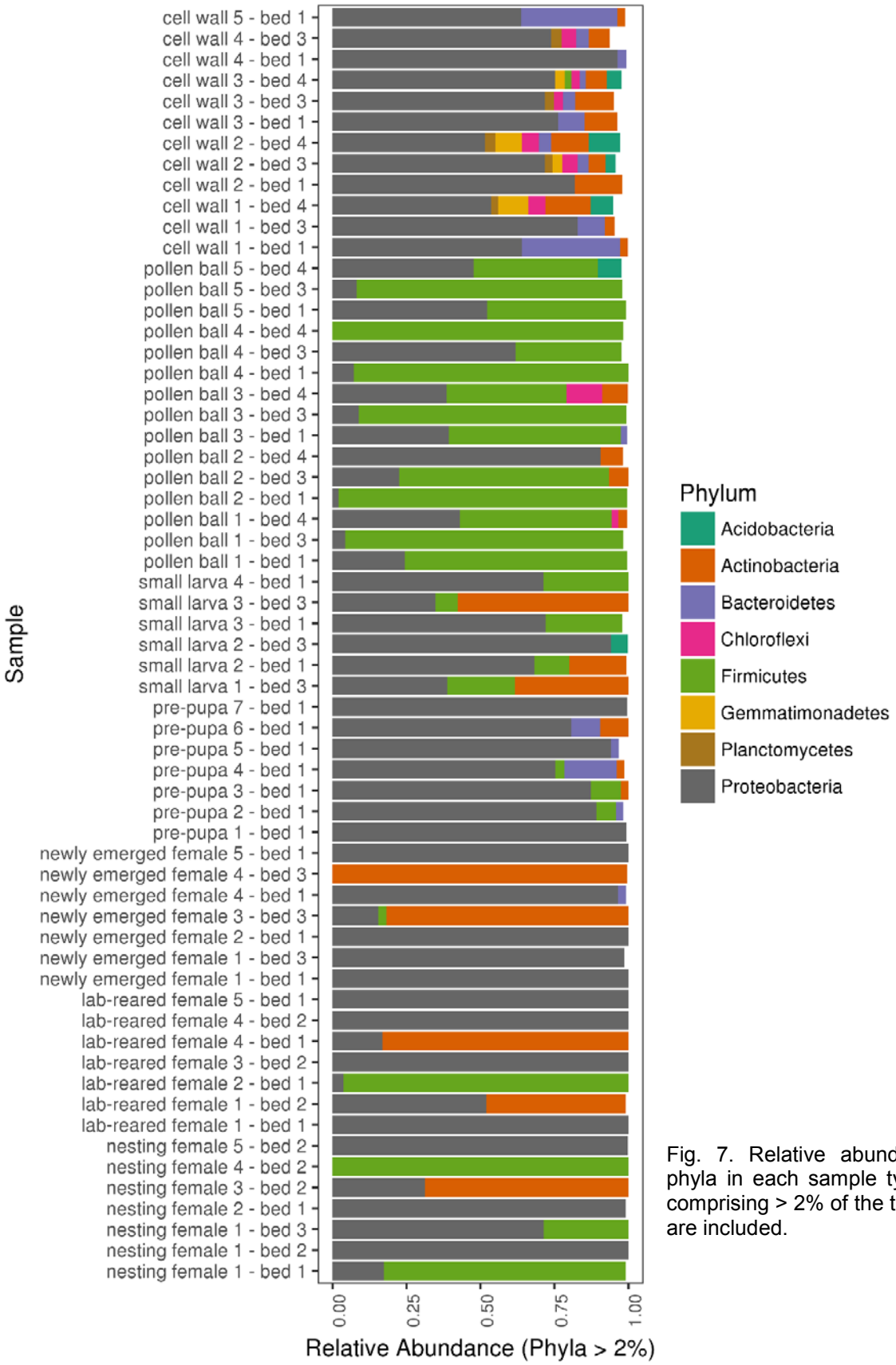


Fig. 7. Relative abundance of major phyla in each sample type. Only phyla comprising > 2% of the total microbiome are included.

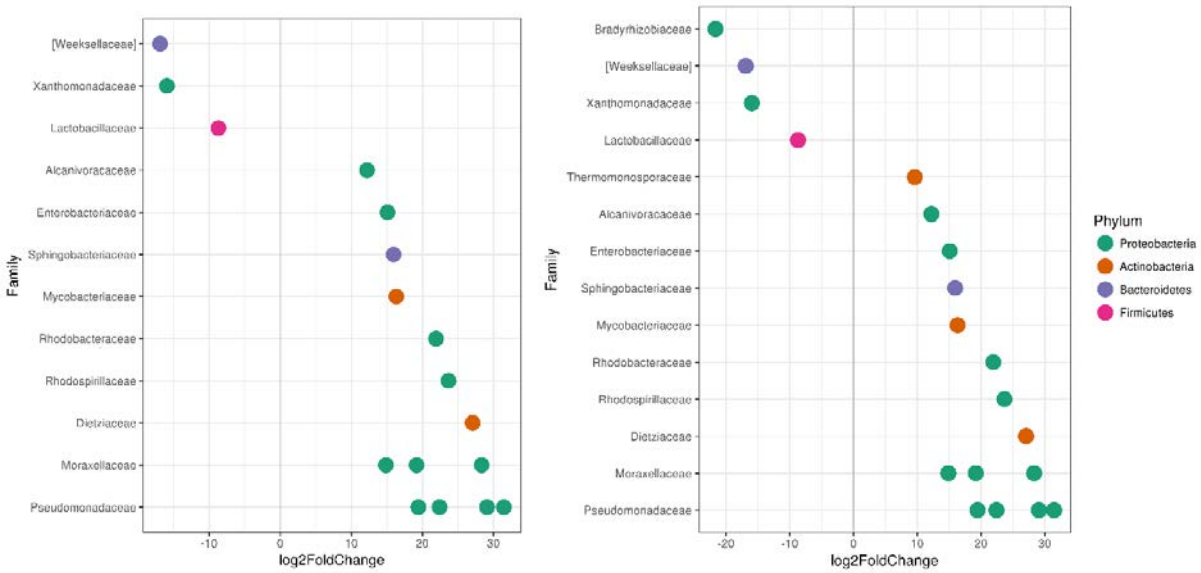


Fig. 8. Differences in abundance of gut bacteria among actively newly emerged (left) or lab-reared females (right) and actively nesting females. Log_2 fold change represents the relative abundance of each taxa in newly emerged or lab-reared females, as compared to nesting females. Points on the left 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.

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A conceptual framework that links pollinator foraging behavior to gene flow

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Introduction

The foraging behavior of pollinators can influence pollen dispersal and gene flow. When moving from flower to flower, pollinators deposit pollen on the stigmas of flowers and pick up pollen from their anthers. Genes are transferred during this process and the transfer of unwanted genes can lead to adventitious presence (Kershen and McHughen 2005). This can occur, for example, when a pollinator moves from a genetically engineered (GE) field to an organic field. In insect-pollinated crops such as alfalfa, a better understanding of how pollinator foraging behavior affects gene flow could lead to the development of management strategies to reduce gene flow and facilitate the coexistence of distinct seed-production markets.

In order to facilitate linking pollinator behavior to gene flow, we divide the process into three separate components. The first component comprises pollinator movement within patches (Fig. 1). Pollinators move between flowers, racemes and plants within patches and one must understand the behaviors that influence within patch movements and the factors that influence such behaviors. Within patch movement is equivalent to a pollinator moving within a continuous landscape. Moreover, as they move from flower to flower, pollinators deposit pollen on stigmas and pollen deposition is the second component of this process (Fig. 2).

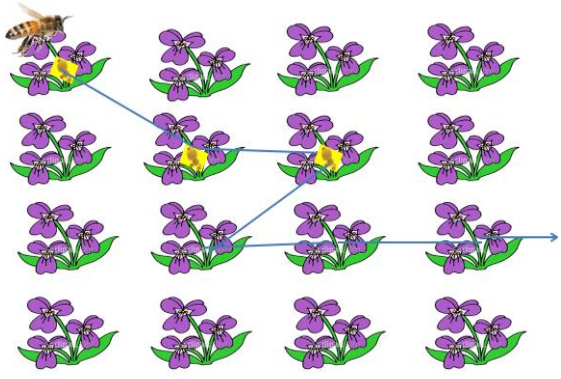

Fig. 2. Pollen deposition

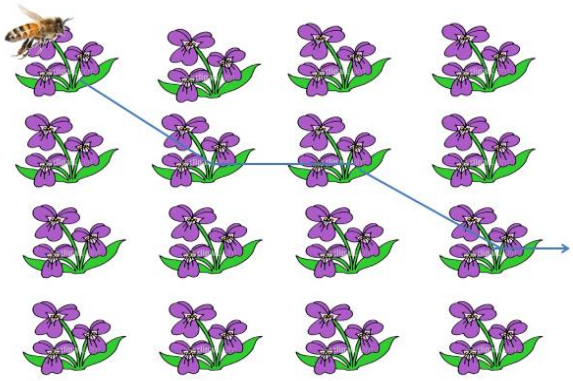

Fig. 1. Pollinator movements within a patch

Pollen deposition curves describe the number of pollen grains from a source that are deposited on stigmas of flowers visited in succession during a foraging bout. The source can represent a specific plant in a field or a patch of GE alfalfa. The steeper the pollen deposition curve, the lower the expected pollen dispersal and ensuing gene flow (Thomson and Plowright 1981; Price and Waser 1982; Castellanos et al. 2003; Richards et al. 2009). The third component of the process includes patch choice or

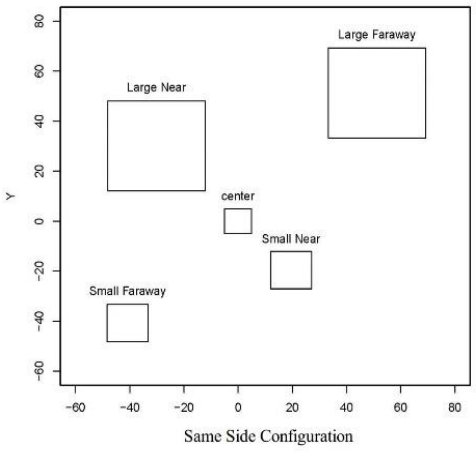

Fig. 3. Agricultural Landscape configuration

pollinator movement between patches (Fig. 3). Pollinators will tend to move between patches over the agricultural landscape and, when doing so, they must decide which patch to move to next. Although this has been a difficult question to tackle, an understanding of the patch choice decision process would greatly improve our ability to predict pollinator movements and subsequent gene flow in discontinuous landscapes. Below, we discuss the foraging behaviors that will influence each of these three components, the factors likely to affect such behaviors and the subsequent potential impact on gene flow. Our goal is to illustrate how genes are moved via pollen by pollinators as they forage over the agricultural landscape and how such process affects patterns of gene flow.

Pollinator behavior and gene flow

Pollinator movements within patches

As they forage within a patch, pollinators move between flowers, racemes and plants and travel a certain distance and direction between pairs of flowers, racemes or plants (Fig. 1). The distance traveled between racemes or plants and whether pollinators exhibit directionality of movement within foraging bouts will affect how far pollinators move in continuous landscapes (Brunet et al. 2018). Directionality of movement indicates that when a pollinator starts moving in one direction it keeps moving in the same direction throughout its foraging bout (Waddington 1980). A foraging bout represents one foraging trip in a patch by one individual pollinator. A pollinator that exhibits directionality of movement will tend to move greater net distances relative to a pollinator that moves randomly between racemes or plants (Levin et al. 1971). Net distance is the distance between where a pollinator started and ended foraging in a patch. Directionality of movement is a characteristic of a pollinator species and while leafcutting bees tend to move randomly among racemes when foraging on alfalfa flowers, honey bees and bumble bees exhibit directionality of movement within foraging bouts (Brunet et al. 2018). A bee species that moves greater net distances will tend to move pollen and genes farther distances, although the distance traveled by pollen will often be shorter than the physical distance traveled by pollinators.

Residence or the number of flowers a pollinator visits during one trip in a patch will also affect pollen dispersal and gene flow (Cresswell et al. 2002). For example, when a pollinator moves from a GE to a conventional patch of alfalfa, it carries GE pollen on its body and, as it moves from flower to flower, it deposits GE pollen on stigmas of conventional alfalfa plants while also picking up conventional pollen. The more flowers a pollinator visits within a patch, the higher its residence and the more likely all GE pollen carried on the pollinator's body will be deposited in the patch before the pollinator moves to the next patch. Therefore, the higher the residence the less gene flow and we therefore expect residence to be inversely proportional to gene flow (Cresswell et al. 2002). It is important to note here that it is the pollen located on the areas of the

pollinator's body that come into contact with the plants' stigmas that is involved in the pollination process and not the pollen in the pollen sacs; the latter is returned to the hive to feed the young and is not used for pollination.

Pollen deposition

A pollen deposition curve is specific to a given plant/pollinator species system and is known to vary among pollinator species foraging on the same plant species or for a given pollinator species foraging on different plant species (Thomson 1986; Waser 1988; Castellanos et al. 2003; Richards et al. 2009). The steeper the rate of pollen decay over successive flowers, the fewer flowers will need to be visited by a pollinator before most of the source pollen grains carried on the pollinator's body get depleted (Holmquist 2005; Karron et al. 2009). Therefore, the steeper the pollen deposition curve, the less gene flow is anticipated (Thomson and Plowright 1980; Price and Waser 1982, Castellanos et al. 2003, Richards et al. 2009) (Fig. 4). If a pollinator coming from a GE patch deposits most of the GE pollen it carries on its body onto the first 30 flowers visited in the conventional patch, relative to a second bee species that deposits most of the GE pollen onto the first 60 flowers visited, other factors being equal, the first bee species will carry pollen farther distances relative to the second. Grooming and floral nectar production may influence the shape of the pollen deposition curves (Thomson 1986; Harder 1990). Pollinators may stay longer on flowers of plant species with more copious nectar and remove more pollen from these flowers and this could influence the shape of the pollen deposition curve. Moreover, steeper pollen deposition curves have been suggested for grooming relative to non-grooming pollinator species because grooming tends to remove pollen from the pollinator's body to place it into the pollen sacs (Thomson 1986). However, distinct pollen deposition curves may also occur among bee species that are all grooming pollinators (J. Brunet, unpublished data).

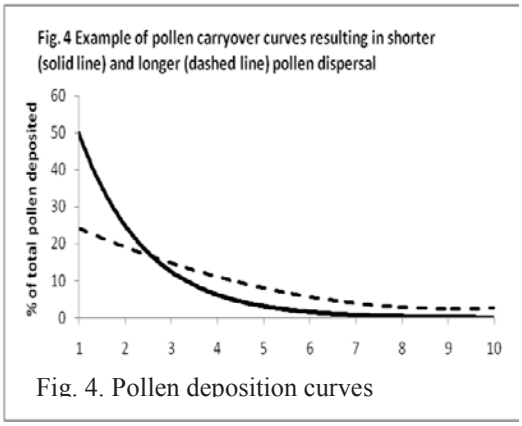

Fig. 4. Pollen deposition curves

The tripping rate can influence pollen dispersal and gene flow (Brunet et al. 2018). In certain plant species, like alfalfa, anthers and pistils must be released by bees in a process called tripping. Tripping is required for pollen deposition on stigmas and subsequent seed set. The tripping rate or the proportion of flowers visited that are tripped can vary among bee species and for a given bee species, the tripping rate can be affected by temperature (Bohart 1957; Cane 2002; Breazeale et al. 2008; Brunet and Stewart 2010; Pitts-Singer and Cane 2011). Because tripping rate influences seed set (Bauer et al. 2017) it will also affect gene flow. Current research in the Brunet laboratory indicates that pollinator species with a lower tripping rate will tend to increase the distance traveled by pollen (Brunet et al. 2018).

Pollinator movements between patches

When foraging in an alfalfa landscape, a pollinator encounters patches of alfalfa cultivars of different sizes and located at distinct distances from one another. We know that patch size, but not so much patch isolation, can affect residence with pollinators visiting more flowers in succession in larger patches (Cresswell and Osborne 2004). Larger patches may also have greater

visitation rates, they attract more pollinators (Sih and Baltus 1987), although this may not translate into more pollinator visits per plant (Kunin 1997). Pollinators must also take a decision when moving between patches and decide which patch to move to next. We know very little about the decision process used by pollinators when moving between patches. For example, pollinators could select patches randomly, select the nearest neighbor patch, or choose patches based on other rules, possibly based on patch size and patch isolation following optimal foraging theory (Pyke et al. 1977; Pyke 1984). Although this process has been difficult to study, predictions can be made for the different models of patch selection and empirically tested. In collaboration with Dr. Murray Clayton, the Brunet laboratory is generating such expectations based on distinct bee decision models and will experimentally test these alternative models of bee decision processes in the coming years. Understanding how pollinators select patches will improve our understanding of how pollinators move pollen in a discontinuous landscape.

Conclusions

The foraging behavior of pollinators can influence how far pollinators move in the agricultural landscape, how far pollinators move pollen along the way, and therefore help explain how pollinator behavior affects gene flow. Factors that influence pollinator foraging behaviors will in turn affect gene flow. Knowledge of the behaviors with a strong impact on gene flow can guide the development of management strategies to reduce gene flow and improve coexistence. Current data indicate that pollinators exhibiting random movements among racemes, high residence, steep pollen deposition curves and high tripping rate will tend to minimize gene flow. Furthermore, the layout of patches over the agricultural landscape can be used as a tool to contain gene flow. Linking pollinator behavior to gene flow can therefore guide management strategies to improve coexistence of alfalfa seed production markets.

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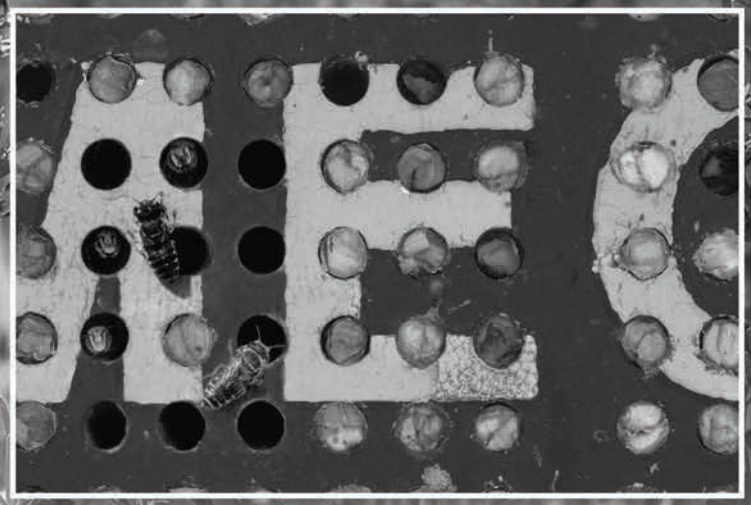

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