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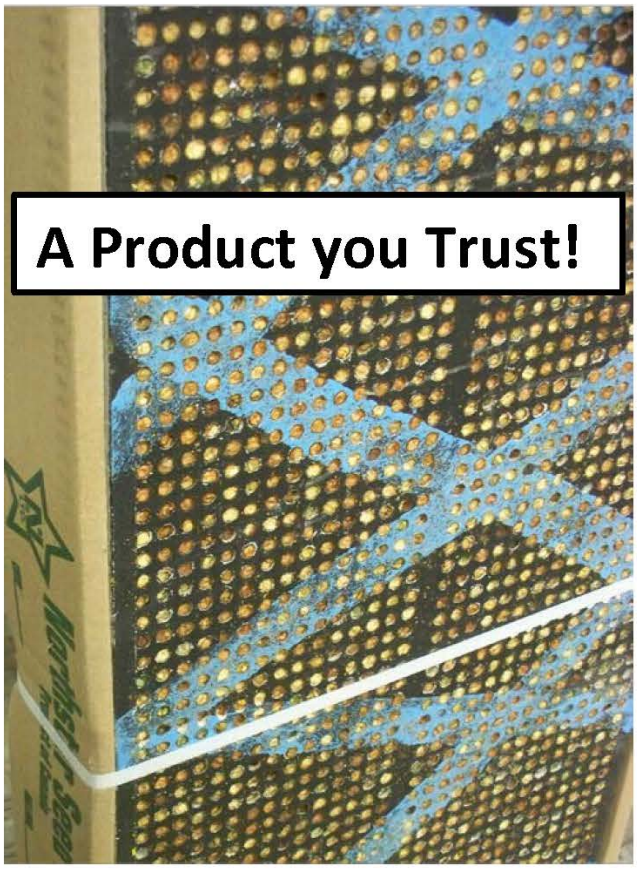

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WESTERN ALFALFA SEED GROWERS
2021 WINTER SEED CONFERENCE
VIRTUAL PROGRAM
January 25-26, 2021

Premier Conference Sponsor: ***S&W Seed Company***

Monday, January 25 ***(All Times are Pacific Time)***

Research Session – Sponsored By: Forage Genetics International

7:30 AM – **Conference Kickoff –** Shane Johnson, Western Alfalfa Seed Growers Association
7:40 AM – **Introduction of Dr. Kelsey Graham** – USDA-ARS PIRU, Logan, UT
7:50 AM – **Impact of Organosilicone Spray Adjuvants and Insecticides Upon the Health & Reproduction of the Alfalfa Leafcutting Bee** - Diana Cox-Foster, USDA-ARS PIRU Logan UT
8:15 AM – **Advanced Monitoring of Established and Emerging Pathogens and Parasitoid Infections of Alfalfa Bee (Megachile rotundata) Nest Cells Using Multiplex Technology** - Justin Clements, University of Idaho
8:40 AM – **Effects of Spring Storage Duration During Development on Reproductive Fitness in the Alfalfa Leafcutting Bee, Megachile rotundata** - Kendra Greenlee, Professor and Chair Biological Science, North Dakota State University
9:05 AM – BREAK

Research Session – Sponsored By: Corteva AgriScience

9:25 AM – **Development of Control Protocols for Melittobia Infesting Populations of Megachile rotundata –** Theresa Pitts-Singer Research Entomologist, USDA-ARS PIRU, Logan UT
9:50 AM – **Enhancing and Protecting Populations of Alfalfa Seed Pollinators –** Doug Walsh, Washington State University
10:15 AM – **Other Alfalfa Seed Research** - Doug Walsh, Washington State University
10:40 AM –**Nozzles, Drift and Understanding Droplet Size** – Mike Powers, Tee-Jet Technologies
11:40 AM – **Day One Conclusion** – Announcements for Tomorrow's Session

Tuesday, January 26 ***(All Times are Pacific Time)***

State Of… Session – Sponsored By: Northstar Seed Ltd.

7:30 AM – **Welcome –** Shane Johnson, Western Alfalfa Seed Growers Association
7:40 AM – **NAFA Update** – Beth Nelson, President, National Alfalfa & Forage Alliance
8:10 AM – **Dairy and Forage Research Center Update** - Dennis Hancock, Director of the Dairy Forage Research Center
8:50 AM – **Grower Survey** – Jerry Neufeld, County Chair, Extension Educator – Crops, University of Idaho
9:30 AM – BREAK

State Of… Session – Sponsored By: Alfalfa Partners, a Brand of S&W Seed Company

9:50 AM – **State of the Industry** – Robin Newell, S&W Seed Company
10:30 AM – **Agriculture and US Trade: Where We Are and What's Ahead Part 2** – Bill Bryant, Founder, Bryant Christie

11:30 AM – 2021 WASGA Winter Seed Conference Adjournment

Project Title: ***Impact of organosilicon spray adjuvants and insecticides upon the health and reproduction of the alfalfa leaf cutting bee***

Year(s) of Study: 2020 –2021

Lead Investigator / Affiliation:
Diana L. Cox-Foster
USDA ARS Pollinating Insects Research Unit

For alfalfa seed production, pollinators are a critical partner in the fertilization of flowers and seed set. For alfalfa, the most commonly used bees are two non-*Apis* (non-honey bee) pollinators, the alfalfa leafcutting bee, *Megachile rotundata*, and the alkali bee, *Nomia melanderi*. This proposed research focuses on the alfalfa leafcutting bee (ALCB). In order to have optimal seed set, it is critical that the bees are healthy and have maximal reproduction (i.e., creating high numbers of nest cells requiring pollen and nectar from the alfalfa plants). The act of gathering pollen and nectar fertilizes the flowers of the alfalfa for seed set. Understanding how crop management practices affect the bee health and ultimate reproduction is critical to ensuring that the bees are performing effectively.

In the last 10 years, dramatic losses of pollinators have been reported. The losses are known to have occurred in honey bees and bumble bee populations. For ALCB, the level of bee return has declined to a reported 25-40% in some areas, reflecting a poor reproductive success and possible lower levels of nesting per bee. For honey bees and the other species, several factors (the 4 P's) are contributing to the high yearly losses- pathogens, poor nutrition (pollen availability) and finally pesticide exposure. These same four factors are also observed to affect other pollinator species, with the parasites being pollinator specific. Evidence from experiments reported worldwide indicates that these factors can interact. Some of the factors that may be common across pollination of all crops are adjuvants and pesticides. Besides the active ingredients in pesticides, the "inerts" or adjuvants may be an issue (Mullin, 2015; Mullin et al, 2015; Chen et al., 2018).

One type of adjuvants being used is organosilicon spray adjuvants (OSS) that are tank mixed with the pesticides. These compounds are called super spreaders and penetrants and are reported by their manufacturers to increase the efficacy of the pesticides. (However, there are very few published studies to support this claim). These adjuvants are tank mixed with fungicides and insecticides for many crops, including alfalfa, with concentrations ranging from 300 to 5000 ppm in the tank. In addition, the organosilicons may be included in the formulated pesticides; given that the formulations are trade-secrets, the concentrations are not known.

For OSS, studies indicate that these are toxic to honey bee adults when fed in 50% sucrose, with oral LC50s for pure commercial trisiloxane surfactants ranging to below 10 ppm, and significant mortality down to 100 ppb (Mullin et al, 2015; Chen et al, 2018). These adjuvants are of concern

since trisiloxane surfactants can be detected in 60% of pollen samples from different crops (Chen and Mullin, 2013b). The impact of the organosilicons at sublethal levels affects honey bees at several levels: 1. Toxicity via oral ingestion (Chen J, Fine JD, Mullin CA. 2018. Sci Total Environ. 612:415-421); 2. Impacts on bee learning and behavior (Ciarlo TJ, Mullin CA, Frazier JL, Schmehl DR. PLoS One. 2012;7(7):e40848.); 3. Interaction with viruses to cause increased toxicity in honey bee larvae (Fine JD, Cox-Foster DL, Mullin CA. Sci Rep. 2017 Jan 16;7:40499); 4. Impacts on queen health in microcolonies and increased viral titers in workers (Cox-Foster, unpublished); and 5. Altered transcriptome with increased viral titers in workers (Thompson and Cox-Foster, unpublished). In the transcriptome work, we asked how the OSS affected the physiology of the bees, looking at gene expression. The three aspects being impacted included genes linked to metabolism, immunity, and genes potentially linked to hormone biosynthesis. For immunity, the genes impacted were those involved in anti-viral and anti-microbial defenses.

In research trials from 2017 through 2019, we began to ask how OSS affects ALCB nesting and health. In particular, we wanted to ask how OSS application affected bees via either leaf pieces used in nest cells or the pollen/nectar in the larval provision. To do this, we used two types of plants, one the bees would use for leaf pieces (buckwheat) and another for pollen/nectar (Phacelia). The plants were grown in pots and given to the bees in small cages with nesting blocks provided. Four treatment groups were used, Control (no spray), Leaf Pieces only (Buckwheat sprayed), Pollen/Nectar only (Phacelia sprayed), or both with OSS. Nesting behavior, nesting, and survivorship were monitored; at the end of the experiment, adult females from the cages and their progeny were sampled and frozen for pathogen analyses. Our findings show that OSS is having an impact by affecting nesting. Analysis of the nesting success demonstrated that there was a significantly decreased amount of nesting by the adult females when the bees were exposed to either Phacelia or both plants that had been sprayed versus the controls or leaf-piece source treated with OSS. In all of the years, OSS in the pollen/nectar resulted in a significant increase in dead larvae (last instar). In one of the years, bees exposed to OSS had a significant increase in *Mellitobia* parasitioids in their nest cells. We are currently asking about impact on viral pathogens and if this is associated with larval death.

We also performed lab exposures to OSS at different concentrations via different routes of exposure to adult females. The toxicities differed depending upon the route with the most toxic route being oral ingestions (via nectar/pollen), then topical (i.e. direct spray), and then contact (spray dried on filter paper lining the bottom of the cage). The latter was unexpected but concerning. Recent information on the fate or silicones in the environment suggests that breakdown products may volatilize and be toxic. The leaf pieces may present this toxicity to the larvae in the cell.

OSS has been shown to synergize the toxicity of a fungicide and insecticide (Propiconazole (Tilt) and Chlorantraniliprole (Altacor)) in honey bees (Cox-Foster unpublished). The 50% survivorship of the bees was significantly decreased from 25 days down to 13 days when OSS, Tilt and Altacor were ingested. This increased toxicity has been suggested for other pesticides as well by Mullin and collaborators.

For the 2020 research, we wanted to ask about the impacts of OSS on the toxicity/impacts of insecticides that are applied at bloom to control pest insects. As part of the integrated pest and pollinator management (IPPM), it will aide growers to know if OSS when added to the tank mix negatively increases the impact of the pesticides being used to control the pests. Unfortunately, COVID-19 disrupted most of our research plans. We did learn about the fate of OSS in the field and its interactions with plants. Our findings suggest that OSS may impact the exposure bees to the pesticides being used to treat pests and allow for greater persistence/exposure to pesticides and the adjuvants via the pollen and potentially the nectar.

Through carefully controlled experiments done in collaboration with Dr. William Doucette and Autumn Slade at Utah State University, we now know how stable organosilicon spray adjuvants are and that they are transported in plants via root uptake. Given this uptake, we also expect that organosilicon spray adjuvants will be absorbed into the plant via foliar application. In the root-uptake studies, the majority of the spray adjuvant and its byproducts were moved to the leaves, and also to flowers and fruit. This uptake and movement may be responsible for greater exposure to bees to pesticides in pollen and nectar, since the OSS would mobilize more hydrophobic or water-insoluble residues and potentially increase their concentrations in pollen and nectar.

Studies were also done in almonds to ask about the concentrations of OSS that bees see after spray and after 24 hours. Samples of the tank mix, flowers, foliage, and pollen (collected by honey bees) were taken from two separate orchards. Samples were either frozen in liquid nitrogen immediately, frozen at -20 °F for an extended time, or left at ambient temperature. For up to 30 hours (last time point sampled), the pollen had concentrations of OSS matching that in the tank mix. The sample breakdown at different temperatures demonstrated that OSS can breakdown at ambient temperature and at normal freezer temperatures. For accurate detections, it is essential to freeze samples using liquid nitrogen for transport back to the analytical lab.

Plants were found to break down OSS. The breakdown process did not inactivate the OSS, but converted the polymeric OSS into smaller components. Some of these components may match forms of organosilicon that have been of concern by different regulatory agencies. Some of the smaller forms can volatilize, and data in mammals suggests toxicity and health issues. Potentially it is these smaller components that underlie some of the toxicity that we have previously found in alfalfa leafcutting bees and other bee species.

Additional research is needed to ask how plant stress impacts the breakdown. Some stressed plants were found to take up OSS at extremely high concentrations. Experiments did find that increased salinity was not linked to this increased uptake/retention.

COVID-19 prevented us from doing the field experiments that we had planned and also from traveling to visit growers to take samples. In future years, we would like to re-initiate the experiments to test other adjuvants and their impacts on bees.

We did initiate other research that could be more easily done in the lab under the restrictions related to COVID-19. We were able to generate preliminary data on modified diets that could be used to sustain and possibly ensure the health of alfalfa leafcutting bees if the bees need to be held in storage to match the bloom of fields. We found that we can feed newly emerged adult female bees either 30% sucrose solution or a mixture of amino acids/30% sucrose.

Nectar of bee-pollinated flowers contains amino acids at a significant, but low concentration. This nectar may help adult alfalfa leafcutting bees to accumulate the necessary protein resources needed to produce eggs. Egg production is likely to be linked to nest cell formation. We hypothesize that female nutrition, her capacity to produce eggs, and nest cell formation are integral to the bee's performance in pollinating seed crops.

In our preliminary studies, we compared single amino acid mixtures to a mixture of essential amino acids. The choices of single amino acids were based on published research that defined the taste preferences of honey bees. We found that female alfalfa leafcutting bees had similar preferences as honey bees. Importantly, we found that a mixture of amino acids at concentrations comparable to the amino acid concentration in flowers gave the best results in terms of promoting life span and also in consumption rate. For both 30% sucrose and the mixture of essential amino acids/30% sucrose, the female bees live over 20 days following emergence and up to 48 days. It is important to note that the mixture of essential amino acids is more easily obtained and less expensive, potentially making this a cost-effective measure.

With Dr. Pitts-Singer, a new project is being proposed to ask how the physiological performance (nutritional makeup) of alfalfa leafcutting bees matches the performance as a pollinator of alfalfa seed crops. The project will ask how release of newly emerged bees can be delayed by storage of adult bees and still preserve their performance as pollinators.

(Growers who would like more information or actual data are encouraged to contact Dr. Diana Cox-Foster. She would be glad to discuss the research findings in greater detail.)

2020 Report of Work for Alfalfa Pollinator Research Initiative

Project Title: Advanced Monitoring of Established and Emerging Pathogens and Parasitoid Infections of Alfalfa Leafcutting Bee (*Megachile rotundata*) Nest Cells using Multiplex Technology

Year(s) of Study: Year 1 (FY2020)

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Introduction

Alfalfa (*Medicago sativa*) is an important agricultural commodity with high production value throughout the United States. For the continued success of alfalfa production, pollination by robust populations of alfalfa-pollinating bees is needed. The alfalfa leafcutting bee (*Megachile rotundata*) is one of the primary pollinators of alfalfa plants. However, multiple pathogens and parasitoids can prey on the developing larvae of this species, resulting in the loss of the future adult bee and reduction in pollination efficiency. The University of Idaho Parma Cocoon Testing Laboratory is a non-profit, extension-oriented service focused on providing statistically accurate estimates of the proportion of live cells, chalkbrood mortality, pollen ball mortality and parasitoids of the alfalfa leafcutting bee nest cells to growers using X-ray diagnostics. In the current proposal, we are investigating whether we can develop a multiplex molecular diagnostic assay to complement the already existing x-ray technology. This technology would allow us to quickly confirm the accuracy of visual assessments and examine samples for newly emerging pathogens and parasitoids.

In 2020 Justin Clements and his technical staff worked together to complete the objectives for year one of this ongoing study.

Objectives for Year 1 & 2

Objective 1) Enhance, confirm, and investigate established and emerging pathogens and parasitoid infections of alfalfa leafcutting bees to provide reliable pollinator health guidelines to growers and add scientific knowledge to the academic community. (Year 1)

- Compile known pathogen and parasitoids nucleic acid sequences of leafcutter bees (as determined through extension and literature searches) to be further analyzed.
- Develop primers to conserved genomic regions of each pathogen and parasitoid. Within the primer set, we will also include universal fungal, bacterial, and invertebrate primers for the detection of new pathogens.
- Analyze alfalfa leafcutting bee nest cells using the diagnostic cocoon x-ray facility at the Parma Research and Extension Center and extract total DNA from leafcutter bee cells from both healthy and infected cells.
- Working with Floodlight Genomics, develop and run a multiplex assay to detect and identify pathogens and parasitoids of leafcutter bee cells. Mine sequencing results for new pathogens and conserved DNA regions to develop a lab based molecular assay.

Objective 2) Validate our multiplex technology over two growing seasons and develop a lab based diagnostic technique to confirm hard to identify pathogens. (Year 2)

- Using sequence data acquired from year one of the study, develop DNA primers to hard to classify pathogens to build a lab based multiplex assay

Objective 3) Our third objective is to have a multiplex technique available for the quick identification of emerging pathogens and parasitoids of leafcutter bees in the future. (Year 2)

- Have primers on hand and methods developed for molecular analysis capable of identifying parasitoids and pathogens of leafcutter bee cells quickly and accurately with a lab-based assay.

Report for Year One (Objective 1)

Towards the completion of objective 1, we have conducted an extensive literature review to explore known pathogen and parasitoids of alfalfa leafcutter bee cells. We compiled genomic sequences obtained from the National Center for Biotechnology Information (NCBI). In total we complied 930 genomic sequences, not including bacterial reads. From the genomic reads, we determined unique regions within the sequences to design multiplex primers. Primers and amplicon sequences were BLASTED against the NCBI reference database to confirm that the nucleic acid sequence could be used to distinguish the desired species. Target species, primers, and the target sequence can be found in Table 1.

Table 1: Pathogen and parasitoid targets for amplification in the multiplex reaction and corresponding primers

Target	Species	Optimal sequence for primer construction	Forward	Reverse
Mites	Muitple mite species	Microsatellite Loci for Varroa destructor VDOO1 (155bp) - Motive ((GA)10. (GA)6. (GA)5)	CCCGCGAACGAAATAAATAGAG	AGCCACCTACGGTGCTCG
Mites	Mutiple mite species	Microsatellite Loci for Varroa destructor VDO15 (143bp) - Motive ((GT)17)	GCGCAAACTTAACGCTCG	TCAAGCCAGAGTGCTGCAG
Bacterial	Mulitple bacteria species	V3-V4 (338f - 518r)	ACTCCTACGGGGAGGCAGCA	ATTACCGCGGCTGCTGG
Bacterial	Mulitple bacteria species	V4-V5 (517f - 798r)	AGCAGCCGCGGTAAT	AGGGTATCTAATCCT
Bacterial	Mulitple bacteria species	V6-V7 (917f - 1110r)	GAATTGACGGGGACCC	GGGTTGCGCTCGTTA
Bacterial	Mulitple bacteria species	V6-V7 (917f - 1110r) -Primer set 2	GAATTGACGGGGGCCC	GGGTTGCGCTCGTTG
Bacterial	Mulitple bacteria species	V8-V9 (1407f - 1541r)	GTACACACCGCCCGTC	AAGGAGGTGATCCAACCGCA
Bacterial	Mulitple bacteria species	V8-V9 (1407f - 1541r) -Primer set 2	GCACACACCGCCCGTC	AAGGAGGTGATCCAGCCGCA
Insects	*Megachile ericetorum*	sc232 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	AGGATGAACAGTTTATCCTCCCT	TGATCCAATAATAGATGAAATTCCCGA
Fungal	*Ascosphaera aggregate*	18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	CGTCATTGCAACCCTCAAGC	AAGAACATGATTGATCTTGG
Fungal	*Ascosphaera larvis*	18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	CGTCATTGCAACCCTCAAGC	AGGCCACAAGAGCGGAAGAC
Fungal	Amplifies both (*Ascosphaera aggregate* and *Ascosphaera larvis*)	5.8S ribosomal Spacer	GTGAAGCGGCAAAAGCTCAA	GTCGAAGGAGCTCTACACGG
Fungal	Amplifies both (*Ascosphaera aggregate* and *Ascosphaera larvis*)	5.8S ribosomal Spacer	AACGTGTGTCTGTGCGG	CTTCGACTGGAGTTCGTTT
Insect parasites	*Tribolium audax*	Cytochrome c oxidase	GGAGGATCATCCGTAGACCT	CCTGCTAGTACGGGTAGAGA
Insect parasites	*Pteromalus venustus*	Cytochrome c oxidase	CAGGATGAACTGTTTATCCACCA	TTGCAGTTAATAATATCGATCATGCT
Insect parasites	*Trichodes ornatus*	Cytochrome c oxidase	CTGGTTGAACTGTTTATCCCCC	TGCTCATACAAATAATGGAGTTCGG
Insect parasites	*Epeoloides pilosula*	Cytochrome c oxidase	TCTGGAATTTCCTCTATTTTAGGTGC	ATTGCTCCAGCTAATACGGGT
Insect parasites	*Monodontomerus obscurus*	Cytochrome c oxidase	ACATATTGCTGGTGTTTCATCAA	AAATAACAATATTGTAATAGCACCAGC
Insect parasites	*Nemognatha lutea*	Cytochrome c oxidase	ATCAACATACGACCTGCAGG	TAGGATCTCCACCTCCTGCA
Insect parasites	*Sapyga pumila*	Cytochrome c oxidase	TCTTCATATTGCAGGAATTTCCTCA	GCACCAGCAAGAACAGGTAA
Insect parasites	*Leucospis affinis*	Cytochrome c oxidase	CCAGTTATAATAGGTGGATTTGGCA	CCAGTTCCTGTACCTGATCCA
Insect parasites	*Tribolium audax*	Cytochrome c oxidase	CACAGTAGGAGGATTAACTGGG	ACATAGTGGAAATGTGCAACTACG
Insect parasites	*Tribolium brevicornis*	Cytochrome c oxidase	CACAGTAGGAGGACTAACAGGAG	ACATAGTGGAAATGTGCAACTACA
Insect parasites	*Monodontomerus obscurus*	Cytochrome c oxidase	TACTGTAGGGGGATTAACAG	ACATAATGAAAATGAGCTAC
Insect parasites	*Epeoloides pilosula*	28s ribosomal RNA	CCTAGTAGAACGTCGCGACC	TGACGCACACACATTCGAGA
Insect parasites	*Leucospis affinis*	28s ribosomal RNA	TCGTCTCGCTCGGATTTACG	CAATGACTCGCGCACATGTT
Insect parasites	*Sapyga pumila*	28s ribosomal RNA	AATGGTATACGGCCGCAGTC	CCCGAGAGTACCCAAAGCAG
Insect parasites	*Tribolium brevicornis*	28s ribosomal RNA	GTAGGACGTCGCGATCCTTT	CACTAGGTCCGATCGCCATC
Insect parasites	*Trichodes ornatus*	28s ribosomal RNA	TCGAGCGACGGTTGATCATT	CCATCTGTCTACCGTCGAGC

Leafcutter bee cells were acquired from multiple agricultural producers from Idaho, Oregon, Washington, and Canada in 2020. Bee cells were x-rayed using a Faxitron machine at the Parma

Research and Extension Center. Approximately 750 bee cells from multiple locations were identified as healthy, infected with different pathogens, or unknown, and placed in 1.5 ml microcentrifuge tubes for DNA extraction.

Preliminary DNA extractions were conducted with multiple different published methods. Methods explored included a kit-based assays from OPS diagnostics, the classical cetyl trimethylammonium bromide (CTAB) methodology, and a modified CTAB extraction method which we developed in-house. Different homogenization techniques were also tested to grind and homogenize bee cells. Each method successfully extracted DNA from bee cells, as determined through the use of a nanodrop machine and running samples on agarose gel.

200 bee cells were extracted using OPS diagnostics SYNERGY™ Plant DNA Extraction Kit, due to the ease of use. A subset of 10 samples was submitted to Floodlight Genomics LLC for preliminary DNA amplification and multiplex amplification using primers in Table 1. Floodlight Genomics conducted the preliminary assay at no charge to the PI.

The multiplex amplification was unable to generate high quality reads. We determined this was caused by contaminants left behind during the extraction. Methods for DNA extraction were reassessed to remove containments from DNA samples. Multiple trials and extractions were conducted. We developed a modified CTAB reaction in which we were able to further purify DNA, as shown in Figure 1 1. From the modified CTAB reaction, we were able to amplify the cytochrome c oxidase gene from leafcutter bee cells using primers developed by Folmer et al. 1994. We were unable to amplify cytochrome c oxidase gene from DNA extracted using OSP kit-based assay or classical CTAB methodology.

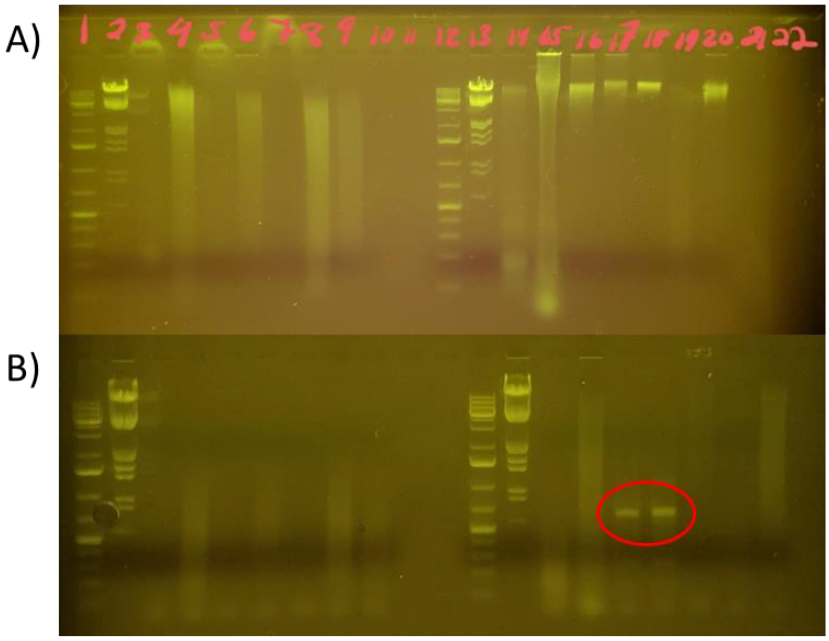

Figure 1: A) DNA extracted using OSP kit-based assay (rows 3-9) and DNA extracted using modified CTAB method (rows 14-20). B) PCR amplification of the cytochrome c oxidase gene of the same samples listed in A. Highlighted in red is the amplified cytochrome c oxidase product.

Even using the modified CTAB reaction, we were unable to amplify the cytochrome c oxidase gene from all preliminary leafcutter bee samples. To confirm that only high-quality DNA samples are being sent to Floodlight Genomics for multiplex analysis, we have started to re-extract DNA from leafcutter bee cell samples and confirm the purity of DNA though gel-based visualization and PCR amplification. Samples are currently being complied to send to Floodlight Genomics for the completion of the multiplex assay, at which point DNA sequence reads will be provided to Justin Clements and analyzed using Geneious software. We anticipate all of year one objectives will be completed with the awarded budget for year 1.

Conclusion

The development of a molecular assay using multiplex technology will advance the current diagnostic techniques to improve the current estimate of pollinator health. With the development of new diagnostic techniques, we will be able to quickly confirm the accuracy of visual assays and examine samples for newly emerging pathogens and parasitoids. We will further use this technology to assess the overall health of populations of native alfalfa pollinators.

Acknowledgements

We would like to acknowledge the support from USDA Alfalfa Pollinator Research Initiative for funding this research, support from local growers for providing bee samples, and Floodlight Genomics LLC for trouble shooting and technical assistance with the multiplex assay.

Effects of spring storage duration during development on reproductive fitness in the alfalfa leafcutting bee, *Megachile rotundata* (Year 1 Report)

Lead Investigator: Kendra J. Greenlee[1]
Collaborating Investigators: Kayla N. Earls[1], Joseph P. Rinehart[2]

[1] North Dakota State University, Biological Sciences Department, Fargo, North Dakota
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Introduction

Developing *Megachile rotundata* may be exposed to low temperature to slow development to more accurately time adult emergence with alfalfa bloom. Exposure to low temperatures can result in chill injuries, ranging in intensity based on the duration and severity of the low temperature exposure. Surviving insects may have long term sub-lethal effects that can reduce reproductive output. Sub-lethal effects can include morphological deformities, reduced flight performance, and altered behavior.

Megachile rotundata pupae were exposed to low temperature treatments that varied in temperature, duration, and thermal profile (fluctuating or static). Treatments included one- and four-week exposures to a fluctuating regime (FTR), consisting of a base temperature of 6 °C with a daily one hour exposure to 20°C, with one hour ramps up and down. Treatments also included static temperature treatments of 6°C and 10°C (static temperature regime, STR). Our previous research showed that one week of fluctuating low temperatures did not reduce reproductive fitness. Because mortality in FTR does not decrease until 4 weeks of exposure, we aimed to determine if longer durations of storage would reduce fitness. Previous research showed that bees exposed to 6°C STR for one week were less likely to nest. We also tested whether reproductive fitness was affected by exposure to 10°C STR treatment, because this temperature is used by farmers in Canada. This research directly relates to multiple APRI research topics, including management, production, and safety. Results may provide insight on seasonal bee loss and identify ways enhance spring storage of *M. rotundata.*

Main APRI Objective: To determine the effects of spring storage length during development on reproductive fitness and adult *M. rotundata* physiology and morphology

APRI Objectives Year 1

1. Measure reproductive fitness of treated bees in the field.
2. Take physiological measurements of treated bees to determine flight capabilities.
3. Examine internal morphological abnormalities in treated bees.

Report and Results for Year 1

1. Due to complications out of our control, including covid-19 and bad weather, there was little reproduction in any of the tents. Despite our previous success with bees nesting in the tents, nesting may have been inhibited by unfavorable environmental conditions inside the tents (variability in light levels) across the various treatments. In addition, the likelihood of nesting in two of the treatments (4-week FTR, 6°C STR) may have been exceptionally low based on the results of the flight performance tests in objective 2. Therefore, we were unable to assess reproduction in year 1. We plan to repeat these experiments in year 2 using nest boxes placed

in the field without tents. We will use emerged adult bees from the three treatments with viable bees (control, 1-week FTR, and 1-week STR at 10°C). Bees will be released at small nesting blocks containing 144 nesting cavities in a 12 x 12 array. Boxes will be placed 200 m apart along the edge of an alfalfa field, with nesting activity assessed twice a week throughout the nesting period.

2. Using a flight performance test, we determined that ability to fly differs significantly depending on temperature treatment and sex. In all treatments, except for 6°C STR, females were more likely to fly. Bees in the 6°C STR and 4-week FTR had the worst performance with 60% of females and 55% of males flying in the STR treatment. Surprisingly, bees exposed to 4-week FTR fared even worse, with only 31% of females and 9.5% of males able to fly.
3. Due to the fact that morphological abnormalities were easy to assess, we decided to pivot away from the proposed method of micro CT, which is very complicated and time consuming, instead focusing on other parameters that could better indicate the overall health and fitness of bees following cold storage. Because bee deformities mimicked phenotypes observed with many insect viruses, we sent samples for preliminary assessment for presence of virus. Emerged adult bees were flash frozen and sent to the National Agricultural Genotyping Center located in Fargo where they were tested for the presence of Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Lake Sinai Virus 2 (LSV-2), American Foulbrood (AFB), European Foulbrood (EFB), Sacbrood Virus (SBV), Israeli Acute Bee Paralysis Virus (IABPV), Slow Bee Paralysis Virus (SBPV), Lake Sinai Virus 1 (LSV-1), Deformed Wing Virus (DWV), Chronic Bee Paralysis Virus (CBPV), Varroa Destructor Virus 1 (VDV1), Kashmir Bee Virus (KBV), Nosema apis (Napis), and Nosema ceranae (Ncera). No bees had detectable levels of virus. To rule out that bees were able to clear infections after pupation, we plan to add additional time points during temperature treatments.

Conclusion

Research this year came with mixed success. Next summer, we will repeat the field experiment in objective 1 with updated methods to get better nesting. Successes include summer undergraduates able to take physiological measurements of treated bees. Videos and pictures of deformities, including examples of how physiological measurements were performed will become available to farmers and researchers. Additional virus testing will be conducted to rule out that the physiological deformities are caused by infection and not low temperature exposure.

Report for 2020 Project: Development of control protocols for *Melittobia* infesting populations of *Megachile rotundata*

Theresa L. Pitts-Singer, USDA ARS Pollinating Insects Research Unit

Introduction

Populations of alfalfa leafcutting bees (ALCB; *Megachile rotundata*) used as pollinators for alfalfa seed production in the western United States often suffer high parasitism rates by various wasps. Parasitism by *Melittobia* (Eulophidae) has greatly alarmed bee managers due to their quick generation time and high biotic potential (witnessed by bee managers and validated in 2019 studies). There are currently no definitive, recommended control measures for this pest.

Our 2019 research yielded the alarming result that one ALCB cell parasitized with *Melittobia* could result in about 90% infestation of 100 healthy ALCB prepupal cells that had been incubated in a common container. Furthermore, an ALCB cell containing several adult female *Melittobia* served as a reservoir from which those females could exit the cell and, over several days, invade other healthy prepupal cells and lay eggs. Lastly, in 2019, we found that the base developmental temperature for *Melittobia* is 12.6°C. These wasps develop from egg to the overwintering prepupal stage at temperatures from 15-25°C, and can complete development to the adult stage at temperatures from 25-35°C. There is also some evidence that storing *Melittobia*-infested cells at constant cold temperatures (4°C) in the fall can greatly increase overwintering wasp mortality compared to similar cells left at fluctuating ambient temperature for most of the storage period.

We chose two phases, incubation and fall-winter, of ALCB management for exploring measures to control *Melittobia*. For the incubation study, we examined the use of dichlorvos (Vapona) pest strips during incubation to kill *Melittobia* as it does the chalcid wasps *Pteromalus venustus* and *Tetrastichus megachilidis* (Fig. 1). For the fall-winter study, we sought to understand whether leaving bee cells in nests at ambient temperature in the fall allows time for *Melittobia* reproduction (i.e., infestation of ALCB cells) before bees are placed into cold storage and whether early cold storage reduces *Melittobia* survival over the winter.

Methods: Control of *Melittobia* and *T. megachilidis* during ALCB Incubation

Two incubators in our quarantined shop (away from other bee rearing equipment) were set at 30°C for emerging the wasps and bees. To mimic ALCB incubation "trays," we employed Ziploc food containers (square, 5 cup-size) and created screened vents on two sides (Fig. 2). In each of four containers, we added approx. 1,700-1,800 healthy ALCB cells so that the container was about half filled, and also added 10 each of overwintered ALCB cells parasitized by *Melittobia* and *T. megachilidis* (Fig. 1). (We did not have enough cells parasitized by *P. venustus* to use for this experiment.) Two of these bee cell containers were placed onto each of four cafeteria trays. We surrounded the containers on the tray with yellow sticky cards (3×5 Sensor Cards, BASF Corp.) for collecting any emerged wasps. Additional sticky cards were hung above the bee cell containers, and several other

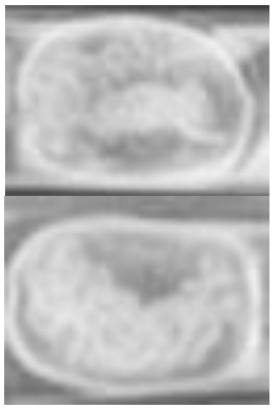

Figure 1. X-radiograph or alfalfa leafcuttng bee cell parasitized by *Melittobia* (top) and *Tetrastichus megachilidis* (bottom).

cafeteria trays were covered in sticky cards in case the wasps dispersed far from the bee cell containers.

Two of the cafeteria trays with bees (total of 4 bee cell containers) were placed on middle shelves of one of two incubators. For one incubator (a large gorwht chamber), a piece of dichlorvos pest strip (Hot Shot No-Pest Strip) was cut to match the size needed for this chamber (2.6187 g of the strip, representing 0.75 strip per 1000 ft^3 recommended by Bitner ALCB Management Calendar). This insecticide was added to the incubator on the third day after incubation began (Day 1, August 3 = incubation start date; Day 4, August 6 = pest strip added). Two oscillating fans inside the incubator were run on the lowest setting to assure insecticide vapors were moved about the chamber. The insecticide was removed on Day 11, August 13, and the incubator door was left open for 24 hours with the fans blowing to flush out vapors. Because it was hot in the shop, the opened incubator temperature reached 34.7°C for a few hours during the time the door was open.

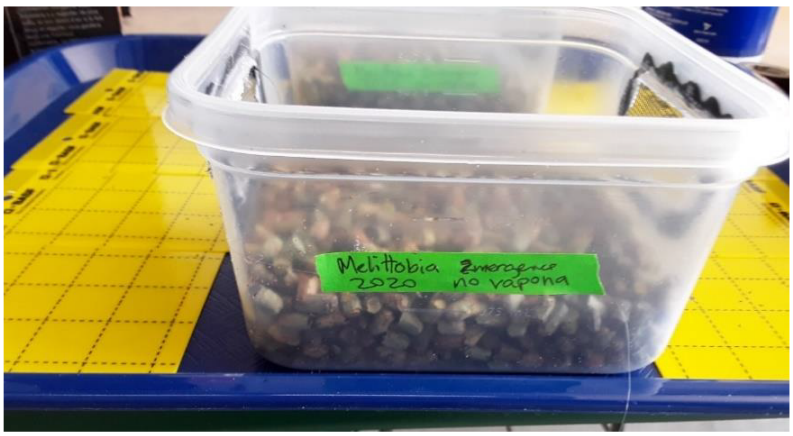

Figure 2. Example of cafeteria tray with two bee cell containers surrounded by sticky cards to trap emerged adult parasitic wasps.

Starting on Day 4, the incubators were inspected for the presence of wasps anywhere in the incubators (on sticky cards or flitting about). Wasps on all cards were counted and recorded daily until numbers were so high (>1000) that this task was not possible; thus, the number of wasps was estimated. Lids were placed onto the containers after the insecticide was removed so that future emerging bees could not escape. The first male bees were observed on 17 August, and the first females observed on 19 August. Some bees chewed through the window screening on the vents and were captured on the sticky cards. Daily, containers were opened to allow bees to quickly fly out of the containers into the local field.

The study ended on Day 26, August 28, when it was assumed that most bees had emerged. The cells in the containers were frozen to kill any live wasps, and inherently any unemerged bees. Then, all the cells from which a bee successfully emerged were counted. Also, cells were examined using x-ray diagnosis or by cutting them open for further inspection so that the number of dead bee adults, dead bee prepupae, dead bee pupae, and newly parasitized cells could be counted. Additionally, for only the incubator that was not treated with dichlorvos, a final total count of all wasps (on only the sticky cards on the cafeteria trays with the bee cell containers) was recorded by upper and lower tray. No wasps were counted on the hanging cards or extra cafeteria trays at the end of the study. Very few wasps were seen at any time in the incubator with the dichlorvos added.

Incubation Study Results

Overwhelmingly, the dichlorvos killed parasitic wasps that emerged from ALCB cells. For the incubator with the dichlorvos added, only 20 *Melittobia* and 17 *T. megachilidis* adults

were found on Days 8-12. In the untreated incubator, wasps on any of the sticky cards were first observed three days after incubation started, and the accumulation of wasps in the incubator

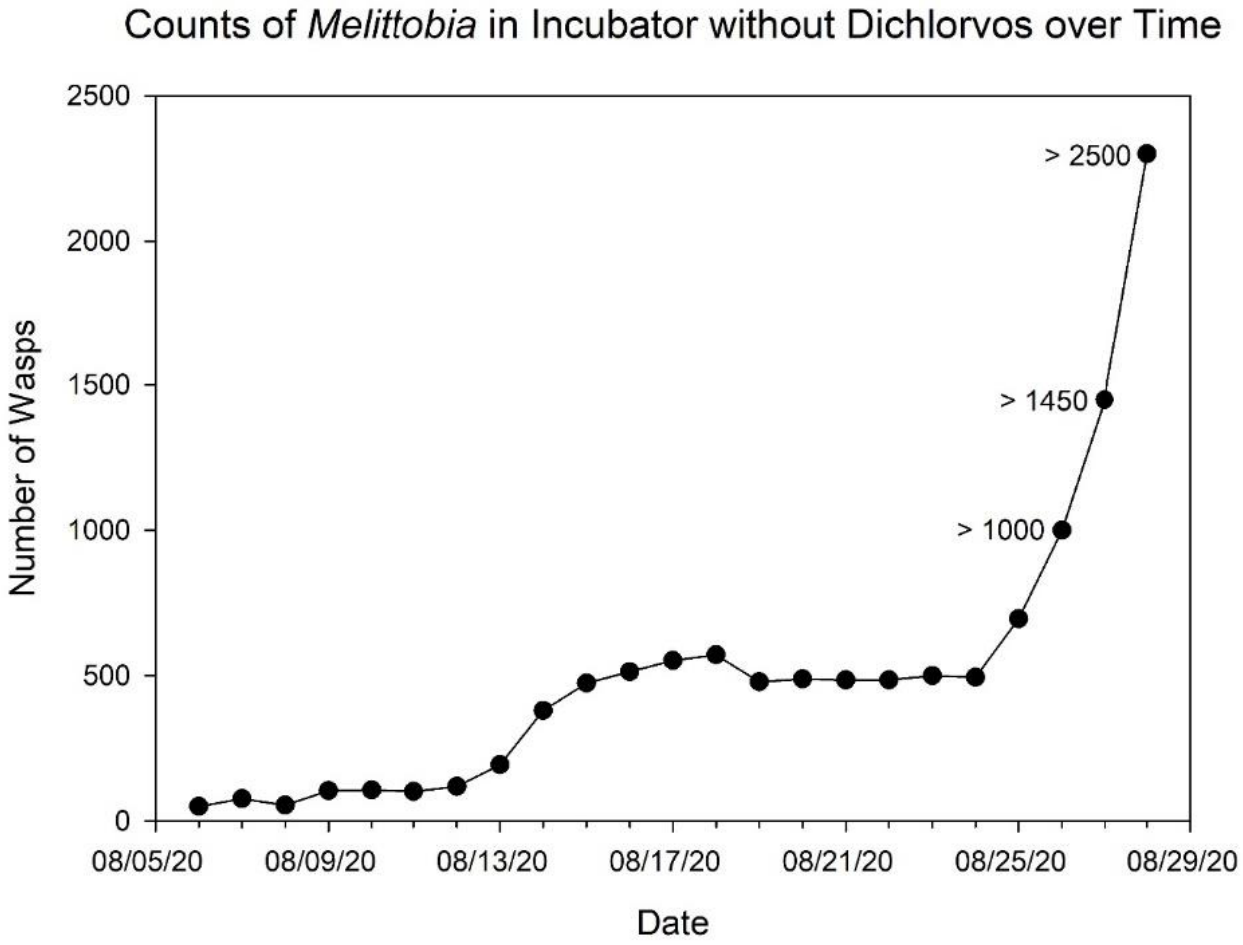

Figure 3. Over time, the cumulative number of adult *Melittobia* observed after ALCB cells in containers inoculated with *Melittobia*-infested cells were placed in 30ºC incubator. High wasp levels were estimated.

increased to over 500 after ~ 14 days and to thousands after ~ 24 days (Fig. 3).

The final total count of wasps on only the sticky cards next to the cell containers on the cafeteria tray revealed thousands of *Melittobia* (n > 5,700) and a few hundred *T. megachilidis* (n > 300) (Fig. 4).

Examination and counts of the contents of bee cells from both incubators frozen after the experiment (Fig. 5) showed very little new parasitism of bee cells in containers in the treated incubator compared to the untreated incubator. 90% of adult bees were accounted for in containers in the treated incubator (emerged adults = 58%, dead adults in cocoons = 31%), while only 61% of adults were accounted for in containers in the untreated incubator (emerged adults = 37%, dead adults in cocoons = 24%). In the incubator where no dichlorvos was used, >29% of cells (n = ~2,000) examined contained developing *Melittobia* that would emergence as adults after bee trays would have been taken to the field (Fig. 5).

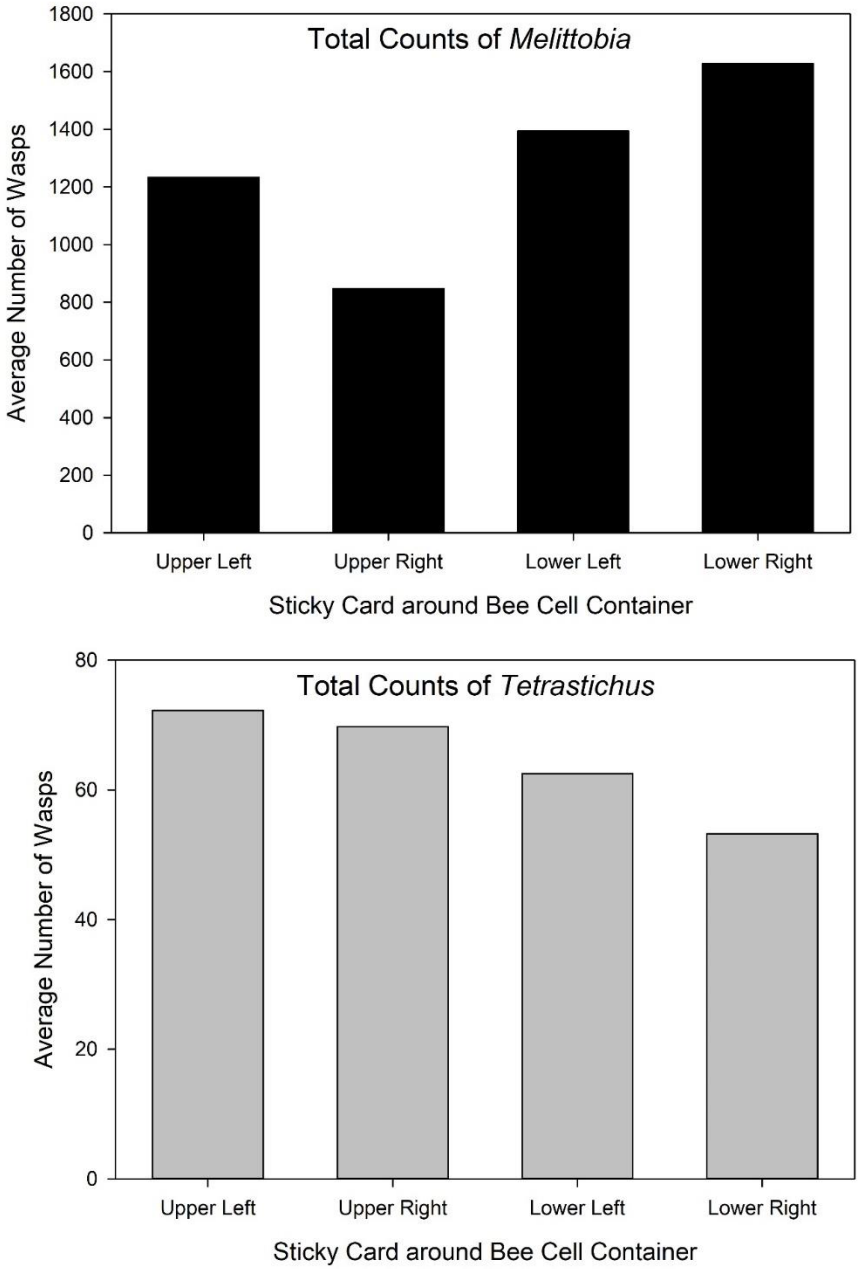

Figure 4. Post-emergence counts of adult *Melittobia* and *Tetrastichus megachilidis* on sticky cards surrounding **left** and **right** sides of the containers of incubated ALCB cells (n = ~1700 cells) each inoculated with *Melittobia*- and *Tetrastichus*-infested cells. There were two bee cell containers on an **upper** cafeteria tray and two containers on a **lower** tray, each with sticky cards.

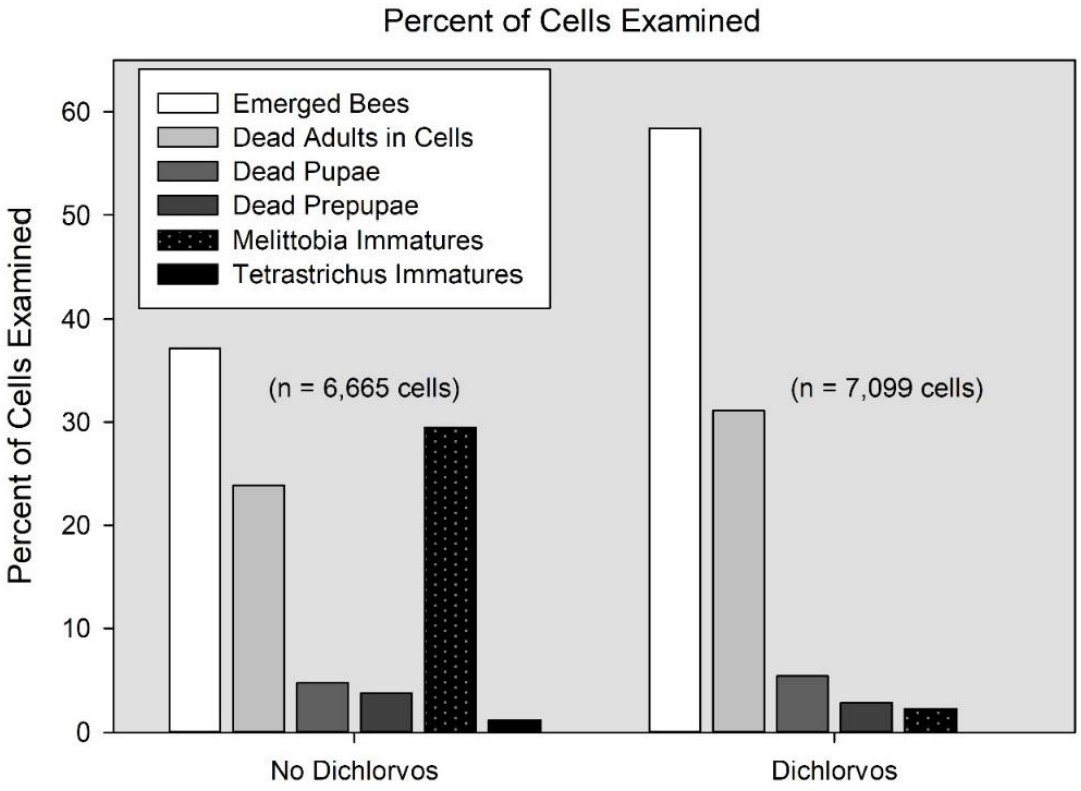

Figure 5. Percent of ALCB cells containing each category of cell contents: empty (bee successfully emerged), dead adult bee, dead bee pupa, dead bee prepupa, developing *Melittobia*, and developing *Tetrastichus* for bee cells in separate incubators – one with dichlorvos treatment and one without. Total number of cells examined for each incubator shown; each incubator had 4 cell containers.

Methods: Fall-Winter Storage

In summer 2020, we collected ALCB nests in paper straws inserted into bee boards at a commercial shelter. We x-rayed nests to sort out healthy prepupae and parasitized cells. We created 15 bee blocks with 121 cavities each (11 × 11 tunnels) (Fig. 6). The blocks were made by sandwiching together slices of polystyrene, with each slice retaining enclosed tunnels. Both ends of the tunnel were open but covered in plastic window screening across one end and stout tissue paper across the other end (Figs. 6 & 7). The stacks were held in place with custom-made cardboard boxes. Use of layers allowed for taking x-radiographs of all cells over time, while simulating the form of a commercial bee board (Fig. 7).

Figure 6. "Bee block" of 11 sandwiched 11-hole sections of polystyrene board. Cells with healthy ALCB prepupae (n = ~725) or developing *Melittobia* (n = 5) were placed inside tunnels. Bee cells were placed in all tunnels, but *Melittobia* were placed only in the back of tunnels (2nd cell) in positions marked with "X."

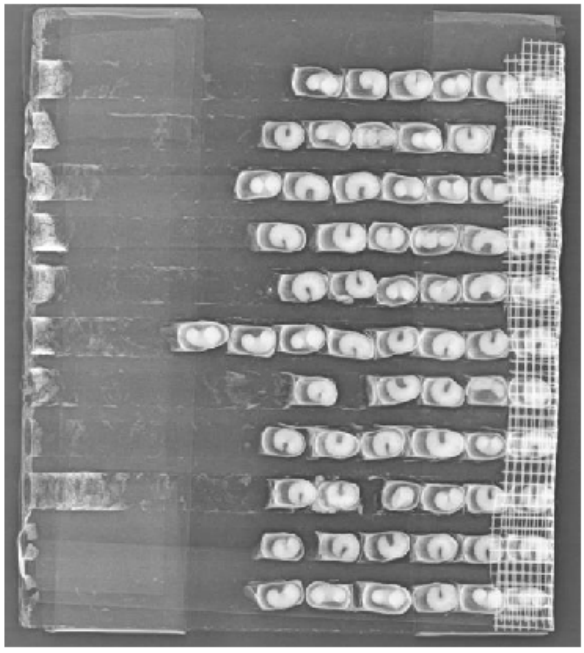

Figure 7. X-radiograph of one layer of a bee block showing bee cells and screen backing.

In the fall, we placed ALCB prepupal cells into each of the tunnels. We also added five *Melittobia*-infested cells among clean bee cells as indicated in Fig. 6, so that once the *Melittobia* adult females emerged, they were distributed across the bee board and had access to all the bee cells. We prepared 15 of these bee blocks as just described in order to assign three blocks to each of five wintering times.

All blocks remained at ambient, outdoor temperatures until moved to cold storage as assigned. Temperatures are being recorded with HOBO dataloggers that remain with each set of blocks. On the 5th day after blocks were stuffed with bee cells (9 October), one set of blocks were placed into cold storage (4-5°C). Another set was placed in cold storage on 14 October, 08 November, and 08 December. The remaining set continues to be sheltered outdoors. To document any cells newly parasitized by *Melittobia*, each layer of all blocks was x-rayed weekly

through 08 December 2020. Any developing second generation bees or non-*Melittobia* parasitized cells were also documented.

In late May or early June 2021 after wintering (while checking for earlier insect emergence for ambient-only sets), all cells will be x-rayed, and the incidence of new *Melittobia*-infested cells will be recorded. Cell sets then will be incubated to examine ALCB and *Melittobia* survival; wasp cells will be incubated separately from bee cells to avoid further ALCB parasitism. Depending on the data that is obtained for each cell set, we will look for effects of cold storage timing on number of infested cells, and then number of emerged bees and wasps (contained separately during incubation).

Conclusions and Discussion

Dichlorvos is highly effective at controlling both *Melittobia* and *T. megachilidis*, and presumably *P. venustus*, if added on the third day bees are placed in an incubator instead of later in the incubation process. Previous APRI-funded work by Utah State University Master's degree candidate Alan Anderson showed that *Melittobia* emerged much sooner than the other wasps in ALCB incubation. Furthermore, *Melittobia* peak emergence was prior to the first emergence of the other wasps which was ~ 10 days after incubation initiation. Newly emerged *Melittobia* were seen for as long as the other wasps (total of 16 days after start of incubation). Therefore, it is important to add dichlorvos as soon as Day 3 of incubation and leave it there through Day 16, if possible. Also, if developing *Melittobia* offspring in newly infested bee cells are present when the bees are taken to the field, as was indicated from our examination of the contents of bee cells from our untreated incubator, then adult female will emerge from those cells with nesting ALCBs and would be able to infest nests in grower fields. More of Anderson's work shows that *Melittobia* can survive in an ALCB cell with a developing larva and wait until the larva spins a cocoon before laying eggs on the prepupa inside.

We will continue to follow the rate of infestation of bee cells in the fall and winter survival of infested cells. Both of our experiments are important for designing protocols for controlling infestation of these fast-producing little wasps.

Enhancing and Protecting Populations of Alfalfa Seed Pollinators - 2020

Doug Walsh
Washington State University

Introduction: Pollination by alfalfa leafcutting bees *(Megachile rotundata,* ALCB) or alkali bees *(Nomia melanderi,* AB) is essential for seed set in alfalfa seed production. Bee mortality that results from inadvertent exposure to pesticides can negatively impact bee survival and/or fitness and potentially reduce seed yields. The first objective of our 2020 project involved facilitating the registration of insecticides for the key pests of alfalfa produced for seed during bloom. In 2020 we focused on several acaricides that have been registered on other crops in recent years. These acaricides included fenazaquin, etoxazole, and spirotetramat .

Our second objective was a victim of the COVID 19 pandemic. We did nothing on developing a bioassay method for quantifying the impact of sulfoxaflor and flonicamid on alfalfa leafcutting bee larval development.

Our third and fourth objectives were completed. We conducted a survey of honey bee hives in Walla Walla County in proximity of fields of alfalfa produced for seed and we completed another year of our annual survey of alkali bees in this same geographic area.

This proposed project addresses 3 of the 5 priorities detailed in the request for plans: I. Management; II. Production; and III. Safety. All the proposed objectives have been developed as a direct result of stakeholder input, through two-way communication with alfalfa seed growers.

Objectives:

1. Conduct topical direct contact bioassays with candidate pesticides on ALCB and AB.
In 2020 we focused on the acaricides fenazaquin (Magister®SC, Gowan Chemical Co.), etoxazole (Zeal® Valent Chemical Co.), and spirotetramat (Movento®, Bayer Chemical Co.). Fenazaquin inhibits the transport of electrons in the mitochondria of exposed spider mites. Etoxazole is a mite growth inhibiting acaricide that inhibits the formation of chitin in spider mite eggs and larvae early in their development. Through experimentation we have documented that etoxazole is most effective on newly laid spider mite eggs and that efficacy decreases as the eggs age towards hatching. Spirotetramat is a lipid biosynthesis inhibitor that has recently been registered on a number of specialty crops. Spirotetramat targets primarily homopteran insects, specifically aphids and whiteflies. However, in hops Walsh has observed some acaricidal activity in that hops treated with spirotetramat experience a week or 2-week delay in the onset of mite outbreaks in hot mid-summer conditions. The candidate acaricides were applied with a R&D CO2 sprayer at 26 gal/A using a hand boom to 0.01-acre plots of alfalfa being produced for seed in the Lowden alfalfa seed-growing district on June 26, 2020. Field-weathered residual test exposures on each insecticide were replicated 5 times per candidate insecticide at 1 hour after the insecticides were applied. Samples consisting of approximately 400 cm^3 of foliage cuttings were taken from the upper 15 cm of the plants and clipped into 2.5 cm lengths. This hay was then placed into individual plastic Petri dish (15 cm diameter) replicates, the tops and bottoms of which were separated by a wire screen (6.7 meshes/cm) insert (45 cm long and 5 cm wide).

Extant ALCB were collected by sweep net from alfalfa fields grown for seed near at the entrance of ALCB domiciles. The bees were tranquilized with CO_2 and put in the Petri dish bioassay cage. Typically we put 10 to 20 ALCB in each Petri dish and wire screen bioassay arena. Bees in cages were held at 75°F for 8 hours and mortality counts were assessed at the conclusion of this time period. Bees were considered "living" if they were capable of flying away after the 8-hour exposure in the bioassay arena. The bees were considered "dead" if they failed to fly away. Bees were considered "moribund" if they wandered aimlessly and didn't fly away. Mortality was corrected against control bioassay arenas. Control mortality was 0%.

These topical bioassays document that the acaricides Magister and Zeal could be considered safe to foraging bees (Table 1) using the criteria long-developed by Dan Mayer and Carl Johansen of <25% mortality in the 1 hr post spray contact bioassay. Zeal is already registered on alfalfa grown for seed via 24C in several states including Washington State. Movento appears safe in topical assays (Table 1). But this was expected. Movento's active ingredient spirotetramat is a very interesting insecticide. It needs to be directly absorbed by the plant and partially metabolized into its enol form before it becomes toxic to the foraging bee. So this topical exposure test is somewhat irrelevant. If there is interest in pursuing registration of Movento or its sister product Ultor® (same active ingredient, different formulation) a different bioassay method will need to be developed because ALCB could be exposed through pollen and nectar and leaf discs harvested for provisioning cell construction.

Table 1. Results of topical exposure bioassays with fenazaquin, etoxazole, and spirotetramat on survivorship and mortality of alfalfa leafcutting bees.

Product	**Rate per acre**	**ALCB % Live (corrected)**	**ALCB % Dead (corrected)**	**ALCB % Moribund (corrected)**
Magister (fenazaquin)	36 fl oz (0.48# ai)	84.2	7.3	8.5
Zeal Miticide (etoxazole)	3.0 oz oz (0.135# ai)	91.3	7.1	1.6
Movento (spirotetramat)	6.0 fl oz (0.09# ai)	93.4	5.0	1.6

Objective 2. Develop a bioassay method to quantify the effects of exposure of ALCB larva to exposure to flonicamid and sulfoxaflor. Our second objective was a victim of understaffing and lack of sufficient lab space due to restrictions relating to the COVID 19 pandemic. We did nothing on developing a bioassay method for quantifying the impact of sulfoxaflor and flonicamid on alfalfa leafcutting bee larval development. I will consider developing a protocol to complete these studies in future years in collaboration with Dr. Kelsey Graham at the USDA-ARS Logan Bee Lab.

Objective 3. Conduct a population survey on the abundance of honey bee hives in proximity to fields of alfalfa produced for seed in Walla Walla County, WA. To accomplish this census, Walsh contacted seed growers by telephone in June and July 2020 regarding locations they had observed bee hives placed near fields of alfalfa produced for seed. Subsequently, these sites were visited and marked with a GPS locater and the number of bee colonies were counted. These data will show us whether the regulations detailed within Walla Walla County Ordinance 345 in regards to the placement of honey bee hives in proximity to fields of alfalfa grown for seed are being adhered to. In total we observed 5 sites where a substantial number of honey bee hives were placed and in total we counted 578 hives (Figure 1).

Figure 1. Location and number of beehives counted with 2-mile diameter circles from hive location.

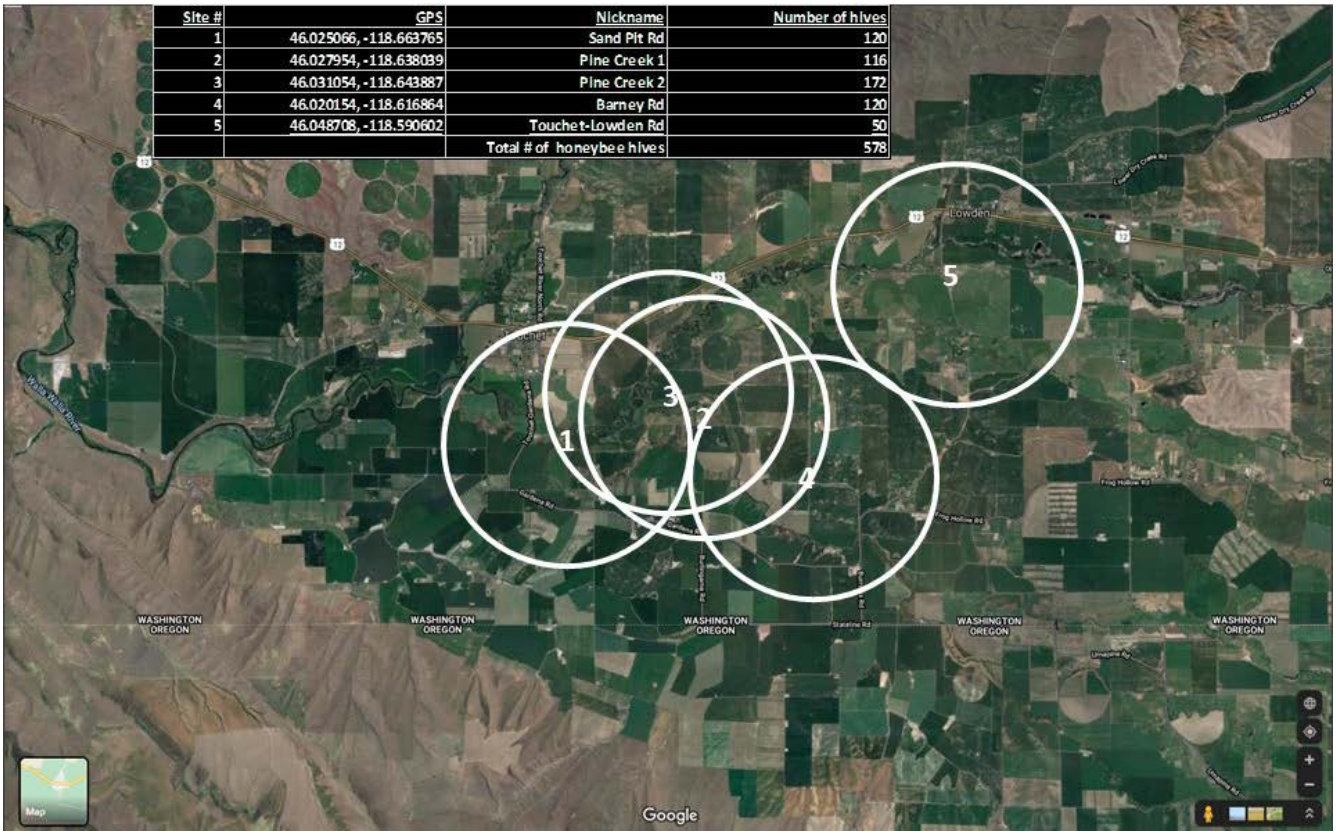

Figure 1 details the GPS coordinates of specific beehive locations and the number of hives placed at each location. The white circles are 2 miles in diameter. Honeybees (*Apis mellifera*) have been documented to have a "hive mind" that has been deemed as a form of swarm intelligence. Honeybees employ what is called a waggle dance in which the older worker bees that are actively foraging for pollen, nectar, and water recruit their like sisters to visit the sites that they have found rewarding with these precious resources. There are several riparian areas in proximity to the 5 locations detailed in Figure 1 and some other crops being produced there as well. These crops included forage alfalfa, pea seed, wheat, wine grapes, onions and other fruiting vegetables. However, under the arid climate of the Touchet-Lowden area the overwhelming floral and nectar resources available to these honeybees in June and July is alfalfa being produced for seed. Specifically, honeybees are seen actively foraging on blossoms of alfalfa being produced for seed. Honeybee hives on average have roughly 30,000 sexually undeveloped female worker bees. In high summer these worker bees typically live for 6 weeks. These worker bees spend the final span of their lives performing the most dangerous tasks assigned to them which includes foraging for pollen, nectar, and water. This foraging period lasts on average about 2 weeks. Thus, by extrapolation about 10,000 honey bees per hive are actively foraging per day. Among these 10,000 foraging bees (again by extrapolation) about 715 die or are killed each day and they in-turn are replaced by younger sisters.

In total we counted 578 honeybee hives among the 5 locations we observed. This would extrapolate to 5,780,000 honey bees foraging every day. For solitary bees like AB and ALCB, each female is on her own and she is not replaced upon her demise by a younger sister. If each honeybee hives replaces 714 worker bees engaged in foraging for pollen, nectar, and water each day this calculates out to 412,692 new foraging bees per day. Multiplying 412,692 new bees per day for the 6 weeks of bloom in alfalfa produced for seed in the Walla Walla Valley this calculates out to 17,333,064 new worker bees over the course of the season. Adding this to the average value of 5,780,000 of hone bees foraging per day this calculates out to a total 23,113,089 honeybees that could potentially forage in fields of alfalfa produced for seed.

From the Section 18 request for sulfoxaflor that Walsh submitted from Washington State, acreage of alfalfa grown for seed averaged about 11,700 acres per year. Most growers release an average of 4 gallons of ALCB per acre. A gallon of ALCB is 10,000 individuals among which 30% or 3,000 bees are female. So by extrapolation, 12,000 female alkali bees per acre are released on 11,700 acres of alfalfa produced for seed in the Touchet-Lowden area. This calculates out to 140,400,000 ALCB.

In Objective 4, following, we detail our annual survey of the alkali bees present in the Walla Walla Valley. From these surveys we calculated that there were 10,293,582 in 2018, 6,559,812 alkali bees in 2019, and 4,564,000 alkali bees in 2020.

Honey bees are the 2nd most abundant managed bees in proximity to alfalfa grown for seed in Walla Walla County.

Objective 4. Conduct an annual census of the alkali bee population abundance in Walla Walla County, WA. Alkali bee population surveys 2010 - 2020. Alkali bee emergence hole counts were recorded annually at the end of the alkali bee nesting season (mid to late July) from 2010 to 2020, in accordance with standardized methods established by Vinchesi and Walsh in 2014. In this method $0.5m^2$ quadrats made of lightweight PVC pipe, with dimensions of 0.7m by 0.7m, are haphazardly tossed across each surveyed bee bed 24 times, and the number of emergence holes contained within each quadrat is counted and recorded. The same 13 bee beds were consistently sampled year-after-year and were initially selected for observation due to known history of alkali bee nesting activity, ease of access, and interest from grower collaborators. New beds have been added to the survey in recent years and added to the total count of bees but for consistency we report these data as a separate value and leave a column in Table 2 to represent the original 13 beds. At each bee bed, special care was taken to ensure that each quadrat landed in a previously unsampled space. Surveyors "calibrated" their counts at the beginning of the survey by counting three quadrats together to ensure that each person counted the same number of bee emergence holes. For continuity, every year at least one of the surveyors had participated in surveys the prior year. The emergence hole counts were used to estimate the number of active nests per bee bed using the following formula:

2 x ([Mean number of quadrat counts per 0.5 m2 ± SE] x [2/3] x [surface area of bee bed])

This formula was first proposed by Jim Cane through video observations of nesting activity that found that two-thirds of nest holes were being actively provisioned. The practice of using surface nest holes to estimate alkali bee populations was then validated by Vinchesi and Walsh in 2014, which confirmed that surface nest hole counts were tightly correlated with the abundance of belowground prepupae. The above formula has been adjusted to rectify an error in Vinchesi and Walsh in 2014, which failed to account for the use of $0.5m^2$ quadrats instead of $1m^2$ quadrats. As a result, all population estimates previously reported by Vinchesi and Walsh in 2014 were doubled before inclusion here.

Results

From 2010 to 2019 the estimated population abundance of alkali bees varied from a low near 4.0 million in 2014 to a high of over 9.4 million in 2012 (Table 2). Unfortunately, the population of alkali bees among the 13 original sampled beds hit a new low at 3,590,000 in 2020. However the addition of 3 fairly new very active bee beds brought the total count to 4,564,000.

Discussion

Alkali bees continue to serve as an important resource for alfalfa seed growers in the Touchet, Gardena, and Lowden alfalfa seed growing areas. The population abundance has dropped over several years. Economic issues and low demand for seed have led to a decrease in acreage over the past several years. This may be contributing to recent declines in alkali bees. We anticipate that 2021 will be a low year for alkali bee abundance. However, we had one event in 2014 in which an individual grower had a mishap and treated their fields with their late spring clean-up spray in 2013. This single event led to a dramatic drop in the total abundance of bees in 2014 to

just over 4 million bees. However, the bee abundance in this bed recovered to its original population abundance by 2015. Well managed alkali bee beds appear to be very resilient.

Table 2. Estimated population abundance of alkali bees from 13 managed bee beds from 2010 through 2017 and 16 managed bee beds from 2018 through 2020 in the Touchet Valley of Walla Walla County, WA.

	Original 13 beds	Plus 3 new beds
2010	8,437,000	
2011	5,335,000	
2012	9,428,000	
2013	6,917,000	
2014	4,005,000	
2015	6,177,000	
2016	8,211,000	
2017	7,053,000	
2018	7,354,000	10,294,000
2019	4,763,000	6,550,000
2020	3,590,000	4,564,000

Lygus Insecticide Studies on Alfalfa Produced for Seed, 2020

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Project Summary: Insecticide resistance has been indicated in Lygus. In particular, pyrethroid resistance has been demonstrated to be a single-gene trait in Lygus populations. In 2020 we captured Lygus at different times during the growing season from sites within and near alfalfa produced for seed and analyzed them for pyrethroid, and sulfoxaflor resistance. We had also also planed to develop and validate a bioassay method for flonicamid. We failed in this objective If we find tolerance and resistance to these insecticides in specific populations, we will determine whether this resistance is due to a single-gene mutation or to upregulated metabolic changes (e.g., elevated esterase or glutathione transferase expression). This work will contribute important new information as to whether Lygus exhibits resistance to multiple classes of insecticides. In addition, establishing the underlying pyrethroid resistance mechanisms within the Lygus populations in alfalfa seed will provide evidence for the intensity of selection from pyrethroid use and the baseline for resistance management.

Project Report, Objective 1. Conduct small scale insecticide efficacy trials pre-bloom and during bloom at WSU IAREC.

Research plots were established on our research alfalfa field on the Roza Unit of WSU IAREC. Treatments were applied by CO_2 powered boom sprays at slightly under the equivalent of 20 gallons per acre. Each treatment was replicated 4 times and each replicate plot was 12 by 20 feet (240 ft^2). Counts are the average number of insects per three 180° sweeps with an 18" sweep net. There were a total of 12 treatments evaluated. These included Beleaf, Brigade, Brigade+Mustang, Carzol, Cobalt, Lorsban, Mustang, Naled, Orthene, Stallion, Transform at a low and a high rate, and Warrior. Insect counts from each of these treatments were compared to insect counts from untreated plots. All the insecticides controlled large and small Lygus nymphs for over 2 weeks. Cobalt, Carzol, Lorsban, Orthene, Stallion, and Transform suppressed Lygus adults for over 2 weeks. Treatment effects were indistinguishable after 3 weeks after treatment.

Project Report, Objective 2. Conduct large-scale demonstration plots with candidate insecticides in Touchet/ Lowden, WA.

A large post-bloom demonstration trial was established at a grower collaborator alfalfa seed post bloom. This field was treated with a post-bloom tank mix treatment of Brigade and

Mustang Max. This treatment was highly effective and controlled Lygus for the 3 week period prior to seed harvest by combining.

Project Report, Objective 3. Quantify dose response curves, LC50 and LC90 values for Lygus populations in and near fields of alfalfa produced for seed and quantify how these values change as the season progresses.

We completed 32 sets of bioassays of the response Lygus bug populations had to the insecticide, bifenthin. With lambda-cyhalothrin we competed 31 sets of bioassays. With sulfoxaflor sulfoxaflor. The Lygus bugs have been collected from 10 distinct geographic regions including, Othello, Prosser, Horse Heaven Hills, Touchet, Gardena, North Pasco, Ash, and Prescott. The Touchet and Lowden sites are truly the sites relevant to this specific WSCPR proposal.

Methods

Lygus bugs were collected by sweepnet and placed in a bug dorm (BioQuip Inc.) and transported to the Environmental and Agricultural Entomology Laboratory located at WSU IAREC. The Lygus are provided with organically produced green beans for sustenance and held overnight. The Lygus were then aspirated into small snap top plastic vials in aliquots of approximately 10 adults and held for approximately 15 minutes. The Lygus were then anesthetized with CO_2 and placed in a petri dish with a piece of filter paper on the base. These individual cohorts of approximately 10 Lygus per petri dish were then subjected to increasing doses of each insecticide in our Potter precision spray tower in dilutions equivalent to 20 gallons per acre. The rates for bifenthrin were 0, 1, 5, 10, and 50% of the labeled field rate of 0.1 pounds active ingredient per acre for bifenthin. The rates for Lambda-cyhalothrin were 0, 1, 10, 20, and 50% of the labeled field rate of 0.03 pounds active ingredient for lambda-cyhalothrin. The rates for sulfoxaflor were 0, 1, 5, 10, and 20% of the labeled field rate of 0.071 pounds active ingredient per acre for sulfoxaflor. Each dilution rate for all three insecticides was replicated 3 times on cohorts of approximately 10 Lygus per Petri dish. The Lygus are provided with a 3 cm piece of green bean and held overnight and assessed for mortality at 24 hours after treatment. For analysis in PoloPLus dose is converted into parts per million. Mortality is corrected in the serial dilutions against the water control treatment by Abbot's Formula and the data is run through Polo Plus™ to calculate LC50 and LC90 values. These values represent the concentration of each insecticide required to kill 50 and 90% of each tested population respectively.

Results

Table 1 provides the geographic region, GPS coordinates, the host plant (Crop) the Lygus population was captured and the day the Lygus were exposed to bifenthrin. Also detailed are the Polo Plus calculated LC_{50} and LC_{90} concentrations for each Lygus population. Blanks in the

data for LC50 and LC90 are where Polo Plus failed to calculate values where the tested rates were either too high or low. Similar information is provided in tables 2 and 3 for the insecticides lambda-cyhalothrin and sulfoxaflor, respectively.

Table 1. Location of sampled Lygus bugpopulation, host plant, spray date and LD50 and LD90 values for bifenthrin

Location	Crop	GPS	Spray Date	LD50	LD 90		
Ash	forage alfalfa	46.2470656, -118.8181542	7/21/2020	30.97	206.14		
Gardena	forage alfalfa	46.006523, -118703113	6/4/2020	22.72	184.54		
Gardena	forage alfalfa	46.006523, -118703113	7/14/2020	33.8	270.17		
Gardena	forage alfalfa	46.006523, -118703113	7/21/2020	62.72	320.34		
Gardena	forage alfalfa	46.006523, -118703113	8/6/2020	54.95	250.78		
Gardena	forage alfalfa	46.006523, -118703113	9/14/2020	11.65	98.77		
Gardena	Seed alfalfa	46.006406, -118.700049	6/23/2020	did not calculate			
Gardena	Seed alfalfa	46.006406, -118.700049	6/23/2020	38.5	168.99		
Horse Heaven Hills	Mustards	46.109963, -119.552128	6/23/2020	9.833	68.6		
Horse Heaven Hills	Mustards	46.0088121, -119.602416	7/1/2020	13.217	123.16		
Horse Heaven Hills	Mustards	46.0088121, -119.602416	7/9/2020	11.5	69.1		
IAREC HQ	forage alfalfa	46.252283, -119.730591	7/24/2020	34.36	231.17		
IAREC HQ	forage alfalfa	46.252283, -119.730591	8/4/2020	84.04	231.76		
N. Pasco	Unmanaged alfalfa	46.401755, -119.113827	6/2/2020	139.3	752.2		
North Pasco	forage alfalfa	46.495270, -119.132557	6/23/2020	52.75	256.59		
North Pasco	forage alfalfa	46.394354, -119.114806	6/23/2020	27.63	71.38		
North Pasco	Unmanaged alfalfa	46.401755, -119.113827	6/23/2020	Did not calculate			
Othello	Mustard	46.785968, -119.037370	6/2/2020	44.5	333.9		
Prescott (potato 1)	Potato (Russian thistle)	46.2958101, -118.6351347	8/13/2020	Did not calculate high mortality at 5%<			
Prescott (potato 2)	Potato (Russian Thistle)	46.2943734, -118.7015200	8/13/2020	21.98	88.74		
Prosser	Alfalfa	46.239924, -119.789354	5/21/2020	25.8	198.7		
Roza	Alfalfa	46.785968, -119.037370	5/27/2020	91.4	412.1		
Roza	forage alfalfa	46.785968, -119.037370	6/19/2020	51.89	196.85		
Roza	forage alfalfa	46.785968, -119.037370	7/9/2020	41.9	500.3		
Roza	forage alfalfa	46.785968, -119.037370	7/24/2020	62.05	156.23		
Roza	forage alfalfa	46.785968, -119.037370	8/4/2020	Did not calculate low Mortality<10%			
Roza	forage alfalfa	46.785968, -119.037370	8/18/2020	16.8	76.32		
Roza	Mustard	46.296866, -119.746376	5/19/2020	Rates too low			
Roza	Mustard	46.296866, -119.746376	5/27/2020	28.2	117.8		
Roza	Mustard	46.296866, -119.746376	6/19/2020	18.57	66.46		
Touchet	Mustard	46.041504, -118.652145	6/4/2020	52.25	327		

The field rate of bifenthrin at 0.10 lb active ingredient diluted in the equivalent of 20 gallons per acre converts to 599 parts per million (ppm). The greatest value for a LD90 was observed in a unmanaged alfalfa field in North Pasco at the intersection of N. Glade Rd and Cedar Rd. The LC90 was 752.2 ppm. The Lygus in this site were certainly tolerant to bifenthrin, but I do not think that this population was truly resistant to bifenthrin. Many of the populations were very susceptible to being killed by bifenthrin. Several of the locations/ populations were sampled multiple times. The forage alfalfa location in Gardena was in close proximity to alfalfa produced for seed. The LC90s in this location was fairly consistent among the dates it was sampled and dropped down quite a bit in September. Overall, the Lygus that were tested among multiple crops/ host plants across a large geography in the Columbia Basin of Washington State were not resistant to bifenthrin.

Table 2. Location of sampled Lygus bugpopulation, host plant, spray date and LD50 and LD90 values for lambda-cyhalothrin							
Location	Crop	GPS	Spray Date	LD50	LD 90		
Ash	forage alfalfa	46.2470656, -118.8181542	7/21/2020	214.41	748.51		
Gardena	forage alfalfa	46.006523, -118703113	6/4/2020	73.2	1014.2		
Gardena	forage alfalfa	46.006523, -118703113	7/21/2020	272.9	684.91		
Gardena	forage alfalfa	46.006523, -118703113	8/6/2020	176.37	2008.2		
Gardena	forage alfalfa	46.006523, -118703113	9/14/2020	80.85	379.95		
Gardena	Seed alfalfa	46.006406, -118.700049	6/23/2020	did not calculate			
Gardena	Seed alfalfa	46.006406, -118.700049	6/23/2020	194.78	713.53		
Horse Heaven Hills	Mustards	46.109963, -119.552128	6/23/2020	Very susceptilble			
Horse Heaven Hills	Mustards	46.0088121, -119.602416	7/1/2020	30.5	180.28		
Horse Heaven Hills	Mustards	46.0088121, -119.602416	7/9/2020	56	360		
IAREC HQ	forage alfalfa	46.252283, -119.730591	7/24/2020	142.9	887.99		
IAREC HQ	forage alfalfa	46.252283, -119.730591	8/4/2020	297.93	1179.75		
N. Pasco	Unmanaged alfalfa	46.401755, -119.113827	6/2/2020	rates too low			
North Pasco	forage alfalfa	46.495270, -119.132557	6/23/2020	Did not calculate			
North Pasco	forage alfalfa	46.394354, -119.114806	6/23/2020	Did not calculate			
North Pasco	Unmanaged alfalfa	46.401755, -119.113827	6/23/2020	286.54	2365		
Othello	Mustard	46.785968, -119.037370	6/2/2020	147.2	1137.8		
Prescott (potato 1)	Potato (Russian thistle)	46.2958101, -118.6351347	8/13/2020	98.04	428.72		
Prescott (potato 1)	Potato (Russian Thistle)	46.2958101, -118.6351347	8/13/2020	98.04	428.72		
Prescott (potato 2)	Potato (Russian Thistle)	46.2943734, -118.7015200	8/13/2020	78.73	526.34		
Prescott (potato 2)	Potato (Russian Thistle)	46.2943734, -118.7015200	8/13/2020	78.73	526.34		
Prosser	Alfalfa	46.239924, -119.789354	5/21/2020	16.8	347.3		
Roza	Alfalfa	46.785968, -119.037370	5/27/2020	Rates not good			
Roza	forage alfalfa	46.785968, -119.037370	6/19/2020	188.38	700.48		
Roza	forage alfalfa	46.785968, -119.037370	7/24/2020	171.62	1029.9		
Roza	forage alfalfa	46.785968, -119.037370	8/4/2020	156.96	563.88		
Roza	forage alfalfa	46.785968, -119.037370	8/18/2020	206.75	1903.4		
Roza	Mustard	46.296866, -119.746376	5/19/2020	Rates too low			
Roza	Mustard	46.296866, -119.746376	5/27/2020	Rates too high			
Roza	Mustard	46.296866, -119.746376	6/19/2020	43.75	255.14		
Touchet	Mustard	46.041504, -118.652145	6/4/2020	96.5	1165.5		

The field rate of lambda-cyhalothrin at 0.03 lb active ingredient diluted in the equivalent of 20 gallons per acre converts to 1,875 parts per million (ppm). The greatest value for a LD90 was observed in a unmanaged alfalfa field in North Pasco at the intersection of N. Glade Rd and Cedar Rd. The LC90 was 2,365 ppm. However there were a greater number of locations including the alfalfa forage field near Gardena and the alfalfa forage field on the Roza that exhibited greater tolerance in early August then on any other dates these sites were sampled. The LD90s were around 1,900 to 2,000 ppm. There were many sites in which the Lygus were very susceptible to being killed by lambda-cyhalothrin at recommended field rates. However, it appears that between the two pyrethroids, bifenthrin and lambda-cyhalothrin that the Lygus in general are more tolerant to the lambda-cyhalothrin.

Table 3. Location of sampled Lygus bugpopulation, host plant, spray date and LD50 and LD90 values for sulfoxaflor							
Location	Crop	GPS	Spray Date	LD50	LD 90		
Ash	forage alfalfa	46.2470656, -118.8181542	7/21/2020	9.19	59.3		
Gardena	forage alfalfa	46.006523, -118703113	6/4/2020	13.14	33.49		
Gardena	forage alfalfa	46.006523, -118703113	7/21/2020	16.74	30.8		
Gardena	forage alfalfa	46.006523, -118703113	8/6/2020	15.97	39.86		
Gardena	forage alfalfa	46.006523, -118703113	9/14/2020	9.03	29.82		
Gardena	Seed alfalfa	46.006406, -118.700049	6/23/2020	21.47	60.61		
Gardena	Seed alfalfa	46.006406, -118.700049	6/23/2020	15.41	39.47		
Horse Heaven Hills	Mustards	46.109963, -119.552128	6/23/2020	7.463	24.451		
Horse Heaven Hills	Mustards	46.0088121, -119.602416	7/1/2020	Did not calculate			
Horse Heaven Hills	Mustards	46.0088121, -119.602416	7/9/2020	24.14	59.76		
IAREC HQ	forage alfalfa	46.252283, -119.730591	7/24/2020	13.01	34.22		
IAREC HQ	forage alfalfa	46.252283, -119.730591	8/4/2020	Did not calculate high mortality >5%			
N. Pasco	Unmanaged alfalfa	46.401755, -119.113827	6/2/2020	55.3	128		
North Pasco	forage alfalfa	46.495270, -119.132557	6/23/2020	7.18	18.3		
North Pasco	forage alfalfa	46.394354, -119.114806	6/23/2020	7.89	15.44		
North Pasco	Unmanaged alfalfa	46.401755, -119.113827	6/23/2020	19.39	46.06		
Othello	Mustard	46.785968, -119.037370	6/2/2020	10.98	28.75		
Prescott (potato 1)	Potato (Russian thistle)	46.2958101, -118.6351347	8/13/2020	13.23	48.96		
Prescott (potato 2)	Potato (Russian Thistle)	46.2943734, -118.7015200	8/13/2020	9.72	32.05		
Prosser	Alfalfa	46.239924, -119.789354	5/21/2020	rates too low			
Roza	Alfalfa	46.785968, -119.037370	5/27/2020	39.6	253.4		
Roza	forage alfalfa	46.785968, -119.037370	6/19/2020	11.23	25.87		
Roza	forage alfalfa	46.785968, -119.037370	7/24/2020	Not calculated high mortality at >5%			
Roza	forage alfalfa	46.785968, -119.037370	8/4/2020	Did not calculate high mortality >5%			
Roza	forage alfalfa	46.785968, -119.037370	8/18/2020	6.38	21.61		
Roza	Mustard	46.296866, -119.746376	5/19/2020	0.177	1.121		
Roza	Mustard	46.296866, -119.746376	5/27/2020	16.1	65.09		
Roza	Mustard	46.296866, -119.746376	6/19/2020	11.6	28.49		
Touchet	Mustard	46.041504, -118.652145	6/4/2020	16.42	62.75		

The field rate of sulfoxaflor at 0.071 lb active ingredient diluted in the equivalent of 20 gallons per acre converts to 406 parts per million (ppm). The greatest LD90 was observed in a Lygus bug population collected from forage alfalfa on the WSU Roza site. The LD90 was 253.4. This was an anomaly because the next greatest LD90 was in Gardena from an field of alfalfa produced for seed at 60.61. This seed field had been treated 1 to 2 times per growing season for the past 5 years with sulfoxaflor. These results provide a good snapshot for the relative susceptibility of Lygus bugs in South Central Washington State to sulfoxaflor at an early period of its registration. We can check periodically in future years to quantify if Lygus are gaining tolerance to sulfoxaflor.

Objective 4. Develop and validate a bioassay method for studying the dose response of Lygus exposed to the insecticide flonicamid.

We attempted several methods. All of them failed. We presently do not have a bioassay method to test the efficacy of flonicamid on Lygus bugs.

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