Annual Progress Report
This is a Final Report for a Two-year project.
For the Washington State Grape & Wine Research Program

Date: 12/15/2014
Project Title: Sampling, Identification & Control of Leafhopper Pests in Washington Vineyards
Principal Investigator(s):
Douglas Walsh, PhD, Professor of Entomology, WSU Prosser, dwalsh@wsu.edu 509-786-9287
Laura Lavine, PhD, Professor of Entomology, WSU Pullman, lavine@wsu.edu 309-335-7907
Collaborator(s):
Holly Ferguson, PhD, Extension IPM Coordinator Specialist, WSU Prosser
Mark Lavine, PhD, Post Doctoral Researcher, Entomology WSU Pullman
Project Budget Number: 13Z-3343-7789, OGRD 124347

I. Project Summary:

Two species of leafhoppers, the Western Grape and the Virginia Creeper Leafhoppers (WGLH and VCLH), are direct pests of grapes due to their feeding damage, nuisance pests for vineyard workers, and suspected vectors for grapevine red blotch, a viral disease recently introduced into Washington State, which causes a dramatic drop in Brix (sugar content) of ripened fruit on infected vines. Disparity may exist between leafhopper species in their ability to vector the disease. In the early 2000s, Walsh conducted sampling studies that determined that most grape growers treated their vineyards for leafhoppers when population abundance exceeded 15 per leaf. No distinctions among leafhopper species were made in these studies. Grape entomologists have paid little attention to leafhopper research for the past ten years, focusing instead on mealybugs. Given the emergence of grapevine red blotch, it is important to reinitiate studies on leafhopper abundance in grapes and follow grower treatment decisions based on leafhopper abundance. In 2013 we initiated leafhopper surveys in Washington State vineyards. We continued surveys in 2014 to further refine leafhopper sampling, identification, and control strategies.

Objectives of Research:
1. Investigate sampling methods for quantifying leafhopper abundance in grape vineyards.
2. Develop a DNA barcode method to differentiate WGLH and VGLH from one another.
3. Conduct insecticide efficacy studies to determine if there are differences between WGLH and VCLH in response to commercially available and prevalent insecticides.

At each vineyard site, field sampling consisted of looking at 100 leaves for presence/absence of leafhoppers, scanning 20 leaves with a hand lens for field counts of leafhoppers, and bringing back ten of those 20 leaves to the lab for counting and identification to species. Leafhopper nymphs identified to species were placed in vials with 95% alcohol and sent to Pullman for DNA barcoding analysis. Since insecticides commonly used against leafhoppers (mostly imidacloprid products) are still very effective, insecticide efficacy studies were deemed not necessary. Growers were kept informed if leafhopper infestations approached economic levels. Results will be reported at industry and entomology meetings. Ultimately, we will provide updated knowledge of leafhopper abundance and species to improve leafhopper management strategies for Washington State grape growers.

II.   Materials, Methods and Experiments Conducted to Meet Stated Objectives:

1. Investigate sampling methods for quantifying leafhopper abundance in grape vineyards:
Working with growers in five AVAs from Zillah to Kennewick, 49 vineyard blocks were sampled for leafhoppers using three methods to estimate abundance (Table 1). Seventeen wine grape varieties were included in the survey, and two Concord grape blocks were sampled in early season. Vineyards with leafhoppers in abundance were sampled weekly till early September or until grower treated the vineyard with insecticide. Vineyards found to have low numbers of leafhoppers were sampled less frequently. At each site, 100 shade leaves picked from several rows were visually scanned in the field for presence or absence of leafhoppers to determine percent infestation, i.e., number of leaves with at least one leafhopper. Twenty shade leaves were chosen randomly from several rows and were scanned with a magnifying hand lens for field leafhopper nymph counts. Counts of leafhopper adults were not accomplished because they usually fly off the leaf once it is picked. Then ten of those 20 leaves were collected and transported to Walsh's Environmental and Agricultural Entomology Laboratory where leaves were examined under a dissecting microscope and leafhopper nymphs were identified to species and counted. Leafhopper nymphs found on these leaves were placed in 95% ethanol, keeping species separate, for use in Objective 2 (detailed below). Regression analyses (Minitab® 16.2.4) were performed to determine the relationships between counts obtained in the field versus counts under the laboratory microscope and between percent infestation and nymphs per leaf obtained with 20 leaves and with ten leaves. Seasonal abundances of the two species were graphed using a composite set of count means over the whole season for Yakima Valley, Horse Heaven Hills, and Columbia Valley AVAs.

2. Develop a DNA barcode method to differentiate WGLH and VGLH from one another.
DNA was extracted from leafhopper samples in Lavine's WSU-Pullman laboratory using Chelex® 100 resin (Bio-Rad). DNA primers were designed to amplify a short length of mitochondrial DNA (mtCOI), which is species-specific. Amplified DNA was then sequenced and aligned with known sequences using MUSCLE, an accurate and rapid multiple sequence alignment method. Phylogeny trees (relationships between species-specific sequences) were constructed with PHYML (an online computer program that estimates relatedness between sequences).

3. Conduct insecticide efficacy studies to determine if there are differences between WGLH and VCLH in response to commercially available and prevalent insecticides: Since insecticides commonly used against leafhoppers (mostly imidacloprid products) are still very effective, insecticide efficacy studies were deemed not necessary and were not conducted.

III.   Major Research Accomplishments

2014 Timeline and Milestones:
April to July: Contacted growers to get vineyard blocks for leafhopper sampling *(Objective 1)*.
Early June: Began sampling vineyards with three methods to estimate population abundance at the beginning of the first generation *(Objective 1)*.
Early August: Sent first box of vials with field-collected leafhoppers to Pullman *(Objective 2)*.
August to November: Conducted DNA analyses in Pullman for speciation of field-collected leafhoppers *(Objective 2)*.

Early September: Concluded field sampling program while leafhoppers were in their third generation *(Objective 1)*.

September to October: Conducted data analysis and wrote report *(Objectives 1 and 2)*.

Early October: Sent larger box of vials with field-collected leafhoppers to Pullman. *(Objectives 1 and 2)*.

Early November: *Objectives 1 and 2 were 100% accomplished.*

Scope of Vineyard Survey

From June to early September, grape blocks were sampled for leafhoppers from Zillah to Kennewick and south to Paterson, covering the AVAs of Rattlesnake Hills, Yakima Valley, Red Mountain, Columbia Valley, and Horse Heaven Hills and including 17 varieties. We were seeking data on leafhopper abundance and distribution across grape production regions, relative abundance of Western Grape Leafhopper versus Virginia Creeper Leafhopper, and grower treatment decisions based on leafhopper abundance. Leafhopper abundance in each block was rated according to the highest level of infestation recorded, and 22 (45%) had high levels, nine (18%) had moderate levels, and 27 (55%) had low levels of infestation during 2014 (Table 1). Interestingly, 63% of the blocks had both WGLH and VCLH species, with VCLH being the predominant species. Grape variety did not appear to be a factor affecting level of leafhopper infestation, as high leafhopper infestations were found in blocks comprised of ten different varieties (Cabernet Sauvignon, Chardonnay, Merlot, Muscat, Pinot Gris, Sauvignon Blanc, Syrah, Port varieties, Viognier, and White Riesling).

Comparison of Leafhopper Sampling Methods

We compared our 2014 leafhopper sampling data with results from a similar sampling study done in 2004. Mean leafhoppers-per-leaf values were log-transformed prior to regression analyses. When field counts and microscope counts of leafhopper nymphs per leaf were compared, we generally found that more leafhoppers per leaf were counted under the microscope. This makes sense because leafhopper nymphs are highly mobile on the leaf and the smallest nymphs are very tiny and difficult to see in the field even with a hand lens. Laboratory counts were regressed against field counts for both 2004 and 2014 data sets, fitting a linear model quite well, especially in 2014 (2004: $r^2 = 81\%$, Fig. 1A; 2014: $r^2 = 91\%$, Fig. 1B). In both years, field samplers missed counting about 20% of the total number of leafhoppers on a leaf.

Lab Counts Vs. Field Counts of Leafhopper Nymphs, 2014
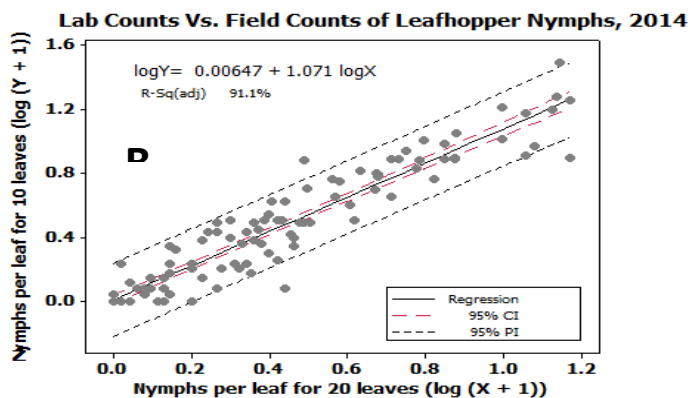
logY= 0.00647 + 1.071 logX
R-Sq(adj)   91.1%

D
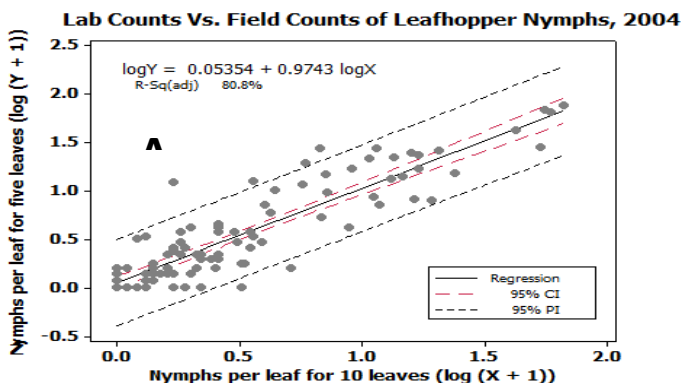
Figure 1. Laboratory counts versus field counts of leafhopper nymphs, 2004 (A) and 2014 (B)

Field and lab counts were regressed against percent infestation in both years (Figs. 2A-D). Percent infestation is often used by growers to assess leafhopper abundance and the need to treat the block for leafhoppers. However, the relationship between percent infestation and leafhopper abundance is poorly understood. Leafhoppers at low abundance are more likely to be missed during presence/absence sampling, while leafhoppers at high abundance are more likely to be greatly underestimated. Economic thresholds are based on numbers of leafhoppers per leaf. In 2004, growers treated for leafhoppers when numbers exceeded 15 per leaf. In recent years, growers are more likely to treat before leafhoppers get that high; they may in fact treat the field at one-tenth of that threshold. In 2014, at least five of the sampled blocks in the Yakima Valley got treated with imidacloprid when counts were equal to or less than one nymph per leaf. Moreover, wine grape blocks not included in the sampling survey were treated with imidacloprid for mealybugs in early season, which effectively wiped out leafhoppers as well. The 2004 data came from conventional wine grape blocks, while the 2014 data set came from both organic and conventional blocks. In both years, the log-transformed data fit a linear model well (2004: $r^2 = 77$ and 71%, Figs. 2A and B; 2014: $r^2 = 77$%, Figs. 2C and D). In 2004, 15 nymphs per leaf meant an infestation level of 72% (Fig. 2A) or 67% (Fig. 2B). However, in 2014, 15 nymphs per leaf corresponded to higher infestation levels: 100% (Fig. 2C) or 95% (Fig. 2D). In 2004, overall field populations of leafhoppers were much greater than in 2014; 14 sites had numbers exceeding 15 per leaf. In contrast for 2014, only five sites had leafhopper counts exceeding 15 per leaf. The decline in overall population abundance may be due a number of factors including (1) increased adoption of cultural practices such as stripping and leafing, which open up the canopy, making it more sun-exposed and less hospitable for leafhoppers; (2) increased prophylactic/early season treatment for mealybugs, which transmit grapevine leafroll virus disease; and (3) increased acreage in organic or biodynamic production, in which parasitization by *Anagrus* spp. wasps help keep leafhopper populations in check.
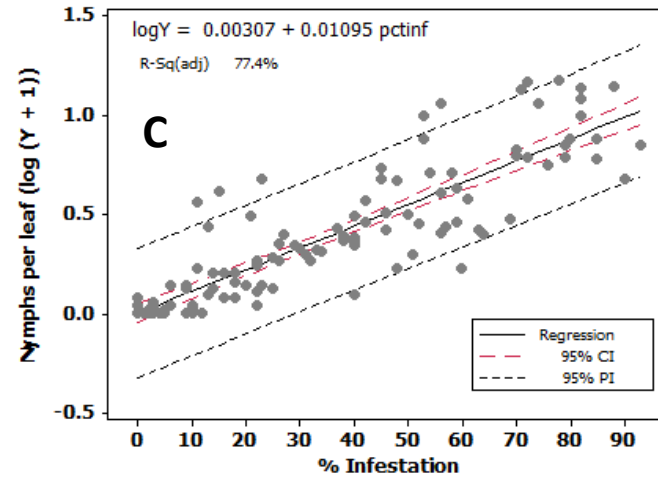
Seasonal Abundance and Leafhopper Species Shift
Leafhopper abundance from early July to early August 2004 and from early June to early September 2014 appear in Figure 3. Great variability existed from week to week in the same blocks because nymph distribution in a block was generally clumped or aggregated. In 2004, the increase in abundance in early August is the beginning of the third generation in the Yakima Valley; limited data were gathered from the Horse Heaven Hills and Columbia Valley AVAs.
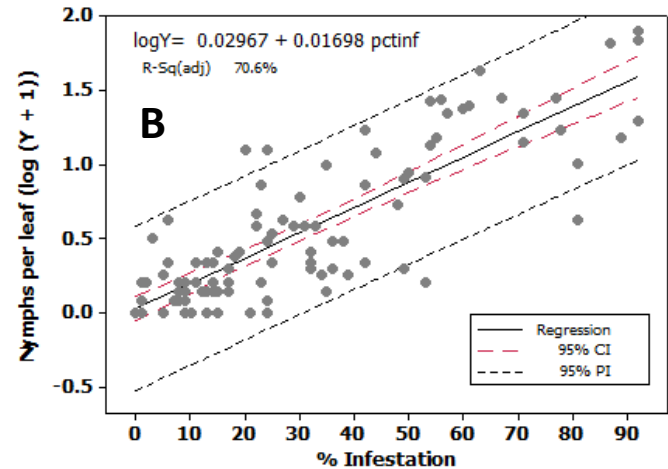
2004 Leafhopper Nymphs Per 10 Leaves Vs. % Infestation

logY = 0.00688 + 0.01630 pctinf
R-Sq(adj)   76.8%

A
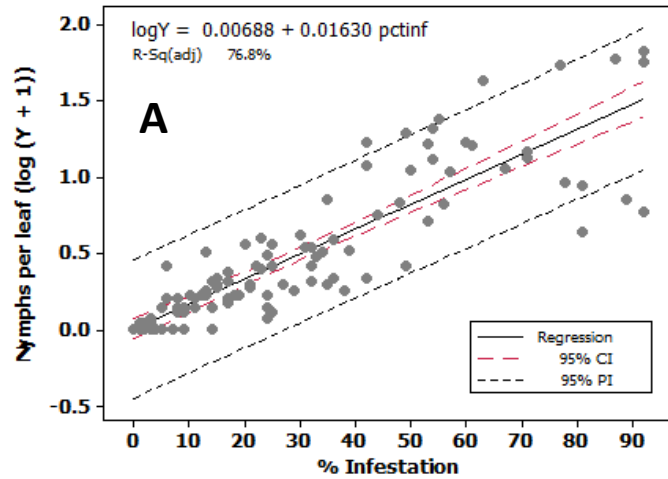
2014 Leafhopper Nymphs per 20 Leaves Vs. % Infestation

logY = 0.00307 + 0.01095 pctinf
R-Sq(adj)   77.4%

C

2004 Leafhopper Nymphs Per Five Leaves Vs. % Infestation

logY= 0.02967 + 0.01698 pctinf
R-Sq(adj)   70.6%

B

2014 Leafhopper Nymphs per 10 Leaves Vs. % Infestation

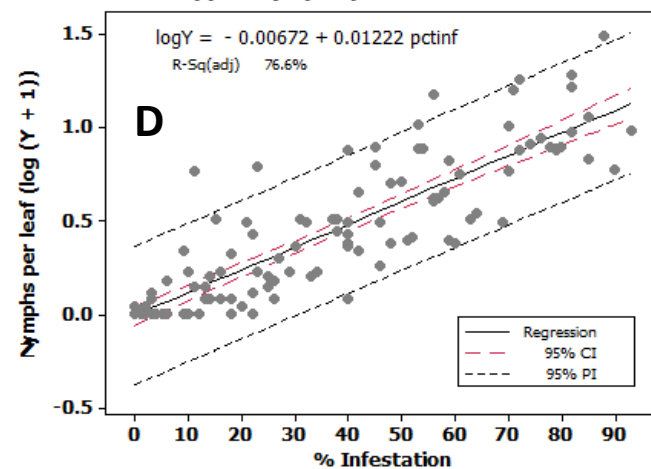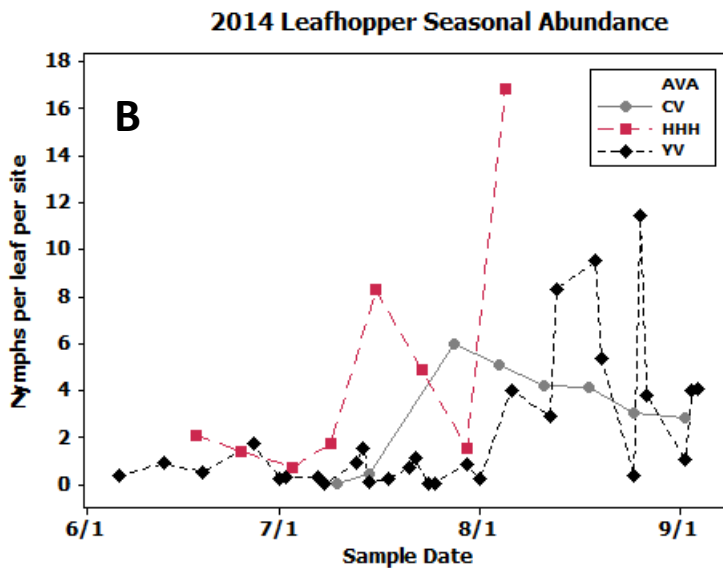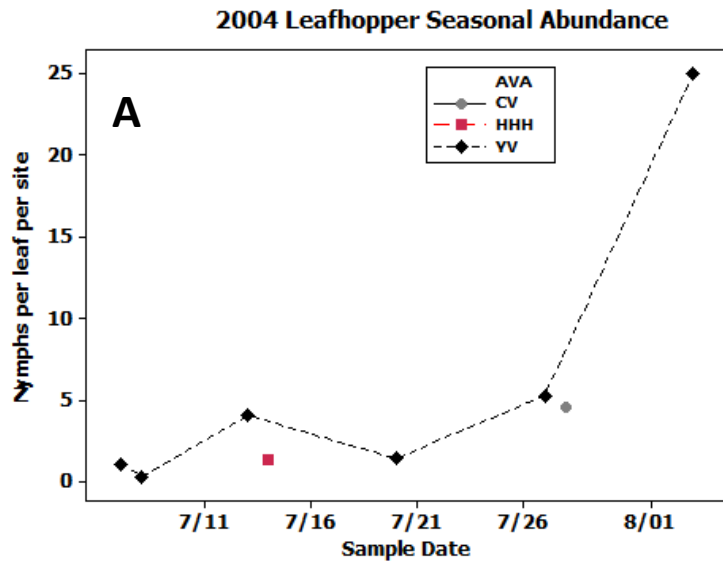logY = - 0.00672 + 0.01222 pctinf
R-Sq(adj)   76.6%

D

Figure 2. Leafhopper nymphs per leaf (log-transformed) versus percent infestation, 2004 and 2014. In 2004, 10 leaves were counted in the field (A), five leaves under the microscope (B). In 2014, 20 leaves were counted in the field (C), 10 leaves under the microscope (D).

In 2014, the first two generations in the Yakima Valley were low in number, while the third generation increased rapidly in August. For Horse Heaven Hills region, populations increased rapidly in July and peaked in August in those organic blocks. In the Columbia Valley region, where most of the blocks have been managed organically for many years, leafhopper numbers increased in late July and then declined to low levels in August because of parasitization by *Anagrus* spp. wasps. The bulk of the leafhoppers collected in 2014 were Virginia Creeper Leafhopper (Fig. 4). Note that the overall seasonal abundance pattern (Fig. 3B) matches the abundance pattern for Virginia Creeper Leafhopper (Fig. 4B). Ten years ago, the Western Grape Leafhopper was by far the predominant species in eastern Washington grape vineyards, according to a viticulturist who managed 5,000 acres in 2004. The reasons behind the displacement of WGLH with VCLH are not clear. Neither species is native to Washington State but have been pests of grapes since before 1955 for WGLH (Wolfe 1955) and before 1983 for VCLH (Wells and Cone 1989). Perhaps the *Anagrus* wasp species complex has changed as well, such that the more abundant species are more effective against WGLH than VCLH. Abundance of alternative host plants preferred by VCLH and reduced abundance of host plants preferred by WGLH would provide a selective advantage to VCLH. It is important to remain vigilant for the increasing abundance and range of VCLH for it has been demonstrated in potted vines that this leafhopper species can transmit the virus that causes grapevine red blotch disease.

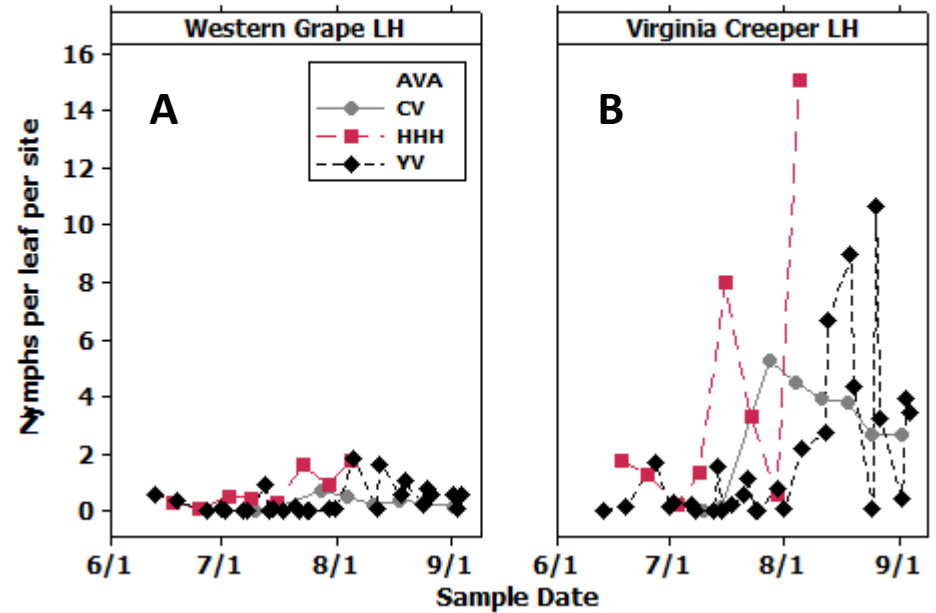Figure 3. Leafhopper seasonal abundance, 2004 (A) and 2014 (B) in
Columbia Valley (CV), Horse Heaven Hills (HHH), and Yakima Valley (YV).

Figure 4. 2014 Leafhopper seasonal abundance, Western Grape
Leafhopper (A) and Virginia Creeper Leafhopper (B).

DNA Barcoding of WGLH and VCLH

In 2014 we developed a DNA barcoding protocol that quickly and reliably identifies individuals of these two leafhopper species prevalent in Washington State species. Genetic analysis of localized populations of *E. ziczac* indicates that there is extensive interbreeding throughout the region, with concomitant implications for insecticide resistance and control. Analysis of an *E. ziczac* population from the Sacramento area of California is consistent with the recently introduced species being a result of introduction from Washington State.

We identified 7 adult *Erythroneura elegantula* and 10 adult *Erythroneura zicac* collected from 2 of our Washington vineyard sites (Patterson and Walla Walla), as well as 13 adult *Erythroneura zicac* from our California vineyard site (Elk Grove), using traditional morphological taxonomy. We extracted DNA from these individuals, as well as 57 randomly chosen nymphs from five vineyard sites. Extracted DNA was used for PCR amplification of the CO1 gene, followed by direct sequencing of a 461bp fragment of this gene from each individual. We then aligned the sequences (MUSCLE v3.7) and constructed a phylogentic tree (PhyML v3.0 aLRT). The tree contained two principal branches, one containing all 7 adult specimens of *E. elegantula*, the other containing all 23 adult specimens of *E. ziczac*. All unclassified nymphs were grouped into one of these two branches. The branch support values for both of these branches were 100%. No leafhoppers were identified as belonging to species other than *E. ziczac* or *E. elegantula*, either by morphological or genetic criteria in the samples collected in Washington State.

In addition we used Kimura's 2 paramater distance model (Kimura 1980) to estimate the percentage of nucleotide sequence divergence for the CO1 region within and between each species. Sequence divergence values were 0.28% within the sampled *E. ziczac*, 0.37% within the sampled *E. elegantula*, and 9.89% between the two congeneric species. These values agree well with CO1 sequence divergence values determined for other insect taxa, calculated by Hebert et al. (Hebert et al. 2003), which ranged from 0.17 – 0.36% for within species divergence, to 5.8 - 9.1% for within genus divergence. These results further support the robustness of our DNA barcoding methodology in identifying these two leafhopper species.

Because the leafhopper nymphs used in establishing our DNA barcoding protocol represented random assemblages of all leafhopper nymphs collected from our 5 vineyard sites, we used our barcoding identification of species to estimate relative numbers of the two species in south central Washington State at the time of collection. *Erythroneura ziczac* was by far the more prevalent species, in all locations, representing from 80% in Quincy to 100% of the nymphs in Patterson, and 91% of the sampled nymphs overall. With the relatively small sample sizes per site we could not determine if the site location or varietal had *any* impact on relative abundance. However, it is clear that at the time of our sampling, *E. ziczac* was by far the dominant leafhopper in vineyards in our sample region.

The genetic structure of *Erthroneura ziczac* populations indicates that leafhoppers are extensively interbreeding between locations. Because *E. ziczac* accounted for most of the leafhoppers we sampled, we investigated whether our samples from the 5 different Washington and 1 California vineyard site exhibited genetic structure. There were 16 haplotypes represented in our sequencing of *E. ziczac* individuals. Most individuals were either haplotypes 1 or 2 (59.7% and 19.5% of total individuals, respectively). The remaining 14 haplotypes were

possessed by only one or two individuals, and were unique to a particular site. Each vineyard site had from 3 – 5 haplotypes observed, and all sites had individuals with haplotype 1, and 4 of the 6 had individuals with haplotype 2.

Analysis of molecular variance (AMOVA) showed that variation within sites accounted for most of the variation represented by these 16 haplotypes: within site was the source for 93.36% of the variation, while among populations was the source for only 6.64% of the variation. Pairwise fixation index (F_{st}) values were low, indicating extensive interbreeding between populations. In fact 10 of the 15 population pairs had had F_{st} values that did not differ significantly from zero, indicative of panmixis.   The five pairwise comparisons that were significantly above zero (Patterson vs Walla Walla, Patterson vs Kennewick, Patterson vs Elk Grove, Quincy vs Kennewick, and Quincy vs Elk Grove) had relatively low F_{st} values, ranging from 0.06672 to 0.20760 (an F_{st} value of 1 would indicate the two populations are isolated and do not share any genetic diversity). Interestingly, there was no greater genetic structure observed between the much more distant California and Washington populations, than between the Washington populations themselves.

Conclusions and Key Outcomes
- Leafhoppers continue to be direct and nuisance pests for wine grape growers and may be vectors of grapevine red blotch disease.
- Based on 2014 sampling data, economic threshold of 15 nymphs per leaf corresponds to 95-100% infestation level.
- In early to mid season, growers often treat with imidacloprid when counts are equal to or less than one per leaf, but at least in early season, the target is more likely mealybugs.
- DNA Barcoding method of distinguishing WGLH from VCLH has been established and will prove useful to determine species of first and second stage nymphs found in the field.
- Virginia Creeper Leafhopper has become the dominant species over Western Grape Leafhopper in Washington wine grape vineyards.
- Key outcome: Recommendations using economic thresholds for leafhopper management stay the same, with added emphasis on remaining vigilant for increasing abundance of Virginia Creeper Leafhopper, a suspected vector of red blotch disease.

References Cited

Wells, J. D. and W. W. Cone. 1989. Biology of *Erythroneura elegantula* and *E. ziczac* (Homoptera: Cicadellidae) in *Vitis vinifera* in southcentral Washington. J. Entomol. Soc. Brit. Col. 86: 26-33.

Wolfe, H. R. 1955. Leafhoppers of the state of Washington. Washington Agric. Exp. Stn. Circ. 277, 37 p.

IV.   Dissemination of Results:
1. Oral presentation at Washington Grape Society, Grandview, Nov. 13-14
2. Oral presentation and/or poster at WAWGG Annual Meeting, Kennewick, Feb. 10-13
3. Article in Good Fruit Grower
4. 2015 Grape Fieldmen's Breakfasts

5. Peer-reviewed manuscript has been submitted to the Bulletin of Entomological Research entitled *DNA barcoding used to identify grape leafhoppers in vineyards in Washington State reveals preponderance of Virginia creeper leafhopper (Erythroneura ziczac) in a geographically broad, interbreeding population.*

Project Budget Status: Due to the unexpected departure of Ashley Johnson, expenditures from this grant did not include the funding of her graduate student salary/benefits for Jan. to May 15 or her summer wages/benefits for late May to early Aug.

V.   Other Sources of Funding: There were no other sources of funding for this project.