TITLE OF REPORT: Renewal Progress Report for CDFA Agreement Number 14-0379-SA

TITLE OF PROJECT: Management of insecticide resistance in GWSS populations using toxicological, biochemical and genomic tools.

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TIME PERIOD: March 2017-February 2018

INTRODUCTION:

Systemic imidacloprid treatments have been the mainstay of GWSS management in citrus, grapes, and commercial nursery operations. The treatments in citrus groves are generally applied post-bloom to suppress the newly emerging spring populations. The use of winter or early spring foliar treatments of pyrethroid or carbamate treatments were introduced to the management program to suppress over-wintering adults and reduce the first early season cohort of egg-laying adults. The combination of early season foliar treatments combined with the more persistent systemic treatments has effectively managed GWSS populations in the Bakersfield area for many years.

In Kern County, GWSS populations have been monitored since the area-wide treatment program was instigated by the CDFA following an upsurge in GWSS numbers and an increase in the incidence of PD. The data shows an interesting pattern of sustained suppression of GWSS populations, following the implementation of the area-wide treatment program, until 2009 when numbers began to increase again, culminating in a dramatic flare-up in numbers in 2012. In 2012, a single foliar treatment with either Lannate® (methomyl: carbamate insecticide class), Assail® (acetamiprid: neonicotinoid insecticide class) or Baythroid® (cyfluthrin: pyrethroid insecticide class) was applied in groves in late March while systemic treatments with imidacloprid (neonicotinoid insecticide class) were applied mid March to early April. The application of systemic imidacloprid during 2012 mirrored the strategy used in 2001 when the imidacloprid treatments were highly effective in suppressing the GWSS populations. Despite the additional foliar treatments in 2012, the insecticide treatments failed to suppress the insect population at a level that had occurred previously. It is a worrying trend that in the 2 years prior to 2012, there was a steady increase in total GWSS numbers, an early indication that the predominant control strategy might be failing. Data collected after 2012 show that trap catches of GWSS numbers have remained high each year up until 2015 (when the most recent data were reported), despite more aggressive implementation of the area-wide treatment program (Haviland and Stone-Smith, 2016). The consequence of the increase in GWSS populations has been a steady increase in the incidence of PD in the region. In the Temecula area, this worrisome increase in GWSS has not occurred, although trap counts for 2016 appeared earlier in the year and at higher levels than those recorded for most years since monitoring began (Daugherty, 2016). Data in the Feb 12, 2018 Glassy-winged Sharpshooter Newsletter distributed by Dr Matt Daugherty (Dept of Entomology, UCR) already show a higher than normal February trap count for GWSS in the region. While the higher numbers are likely due to the mild Winter, it will be important to continue to monitor the susceptibility of the insects to the insecticides used for control.

There is also significant concern for the development of insecticide resistance arising from the management of GWSS in commercial nursery production. The majority of commercial nurseries maintain an insect-sanitary environment primarily through the use of regular applications of soil-applied imidacloprid or other related systemic neonicotinoids. For nursery materials to be shipped outside of the Southern California glassy-winged sharpshooter quarantine area, additional insecticidal applications are required. Applications of fenpropathrin (pyrethroid insecticide class) or carbaryl (carbamate insecticide class) must be applied to all nursery stock shipped out of the quarantine area. As with citrus and vineyard production, the potential for the development of insecticidal resistance in nursery populations of GWSS to these three classes of insecticides (neonicotinoids, pyrethroids, and carbamates) is high.

The focus of this study is to investigate the role of insecticide resistance as a contributing factor to the increased numbers of GWSS that have been recorded since 2009 in commercial citrus and grapes in Kern County. Although the primary focus of our research is in Kern County, we have broadened the scope of the project to include populations from agricultural, nursery and urban settings. This broader approach will enable us to provide a more comprehensive report on the overall resistance status of GWSS within southern California and develop more effective resistance management plans.

OBJECTIVES:

1. For commonly used pyrethroid, carbamate, and neonicotinoid insecticides, determine LC_{50} data for current GWSS populations and compare the response to baseline susceptibility levels generated in our previous studies.
2. Define diagnostic concentrations of insecticides that can be used to identify increased tolerance to insecticides in insects sampled from other locations (where numbers are relatively low).
3. Monitor populations for known molecular markers of resistance to pyrethroids
4. Monitor populations for target-site insecticide resistance, by testing enzymatic activity against carbamates using the AChE biochemical assay
5. Monitor populations for broad-spectrum metabolic resistance, by comparing esterase levels in current populations of GWSS to baseline susceptibility levels we previously recorded.
6. Develop assays for additional resistance mechanisms not previously characterized in GWSS.

ACTIVITIES:

Objective 1: For commonly used pyrethroid, carbamate, and neonicotinoid insecticides, determine LC_{50} data for current GWSS populations and compare the response to baseline susceptibility levels generated in our previous studies.

AND

Objective 2: Define diagnostic concentrations of insecticides that can be used to identify increased tolerance to insecticides in insects sampled from other locations (where numbers are not so high).

<u>Neonicotinoids – imidacloprid and acetamiprid</u>

During 2017, an extensive bioassay program was undertaken that evaluated the responses of different Central Valley and Southern California GWSS populations to imidacloprid, acetamiprid and fenpropathrin. The data generated from topical application bioassays were compared with similar bioassays from studies conducted in 2003 with Riverside County populations, and with data generated during our resistance monitoring effort in 2016. The 2003 data serve as a useful historical reference against which current populations can be compared. In bioassays, insecticide is topically applied to the abdomen of adult GWSS and mortality is assessed at 24 and 48 hours post treatment (Byrne and Toscano, 2005). Although imidacloprid is used systemically under field conditions to target GWSS feeding on citrus and other host plants, topical application of insecticide to individual insects ensures that the insect receives a uniform dose, and eliminates any behavioral factors that might occur when the insect encounters the insecticide (either through direct contact or during feeding). Imidacloprid is one of the most important insecticides used for the control of GWSS, and this insecticide has been shown to elicit anti-feedant effects in several pest species (Nauen et al., 1998). Acetamiprid also belongs to the neonicotinoid insecticides class, but unlike imidacloprid, it is applied as a foliar treatment, rather than as a systemic treatment. Thus, the bioassay data represent a direct comparison of the toxic effects of both neonicotinoids.

In 2016, we were unable to generate full dose-response lines in bioassays with Temecula Valley GWSS due to the availability of low numbers of insects (Redak et al, 2016). The only data we were able to obtain was with discriminating doses of imidacloprid (chosen based on the original dataset generated for Ag-Ops 2003), and they indicated that the Temecula insects were susceptible to imidacloprid. In 2017, the extremely high numbers of GWSS in the region facilitated evaluations of imidacloprid, acetamiprid and fenpropathrin in bioassays.

The response of the Temecula population (TEM 2017) to imidacloprid mirrored that of the Tulare 2016 population, with a noticeable, yet modest, shift in response compared with the Ag-Ops 2003 data **(Figure 1** & **Table 1)**. The location of the TEM2017 population was well removed from the site where the 2016 insects were collected, so the data suggest some degree of variation in Temecula Valley populations in their response to imidacloprid. In the Central Valley's Edison 2017 population, insects exhibited strong resistance to imidacloprid (153-fold). Interestingly, the 500 ng/insect dose did elicit a response in this population (close to the LD_{50}), whereas in the GBR 2016 population that was collected from the General Beale Road area (several miles east of the Edison collection site), there was no measureable toxic effect. Again, these data demonstrate the variable expression levels of resistance in GWSS populations from conventionally managed citrus.

Table 1. Imidacloprid and acetamiprid bioassay data for GWSS collected from Central Valley and Southern California citrus groves. Data for Ag-Ops 2003 are included for reference and were generated in 2003 from bioassays on susceptible insects collected on the UCR campus citrus. Data for HWY65 2016 and GBR 2016 were generated during the 2016 season. RR is the resistance ratio determined from the LD_{50} for field popuations relative to the Ag-Ops 2003 strain.

Population	Imidacloprid LD_{50}	RR	Acetamiprid LD_{50}	RR
Tulare 2016	11 ng	4		
HWY65 2016	50 ng	17		
HWY65 2017	27 ng	9	3 ng	3
GBR 2016	>10,000 ng	>3,333		
Edison 2017	460 ng*	153	Insufficient data	
TEM2017	14 ng	5	1 ng	1
Ag-Ops 2003	3 ng		1 ng	

*Based on partial dataset (see Figure 1)

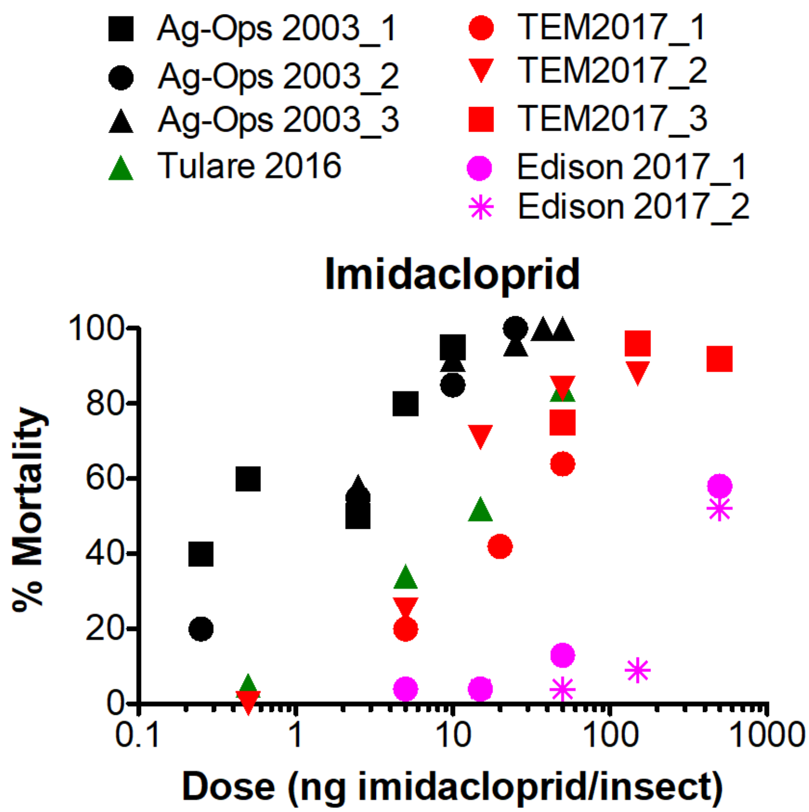

Figure 1. Dose response of GWSS adults to imidacloprid applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. Tulare 2016 (green symbols) was collected from an organic grove in Tulare County during the 2016 monitoring program. The Edison 2017 populations (pink symbols) originated from conventionally managed groves east of Bakersfield in Kern County. TEM2017 (red symbols) was collected from an organic grove in Temecula Valley. For the latter, three separate collections of insects were evaluated by bioassay to generate the dose-response line.

In 2017, we were interested to determine whether resistance to imidacloprid conferred cross-resistance to acetamiprid, as this is an important consideration when developing resistance management strategies, and in evaluating resistance mechanisms. The TEM2017 population exhibited a similar dose-response to acetamiprid as Ag-Ops 2003 (**Figure 2**), indicating that it was fully susceptible to the insecticide. The responses of HWY65 2017 and Edison 2017, both Central Valley populations, indicated some degree of resistance, particularly in the Edison 2017 population **(Figure 2 & Table 1)**. We have yet to generate full dose-response lines for the Edison population (dwindling insect numbers late in September did not allow us to test a broad enough range of doses to generate a complete line), but the shift in response at the doses tested is a clear indication of cross-resistance likely caused by the widespread use of imidacloprid in the region. The response of HWY65 2017 was intermediate between those of the Ag-Ops/TEM and Edison 2017 populations, and this is likely a reflection of the mixed management systems that occur in the area, located north of Bakersfield, where the HWY65 insects originated.

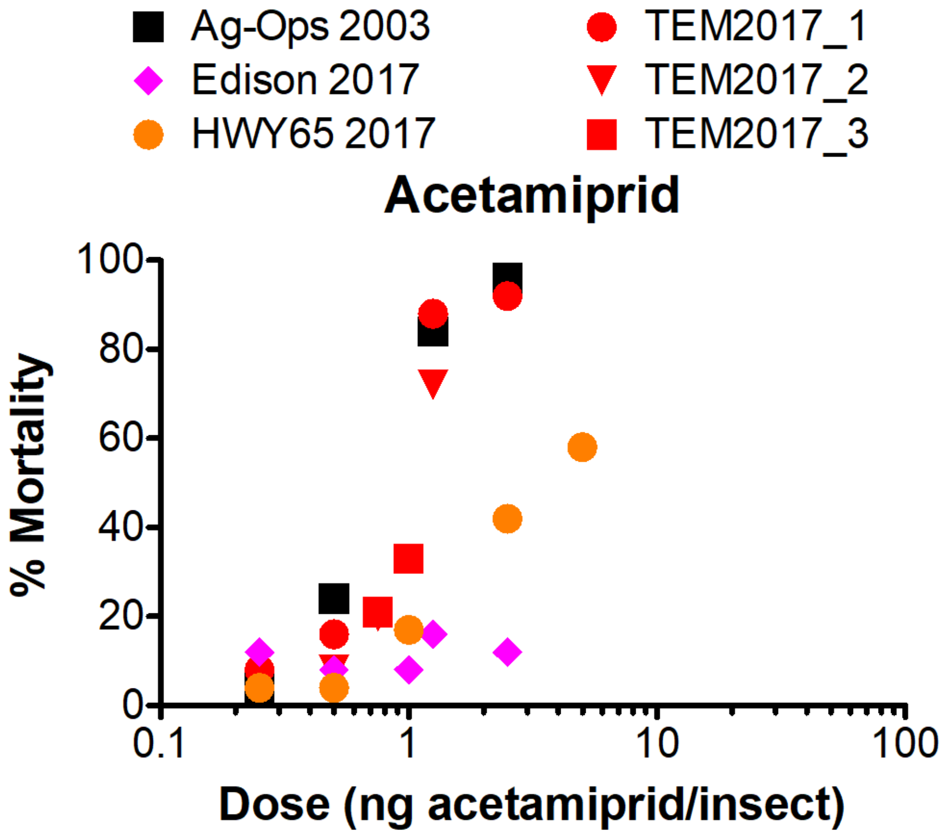

Figure 2. Toxicological response of GWSS adults to the neonicotinoid acetamiprid applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. The Edison 2017 population (pink symbols) originated from conventionally managed groves west of Bakersfield in Kern County. TEM2017 (red symbols) was collected from an organic grove in Temecula Valley, Riverside County. The HWY65 2017 insects (orange symbols) were collected from a grove north of Bakersfield in Kern County.

Pyrethroids

In bioassays with the pyrethroid fenpropathrin, the response of TEM2017 was similar to Ag-Ops 2003 and Tulare 2016, indicating that these three populations were highly susceptible to the insecticide **(Figure 3)**. In contrast, the insects collected from conventionally managed citrus (Edison 2017) exhibited slight tolerance to the pyrethroid. Although the marginal shift in response does not seem to have compromised the efficacy of the insecticide under field conditions, where it has been effectively used to suppress GWSS populations, it is imperative to continue monitoring for resistance to this insecticide to ensure that it remains an effective chemistry for use by growers in the region. An effective rotational strategy will help achieve this goal.

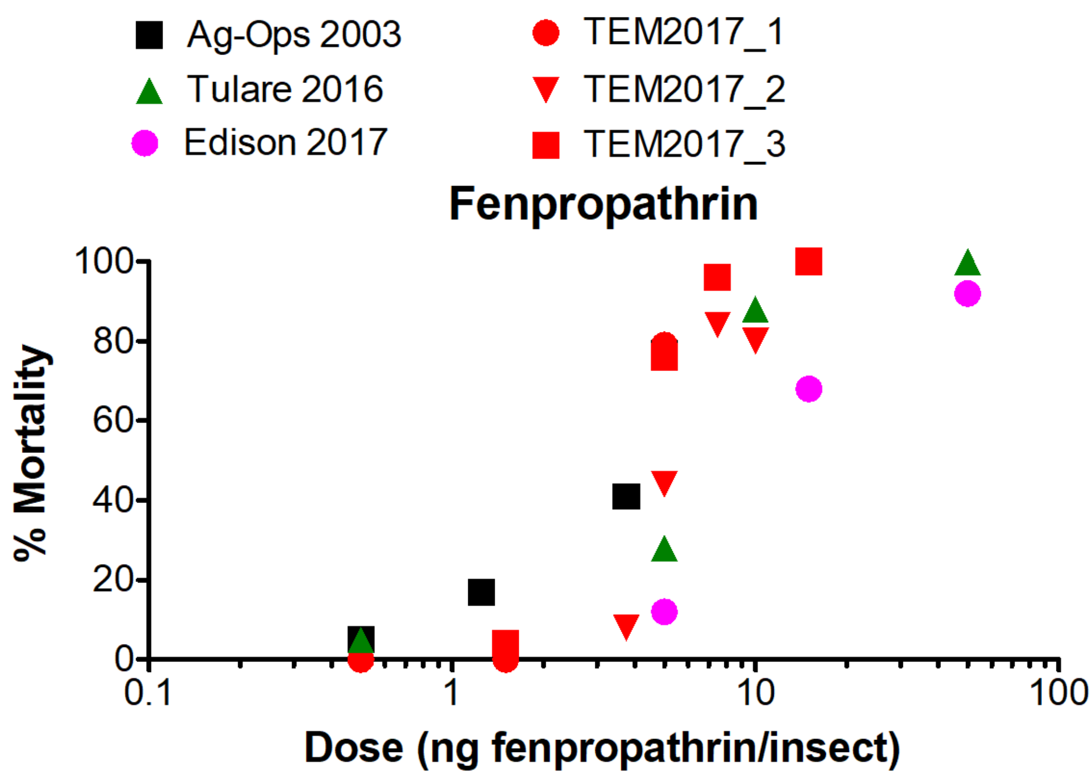

Figure 3. Toxicological response of GWSS adults to the pyrethroid fenpropathrin applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. Tulare 2016 (green symbols) was collected from an organic grove in Tulare County and tested during the 2016 monitoring program. The Edison 2017 population (pink symbols) originated from conventionally managed groves west of Bakersfield in Kern County. TEM2017 (red symbols) was collected from an organic grove in Temecula Valley. For the latter, three separate collections of insects were evaluated by bioassay. Data analyzed using POLO Plus.

Table 2. Fenpropathrin bioassay data for GWSS collected from Central Valley and Southern California citrus groves. Data for Ag-Ops 2003 are included for reference and were generated in 2003 from bioassays on susceptible insects collected from UCR campus citrus. Data for HWY65 2016 and GBR 2016 were generated during the 2016 season. RR is the resistance ratio determined from the LD_{50} for field popuations relative to the Ag-Ops 2003 strain.

Population	Fenprpathrin LD_{50}	RR
Tulare 2016	6 ng	2
HWY65 2016	14 ng	4
HWY65 2017	5 ng	1
GBR 2016	26 ng	7
Edison 2017	8 ng	2
TEM2017	3 ng	1
Ag-Ops 2003	4 ng	

Objective 3: Monitor populations for known molecular markers of resistance to pyrethroids.

This objective was comprehensively addressed during the previous two years of the project using insects from Tulare and Kern Counties that expressed differential responses to fenpropathrin in bioassays. In our investigations, we did not find the classic leucine to phenylalanine (L to F) mutation in the domain II region of the sodium channel gene that confers kdr resistance in houseflies and other species. We identified several synonymous and non-synonymous mutations in these populations, but a causal link between these mutations and resistance warrants further investigation before they can be used as markers for resistance.

During 2017, samples of GWSS from residential and nursery settings were collected and stored for later genetic analysis. For each collection, the plant host and GPS coordinates were noted.

Objective 4: Monitor populations for target-site insecticide resistance, by testing enzymatic activity against carbamates using the AChE biochemical assay.

AND

Objective 5: Monitor populations for broad-spectrum metabolic resistance, by comparing esterase levels in current populations of GWSS to baseline susceptibility levels we previously recorded.

These objectives were largely addressed during the 2016 monitoring season (Redak et al, 2016), during which an assay was developed for GWSS that enabled the measurement of both the total esterase activity and the sensitivity of the AChE to paraoxon in an individual insect. In populations sampled from the Central Valley (GBR, HWY65, Tulare 2016) and Southern California (TEM2016), all insects were sensitive to the diagnostic concentration of 30 µM paraoxon. Insects were also tested from a nursery location in Orange County, and these insects were also sensitive to the OP.

Esterase activity was measured in GWSS collected from the Kern, Riverside, and Tulare County populations in 2016, and compared with data from our studies in 2003 (Riverside County) and 2015 (Kern County). We found no significant differences in esterase levels between the 5 populations, including the 2003 Ag-Ops population, and concluded that elevated levels of esterase activity cannot be used as a marker for pyrethroid resistance, as no causal link was established.

Objective 6: Develop assays for additional resistance mechanisms not previously characterized in GWSS.

We are using RNA-seq analysis to identify potential roles for detoxification enzymes, such as esterases, cytochrome P450, and glutathione S-transferase, and to identify GWSS ABC transporter genes that could play a role in conferring resistance to a broad range of insecticides.

Nicotinic acetylcholine receptor (nAChR)

Based on the study of the aphid *Myzus persicae*, the mutation (R81T) in the loop D region of the nicotinic acetylcholine receptor beta subunit is associated with resistance to neonicotinoid insecticides. The arginine residue is important for binding of the insecticide to the receptor, and any change at this residue decreases the binding capacity of ligands, including insecticides. We identified one nicotinic acetylcholine receptor beta-like gene from the GWSS, with a single open reading frame of 1587 bp that encodes a protein of 529 amino acids, a 5' untranslated region (UTR) located 337 bp upstream of the putative start codon (ATG) and a 3' UTR of 314 nucleotides that ended in a poly (A) tail. DNA was extracted from Tulare 2016, HWY65 2016 and GBR 2016 GWSS. Sequence analysis revealed four synonymous mutations and one non-synonymous mutation in individuals expressing different imidacloprid resistance levels. However, the R to T mutation was not detected in GWSS, indicating that target site modifications are unlikely to confer resistance to imidacloprid in GWSS.

RNA-seq analysis

We identified several cytochrome P450, glutathione S-transferase and ABC transporter genes based on the genome database of GWSS. In order to facilitate a more comprehensive analysis of their potential involvement in conferring resistance to imidacloprid and fenpropathrin, we are conducting RNA-seq analysis to compare individuals sampled from the Riverside, Tulare and Kern County locations where differences in toxicological response to the insecticides were measured. In addition, we are including in our RNA-seq analyses, survivors from the topical application bioassays, as these individuals are more likely to express resistance-causing genes. Metabolism by cytochrome P450 (Cyt P450) enzymes is of particular interest because these enzymes are known to confer resistance to imidacloprid in several insect species. Selection experiments can contribute to investigations of a potential role for these enzymes in resistance.

Cytochrome P450

To complement the RNA-seq analysis, we will also evaluate the efficacy of imidacloprid in bioassays that incorporate a pre-treatment with the synergist piperonyl butoxide. Initially, we will need to determine the toxicological effects of PB on GWSS, to ensure that the dose chosen is not toxic to GWSS. PB is a known inhibitor of Cyt P450 activity, so if we can identify a dose that does not kill GWSS, but enhances the efficacy of imidacloprid, then we will be closer to establishing a causal link between Cyt P450 activity and imidacloprid resistance.

In addition to the synergist bioassay, we will also modify existing biochemical assays for quantifying cyt P450 activity. Assays with individual insect homogenates were unsuccessful when 7-ethoxy coumarin was used as substrate. High background fluorescence (from NADPH), together with possible inhibition of enzyme activity through the release of endogenous inhibitors during homogenization are possible stumbling blocks to using individual insect preps to measure activity. It will be necessary to generate microsomal preparations from mass homogenates of GWSS and then compare cyt P450 activity between insects from both susceptible (Tulare organic site) and resistant (General Beale Road conventional site) populations. While this approach may still suffer from the release of inhibitors, microsomal preps from insect abdomens (as are used in housefly work) may overcome this problem.

PUBLICATIONS:

Redak, R., White, B., Byrne, F. (2017). Management of insecticide resistance in glassy-winged sharpshooter populations using toxicological, biochemical and genomic tools. In: Esser, T. (Ed), Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes. December 2017. California Department of Food and Agriculture, Sacramento, CA, pp. 163-169.

Redak, R., White, B., Li, M., Byrne, F. (2017). Developing insecticide resistance in the glassy-winged sharpshooter (*Homalodisca vitripennis* (Gemar)). Poster Presentation at the Annual Meeting of the Entomological Society of America. Nov 8, 2017.

RESEARCH RELEVANCE STATEMENT:

Bioassay techniques used in this project have identified high levels of resistance to imidacloprid, cross-resistance to acetamiprid, and moderate levels of resistance to the pyrethroid fenpropathrin in Central Valley GWSS populations. The data generated over the past three years confirm a major shift in toxicological response of sharpshooters to insecticides that are routinely used for their control. The consequence of using ineffective insecticides, or insecticides whose efficacy has been compromised by resistance (or cross resistance, in the case of acetamiprid), is that insects will survive treatments and then have the potential to act as vectors of Pierce's Disease. We have made good progress in developing assays that measure qualitative and quantitative changes in putative insecticide resistance-causing enzymes. The use of these assays has eliminated several mechanisms from those that could be potentially involved, thereby allowing us to hone in on the most likely contenders. These assays will allow us to evaluate the incidence of insecticide resistance in agricultural, nursery, and urban populations of GWSS. Data derived from this project will enable growers, pest managers and regulatory agencies to better manage and limit the spread of GWSS populations. During the 2018 season, we will continue to monitor for resistance to imidacloprid, acetamiprid and fenpropathrin in GWSS populations, and incorporate a carbamate insecticide into the testing. The cross-resistance data have already been communicated to Beth Grafton-Cardwell and other extension experts, so that they can make recommendations to growers on how to overcome problems with resistance and improve GWSS management.

LAYPERSON SUMMARY OF PROJECT ACCOMPLISHMENTS:

Failure to control GWSS has led to an increased incidence in PD in the Central Valley. Insecticide resistance is one of the major causes of pest control failures for growers, and is most likely to occur where there is reliance on one insecticide. In many cases, the selection for resistance to the principal insecticide used for pest management within a system may also confer cross-resistance to other insecticides. Our project addresses the recent upsurge in GWSS numbers in Kern County where reliance on a small number of insecticides (most notably imidacloprid) has selected for resistance. Within the past year, we have confirmed that resistance to imidacloprid confers cross-resistance to acetamiprid. In addition to our work in the Central Valley, we are investigating whether heavy insecticide use has selected for resistance in Western Riverside County (Temecula area) and in Orange County (commercial nursery industry). There has been a slight change in response to imidacloprid in the Temecula region, although the change is unlikely to affect the efficacy of imidacloprid treatments under field conditions. Acetamiprid and fenpropathrin remain fully effective. We are using diagnostic tools to detect resistance, and the information generated will enable pest managers to refine existing control strategies and minimize the impact that resistance has on future management efforts.

Accomplishments of this project to date include the confirmation of imidacloprid and pyrethroid resistance in Central Valley populations of the GWSS, particularly in the Bakersfield area. Such high levels of resistance have not been detected in the Riverside County area. We have been able to show that there is a direct link between the levels of imidacloprid resistance and the degree to which insects have been exposed. Our data suggest that the high levels of imidacloprid resistance are responsible for conferring cross-resistance to acetamiprid and the pyrethroid, and it is therefore not inconceivable that cross-resistance to other non-neonicotinoid insecticide classes could also arise. Thus far, there does not appear to be a major shift in resistance to organophosphate and carbamate insecticides.

STATUS OF FUNDS:

$201,181 (total direct and indirect) remain in the budget at this time (12/31/17).

SUMMARY AND STATUS OF INTELLECUAL PROPERTY:

Not relevant.

LITERATURE CITED:

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Redak, R., White, B., Byrne, F. (2016). Management of insecticide resistance in glassy-winged sharpshooter populations using toxicological, biochemical and genomic tools. In: Esser, T. (Ed), Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes. December 2016. California Department of Food and Agriculture, Sacramento, CA, pp. 230-236.