Title: Implementing Lygus Management Strategies in Alfalfa Seed Production

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Program Code: AFRP

Program Name: Alfalfa and Forage Program

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Performing Department
Agricultural Research Center

Co-Project Directors
Davison, Jason
Barbour, James

Departments
Sponsored Projects
Plant Soil & Entomol Sciences

Non-Technical Summary
We propose to conduct a comprehensive investigation on the biology and management of Lygus bug populations infesting fields of western US alfalfa produced for seed. We will investigate pest and beneficial arthropod species constituency and abundance, predatory insect feeding behaviors, and dispersal patterns of Lygus. Simultaneously we plan to investigate the genetic basis of insecticide resistance, using techniques recently developed by our team of scientists. We will create a matrix of these factors and the resulting seasonal insecticide recommendations, based on efficacy on Lygus and safety to beneficial arthropods and pollinators, and make it available to growers via traditional and state-of-the-art electronic push extension technologies.

Accomplishments
Major goals of the project
We propose to conduct a comprehensive investigation on the biology and management of Lygus bug populations infesting fields of western US alfalfa produced for seed as a non-food/non-feed crop. We will investigate pest and beneficial arthropod species constituency and abundance, predatory insect feeding behaviors, and dispersal patterns of Lygus. Simultaneously we plan to investigate the genetic basis of insecticide resistance. We will create a matrix of these factors and the resulting seasonal insecticide recommendations, based on efficacy on Lygus and safety to beneficial arthropods and pollinators, and make it available to growers via traditional and recently developed electronic push extension technologies.

1. Utilize a standardized DNA region as a tag (DNA barcode) for rapid and accurate species identification for Lygus populations infesting alfalfa seed fields in Idaho, Oregon, Nevada, and Washington.
2. Develop and implement DNA-based diet analyses to identify prey from the digestive systems of the generalist predatory arthropod guild present in the candidate crops. These predatory arthropods will include big-eyed bugs, minute pirate bugs, assassin bugs, and spider species.
3. Use standard trapping methods (e.g. pan traps, sweep net samples) and DNA markers (i.e. RR) to identify dispersal patterns of Lygus among alfalfa seed and other Lygus crop and non-crop hosts in Idaho, Oregon, Nevada and Washington.
4. Develop an insecticide resistance monitoring system using molecular techniques to quantify increased tolerance and
resistance to specific insecticides in Lygus populations in alfalfa produced for seed.
5. Create a matrix of insecticide chemistries and insect susceptibility profiles that reflects pest susceptibility and safety to non-target predatory, parasitic, and pollinating arthropods.
6. Disseminate educational outreach information in a multi-modal, timely, and targeted fashion appropriate to alfalfa seed growers and other stakeholders.

What was accomplished under these goals?

Goal 1. Utilize a standardized DNA region as a tag (DNA barcode) for rapid and accurate species identification for Lygus populations infesting alfalfa seed fields in Idaho, Oregon, Nevada, and Washington. This objective has been accomplished. We do have a complex of Lygus species persisting in the west, but typically once an insecticide is introduced into the agroecosystem during the growing season Lygus hesperus becomes the predominant species in fields of alfalfa produced for seed.

In evaluating the genetic diversity of Lygus populations among western US states we have determined that there is very little genetic diversity and it appears that the center of origin for Lygus hesperus is central California. As irrigation was developed in other arid regions of the west and crops like alfalfa were planted in these areas L. hesperus migrated into these new habitats and established endemic populations in these regions.

Goal 2. Develop and implement DNA-based diet analyses to identify prey from the digestive systems of the generalist predatory arthropod guild present in the candidate crops. These predatory arthropods will include big-eyed bugs, minute pirate bugs, assassin bugs, and spider species. We have initiated these studies and have collected surveys of insect populations from 50+ site/field locations. These samples have been sorted. A generalist predatory insect called the big-eyed bug was often present in the greatest abundance among all the generalist predators in the sampled locations. Therefore we are focusing our initial efforts at developing primers for the supposed prey of big-eyed bugs. This includes cowpea aphid and Lygus bugs.

We have proved that cow pea aphids are the preferred prey species of most beneficial arthropods. Lygus are far more mobile and difficult to capture for prey species. In summer 2015 we treated a field of alfalfa produced for seed with a selective aphicide. There was a greater abundance of big-eyed bugs then either Lygus or aphids. In this situation the big-eyed bugs fed on Lygus, but we also noted a substantial amount of cannibalism among the big-eyed bugs in the absence of prey.

Goal 3. Use standard trapping methods (e.g. pan traps, sweep net samples) and DNA markers (i.e. RR) to identify dispersal patterns of Lygus among alfalfa seed and other Lygus crop and non-crop hosts in Idaho, Oregon, Nevada and Washington. This project is following directly Goal 2 in that we have now sampled 50+ populations from late winter through high summer in and around fields of alfalfa produced for seed.

We have determined that Lygus tend to stay-put within fields of alfalfa produced for seed. A mass migration occurs into fields of alfalfa produced for seed in late spring as rain-fed spring and winter weeds dry down and their value as host plants diminish. Subsequent migrations occur in the growing season from adjacent crops, most notably forage alfalfa when these fields are disturbed by mechanical activity. For forage alfalfa this is when the fields are swathed. Adult Lygus leave the swathed field and migrate into alfalfa fields being produced for seed.

Goal 4. Develop an insecticide resistance monitoring system using molecular techniques to quantify increased tolerance and resistance to specific insecticides in Lygus populations in alfalfa produced for seed.

We focused on the organophosphate insecticide chlorpyrifos and the pyrethroid insecticide bifenthrin. With both insecticides we did not observe any true resistance within the Lygus populations we surveyed. The registered field rates of these two insecticides killed all the populations we tested under controlled conditions. We observed some isolated incidences of increased tolerance and we observed an increase in the titer of housekeeping genes in these populations, but we have not observed any direct point mutations in L. hesperus. We do conclude that growers have been good stewards of the insecticides that are registered on alfalfa produced for seed and that these growers are following our research-based resistance management programs.

5. Create a matrix of insecticide chemistries and insect susceptibility profiles that reflects pest susceptibility and safety to non-target predatory, parasitic, and pollinating arthropods.

This matrix will be published in the proceedings of the Western Alfalfa Seed Growers meeting in January 2017 and then place on the seed grower's association's website. This will also be published in the PNW Insect Control Handbook for 2017.

6. Disseminate educational outreach information in a multi-modal, timely, and targeted fashion appropriate to alfalfa seed growers and other stakeholders. We continued to do this via annual meetings and field days throughout the project. Most notably we host a workshop annually at the Western Alfalfa Seed Grower's Conference.

What opportunities for training and professional development has the project provided?

Our biggest challenge for this project was the untimely death of the PhD student that was working on this project in March 2015. Subsequently we had to regroup our efforts and we hired a post doc to finish the project. The post-doc worked on the project and finished the molecular-based research in fall 2015 and spring 2016. The post-doc has now separated from Washington State University and accepted a position with the University of California. A manuscript based on the gut-content
studies of big-eyed bugs is being finalized for submission to the Journal of Economic Entomology.

How have the results been disseminated to communities of interest?
Yes the results have actively been distributed to the growers of alfalfa (and other) seed producers.

What do you plan to do during the next reporting period to accomplish the goals?
{Nothing to report}

Participants
Actual FTE's for this Reporting Period

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Target Audience
The target audience consists of seed producers with an emphasis on alfalfa seed growers. However producers of other small seeded vegetable seed benefit from this program because most of the data we collect will extrapolate readily.

Products

Type             | Status      | Year Published | NIFA Support Acknowledged |
Journal Articles | Submitted   | 2015           | YES                        |

Citation

Amalia, D., M.D. Lavine, L. Corley-Lavine1, & D.B. Walsh. Quantifying tolerance to organophosphate and pyrethroid insecticides among populations of the western tarnished plant bug (Lygus hesperus) in central Washington State, USA. Submitted to Pesticide Science

Type             | Status      | Year Published | NIFA Support Acknowledged |
Journal Articles | Published   | 2014           | YES                        |

Citation

Transcriptome-based identification of ABC transporters in the western tarnished plant bug Lygus Hesperus. PLoS 1. DOI: 10.1371/journal.pone.0113046
### Accession No. 1004706  Project No. WNP04616

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**Citation**

Pest and Pollinator Management on Alfalfa Seed 2015
Western Alfalfa Seed Growers Conference, Las Vegas, NV 2016

**Other Products**

**Product Type**
Other

**Description**

Section 18 Emergency Exemption from Tolerance requests for the use of sulfoxaflor on alfalfa produced for seed in Washington State, Idaho, Oregon, and Montana.

**Changes/Problems**

Our biggest challenge for this project was the untimely death of the PhD student that was working on this project in March 2015. Subsequently we had to regroup our efforts and we hired a post doc to finish the project. The post-doc worked on the project and finished the molecular-based research in fall 2015 and spring 2016. The post-doc has now separted from Washington State University and accepted a position with the University of California. A manuscript based on the gut-content studies of big-eyed bugs is being finalized for submission to the Journal of Economic Entomology.
Dietary choices of big-eyed bugs (Hemiptera: Geocoridae) and damsel bugs (Hemiptera: Nabidae) in response to fluctuating pest populations

Amber C. Vinchesi and Douglas B. Walsh

Abstract

Lygus bugs are a major pest of alfalfa grown for seed. Many generalist predators also exist in alfalfa fields. We conducted a survey of the pest and predator species present in alfalfa seed, alfalfa forage, and mixed weeds/mustard to determine the predator/prey composition. A gut content analysis was conducted on the predator species collected, specifically damsel bugs and big-eyed bugs, to determine whether they were eating important pest species, such as Lygus and aphids. At one of the alfalfa seed sites, a new candidate insecticide was applied; the insect complex before application and up to three-weeks post-application was collected. The insecticide was effective at reducing aphid populations for up to two weeks, but did not reduce predator populations or Lygus nymphs. Our objective was to determine the dietary choices of damsel bugs and big-eyed bugs when aphids were not available, specifically whether or not these predators ate more Lygus bugs. If predators are actively controlling pests in alfalfa seed fields, then a pest management program should include protection of beneficials.

Introduction

Over 30,000 ha of alfalfa seed are produced annually in the United States with an average production of 36 million kg of seed. Most of the production takes place in the western United States (Anonymous 2005). Considered a minor crop, alfalfa seed is the foundation of the alfalfa forage industry which ranks third in planted agricultural acres in the United States at 9.3 million ha annually and subsequently impacts the dairy and beef cattle industries as a major feedstock (Anonymous 2005).

A complex of Mirid plant bug species that is reported to be dominated by *Lygus hesperus* Knight (Hemiptera: Miridae) are key direct pests in a broad range of crops in the Western United States. Alfalfa grown for seed is annually at risk of economic damage from Lygus feeding (Kelton 1975, Mayer et al. 1998). Feeding damage and economic loss from Lygus is a common concern among all producers of alfalfa in the western US. Several generalist predators including big-eyed bugs (*Geocoris* spp.) and damsel bugs (*Nabis* spp.) are among the predators often cited as providing some biological suppression of Lygus bug populations (Johansen and Eves 1973, Berry 1998, Waters 2009). Many non-crop and crop hosts of Lygus are grown in close proximity to alfalfa seed fields in Washington state, including alfalfa forage fields. When forage fields are cut, dry out, or are disturbed by spraying or mowing, Lygus will migrate into the adjacent alfalfa seed fields. Seasonal weeds can also be problematic when flowering.

Lygus is the primary insect pest of alfalfa seed in the Pacific Northwest, and if left uncontrolled, will cause reduced seed yields (Sorenson 1936). Lygus inject their mouthparts into plant tissues to ingest the contents of these tissues (Mayer and Johansen 1991). Floral bud
abortion is caused when Lygus feed on flowers (Strong 1970). The major crop loss caused by Lygus within alfalfa seed production systems is due to seed feeding which leads to damaged and unviable seed pods (Strong 1970, Mayer and Johansen 1991).

Aphid feeding in alfalfa seed can cause plants to wilt, turn yellow, or die. They can severely damage plant stands of susceptible varieties (Baird et al. 1991). Some aphid species may inject a toxic secretion while feeding, causing yellowing at the feeding site and along leaf veins. Honeydew secreted by aphids can lead to the development of sooty mold and reduced plant productivity (Sloderbeck and Whitworth 2008).

The Western big-eyed bug, Geocoris pallens Stäl (Hemiptera: Geocoridae) is an important predator of Lygus and aphids in alfalfa seed production and the most common and important predator of Lygus in Washington state. Big-eyed bugs seem to prefer aphids, so when aphids are abundant, big-eyed bugs feed less on Lygus (Baird et al. 1991). Both adults and nymphs are predacious and extract the contents of their prey with their piercing-sucking mouthparts (Schuh and Slater 1995).

The damsel bug, Nabis spp. Latreille (Hemiptera: Nabidae), is another important predator of aphids and Lygus in alfalfa fields. They are avid predators, grasping their prey and sucking out their body fluids. Both nymphs and adults attack aphids and Lygus. In a study by E. J. Taylor (1949), when damsel bugs were offered both aphids and Lygus prey, they attacked both prey options (Taylor 1949). On a daily basis, 5th instar damsel bugs may consume 2 or 3 Lygus each (Berry 1998). Damsel bugs are encouraged to multiply when spring weather is warm, but cooler springs lead to rises in aphid populations and the damsel bug population is not large enough to provide control (Berry 1998).

In alfalfa seed production, management of Lygus relies almost exclusively on insecticides. Insecticides commonly used to control Lygus bugs are pyrethroids, organophosphates, carbamates, and neonicotinoids. Insecticide applications to control Lygus are usually recommended three times per season: before alfalfa bloom, during bloom, and the last after bloom during seed set and maturation (Waters 2009). Applications before bloom can be made during the day, but during bloom applications should only be made in the evening to avoid impacts and mortality to pollinators. Alfalfa seed growers have to balance Lygus control with pollinator safety, which leads to growers using chemicals with shorter residuals and reduced Lygus efficacy (Waters 2009).

Unfortunately, Lygus has become increasingly resistant to chemical insecticides over the years. Snodgrass (2009) detected acephate resistance in the population of the tarnished plant bug, Lygus lineolaris Palisot de Beauvois (Hemiptera: Miridae). Zhu (2011) also reported that L. lineolaris has become increasingly resistant to organophosphate insecticides in recent years. A new candidate insecticide, Sivanto®, was applied in test plots on an alfalfa seed grower’s field in Touchet, WA, located in the southeastern part of the state. This insecticide is classified as a Group 4D chemical and has the same action site as a neonicotinoid, targeting nicotinic acetylcholine receptors. Arthropod collections were made before the application and up to three weeks after
the spray for gut content analysis. Insects were also field-counted so that the impact of this new insecticide on the pest and predator complex could be identified. In this chemical trial, the new candidate insecticide knocked down aphid populations significantly for two weeks in the alfalfa test plots. We aimed to determine if generalist predators, such as Geocoris spp. and Nabis spp. switch their prey choices to Lygus while aphid populations were reduced.

The analysis of predator gut contents using molecular tools has become an important new technique that enables researchers to determine whether or not a particular prey item has been consumed by a particular predator without having observed the event (Symondson 2002; Harwood et al. 2009; King et al. 2008; Dunshea 2009). This approach relies on the use of Polymerase Chain Reaction (PCR) to amplify specific DNA sequences from the prey that have been ingested by a predator (Symondson 2002). We developed our own target DNA sequences for rapid and accurate identification of prey from predator gut contents. Generalist insect predators consume many different prey species, therefore determining the entire range of their diets is not possible with species-specific primers (Sint et al. 2014). Our focus was on Lygus spp. and Aphis spp. Linnaeus (Hemiptera: Aphididae) due to the negative impact and prevalence of these pest species on alfalfa grown for seed. The use of real-time PCR allowed for higher accuracy and more specificity when determining pest DNA from the guts of predator DNA in a presence/absence assay.

Our objective was to develop and implement DNA-based diet analyses to identify prey (aphids and Lygus) from the digestive systems of the generalist predators present in alfalfa produced for seed, specifically damsel bugs and big-eyed bugs. Damsel bugs and big-eyed bugs were chosen because they were the predominant natural enemies present in our sweep net collections.

Materials and Methods

Field collection

Specimen were collected from multiple field sites in southeastern and south central Washington, including feral alfalfa, alfalfa forage fields, alfalfa seed fields, mustards and weeds throughout May and early June 2015. The different sites were divided into alfalfa seed, alfalfa forage, and weeds/mustards for data analysis. Some of the sites were visited weekly, but given a new site number each visit. Date, time of day, and coordinates were specified at each collection and at each site. Specimen were sweep-netted, placed into a plastic tank, exposed to CO₂ to prevent them from continuing to feed on each other, and then placed in 50 mL Falcon tubes with 70-95% ethanol and labeled by site number. The tubes were transported back to the laboratory in a cooler and placed in the freezer. Arthropods were then sorted to family and transferred into 15 mL Falcon tubes with 95% ethanol for later DNA extraction.

Pesticide Trial
The pesticide trial was conducted on a large test plot (0.99 acre) where the grower applied the candidate insecticide, Sivanto® 200 SL, at 14 oz/acre on May 18, 2015. Each arthropod collection sample equaled five 180° sweeps with a standard sweep net (BioQuip Products Inc. Rancho Dominguez, CA). Samples were taken before the treatment was applied on May 18, four days post-application, 15 days post-application, and 22 days post-application. By sampling pre-treatment on the same day as the spray, there is an accurate picture of the arthropods that were in the field directly before the insecticide spray. Insect counts were averaged per 15 samples (Table 2).

Molecular Methods

DNA was extracted from 238 big-eyed bugs and 128 damsel bugs. Specific primers were developed using online molecular databases (BLAST and NCBI). *Lygus* was the only species-specific primer pair developed due to the presence of *Lygus hesperus* (the target species) in the database, all other primers were developed to be genus-specific. *Geocoris* primers were developed from the *Geocoris pallidipennis* genome, though *G. bullatus* and *G. pallens* are found in alfalfa fields in southeastern Washington. The *Nabis apicalis* genome was used to develop primers for *Nabis* spp. and the *Aphis gossypii* (cotton aphid) was used for aphid primer development, though the target species was *Aphis craccivora*, or the cowpea aphid. Primer design was done using PrimerQuest Tool from IDT (Integrated DNA Technologies, Inc. Coralville, IA) with sequences from the NCBI database, as explained previously.

The type of PCR used was real-time PCR, or qPCR. This protocol uses probes that are designed to match the specific primers developed for each species. Each probe contains a specific fluorescent dye and a “quencher”. In the first step, called the annealing step, the primers and probe attach to their matching DNA strand (Lygus or aphid), if it is present. While the probe is intact, the quencher part of the probe absorbs the fluorescent dye so it cannot be released. In the second step, the polymerase enzyme, which multiplies the DNA, also extends the primers. Once the enzyme reaches the probe as it extends the primer, it splits the probe, separating the fluorescent dye and the quencher. The quencher is now no longer absorbing the dye and the fluorescent dye is released. The thermal cycler machine used to conduct qPCR, produces a value and a computer read-out of the level of fluorescence. The level of fluorescence demonstrates the presence or absence of Lygus or aphid DNA within the gut DNA of the damsel or big-eyed bugs. Once the primers were tested the matching probes were ordered. Each probe was labeled with a different fluorescent dye to determine which insect was being detected with no overlap. Since each dye is assigned to a specific primer pair, if that DNA was not detected, then that specific dye would not be seen in the results. If the fluorescent dye is released and read by the thermal cycler program, then a specific species’ DNA has been detected in the guts of the predator. For example, if Dye A (aphids) is not released but Dye B (Lygus) is released, then Lygus DNA was detected within the predator guts, but aphid DNA was not.

Results

Gut Content Analysis
Aphids were detected more often than Lygus in the guts of both big-eyed bugs and damsel bugs, suggesting that aphids are eaten more often than Lygus, or that they are more available (Table 1). Aphids and Lygus were detected in higher percentages in the guts of damsel bugs than in big-eyed bugs, suggesting that damsel bugs eat more of each prey type (Table 1). This could be attributed to their larger size relative to big-eyed bugs. Damsel bugs are larger than Lygus nymphs and Lygus adults whereas big-eyed bugs are similar in size to Lygus nymphs.

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Table 1. Numbers of big-eyed bugs (BEB) and damsel bugs (NAB) collected in May and June 2015 from each type of site and mean numbers of Lygus bugs (LYG) and aphids (APH) that were detected in the guts of the predators.

Approximately 75% of the damsel bugs tested ate aphids and over 50% ate Lygus (Figure 2D). Predators were collected in higher numbers in alfalfa fields than in weedy sites, though the majority of damsel bugs were collected from seed fields and not alfalfa forage fields (Figure 1). Lygus were detected in 20% of the big-eyed bugs sampled and aphids were detected in 35% (Figure 2D). Big-eyed bugs were more commonly collected (238) than damsel bugs (128), but both were abundant (Figure 1).

Big-eyed bugs were much more abundant in alfalfa forage and seed fields (equally represented in both) than at weedy sites. Only one damsel bug was collected from weeds (Figure 2A), and most were collected from alfalfa seed fields and not forage fields (Figures 1 and 2). Because of the specific primers, we could not determine if big-eyed bugs and damsel bugs were cannibalistic. Both are generalist predators and it would not be surprising if they also fed on each other. We would not have been able to differentiate whether a positive detection was because the predator ate one of its own species or if we were detecting the individual predator’s DNA. No positives were detected for big-eyed bugs within the guts of damsel bugs, but this is likely due to primer error rather than damsel bugs’ actual food preference since they are generalist predators, though this study cannot confirm either theory.

Pesticide Trial

Four days post-application of Sivanto®, there was an immediate knockdown of aphids that lasted two weeks (Table 2). The predator numbers remained consistent, though two weeks post-application all insect numbers decreased except for Lygus adults which were on the rise (Table 2). By three weeks post-application, Lygus adult numbers were still climbing, but predators had returned, especially big-eyed bugs. Aphids were also back in higher numbers by 22-days post-application on June 9 (Table 2). Sivanto® was effective at knocking down aphids for two weeks.
post-treatment while not having a detrimental effect on the beneficial predators present in the alfalfa seed field (Figure 3).

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</tr>
<tr>
<td>Jun-9</td>
<td>12.47</td>
<td>0.40</td>
<td>0.87</td>
<td>49.00</td>
<td>16.20</td>
<td>1.73</td>
</tr>
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</table>

Table 2. Mean number of insects collected from the Sivanto® field trial at each sampling date. Insect counts were averaged per 15 sweep net samples. * represents the pre-treatment sample.

For the gut content analysis field collection at the Sivanto® site, big-eyed bugs and damsel bugs were only sampled on June 2 and June 9, two and three-weeks post-treatment of Sivanto®. Of these 31 big-eyed bugs collected, ten were collected 15-days post-treatment and 21 were collected 22-days post-treatment (Figure 4A). Positive detection of Lygus bugs was only observed three-weeks post-Sivanto® application, with three big-eyed bugs testing positive for Lygus (Figure 4A). Three different big-eyed bugs tested positive for aphids on the same date, and one big-eyed bug tested positive for aphids 15-days post-Sivanto® application (Figure 4A). Eight damsel bugs were collected from the pesticide trial field site, two on June 2 and six on June 9 (Figure 4B), but positive detections of Lygus and aphids were only observed from one damsel bug collected three weeks post-treatment (Figure 4B). Both positive detections were made from the same damsel bug specimen, suggesting it ate both Lygus and aphids shortly before it was collected.

The number of predators collected for gut content analysis in the pesticide field trial, suggests that Sivanto® did have a negative effect on predators (Figure 4), but from the general collections at the field site, this is not the case (Table 2). It is difficult to specifically state whether or not the positive detections of Lygus bugs and aphids from the guts of the predators three-weeks post-application is because the pests were suppressed until this point and not available to the predators, or due to the generalist diet of the predators.

Discussion

If new insecticides work on aphids and the predators begin eating more Lygus, protecting predators would be key. This is an example of biological control and chemical control working together. The chemical program and natural enemy status supplement each other. Beneficial insects provide decent control as long as pest populations remain at manageable numbers. The correct insecticide could provide the lower pest numbers without killing natural enemies and pest control would be sufficient. If predators are eating pests and effectively controlling them, then the insecticide regimen will need to protect the beneficial insects in the field.

We know that damsel and big-eyed bugs are consuming both aphids and Lygus and are predominant predators in southeastern Washington alfalfa fields, whether grown for forage or
seed. It is worth noting that these are only two of the predator species found in alfalfa fields. Minute pirate bugs, syrphid fly larvae, lacewings, lady beetles and spiders are also common natural enemies found in alfalfa production systems. Protecting these predatory arthropods could only benefit alfalfa producers.

This project allows alfalfa seed growers to become aware of predator-prey interactions after chemical sprays in order to effectively apply integrated pest management strategies. This process will assist in developing more economical procedures for controlling the pest, conserving the predator, and achieving better crop yield. By reducing pest numbers without reducing predators, more efficient control of pests can be achieved. More field research needs to be done on new chemistries, like Sivanto®, to understand the impacts to both pests and predators.

Acknowledgements

We would like to thank Russ Walsh for the insect collections from various sites and the Walsh lab for assistance with sorting the insects from each site. A special thanks to Mark Lavine for his assistance and guidance in developing protocols for this study and Sally O’Neal for review of this manuscript. We would also like to thank the Touchet-Lowden-Gardena alfalfa seed growers for the use of their fields to collect insects, especially Mark Wagoner for contributing his field and time for the pesticide trial. Final thanks to the NIFA-ARFP for funding.

References


**Figures**

Figure 1. Percentage of big-eyed bugs (BEB) represented in red and damsel bugs (NAB) represented in yellow collected in each type of site (weeds, alfalfa seed, alfalfa forage, and miscellaneous) in May and June 2015.
Figure 2. A.) Percentage of Lygus bugs (blue) and aphids (orange) detected within the guts of big-eyed bugs (BEB) and damsel bugs (NAB) that were collected from weedy sites. B.) Percentage of Lygus bugs (blue) and aphids (orange) detected within the guts of big-eyed bugs (BEB) and damsel bugs (NAB) that were collected from alfalfa seed fields. C.) Percentage of Lygus bugs (blue) and aphids (orange) detected within the guts of big-eyed bugs (BEB) and damsel bugs (NAB) that were collected from alfalfa forage fields. D.) Total percentages of Lygus bugs (blue) and aphids (orange) detected within the guts of big-eyed bugs (BEB) and damsel bugs (NAB) collected from all sites.
Figure 3. Mean ± SE of Lygus adults, large Lygus nymphs, small Lygus nymphs, aphids, big-eyed bugs, and damsel bugs sampled from the pesticide trial field site on May 18 (pre-treatment in blue), May 22 (4 days post-treatment in orange), June 2 (15 days post-treatment in gray), and June 9 (22 days post-treatment in yellow), 2015.
Figure 4. Total number of predators collected over the course of the Sivanto® trial for gut content analysis and the positive detections of Lygus and aphids from the gut content analysis. A.) Total number of big-eyed bugs (BEB) collected at specific dates in the Sivanto® trial and the number of positive Lygus (+ LYG) and positive aphid (+ APH) detections within the guts of 31 big-eyed bugs. B.) Total number of damsel bugs (NAB) collected at specific dates in the Sivanto® trial and the number of positive Lygus (+ LYG) and positive aphid (+ APH) detections within the guts of 8 damsel bugs. Note that predators were only collected 15 and 22 days post-application.
Increased tolerance to organophosphate and pyrethroid insecticides in populations of the western tarnished plant bug (*Lygus hesperus*) in central Washington State.

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Abstract

BACKGROUND: *Lygus hesperus* is considered the most important pest of alfalfa throughout the western U.S. Because of continued reports of large numbers of these pests despite the use of registered pesticides, we investigated potential resistance to two pesticides commonly used for lygus control in central Washington State.

RESULTS: We found evidence of increased tolerance to both an organophosphate (chlorpyrifos) and a pyrethroid (bifenthrin). However, bioassays revealed that lethal concentrations required to kill 90 percent of these field populations of *L. hesperus* were still below recommended application rates for both pesticides. Synergist studies indicated that increased field tolerance to chlorpyrifos was mediated by multiple metabolic detoxification pathways, particularly glutathione S-transferases; we could not identify similar mediators of bifenthrin tolerance.

CONCLUSION: Failures of chlorpyrifos and bifenthrin to control *L. hesperus* in Washington State do not appear to be due to resistance to either insecticide. Care should be taken to follow recommended pesticide application rates and schedules, and fields should be monitored to ensure elevated tolerance to these insecticides does not rise to the level of field resistance.

1 INTRODUCTION

The western tarnished plant bug, *Lygus hesperus*, is a key pest of field, vegetable, fruit, and seed crops in the western United States. This species feeds on more than 100 plant species from 24 families {Young:1986wb}, and causes major economic damage to crops such as alfalfa grown for seed {Hutchins:1990bp}, cotton {Cooper:2012bb, Cooper:2013fh}, and strawberries {Laning:2013ug}. In alfalfa, where they are the most important pest throughout the western U.S. {Hyrnick:2005uh}, lygus bugs cause several types of bud and flower damage {Shackel:2005jv}, and can also cause seed deformation and reduce seed viability {Gupta:1980wv}. Yield losses from lygus bugs feeding in alfalfa in the absence of control can range from 50% to over 90% {Hyrnick:2005tx}.

Control of lygus bugs is heavily dependent on insecticides {Hyrnick:2005uh, Zhu:2012bi}. As with many other pest insects, lygus bugs have become increasingly resistant to chemical insecticides. Populations of the tarnished plant bug (*Lygus lineoralis*) collected from cotton in the Mississippi River delta have exhibited resistance
to pyrethroids, carbamates and organophosphates \cite{Snodgrass:2000uj, Snodgrass:2003wa, Snodgrass:2009ve}. However, despite the continuing economic damage caused by \textit{L. hesperus}, there have been almost no studies of field resistance in this pest since reports of resistance to organophosphates in southern Idaho and northern Utah two decades ago \cite{ZHU:1990fw, XU:1994kp, ZHU:1992dt, XU:1993ub}.

Identifying insecticide resistance and understanding its mechanisms is critical to the effective management of insect pests. A lack of understanding of the prevailing resistance mechanism in a particular insect population may result in use and overuse of inappropriate insecticides, leading to ineffective control and even compounding levels of resistance. The study of resistance in the field is also of special importance given that, although laboratory reared and selected populations are often popular and valuable research tools, the mechanisms of resistance that occur naturally in the field are typically unique and understudied \cite{FfrenchConstant:2013eq}.

In this study we investigated levels of insecticide resistance in \textit{L. hesperus} collected from locations throughout the Columbia Basin region of Washington State. The Columbia River Basin in Washington and Oregon is a major production region for alfalfa seed, producing 4.5% of the U.S. total \cite{Hyrnick:2005uh}. Hyrnick and Downey \cite{Hyrnick:2005uh} list 16 insecticides registered for use on lygus in the Western U.S. We chose to focus on potential resistance to two of these insecticides, both commonly used on alfalfa in Washington state and both of which are considered to provide good efficacy (80-90% control) on lygus bugs: chlorpyrifos, an organophosphate, and bifenthrin, a pyrethroid. We also investigated potential mechanisms of resistance in these insects, principally by testing inhibitors of major metabolic resistance pathways to synergize the effectiveness of the insecticides on the most resistant populations.

2 MATERIALS AND METHODS
2.1 Insect collection

Lygus bugs were collected from alfalfa forage, alfalfa seed, and mustard fields in Prosser, Touchet, Yakima, and Othello, in Washington State (Fig. 1). Intensive sampling was conducted from June through August 2013 (for bifenthrin assays) and June through August 2014 (for chlorpyrifos assays). The Roza population from 2014 was also tested for bifenthrin resistance. The lygus bugs were collected with a sweep net from the upper parts of plants. The bugs were then aspirated from the sweep net collection and identified to species using an identification key \cite{Mueller et al. 2009}. \textit{L. hesperus} were then placed in a container with green bean pods (\textit{Phaseolus vulgaris}), for immediate (within 2 hrs of collection) use in bioassays or preservation in RNA \textit{later}™ RNA stabilization reagent (Qiagen), for subsequent RNA extraction. A pesticide-susceptible laboratory-reared \textit{Lygus hesperus} population \cite{Hull:2013ch} was obtained from Joe Hull at the USDA-ARS Arid Land Agricultural Research Center (Maricopa Arizona) and reared on green bean pods.

2.2 Pesticide bioassays

Adults of \textit{L. hesperus} from each field location and the laboratory-reared colony were tested for resistance to chlorpyrifos or bifenthrin using a Potter Spray Tower.
Chlorpyrifos (Lorsban 4E, Dow AgroSciences) was dissolved in water and tested at the recommended field rate (600 ppm ai), 30 ppm ai, 60 ppm ai, 120 ppm ai, and 300 ppm ai. Bifenthrin (Capture 2 EC, FMC Agricultural Products Group) was dissolved in water and tested at the recommended field rate (120 ppm ai), 1.2 ppm ai, 12 ppm ai, 60 ppm ai, and 240 ppm ai. Each test was replicated three times. Each individual test consisted of 30 (undamaged, active) adult *L. hesperus* placed in a plastic petri dish with green beans. Mortality was determined 48 hours after treatment (HAT). Adults were considered dead if they were unable to move or there was no movement when prodded. Dose-response curves were plotted for each sample population. Data from the tests was analyzed using probit analysis to calculate LC$_{50}$ and LC$_{90}$ (the lethal concentration that will kill 50% and 90% of the population, respectively). The LC$_{50}$ and LC$_{90}$ values from each population were then compared with the LC$_{50}$ and LC$_{90}$ value from the susceptible population to obtain resistance ratios (RR$_{50}$ and RR$_{90}$). Lack of overlap of 95% confidence limits on estimated LC$_{50}$ and LC$_{90}$ values between susceptible and field populations denoted significant differences in tolerance to bifenthrin.

Synergist bioassays were conducted using pharmalogical inhibitors of the major classes of metabolic resistance pathways: verapamil (Sigma-Aldrich), an inhibitor of ABC transporters (Lanning:1996tm, Chouaibou:2013ci); piperonyl butoxide (PBO) (Sigma-Aldrich), an inhibitor of P450s (Jacobson:2009it, Bass:2011ey, Perry:2011fh, Chouaibou:2013ci); diethyl maleate (DEM) (Sigma-Aldrich), an inhibitor of GSTs (Tiwari:2011bx, Chouaibou:2013ci); and triphenyl phosphate (TPP) (Sigma-Aldrich), an inhibitor of COEs (Tiwari:2011bx, Zhang:2014uy). All inhibitors were used at a concentration of 0.1% and were dissolved in water, except for TPP, which was dissolved in 10% acetone. Insects were pre-exposed to the inhibitor by Potter spray tower and allowed to recover for 1 hour before insecticide application. Insects were scored for mortality 24 hours after insecticide application. For the most effective synergist (PBO) we then repeated performed complete bifenthrin dose response curves as above, both with and without pre-exposure to 0.1% PBO. These dose response curves were then used to calculate the synergism ratio and % suppression for PBO.

**2.3 DNA cloning and sequencing**

Individual adult *L. hesperus* were used for total RNA extraction (RNeasy mini kit – Qiagen). First strand cDNA was synthesized from RNA template by reverse transcription reaction using iScript cDNA synthesis kit (Bio-Rad). Two separate regions of the *L. hesperus* VGSC (Hull:2013ch) that contain most of the known mutations that confer pyrethroid resistance in insects {Dong:2007hm, Soderlund:2008bz, Liu:2012ty} were amplified by PCR (Fig. 2): a 627bp region in domain I that contains two known mutations sites and 428bp region of domain II that contains 5 mutation sites. The primers used were domain I forward primer 5'- TTTCCCCGAAGACGGTTCAG -3'; domain I reverse primer: 5'- GTTGCCCTTCTCTTGTCCGA -3' domain II forward primer: 5'- AAGGCTGTGACTTTTGCGA -3'; domain II reverse primer: 5'- ATCGAAGGCTACGAGATTT -3'. PCR cycling parameters were: initial denaturation 95°C for 2 min; 40 cycles of 95°C for 30 sec, 55°C annealing for 1 minute, and 72°C extension for 2 min. Amplified gene fragments were purified (DNA Clean & Concentrator -5, Zymo Research) and Sanger dye
RESULTS

3.1 L. hesperus populations in central Washington show decreased sensitivity to chlorpyrifos and bifenthrin.

We chose to examine whether any resistance was evident in the field to two of the more common insecticides registered for use against lygus bugs, chlorpyrifos (and organophosphate) and bifenthrin (a pyrethroid). Because lygus bugs are extreme generalists and considered to move readily between host plants {Scott:1977vn, Young:1986wb, Carcamo:2003ul, Swezey:2013db} we were also interested to see whether insects collected from hosts that are treated more heavily with insecticides showed higher resistance levels than those collected from hosts receiving lesser or no insecticide treatments. We thus collected L. hesperus adults from three host plants that are subjected to differing insecticide regimes: 1) wild mustard (various Brassicaceae, especially Sisymbrium spp.) – these are weeds that occur on the margins of cultivated fields and may serve as refuges for lygus bugs; they are not treated with insecticides; 2) alfalfa (Medicago sativa) grown for forage – because alfalfa grown for forage is harvested earlier than alfalfa grown for seed it receives fewer insecticide treatments (typically only one); 3) alfalfa grown for seed – a longer growing period required for the plants to produce seed, coupled with the damaging effect of lygus bugs on seed production, means seed alfalfa receives the heaviest insecticide exposure of the three hosts from which we collected lygus. We collected L. hesperus from these host plants at various field sites in central Washington (Fig. 1), as well as a laboratory-reared population that has never been exposed to insecticides. The L. hesperus adults collected from these sites were used in bioassays to generate dose response curves. We tested populations from 15 field sites for their resistance to chlorpyrifos and populations from 10 field sites for their resistance to bifenthrin. The dose response curves were then used in probit analysis to calculate LC_{50} and LC_{90} values for each site, and all sites were compared to the susceptible (lab-reared) population to generate resistance ratios (RR). These results are summarized in Table 1. For chlorpyrifos, 10 of the 15 (67%) field sites had LC_{50} significantly greater than the susceptible (lab-reared) population, and 13 of 15 (87%) had LC_{90} significantly greater than the susceptible population. However, despite elevated levels of resistance, all of the mean LC_{90} values were still below 600ppm ai, the recommended application rate for chlorpyrifos. For bifenthrin, 6 of the 10 (60%) field sites had LC_{50} greater than susceptible population, and 8 of 10 (80%) had LC_{90} greater than the susceptible population. Again though, all of the mean LC_{90} values were below 120ppm ai, the recommended application rate for bifenthrin, except for the Roza site, which had a slightly higher LC_{90} (142.471 ppm ai), although the lower confidence level (86.311 ppm ai) was below the recommended application rate. These increased LC values would therefore not meet the definition of resistance used by the Insecticide Resistance Action Committee (“a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of
control when used according to the label recommendation for that pest species”). We will thus refer to increased sensitivity toward pesticides, rather than resistance, where appropriate, in order to avoid confusion with field failure of pesticides (i.e. field resistance).

There was an increase in mean RR$_{50}$ and RR$_{90}$ values from insects collected from wild mustard to insects collected from forage alfalfa to insects collected from seed alfalfa, for both chlorpyrifos and bifenthrin (Fig. 3). However, due to high variance in resistance ratios between different sites, only for the RR$_{50}$ for chlorpyrifos was there a statistically significant effect of host plant on RR (Table 2). Tukey HSD testing showed that for this comparison, the RR$_{50}$ values for insects collected from seed alfalfa were significantly higher than those collected from mustard or forage alfalfa (P<0.01 for both comparisons), which were not significantly different from each other. Thus our data indicates that host plant is only very weakly related to level of sensitivity.

3.2 Synergist bioassays indicate that sensitivity to chlorpyrifos is mediated by several metabolic detoxification enzymes, especially GSTs.

Despite the lack of field resistance, the decreased sensitivity to chlorpyrifos and bifenthrin that we observed is still of concern. Over time, depending on changes in selection pressure and management techniques, such decreased sensitivity may evolve into true field resistance. Understanding the mechanism for the decrease in insecticide sensitivity we observed would aid in predicting the likelihood of field resistance and in management strategies (McKenzie:1998cf). In order to begin understanding these mechanisms we conducted synergist bioassays. We used inhibitors of the major classes of metabolic detoxification enzymes to see if they would act as insecticide synergists, increasing the sensitivity of _L. hesperus_ to chlorpyrifos or bifenthrin, thus implicating the corresponding enzyme class in resistance. For these experiments we used the _L. hesperus_ population that exhibited the highest resistance levels to both chlorpyrifos and bifenthrin: Roza (from seed alfalfa). We performed bioassays on insects exposed to chlorpyrifos (at 180 ppm ai, approximately the LC$_{50}$ for the Roza population) and bifenthrin (at 27.024 ppm ai, again approximately the LC$_{50}$ for this population), either alone or after a one hour pre-exposure to one of the following insecticidal synergists: piperonyl butoxide (PBO) (an inhibitor of cytochrome P450s), triphenyl phosphate (TPP) (an inhibitor of carboxyl esterases - COEs), diethyl maleate (DEM) (an inhibitor of glutathione S–transferases - GSTs), and verapamil (an inhibitor of ATP-binding cassette [ABC] transporters).

Only verapamil had a significant effect on chlorpyrifos-induced mortality (T-test, $t=3.1623$, $P=0.0341$) (Fig. 4A), and none of the inhibitors had a significant effect on bifenthrin-induced mortality (Fig. 4B). However, although not significant, mean mortality did show an increase for almost all inhibitors for both pesticides. Thus we decided to examine possible synergism more closely by conducting bioassays to generate dose-response curves and calculate LC$_{50}$ and LC$_{90}$ values for both insecticides and insecticides plus inhibitors (all inhibitors were again used at 0.1%). When LC$_{50}$ or LC$_{90}$ differed significantly between insecticide alone and insecticide plus inhibitor we also calculated suppression ratio and percent suppression. For chlorpyrifos at lower
(LC$_{50}$) concentrations all four inhibitors resulted in significantly lower LC$_{50}$ values (Table 2A). At higher (LC$_{90}$) chlorpyrifos concentrations only DEM and verapamil had significant synergistic effects. Thus at lower chlorpyrifos concentrations P450s, COEs, GSTs, and ABC transporters may all play a role in metabolic detoxification. At higher concentrations ABC transporters and especially GSTs seem to be important. In fact GST transporters were the strongest candidates for metabolic detoxification of chlorpyrifos, as DEM produced the strongest synergistic effects for both lower and higher chlorpyrifos concentrations (Table 2A). For bifenthrin, several of the pesticide plus inhibitors produced lower mean LC$_{50}$ and/or LC$_{90}$ than bifenthrin alone, especially TPP (Table 2B). However, none of these comparisons was statistically significant, and at this point we cannot conclude that metabolic detoxification plays a role in our observed decreased sensitivity to bifenthrin.

### 3.3 Resistance to bifenthrin does not correlate with known mutations in the voltage-gated sodium channel target site.

Because we did not find evidence of metabolic detoxification of bifenthrin, and we had a number of preserved specimens from all our populations, we decided to see if there was any evidence for target site immunity to bifenthrin. The target site of pyrethroids is the voltage-gated sodium channel (VGSC), an integral membrane protein containing four repeated domains. Amino acid substitutions that have been confirmed to reduce VGSC sensitivity to pyrethroids have been found in at least 9 loci in the VGSCs of different insect taxa {Dong:2007hm, Soderlund:2008bz, Liu:2012ty}. These mutations all occur in the first two repeat domains. By far the most common of these is the kdr (knock-down resistance) mutation, a substitution of leucine (at position 1014 in L. hesperus) in domain II. Thus we cloned and sequenced two stretches of the L. hesperus VGSC containing the kdr site and six of the other eight mutation sites from insects collected at our different sites (a (Fig. 2), looking for evidence of target site insensitivity-mediated resistance. We cloned and sequenced the 627bp fragment from domain I from 64 individuals: 9 from our susceptible lab population, 10 collected from IAREC Prosser (alfalfa forage), 10 from Touchet (alfalfa forage), 7 from Gardena Rd (alfalfa seed), 9 from Grandview (mustard), 9 from Yakima (alfalfa seed), and 10 from Othello (alfalfa forage) (see Table 1B for bifenthrin susceptibilities for these populations). None of the 64 individuals showed any variation in the 209 amino acid sequence of this region, including Y421 and E434, which are sites where mutations are known to confer resistance in other insects. The other region cloned and sequenced was a 428bp fragment of domain II, which was sequenced from 31 individuals: 9 from the susceptible lab colony, 6 from IAREC Prosser (alfalfa forage), 1 from Othello (alfalfa forage), 7 from Touchet (alfalfa forage), 2 from Yakima (alfalfa seed), 2 from Lowden (alfalfa seed), 2 from Olsen Bros Prosser (mustard), and 2 from Grandview (mustard). No individuals had substitutions in the kdr (L1014) or any of the other four known pyrethroid resistance-mediating sites in this fragment (M918, T929, M932, and V1016). There was one polymorphic site, however, a single base substitution that resulted in an L1017M substitution. Although close to some amino acid sites known to mediate pyrethroid resistance, this site has not been implicated in resistance. In addition, the
polymorphism was evenly distributed throughout the populations: in the susceptible laboratory population 3 individuals were L1017, 4 were M1017, and 2 had sequencing chromatograms with two strong peaks (A and T) at the nucleotide substitution position, consistent with heterozygosity; the IAREC Prosser population had 1 L1017, 4 M1017 and one probable heterozygote; the Othello individual was M1017; Touchet had 3 with L1017 and 4 with M1017; Yakima had 1 with L1017 and 1 with M1017; Lowden had 1 with L1017 and 1 with M1017; Olsen Bros Prosser had 2 with L1017; and Grandview had 2 with M1017. Thus roughly half the insects had the L1017M substitution, both in control and field populations, and there was no correlation between the substitution and bifenthrin susceptibility.

4 DISCUSSION

We have found decreased sensitivity to both the organophosphate chlorpyrifos and the pyrethroid bifenthrin in *L. hesperus* collected from field sites in central Washington State. However, resistance ratios were not high (the highest was 12), and importantly LC<sub>90</sub> values for all populations did not exceed recommended field rates (Table 1). We also collected *L. hesperus* from different host plants that face different intensities of insecticide exposure, with the hypothesis that resistance would be highest when collected from host plants with the highest exposure and thus greater selective pressure. Although the data tended to trend in this direction (Fig. 3), there was for the most part no significant relationship between host plant and resistance. We found decreased levels of sensitivity to insecticides not only in lygus from alfalfa fields that were subjected to insecticide spraying, but in lygus from wild mustards that were never treated with insecticides. Lygus bugs are extreme generalists {Scott:1977vn, Young:1986wb}, and may move between multiple host plants {Carcamo:2003ul, Swezey:2013db}. Such movements insure that individual lygus bugs collected off a particular host plant may not have been at the same location at the time(s) of insecticide application, and insects collected from non-sprayed fields may have come from populations that were exposed to insecticides at a previous location. Our data indicates that if there are any population-level effects on resistance due to greater pesticide exposure on some host plants they are likely offset by movement of individual lygus bugs between different hosts.

It is important to note again that despite increased resistance levels to chlorpyrifos and bifenthrin in most of our study sites, the mean LC<sub>90</sub> values of all our populations were below the recommended field rate of chlorpyrifos and all but one were below the recommended rate for bifenthrin, and no LC<sub>90</sub> lower confidence limits that exceeded the recommended field rates for either insecticides. Thus failures to control *L. hesperus*, and concomitant economic damage, that have been reported on alfalfa in central Washington do not appear to be a consequence of resistance to chlorpyrifos or bifenthrin. Although we do not know the complete exposure histories of all of the sites used in this study, correct application of either of these insecticides at recommended rates should remain effective in control of *L. hesperus*. Failure of chlorpyrifos or bifenthrin to control *L. hesperus* in specific locations may result more
from applicator errors, such as improper application rates (miscalibrated sprayers, etc.), than from insecticide-resistant pests.

Even the low levels of insecticide resistance we observed in this study indicate the need for continued resistance monitoring. Although proper application of chlorpyrifos or bifenthrin should effectively control *L. hesperus*, many different insect populations have become extremely resistant to organophosphates and pyrethroids, often to the point of complete refractoriness (Dong:2007hm, Soderlund:2008bz, Liu:2012ty, Casida:2013it). Thus we investigated the underlying mechanisms for the resistance we did observe in our field populations, mechanisms that may become significant if stronger levels of resistance should develop in the future. Four inhibitors of different metabolic detoxification pathways had at least some significant synergistic effects when used with chlorpyrifos (Fig. 4A, Table 2A). Our data indicates that P450s, COEs, ABC transporters, and GSTs may all play a role in detoxification at lower chlorpyrifos concentrations, and ABC transporters and especially GSTs may be most important, especially at higher chlorpyrifos concentrations. Previous research has focused on a potential role for COEs in detoxification of organophosphates by *L. hesperus* (ZHU:1990fw, XU:1994kp, ZHU:1992dt, XU:1993ub), although a microarray study of organophosphate (acephate) resistant *Lygus lineolaris* found not only increased expression of six esterases, but also three P450s and one GST (Zhu:2012bi). Multiple mechanisms of detoxification seem to be functioning in the *L. hesperus* response to chlorpyrifos as well.

We have no strong candidates for mediating decreased sensitivity to bifenthrin. Our synergism studies did not show a significant relationship between any of our inhibitors and bifenthrin-mediated mortality. This was somewhat suprising to us because the Roza population used in these studies had the highest LC$_{50}$ and LC$_{90}$ of any population for both bifenthrin and chlorpyrifos. Thus we hypothesized that a similar mechanism was responsible for this population’s reduced sensitivity to both insecticides. Of course we cannot rule out any of these mechanisms - we did not test a range of inhibitor concentrations, and do not have measures of the level of enzyme activity at the inhibitor concentrations tested. And there are other classes of enzymes that can mediate resistance, such as UDT-glycosyltransferases (Perry:2011fh), that we were unable to test. But currently we do not have evidence supporting a role for P450s, COEs, GSTs, or ABC transporters in reducing the effectiveness of bifenthrin on our field populations.

We also have no evidence that target-site insensitivity mediates our observed decreased susceptibility to bifenthrin. We found no mutations at any of seven loci in the VGSC known to mediate decreased sensitivity to pyrethroids in insects, including at the kdr position, which is the most frequent such mutation in insects. We did find a find an L1017M amino acid substitution, but this amino acid site has not been previously implicated in pyrethroid resistance, nor was there correlation between bifenthrin sensitivity and the substitution. Thus it does not seem likely that this substitution mediates target site insensitivity to pyrethroids. Certainly our search for resistance associated-mutations in the *L. hesperus* VGSC was not exhaustive. We did not sequence the region associated with two other known mutations, C764R from *Blatella germanica*
or M827I from *Musca domestica* and *Haemotobia irritans* (although the latter mutation only seems to occur in conjunction with the kdr mutation) {Dong:2007hm, Soderlund:2008bz, Liu:2012ty}. And there could be novel mutation sites anywhere within the VGSC that mediate resistance. It should also be noted that the resistance ratios we found in field populations were much lower than those typically reported for insects with target site mutations {Dong:2007hm, Soderlund:2008bz, Liu:2012ty}. However, until we have sequenced the VGSC from multiple individuals from populations with different levels of susceptibility we cannot completely rule out the possibility of this mechanism being a factor.

5 CONCLUSION

Field populations of *L. hesperus* from central Washington State show decreased susceptibility to chlorpyrifos and bifenthrin, two insecticides commonly used for their control on alfalfa. However, bioassays indicate that this decreased susceptibility has not yet evolved to the level of field resistance. Metabolic resistance enzymes, especially glutathione S-transferases, appear to be mediating decreased susceptibility to chlorpyrifos; for bifenthrin resistance mechanisms are unclear. These results indicate the need to adhere to recommended pesticide application rates and to monitor resistance in field populations, in order to prevent decreased susceptibility to insecticides from evolving into full field resistance.
Table 1: Lethal concentration and resistance ratios of A) chlorpyrifos and B) bifenthrin for field populations of *L. hesperus*.

<table>
<thead>
<tr>
<th>Location</th>
<th>Host Plant</th>
<th>Slope±SE</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt;(ppm ai)(95%CL)</th>
<th>LC&lt;sub&gt;90&lt;/sub&gt;(ppm ai)(95%CL)</th>
<th>RR&lt;sub&gt;50&lt;/sub&gt;</th>
<th>RR&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible lab colonies</td>
<td>-</td>
<td>2.263 ± 0.384</td>
<td>3.292 (2.352–4.492)</td>
<td>12.131 (8.108–24.466)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Olsen Bros, Prosser</td>
<td>Mustards</td>
<td>1.390±0.238</td>
<td>3.651 (1.794–6.249)</td>
<td>30.503 (16.686–80.149)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Grandview</td>
<td>Mustards</td>
<td>1.979±0.330</td>
<td>10.729* (5.957–16.434)</td>
<td>47.642* (30.805–89.447)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Wapato, Yakima</td>
<td>Mustards</td>
<td>2.295±0.518</td>
<td>12.112* (5.959–3.776)</td>
<td>43.819* (29.605–88.434)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>IAREC Prosser</td>
<td>Alfalfa forage – no pesticide</td>
<td>1.508 ± 0.257</td>
<td>3.579 (2.056–5.569)</td>
<td>25.323 (14.652–64.014)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Touchet</td>
<td>Alfalfa forage</td>
<td>1.855 ± 0.253</td>
<td>18.393* (11.931–26.347)</td>
<td>90.263* (60.210–160.561)</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Othello</td>
<td>Alfalfa forage</td>
<td>2.155 ± 0.480</td>
<td>9.700* (5.368–14.883)</td>
<td>38.155* (25.076–78.705)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Yakima</td>
<td>Alfalfa seed</td>
<td>1.891±0.400</td>
<td>9.659 (4.134–15.536)</td>
<td>46.022* (29.314–95.159)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Lowden</td>
<td>Alfalfa seed</td>
<td>1.460±0.231</td>
<td>9.602 (1.904–22.292)</td>
<td>72.487* (30.939–425.063)</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Gardena Rd, Touchet</td>
<td>Alfalfa Seed</td>
<td>2.056±0.386</td>
<td>21.597** (11.906–31.586)</td>
<td>90.730* (61.643–168.804)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Roza</td>
<td>Alfalfa Seed</td>
<td>1.918±0.477</td>
<td>30.590* (13.789–47.851)</td>
<td>142.471* (86.311–432.692)</td>
<td>9</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 2: Effect of metabolic resistance enzyme inhibitors on lethal concentration and resistance ratios of A) chlorpyrifos and B) bifenthrin for a field population (Roza) of *L. hesperus*.

<table>
<thead>
<tr>
<th>Insecticide + Inhibitor</th>
<th>Slope ± SE</th>
<th>LC50(\text{g/L}(95%\text{CL}))</th>
<th>LC90(\text{g/L}(95%\text{CL}))</th>
<th>SR(_{50})</th>
<th>% Suppression(_{50})</th>
<th>SR(_{90})</th>
<th>% Suppression(_{90})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>6.571 ± 1.632</td>
<td>183.621 (128.223-227.546)</td>
<td>287.706 (232.212-407.629)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorpyrifos + PBO</td>
<td>2.770 ± 0.437</td>
<td>61.276* (44.092-80.727)</td>
<td>177.705 (129.646-289.039)</td>
<td>3.00</td>
<td>66.63</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorpyrifos + TPP</td>
<td>2.941 ± 0.482</td>
<td>72.653* (51.724-95.676)</td>
<td>198.186 (145.916-317.965)</td>
<td>2.53</td>
<td>60.43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorpyrifos + DEM</td>
<td>3.867 ± 0.824</td>
<td>40.320* (30.064-52.910)</td>
<td>86.475* (63.642-158.748)</td>
<td>4.55</td>
<td>78.04</td>
<td>3.33</td>
<td>69.94</td>
</tr>
<tr>
<td>Chlorpyrifos + VER</td>
<td>9.595 ± 3.205</td>
<td>93.665* (74.524-103.119)</td>
<td>127.393* (113.324-198.953)</td>
<td>1.96</td>
<td>48.99</td>
<td>2.26</td>
<td>55.72</td>
</tr>
</tbody>
</table>
Fig. 1. Central Washington collection sites for *L. hesperus*

Sampling location  WASHINGTON

A. UAREC, Prosser
B. Ross, Prosser
C. Olson Bro, Prosser
D. Sand Pit Rd, Touchet
E. Gardena Rd, Touchet
F. Lowden
G. Othello
H. Yakima
I. Wasco
J. Grandview
Fig. 2. Voltage-gated sodium channel (VGSC) regions cloned and sequenced in this study. Schematic of the approximate positions of amino acid substitutions known to confer decreased sensitivity to pyrethroids in other insects within the two VGSC fragments cloned and sequenced from *L. hesperus*. Substitutions are numbered according to the amino acid sequence of *L. hesperus*.

<table>
<thead>
<tr>
<th>Domain I PCR fragment</th>
<th>627bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Domain II PCR fragment</th>
<th>428bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 4</td>
<td>5 6 7</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V417L/M</td>
<td><em>Cimex lectularius, Heliothys virescens</em></td>
</tr>
<tr>
<td>2</td>
<td>E430K</td>
<td><em>Blattella germanica</em></td>
</tr>
<tr>
<td>3</td>
<td>M918T</td>
<td><em>Musca domestica, Haemotobia irritans</em></td>
</tr>
<tr>
<td>4</td>
<td>T929I/C/V</td>
<td><em>Plutella xylostella, Pediculosis capitis, Eriophyidae occidentalis, Ctenocephalides felis</em></td>
</tr>
<tr>
<td>5</td>
<td>L932F</td>
<td><em>Pediculosis capitis</em></td>
</tr>
<tr>
<td>6</td>
<td>L1014F/H/S (kdr)</td>
<td><em>Many insects</em></td>
</tr>
<tr>
<td>7</td>
<td>V1016G</td>
<td><em>Aedes aegypti</em></td>
</tr>
</tbody>
</table>
Fig. 3. Mean resistance ratios (RR) to A) chlorpyrifos and B) bifenthrin for *L. hesperus* recovered from specific host plants. Error bars indicate one standard deviation. There were no significant differences between different host plants, except the RR$_{50}$ for bifenthrin from insects collected from seed alfalfa was significantly higher than for insects collected from forage alfalfa or wild mustard. See text for details.
Fig. 4. Effect of metabolic detoxification inhibitors on mortality of *L. hesperus* adults exposed to A) chlorpyrifos and B) bifenthrin. PBO (P450 inhibitor), TPP (COE inhibitor), DEM (GST inhibitor), and verapamil (ABC transporter inhibitor) were applied to *L. hesperus* one hour before pesticide application. Mortality was measured 48 hours later.