TITLE OF REPORT: Interim Progress Report for CFA 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: January 2016-July 2016

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. The consequence of the increase in GWSS populations has been an increase in the incidence of PD. In the Temecula area, this worrisome increase in GWSS has not occurred (yet); however the selection pressure in this area remains high as similar management approaches are in use here as in Kern County.

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 application 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 materials (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 will be in Kern County, we propose broadening 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:

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.

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).

Neonicotinoids

Topical application bioassays were conducted during 2015 using insects collected from citrus in the General Beale Road area of Kern County, and the data compared with similar bioassays from studies conducted in 2003. 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). Topical application of insecticide to an individual insect ensures that the insect receives the required dose, and eliminates any behavioral factors that could 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).

In 2003, the bioassays were conducted using populations from Riverside (Agricultural Operations, UCR) and Redlands (commercial citrus grove). At the time the bioassays were conducted, the neonicotinoid insecticide imidacloprid was not being used at Agricultural Operations to control populations, and so the data from those bioassays were considered to represent baseline susceptible levels for GWSS. The response of insects from the Redlands grove, where imidacloprid was incorporated as part of the area-wide management of the GWSS, was similar to Agricultural Operations, indicating that no tolerance to imidacloprid had arisen despite its use as part of the control program. In our view, those early data serve as a useful reference against which current populations can be compared.

In all bioassays, the Kern populations were considerably more tolerant to imidacloprid than the reference population **(Figure 1)**. In the first bioassay, we treated insects over the range 0.25 – 10 ng imidacloprid per insect (n = 150), and there was no insecticide-related mortality at the highest concentration. A second bioassay was conducted with the same source of insects (n = 130) to check the response at 10 ng imidacloprid (using a freshly prepared stock of imidacloprid). Similar to the first bioassay, there was no imidacloprid-related mortality. Based on the reference data set, the 10 ng dose should result in *ca*. 80% mortality, so the bioassays indicated some degree of tolerance to imidacloprid in the Kern insects. The dose range was adjusted to 1.5 – 150 ng imidacloprid per insect for the third bioassay, and a new supply of technical grade imidacloprid was used to prepare the working stock solutions. Although there was a dose response in this bioassay, complete mortality at the higher doses was not achieved, and the level of mortality at the 10 ng dose was minimal.

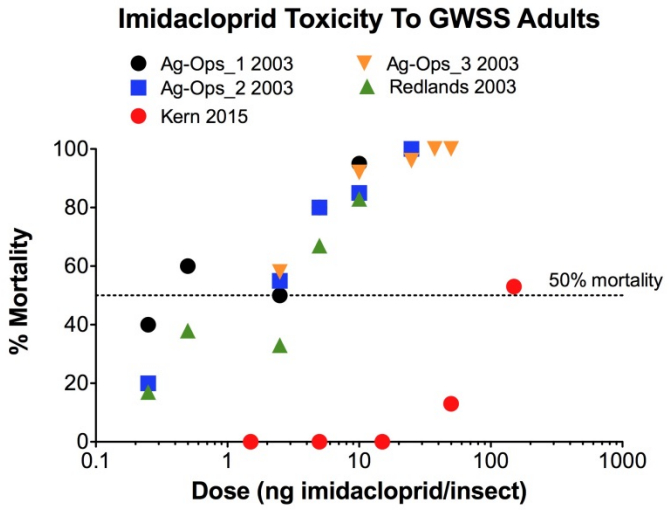

Figure 1. Dose response of GWSS adults to imidacloprid applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for the Kern population (General Beale Road) were generated in October 2015 and compared with bioassay data for insects tested in 2003 from research citrus plots at Agricultural Operations (Ag-Ops) at UCR, and a commercial citrus grove in Redlands.

Pyrethroids

Bioassays were conducted at diagnostic concentrations for 2 pyrethroid insecticides (bifenthrin and fenpropathrin) using populations collected from citrus at General Beale Road **(Figure 2)**. Bioassay data were originally generated in 2004 and 2005 for populations sampled from citrus at Agricultural Operations, and these data are used to represent a reference susceptible.

Both pyrethroids were tested at 5 ng pyrethroid per insect by topical application. Bifenthin was more toxic than fenpropathrin; however, the levels of mortality were lower for both insecticides compared to the Agricultural Operations population in 2005. Despite the lower toxicity at this single dose, it will be important to develop complete dose-response curves for the two pyrethroids to determine whether these differences in toxicity indicate tolerance to the pyrethroids.

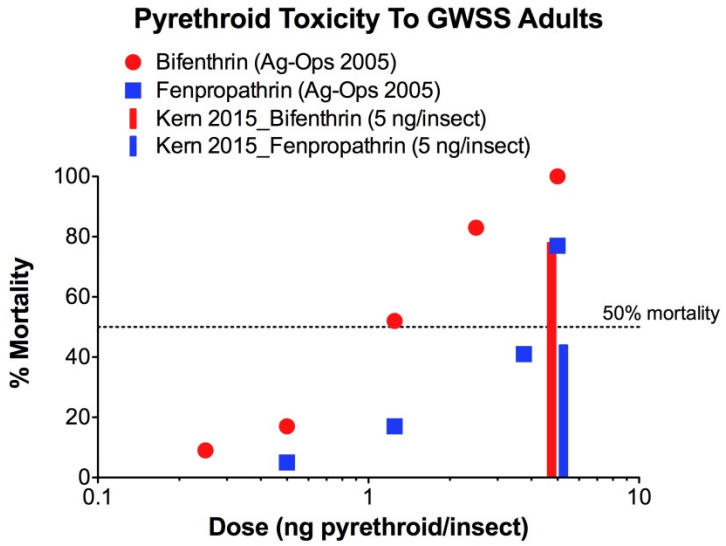

Figure 2. Toxicological response of GWSS adults to the pyrethroids bifenthrin and fenpropathrin applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. The bars show data for the Kern population (General Beale Road) that were generated in October 2015, using a diagnostic concentration of 5 ng/insecticide for both chemicals. Bioassay data for insects tested in 2005 from research citrus plots at Agricultural Operations (Ag-Ops) at UCR are included for comparison.

We are continuing to investigate the significance of the differences in bioassay results between the two regions. Since the last report (data from Dec. 2015), we have conducted four bioassays (two each in November and December), focusing on increased dose rates to try and derive a full dose-response line. The bioassays were confounded by high control mortality at the 48-hour time-point (when final readings are taken); as a consequence, it is difficult to clearly interpret the data. However, at 24 hours after treatment, there was negligible mortality both in the controls and at dose rates as high as 0.5 µg imidacloprid per insect.

The 2016 bioassay program is now getting underway. The most recent trapping data for Kern and Tulare counties indicate that the GWSS numbers are increasing (as of June, 2016). With the beginning of the Summer upsurge in populations now clearly evident, we are making weekly trips to Kern and Tulare Counties to collect adult GWSS for bioassays. Until the adult numbers peak in mid-July, we are focusing our attention on 1st instar bioassays. Adults collected from hot spots (identified from the CDFA maps), and from locations recommended by Russ Carlsen (South Kern Treatment Coordinator) and Judy Stewart-Leslie (Treatment Coordinator, Tulare, Fresno, and North Kern Counties) are placed in cages with cotton and basil plants. Leaves containing fresh egg masses are harvested daily and used in our 1st instar bioassay. The bioassays are currently in progress and the data will be reported in the next update.

In addition to the bioassays, a proportion of the adults from the weekly collections will be used for biochemical and molecular work. Esterase and AChE assays are already refined from past work and these important resistance-causing mechanisms will be monitored routinely throughout the Summer and Fall.

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

In order to identify markers that could be easily typed to determine insecticide resistance in field collected GWSS, it was first necessary to identify the genic targets of commonly used insecticides within the GWSS genome. We utilized gene sequences from housefly and mosquito to identify contigs (via BLAST) within the GWSS assembly that contained genic targets of insecticides. Based on the additional analysis of the GWSS genome using housefly and *Aedes aegypti* (mosquito) as algorithmic models, we identified nine different contigs that contained portions of the GWSS para sodium-gated channel gene, which is the molecular target of pyrethroids (HVIT018256-PA, HVIT018257-PA, HVIT018258-PA, HVIT018259-PA, HVIT018260-PA, HVIT018261-PA, HVIT018262-PA, HVIT018263-PA, HVIT018265-PA). The exon/intron boundaries of the sodium channel gene were annotated using algorithms developed in house. To date, we now have identified two novel sodium channel gene sequences (1975 and 2121 amino acids) that are alternatively spliced. Additionally, we began identification of P450 genes, which are an extremely large gene family involved in detoxification processes, including metabolism of insecticides. Thus far, we have identified more than thirty P450 genes (e.g. HVIT028002, HVIT002146, HVIT014023, HVIT027839, HVIT014027, HVIT028006, HVIT028005, HVIT028857, HVIT002149, HVIT003205, HVIT008014, HVIT008180). Protein products of the genes range in size from 357 to 563 amino acids. Finally, we computationally identified several new nicotinic acetylcholine receptor subunit sequences (HVIT01577, HVIT032410, HVIT018225). Partial sequence of two additional nicotinic acetylcholine receptor beta-like genes have been identified. Several synonymous and non-synonymous mutations have been found in different individuals. We are conducting 3’ and 5’ rapid amplification of cDNA ends (RACE) now.

Based on the above and ongoing analyses of the GWSS genome and transcriptome, gene specific primers have been designed and synthesized to conduct potential target gene full length amplification and detect genetic variations of single nucleotide polymorphisms (SNPs) among GWSS populations located in different areas. We have extracted RNA from GWSS individuals that came from two different locations (Riverside and Corona). cDNA was synthesized from RNA and set up as the template for PCR. The first nicotinic acetylcholine receptor beta-like gene sequence has been identified. The open reading frame of the gene is 1593 bp, which encodes a protein of 531 amino acids. One non-synonymous mutation and three synonymous mutations have been identified among individuals between Riverside and Corona. Sequences have been sent to I-TASSER online server to build the 3 dimensional structure of the protein. The protein structure and binding models with different insecticides will be studied later. Previously (Oct. 2015), we reported that 4551 bp of the sodium channel gene have been confirmed, and 7 synonymous mutations have been found among individuals between Riverside and Corona. Based on the confirmed gene sequence, specific primers have now been used to conduct the 3’ and 5’ rapid amplification of cDNA ends (RACE) of this gene. The full length of the sodium channel gene has been isolated, with a single open reading frame (ORF) of 6315 bp that encoded a protein of 2105 amino acids, a 5’ untranslated region (UTR) located 424 bp upstream of the putative start codon (ATG) and a 3’ UTR of 380 nucleotides that ended in a poly (A) tail. Specific primer pairs have been designed to detect the mutations on sodium channel gene in different individuals with different resistance levels and locations. Several synonymous and non-

synonymous mutations have been found, but the classic leucine to phenylalanine (L to F) mutation in the domain II region of the channel has not been detected.

Additionally we are now comparing sequence data for the β-subunits from different populations to determine if a mutation known to confer insensitivity to neonicotinoids occurs in the more tolerant populations. Sequence analysis has identified a positively charged residue that is conserved in loop D of insect β-subunits that is replaced with uncharged or negatively charged residues in species showing resistance or tolerance (including vertebrates) to neonicotinoids. In *Myzus persicae*, the first report of field-evolved resistance to neonicotinoids due to target-site insensitivity at the nAChR indicated that resistance was a result of a mutation of a key amino acid residue (R81T) in the loop D region of a nAChR β1 subunit (Bass et al, 2011). In tick species, which are known to be naturally tolerant to neonicotinoids, researchers found that instead of the conserved arginine found in insects, a glutamine was present in all the tick sequences (Erdmanis et al., 2012). If we find a mutation at this site in GWSS, we will develop a rapid, high-throughput real-time PCR assay for the detection of the mutation.

We are continuing to collect sharpshooters from urban homeowner yards in Orange, Los Angeles, and Riverside Counties. These collections are from areas with little to no insecticide applications. Insects from these collections are currently undergoing genetic analyses to determine if they have mutations known to confer resistance in other insects.

Objective 4: Monitor populations for metabolic insecticide resistance, by testing enzymatic activity against organophosphates using the AChE biochemical assay

Acetylcholinesterase (AChE) Sensitivity to Paraoxon

Organophosphate (OP) and carbamate insecticides target the neurotransmitter acetylcholinesterase (AChE). Target-site resistance arises as a consequence of mutations in the enzyme that affect the binding efficiency of the insecticide. An assay was developed for GWSS that enabled the measurement of both the sensitivity of the AChE to paraoxon (**Figure 3)** and the total esterase activity (**Figure 4)** in an individual insect.

We tested a large number of insects from the General Beale Road area and all the insects were sensitive to the diagnostic concentration of 30 µM paraoxon **(Figure 3)**. Insects were also tested from locations in Orange County and Tulare County, and these insects were also sensitive to the OP. We are continuing to test for AChE sensitivity in populations throughout the state.

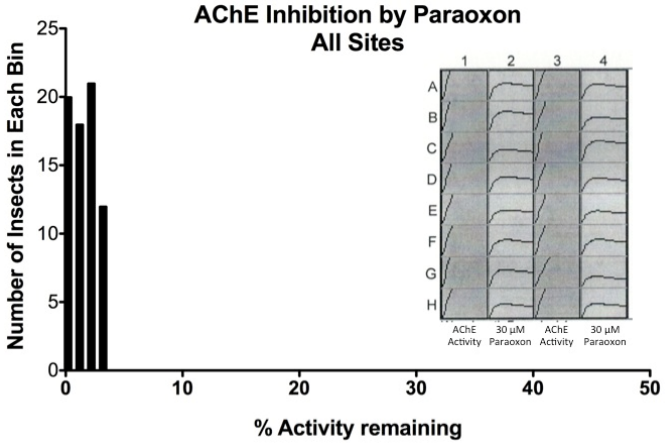

Figure 3. Sensitivity of GWSS AChEs to 30 µM paraoxon. The insert shows the computer output from an assay in which total AChE activity was measured over 20 minutes in the absence (columns 1 and 3) and presence (columns 2 and 4) of the OP. Duplicate aliquots from the same insect homogenate are assayed side-by-side in the absence and presence of the OP (thus boxes A1 and A2 represent the same insect AChE source), providing a visual display of the AChE sensitivity. The main graph shows the distribution of AChE inhibition in GWSS adults collected from multiple sites in California. All assays resulted in complete inhibition of activity within the 20 minute assay, denoting a lack of AChE insensitivity within GWSS populations.

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.

Pyrethroid insecticides are ester-based insecticides and can act as substrates for pyrethroid-hydrolyzing esterases. Total esterase activity was measured in individual GWSS using a colorimetric assay that utilizes naphthyl ester substrates. Although the substrates are non-insecticidal, naphthyl esters can be hydrolyzed by resistance-causing esterases, and they have been used for several decades to identify pyrethroid resistance in agricultural, medical and veterinary pests. We determined the esterase activity in GWSS collected from several citrus groves, and compared the new data with data from our studies in 2003 **(Figure 4)**. Unfortunately, we do not have esterase activity levels from Kern county populations from previous years, so we must rely on available data for Riverside and Redlands populations to make comparisons.

Large numbers of insects were collected from the General Beale Road area of Kern Co., and the mean esterase activity was significantly higher than the reference value for the Riverside population (data from 2003) and a population collected in 2015 from organic citrus in Tulare County **(Figure 4)**.
During 2016, we will continue to measure the esterase activity in GWSS populations collected from various field sites in conjunction with bioassays. In that way, we will be able to better understand the impact, if any, of subtle changes in esterase activity on pyrethroid toxicity. In addition, we will measure the activity in insects that survive bioassay treatments to determine if the survivors have elevated levels of activity compared with control (untreated) insects. Individual esterases that contribute to elevated levels of total esterase activity in GWSS can be identified using polyacrylamide gel electrophoresis (Byrne and Toscano, 2005).

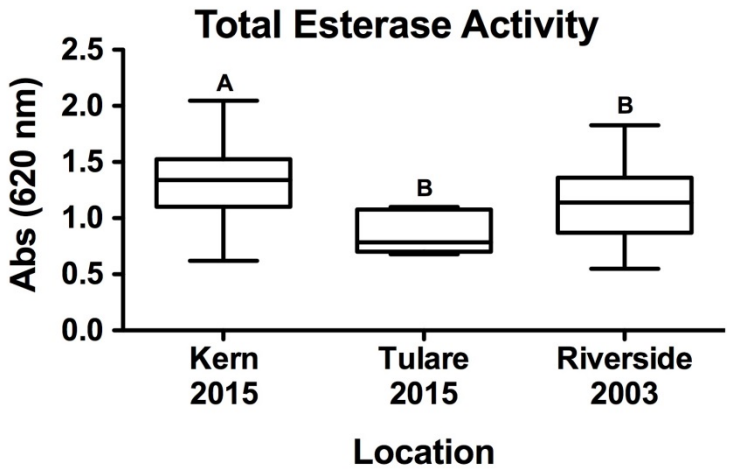

Figure 4. Total esterase activity measured in individual GWSS adults. Activity is represented as absorbance units (320 nm) measured after 30 min incubation with 0.3 mM 1-naphthyl acetate. Homogenates of individual heads were prepared in 0.1 M phosphate buffer, pH 7.5, and then an aliquot (equivalent to 0.01 head) used directly for assay. Levels not connected by the same letter (located above the box plot) are significantly different.

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

Metabolism by Cytochrome P450 (Cyt P450) enzymes is an important mechanism 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. We conducted a selection experiment in which insects were treated at a fixed concentration of imidacloprid (50 ng/insects). At several intervals (3, 6, 12, 24, 48 hours) post-treatment, surviving insects were collected and frozen for later genetic analysis. Control insects were collected at the same time intervals. By determining the expression levels of Cyt P450 genes in treated versus untreated insects, we hope to be able to determine if certain enzymes within this family are preferentially expressed in insects surviving the treatment. Additionally, see work performed under Objective 3 for developments of possible new genetic assays to detect resistance.

PUBLICATIONS:

No publications to date.

RESEARCH RELEVANCE STATEMENT:

The bioassay techniques that were developed and the baseline toxicological data that were generated in early studies determining response to insecticides by GWSS can be used here as a reference point in our efforts to detect any shifts in susceptibility that have occurred as a consequence of the continued use of insecticides for the statewide control of GWSS. We intend to develop new assays that measure qualitative and quantitative changes in putative insecticide resistance-causing enzymes such that an

accurate evaluation of the incidence of insecticide resistance in agricultural, nursery, and urban populations of GWSS can be made. Data derived from this project will enable growers and regulatory agencies to better manage and limit the spread of GWSS populations.

LAYPERSON SUMMARY OF PROJECT ACCOMPLISHMENTS:

Insecticide resistance is one of the major causes of pest control failures for growers. It 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 will address the recent upsurge in GWSS numbers in Kern County where reliance on a small number of insecticides may have selected for resistance. Associated with this work, we will also investigate whether heavy insecticide use has selected for resistance in the Western Riverside County (Temecula area) and in Orange County (commercial nursery industry). We will use diagnostic tools that 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 partial identification of the genes responsible for possible insecticide resistance in GWSS to neonicotinoid, pyrethroid and carbamate insecticides. Our work is ongoing to determine if resistance is actually occurring in this insect in areas of the state in which these compounds are heavily utilized.

STATUS OF FUNDS:

$275,483 remain in the budget at this time (May 31, 2016…latest data available to us).

SUMMARY AND STATUS OF INTELLECUAL PROPERTY:

Not relevant.

LITERATURE CITED:

Not applicable.