**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 2018-February 2019

**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 show 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 between mid March and 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, and management efforts are generally effective when implemented (Daugherty, 2016).

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.

<div align="center">AND</div>

**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).


**Neonicotinoids – imidacloprid and acetamiprid**
During 2018, a bioassay program was undertaken that evaluated the responses of different Central Valley GWSS populations to imidacloprid and acetamiprid. The main goal was to determine whether resistance to imidacloprid conferred cross-resistance to acetamiprid. Acetamiprid belongs to the neonicotinoid insecticides class, but unlike imidacloprid, it is applied as a foliar treatment, rather than as a systemic treatment.


Kern County
In 2017, we were unable to derive full dose-response lines for the most resistant strains due to declining numbers in September that prevented us from conducting sufficient bioassays to evaluate a higher dose range than originally anticipated. However, in 2018, we completed the dose-response line for acetamiprid against the Edison and GBR populations, and generated additional data for imidacloprid against these populations. 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 and 2017. The data confirmed that resistance to imidacloprid confers cross-resistance to acetamiprid (**Table 1** and **Figure 1**). The responses of the Edison and GBR populations were similar (**Table 1**). However, the responses of insects from the HWY65_2017 population, measured during the previous season, were intermediate between those of the Ag-Ops (susceptible strain from 2003) and Edison/GBR populations, and this is likely a reflection of the mixed management systems that occur in the area.
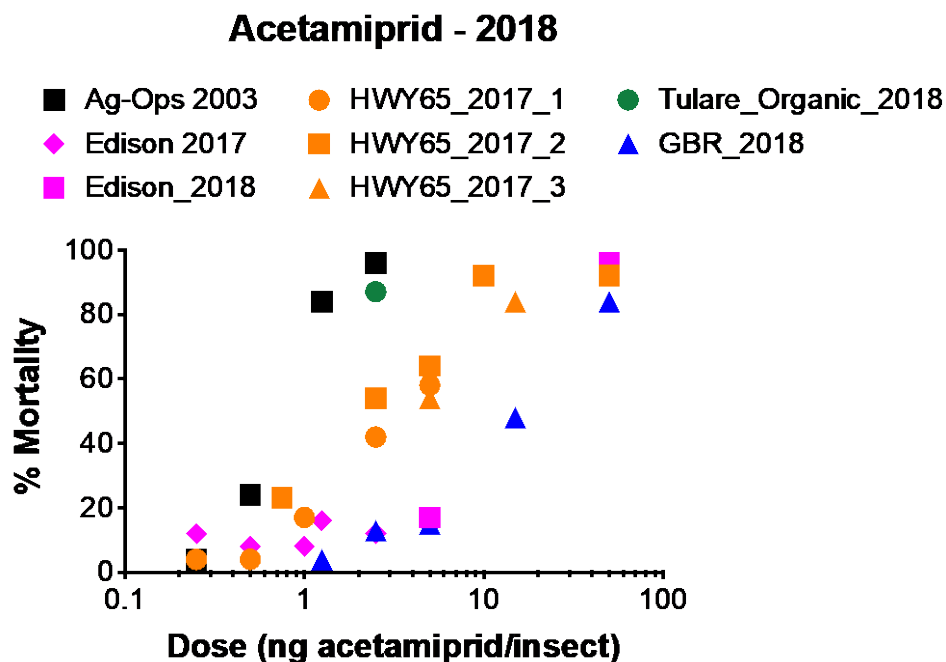


*Figure 1.  Dose response of GWSS adults to acetamiprid applied topically to the abdomen.  Mortality was assessed at 48 h post-treatment.  Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. Tulare_Organic_2018 (green symbols) was collected from an organic grove in Tulare County during the 2018 monitoring program.  The Edison 2017 and 2018 populations (pink symbols) originated from conventionally managed groves east of Bakersfield in Kern County in 2017 and 2018.  The three HWY65_2017 populations (orange symbols) were collected in 2017 from an area on Highway 65 where there is mixed management (conventional and organic) of citrus.  For the latter, three separate collections of insects were evaluated by bioassay to generate the dose-response line.  GBR_2018 was collected from the General Beale Road area, where imidacloprid resistance was first detected in GWSS.*

In conjunction with the acetamiprid bioassays, we conducted further imidacloprid tests against the Edison population and found that the population was highly resistant to the insecticide, although not as resistant as the GBR populations sampled further east (**Figure 2**). With data from 2016 through 2018, we now have compelling evidence of the enormity of the resistance problem in the citrus-growing region east of Bakersfield.  Resistance has also spread to the north of Bakersfield as evidenced by the response of populations from the HWY65 sampling area.

Tulare County
In 2018, we conducted further tests with insects from the organic site (Tulare_Organic_2018; **Figure 2**), where GWSS were generally susceptible to all insecticides tested. Using a discriminating dose bioassay, the insects from this area appear to be still susceptible to imidacloprid. In conjunction with this test, we

collected insects from a site just a few miles south where imidacloprid was part of the management regimen, and where the CDFA monitoring data showed there to be high numbers of insects. For the first time, we can confirm that imidacloprid-resistant GWSS occur in Tulare County, with levels of resistance similar to those in the Edison region (**Figure 2**).
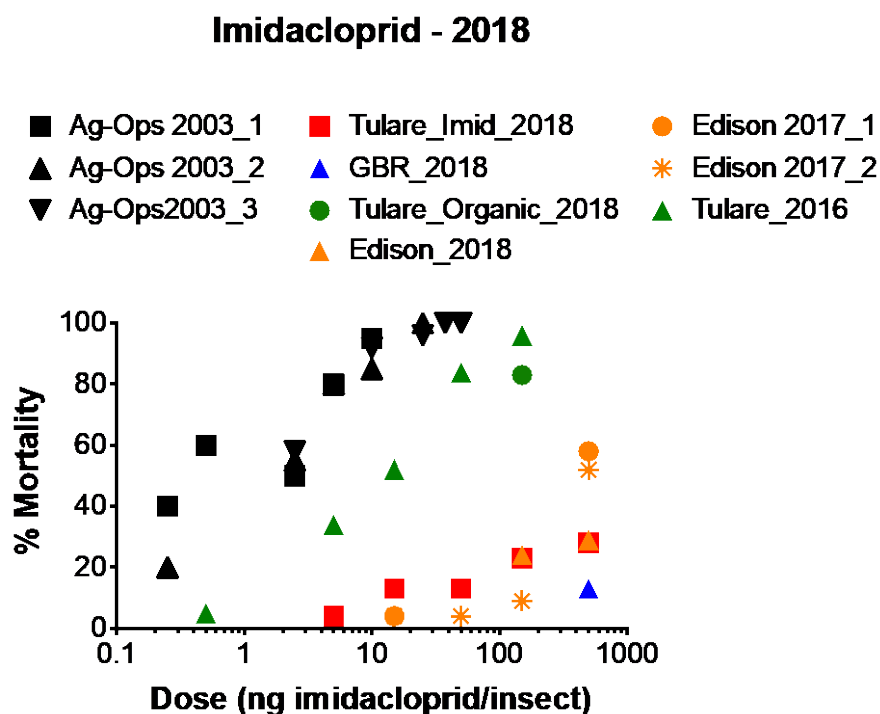


*Figure 2. Dose response of GWSS adults to imidacloprid applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 (3 separate bioassays) and are included for comparison. The Edison 2017 and 2018 populations (orange symbols) originated from conventionally managed groves east of Bakersfield in Kern County in 2017 and 2018. GBR_2018 was collected from the General Beale Road area, where imidacloprid resistance was first detected in GWSS. Tulare_Organic_2018 (green symbols) was collected from an organic grove in Tulare County during the 2018 monitoring program, and tested using a discriminating dose bioassay. Tulare_Imid_2018 is a new conventionally-managed citrus grove in Tulare County.*

**Table 1.** Updated 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. 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 2018 | >10,000 ng | >3,333 | 13 ng | 13 |
| Edison 2018 | 460 ng* | 153 | 9 ng | 9 |
| TEM2017 | 14 ng | 5 | 1 ng | 1 |
| Ag-Ops 2003 | 3 ng | | 1 ng | |

*Based on partial dataset (see Figure 1)

**Pyrethroids**

Prior to 2018, bioassay data for the pyrethroid fenpropathrin indicated that this compound was still largely effective against GWSS in the valley, despite some degree of separation between populations in the General Beale Road area and those further north in Tulare County (Redak et al., 2016, 2017). During 2018, we conducted further bioassays with fenpropathrin in order to compare its efficacy with pyrethrum extract. Organic growers use Pyganic® as a GWSS management tool, and the active ingredients in Pyganic (a mix of pyrethrins) have the same mode of action as fenpropathrin. We are concerned that tolerance to fenpropathrin may affect the efficacy of the pyrethrins against resistant insects should they migrate into organic groves. To address this issue, we conducted a series of bioassays in which we compared the efficacy of fenpropathrin with the pyrethrins, using a commercially-available pyrethrum extract as the source of the latter. Data are summarized in **Figure 3**.
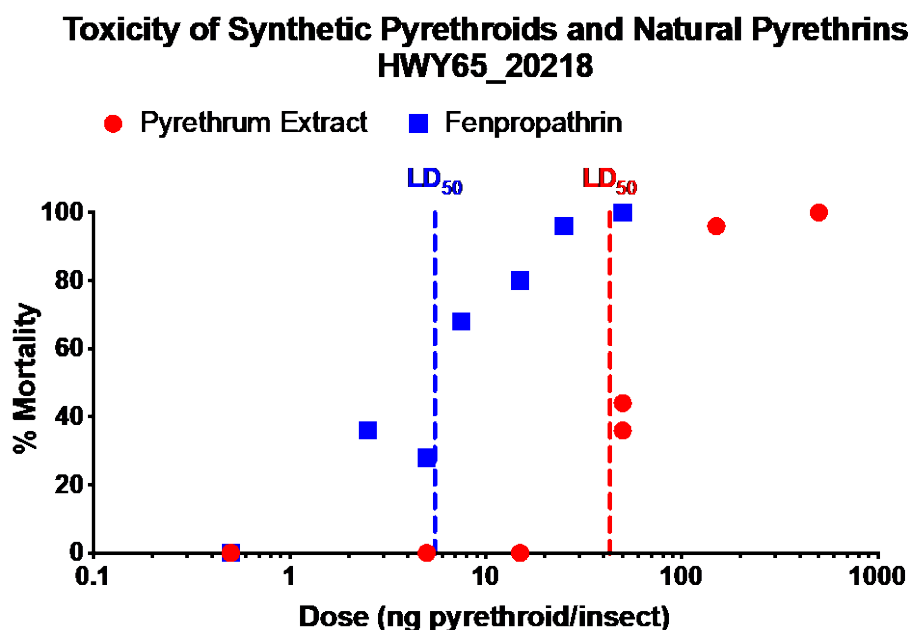


*Figure 3. Toxicological responses of GWSS adults to the synthetic pyrethroid fenpropathrin and the natural pyrethrins (pyrethrum extract) applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. The dose-response of the HWY65 population was the same as in 2017, so data are not included for that year. The dashed lines indicate the $LD_{50}$ values for both compounds that were derived using POLO Plus.*

There was a significant shift in toxicity between the synthetic and natural pyrethroids, with the synthetic compound being more toxic. It will be important to establish the significance of this result because the underlying toxicity of the two compounds indicates that resistance would have a far greater impact on the efficacy of the organic product due to its intrinsically lower toxicity.

**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 (Redak et al., 2017, 2018). 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.
<div align="center">AND</div>
**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.

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 further RNA-seq analyses to compare individuals sampled from the Riverside, Tulare and Kern County locations where differences in toxicological response to the insecticides were measured. In the analysis, we have included survivors from 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.

With the departure of Dr Brad White from UCR, Dr Jason Stajich of the Dept of Microbiology and Plant Pathology at UCR is collaborating with us on the RNAseq analysis. He has completed a preliminary analysis of the data. From the analysis thus far, it appears that the sequencing data generated for the different GWSS populations is separating out according to their toxicological phenotypes.


## Cytochrome P450
We have established a protocol to determine the toxicological effects of piperonyl butoxide (PB) on GWSS resistance to imidacloprid. PB is a known inhibitor of CytP450 activity in insects, and is included in bioassays to test for a possible role for CytP450 in conferring resistance. In preliminary bioassays, PB was not toxic to GWSS by topical application at concentrations of 0.25 and 2.5 ng/insect.

Bioassay 1

The Tulare_Imid_2018 strain, which expresses strong resistance to imidacloprid (**Figure 2**) was treated with 0.25 ng PB. After 24 h, the insects were treated with 50 ng imidacloprid, and mortality was assessed at 48 h. The data show that pre-treatment with PB increased the toxicity of imidacloprid to GWSS, confirming a role for CytP450 in resistance (**Figure 4**).
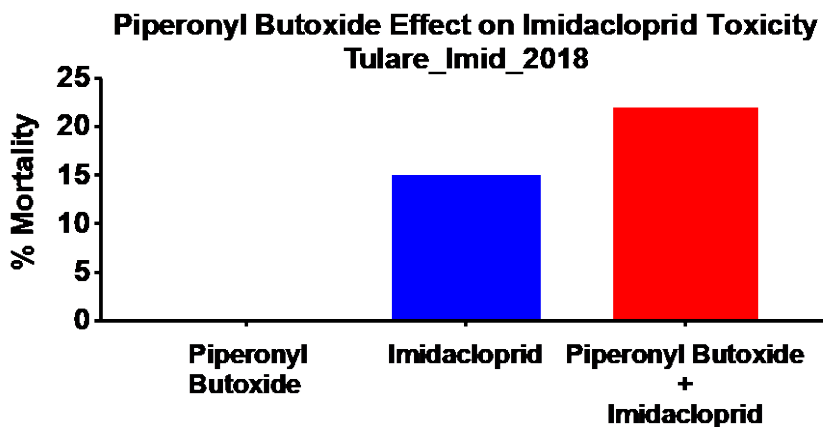
**Piperonyl Butoxide Effect on Imidacloprid Toxicity**
**Tulare_Imid_2018**

*Figure 4.* *Effect of piperonyl butoxide (PB) on the toxicity of imidacloprid to imidacloprid-resistant GWSS collected from Tulare County (Tulare_Imid_2018). Insects were either pre-treated with acetone or PB, and then treated with imidacloprid at the indicated doses 24 h later. Mortality was assessed at 48 h. PB was not toxic to insects.*

Bioassay 2

The HWY65_2018 population was treated with 2.5 ng PB. After 24 h, groups of insects were treated with 0.5, 5, and 50 ng imidacloprid, and mortality was assessed at 48 h. The data show that pre-treatment with PB increased the toxicity of imidacloprid to GWSS, confirming a role for CytP450 in resistance (**Figure 5**).
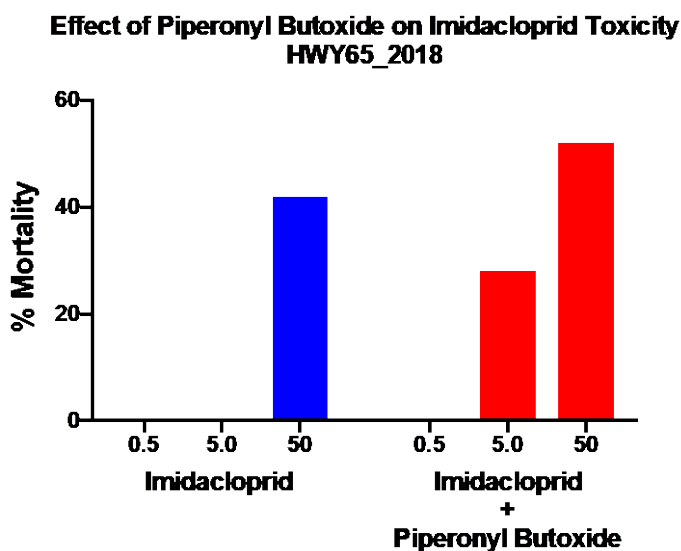
**Effect of Piperonyl Butoxide on Imidacloprid Toxicity**
**HWY65_2018**

*Figure 5.* *Effect of piperonyl butoxide (PB) on the toxicity of imidacloprid to GWSS collected from the General Beale Road (GBR_2018) area of Kern County in the Central Valley. Insects were either pre-treated with acetone or PB, and then treated with imidacloprid at the indicated doses 24 h later. Mortality was assessed at 48 h.*

Bioassay 3

Separate groups of insects from the GBR_2018 population (**Figure 2**) were treated with either 0.25 ng or 2.5 ng PB. After 24 h, all insects were treated with 50 ng imidacloprid, and mortality was assessed at 48 h. The data show that pre-treatment with PB increased the toxicity of imidacloprid to GWSS, and that there was a dose-response with the PB (**Figure 6**).
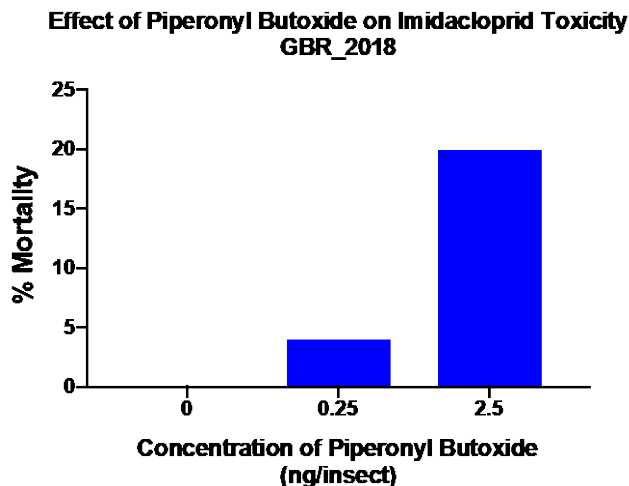
**Effect of Piperonyl Butoxide on Imidacloprid Toxicity**
**GBR_2018**



*Figure 6.* *Effect of piperonyl butoxide (PB) on the toxicity of imidacloprid to GWSS collected from the General Beale Road (GBR_2018) area of Kern County in the Central Valley. Insects were either pre-treated with acetone or PB at 2 concentrations, and then treated with 50 ng imidacloprid 24 h later. Mortality was assessed at 48 h.*

Biochemical Assay

In addition to the synergist bioassay described above, we will also use a biochemical assay for quantifying CytP450 activity. Several published studies proclaim to have measured CytP450 activity in individual insects and mites. The fluorescence assay is prone to misinterpretation, however, due to background fluorescence from NADPH and other fluorescing compounds that may be released after insect homogenization. Therefore, we conducted a thorough evaluation of the assay using commercially available CytP450 (in the form of rat microsomal preps). We developed a working kinetic assay (continuous) that will measure 7-ethoxy coumarin metabolism (O-demethylation) in rat microsomes, both in the presence and absence of GWSS homogenates. The CytP450 activity was similar in rat microsomes, regardless of whether the microsomes were mixed with GWSS homogenate or not. This result indicates that we should be able to detect CytP450 activity in GWSS homogenates above the normal background 'activity'.

**PUBLICATIONS:**

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, San Diego, CA, pp. 230-236.

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., Byrne, F. (2018). 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. 161-166.

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 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. In addition, incursions of resistant insects into organic groves and nurseries could thwart hitherto successful management efforts. We have developed 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 2019 season, we will continue to monitor for resistance to imidacloprid, acetamiprid and fenpropathrin in GWSS populations, and incorporate a carbamate insecticide into the testing. Further testing will also be done to evaluate the synergistic effects of piperonyl butoxide on imidacloprid toxicity. 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. We have also confirmed that resistance to imidacloprid confers cross-resistance to acetamiprid. Most recently, we have detected high levels of resistance in a population in Tulare County, indicating that resistance may be more widespread than originally thought. 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 (Redak et al., 2018), although the change is unlikely to affect the efficacy of imidacloprid treatments under field conditions. Acetamiprid and fenpropathrin remain fully effective. We are developing new 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 (very high) and pyrethroid (low) 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. Our most recent work has identified one potential cause of the resistance to imidacloprid, and we will conduct further investigations during the 2019 season to confirm our hypothesis.

**STATUS OF FUNDS:**

$140,169.13 (direct) $14,169.07 (indirect) remain in the budget at this time.

**SUMMARY AND STATUS OF INTELLECUAL PROPERTY:**

Not relevant.

**LITERATURE CITED:**

Daugherty, M.   2016.   The Riverside County glassy-winged sharpshooter program in the Temecula Valley.   In: Esser, T. (Ed.), Proceedings of the Pierce's Disease Research Symposium, 12-14 December 2016, San Diego, California, pp. 195-198.

Haviland, D. and B. Stone-Smith.  2016.  Monitoring glassy-winged sharpshooter and Pierce's Disease in Kern County, California.  In: Esser, T. (Ed.), Proceedings of the Pierce's Disease Research Symposium, 12-14 December 2016, San Diego, California, pp. 75-80.

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.

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., Byrne, F. (2018). 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 2018. California Department of Food and Agriculture, Sacramento, CA, pp. 161-166.