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: January 2016-February 2017

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).

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 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:

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).

Neonicotinoids

An extensive bioassay program was undertaken during 2016 to evaluate the responses of different Central Valley GWSS populations to imidacloprid. The data generated from topical application bioassays were compared with similar bioassays from studies conducted in 2003 with Riverside County populations. 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).

In 2003, 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 being used 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 represent the best baseline reference dataset against which current populations can be compared.

During the 2015 season, bioassays were conducted with insects collected from the General Beale Road (GBR) citrus region. The insects were considerably more tolerant to imidacloprid than the reference populations (Redak et al. 2015). In bioassays conducted over the dosage range 0.25 – 150 ng imidacloprid per insect (n = 280), there was a dose-response, although complete mortality at the higher dose was never achieved. Based on the reference dataset from 2003, a 10 ng dose should result in *ca*. 80% mortality of a susceptible insect, so the bioassays showing low mortality at the 15 ng dose provided the first evidence that the insects were tolerant to imidacloprid.

During the 2016 season, we completed a more comprehensive series of bioassays on insects collected from several sites within the Central Valley (Kern and Tulare Counties), and from one site in Temecula Valley (Riverside County). In the Tulare County population that was collected from organic citrus, there was a *ca.* 4-fold increase in tolerance to imidacloprid relative to Ag-Ops_2003 **(Table 1 & Figure 1)**. In contrast, insects from conventionally managed citrus in the General Beale Road area exhibited high levels of resistance (>3,000-fold), with minimal survival of insects when treated with doses as high as 5,000 ng imidacloprid per insect **(Figure 1)**. The response of the HWY65 population was intermediate between the Tulare and GBR populations. While the HWY65 insects were collected from organically managed citrus, we believe that the 17-fold resistance measured in this population in 2016 was likely due to the introgression of resistance genes from insects that migrated from nearby conventionally-managed groves. Our molecular studies will help us to investigate the role of migrating insects on the spread of resistance genes from the GBR area. Our results highlight the dynamic nature of imidacloprid resistance in the Bakersfield area, and the likelihood that resistance could be a contributing factor in the upsurge in GWSS numbers in the region and the associated increase in PD incidence.

Table 1. Bioassay data for GWSS collected from Central Valley 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 ration determined from the LD50 for field popuations relative to the Ag-Ops_2003 strain.

Population	Imidacloprid LD50	RR	Fenpropathrin LD50	RR
Tulare_2016	11 ng	4	6.3 ng	2
HWY65_2016	50 ng	17	14 ng	4
GBR_2016	>10,000 ng	>3,333	26 ng	6
Ag-Ops_2003	3 ng		4 ng	

Insect numbers were very low throughout the year in Temecula Valley, and so it was not possible to generate full dose-response lines. Instead, we conducted bioassays to determine toxicity at discriminating doses (15 ng and 50 ng per insect) that should kill up to 95% of a susceptible population (based on data generated from Ag-Ops and Redlands bioassays in 2003). The data from two tests on insects collected from an organic grove overlapped with those from the susceptible strains indicating no shift in susceptibility to imidacloprid **(Figure 1)**.

The CDFA maps showing the trap catches have already indicated that the GWSS numbers are on the rise early in 2017 in the Central Valley. We have already visited the HWY65 and Tulare sites where we collected insects during our 2016 campaign. We used a discriminating dose bioassay on the HWY65 insects and found that at 500 ng imidacloprid per insect, there was 80% mortality. This number is in close accord with our 2016 data. The survival of insects at this concentration is of concern and indicates that resistance may affect the efficacy of insecticide treatments in this region during 2017.

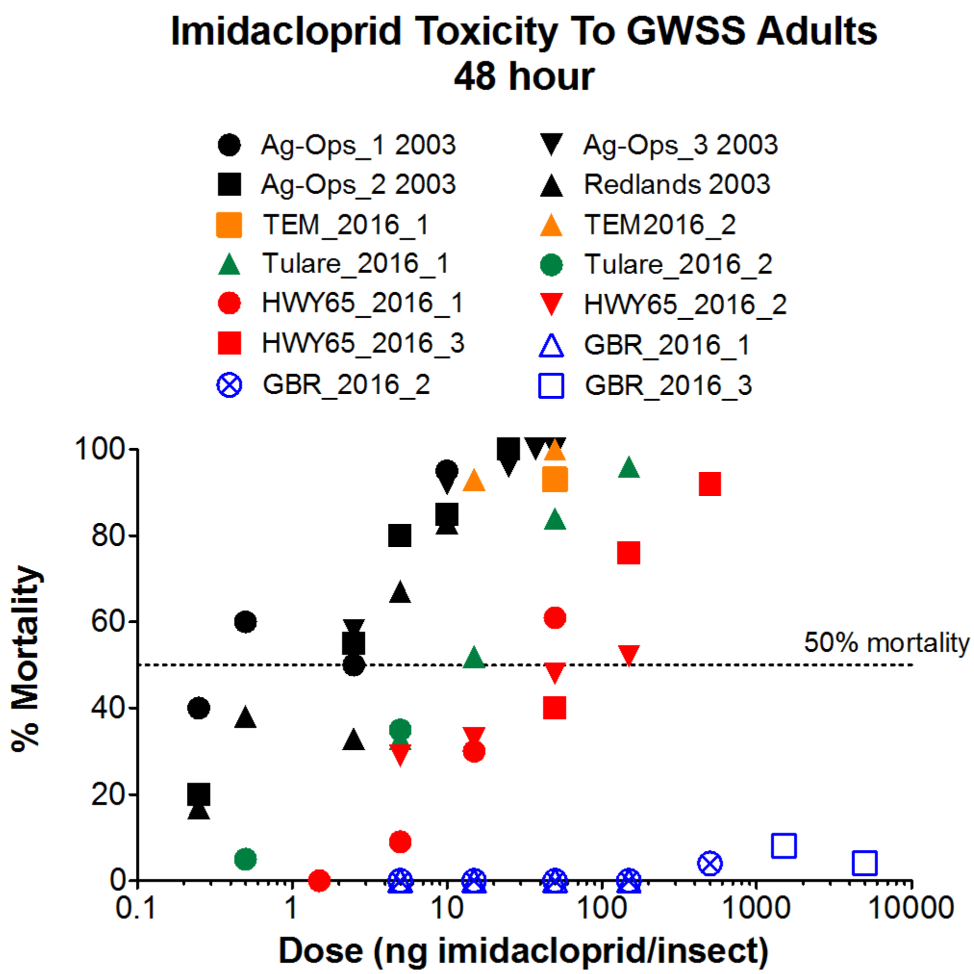

Figure 1. Dose response of GWSS adults to imidacloprid applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for Ag-Ops and Redlands (black symbols) were generated in 2003 and are included for comparison. HWY65 (red symbols) and GBR (blue symbols) are populations collected from organic and conventional groves, respectively, in Kern County. TEM (orange symbols) was collected from an organic grove in Temecula Valley. Tulare (green symbols) was collected from an organic grove in Tulare County.

Pyrethroids

We completed full dose-response bioassays (topical application) with fenpropathrin using the GBR, HWY65 and Tulare populations **(Figure 2)**. Bioassay data that were originally generated in 2003 for populations sampled from citrus at Agricultural Operations, UCR (Ag-Ops) were used as the reference susceptible. LD_{50} data and resistance ratios are summarized in Table 1.

The levels of mortality observed in the GBR, HWY65, and Tulare populations were lower than those of the Ag-Ops population in 2003. While the resistance ratios were not as dramatic as those recorded for imidacloprid, resistance was clearly present, particularly in insects collected from the General Beale Road area. We observed the same trend in the level of response of the insects, with the most susceptible insects occurring at the Tulare organic grove, increased levels of resistance at the HWY65 insects and the highest resistance ratios in the GBR insects. The data confirm the presence of pyrethroid resistance in Central Valley GWSS populations.

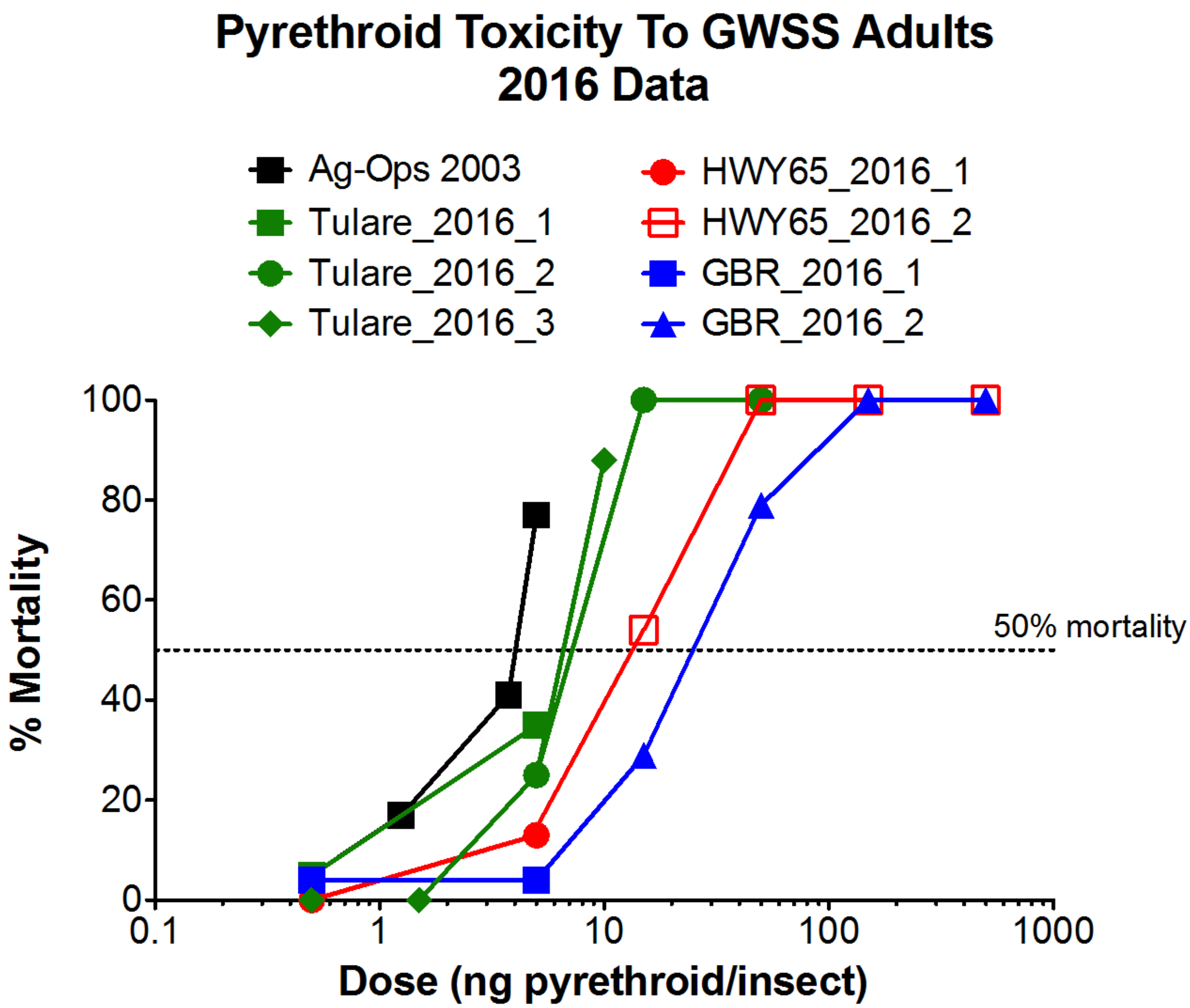

Figure 2. Toxicological response of GWSS adults to the pyrethroid fenpropathrin applied topically to the abdomen. Mortality was assessed at 48 h post-treatment. Data for Ag-Ops (black symbols) were generated in 2003 and are included for comparison. HWY65 (red symbols) and GBR (blue symbols) are populations collected from organic and conventional groves, respectively, in Kern County. Tulare (green symbols) was collected from an organic grove in Tulare County.

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

AND

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

A large number of studies have shown that decreased sensitivity of the target site gene and increased metabolic detoxification of insecticides are two major mechanisms involved in insecticide resistance. More recently, there has been a lot of interest in the role that activation of ABC transporter genes might play in resistance. To elucidate the molecular mechanisms of resistance to imidacloprid (neonicotinoid) and fenpropathrin (pyrethroid) in GWSS, we are checking for the presence of target site mutations in sodium channel (the target site of pyrethroids) and nicotinic acetylcholine receptor (nAChR; the target site of the neonicotinoids) genes that are known to confer resistance in other pest species. 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. We have 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 has been extracted from Tulare, HWY65 and GBR GWSS. Sequence analysis revealed four synonymous mutations and one non-synonymous mutation in individuals expressing different imidacloprid resistance levels. Although the R to T mutation has not been detected in GWSS **(Figure 3)**, further studies will determine whether there is a causal link between other mutations we have identified and imidacloprid resistance.

	Amino acid number of the insect nicotinic acetylcholine receptors				
Species	79	80	81	82	83
Drosophila melanogaster	W	L	R	Q	E
Anopheles gambiae	W	L	R	Q	E
Bemisia tabaci	W	L	R	Q	E
Locusta migratoria	W	L	R	Q	E
Heliothis virescens	W	L	R	Q	E
Myzus persicae	W	L	T	Q	E
Homalodisca vitripennis **(HWY 65)**	W	L	R	Q	E
Homalodisca vitripennis **(Tulare)**	W	L	R	Q	E
Homalodisca vitripennis **(GBR)**	W	L	R	Q	E
Homalodisca vitripennis **(Riverside)**	W	L	R	Q	E
Homalodisca vitripennis **(Corona)**	W	L	R	Q	E

Figure 3. Comparison of the amino acid sequences in several insect species, including GWSS from multiple locations in California. The figure shows the highly conserved nature of the arginine residue (R) between species, which is important for binding of the insecticide to the receptor. A mutation (arginine to threonine at position 81 in loop D) in the nicotinic acetylcholine receptor confers target-site resistance to imidacloprid in *Myzyus persicae*. This mutation was not present in any of the GWSS populations we examined, or in any of several other insect species for which sequence data is available.

Sodium channel

In our initial investigations, we have not found 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 **(Figure 4)**. Furthermore, the L to F mutation was not detected in several Tulare and Kern County populations showing differential responses to fenpropathrin in bioassays **(Figure 2)**. We are currently evaluating several synonymous and non-synonymous mutations that have been found in individuals from these populations **(Figure 4)** to determine whether they play a significant role in conferring resistance.

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Md         CMYVGDVSCIPFFLATVVIGNLVVLNLFLALLLSNFGSSSLSAPTADNDTNKIAEAFNRIARFKNWVKR
Md-R       CMYVGDVSCIPFFLATVVIGNFVVLNLFLALLLSNFGSSSLSAPTADNDTNKIAEAFNRIARFKNWVKR
HWY65      CMYVGDVSCIPFFLATVVIGNLNVLNLFLALLLSNFGSSSLSAPAADNETNKIAEAFDRIGRFSAWVKR
Tulare     CMYVGDVSCIPFFLATVVIGNLNVLNLFLALLLSNFGSSSLSAPAADNETNKIAEAFDRIGRFSAWVKR
GBR        CMYVGDVSCIPFFLATVVIGNLNVLNLFLALLLSNFGSSSLSAPAADNETNKIAEAFDRIGRFSAWVKR
Riverside  CMYVGDVSCIPFFLATVVIGNLNVLNLFLALLLSNFGSSSLSAPAADNETNKIAEAFDRIGRFSAWVKR
Corona     CMYVGDVSCIPFFLATVVIGNLNVLNLFLALLLSNFGSSSLSAPAADNETNKIAEAFDRIGRFSAWVKR
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HWY65      SRNSTSTVTHQPFPDKHYRDFDT   FVSPTLLRVVRVAKVGRVL   GYEPVSSTLWRQREEYC
Tulare     SRNSTSTVTHQPFPDKHYRDFDT   FVSPTLLRVVRVAKVGRVL   GYEPVSSTLSRQREEYC
GBR        SRNSTSTVTHQPFPHKHYRDFDT   FVSPTLLRVVRVKKVGRVL   GYEPVSSTLSRQREEYC
Riverside  SRNSTSTVTHQPFPDKHYRDFDT   FVSPTLLRVVRVAKVGRVL   GYEPVSSTLSRQREEYC
Corona     SRNSTSTVTHQPFPDKHYRDFDT   FVSPTLLRVVRVKKVGRVL   GYEPVSSTLWRQREEYC
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Md: *Musca domestica*

Md-R *Musca domestica* pyrethorid resistance strain

Figure 4. Comparison of the sodium channel amino acid sequences for 5 GWSS populations (HWY65, Tulare, GBR, Riverside, Corona) with those from susceptible (Md) and pyrethroid-resistant (Md-R) houseflies (*Musca domestica*). The leucine to phenylalanine mutation at position 1014 in domain II of the Na channel gene was first detected in houseflies. This mutation was not present in any of the GWSS populations we tested. We have identified other mutations within the GWSS gene (in red font) and are determining the significance of these for conferring resistance in selection experiments.

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.

The first step in our RNA-seq experiments has been to assemble a *de novo* transcriptome for GWSS. A total of 10854 contigs, 49040 unique genes have been generated from the GWSS transcriptome *de novo* assembly **(Figure 5)**.

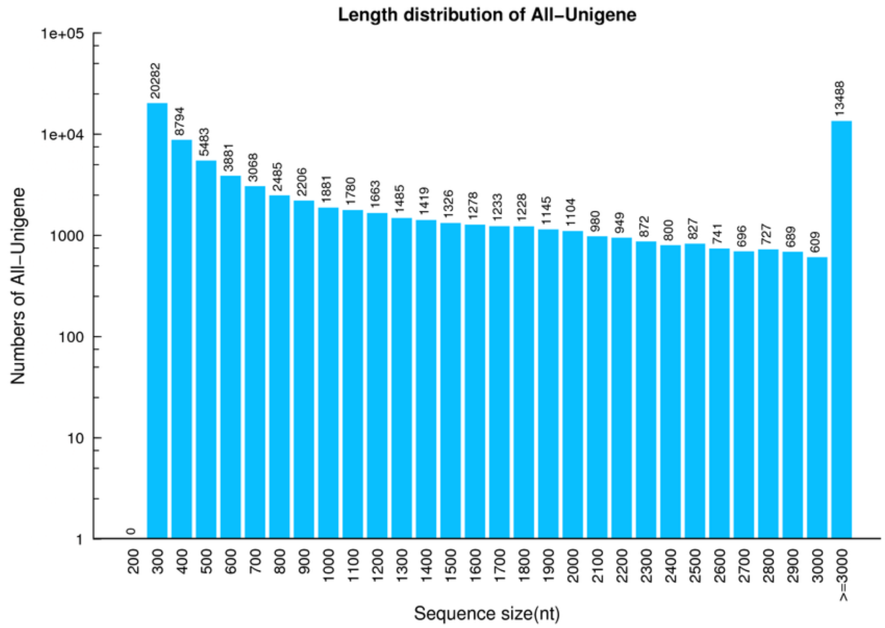

Figure 5. Size distribution of unique genes identified in GWSS.

These unique genes have been annotated using different annotation procedures – the Non-redundant protein database (NR), Kyoto encyclopedia of genes and genomes (KEGG), InterPro, SwissProt and Function annotation (COG) database. **Figure 6** shows the Blast2go functional annotation of GWSS genes. The annotated genes were separated into three functional groups based on biological process, cellular component and molecular function. The combined use of the various databases identified 13,338 unique genes having the same annotation **(Figure 7)**. Using these various annotating tools, our objective is to produce a consensus annotation for GWSS genes that we can then use to analyze the effects of insecticide selection (using bioassays) on gene expression.

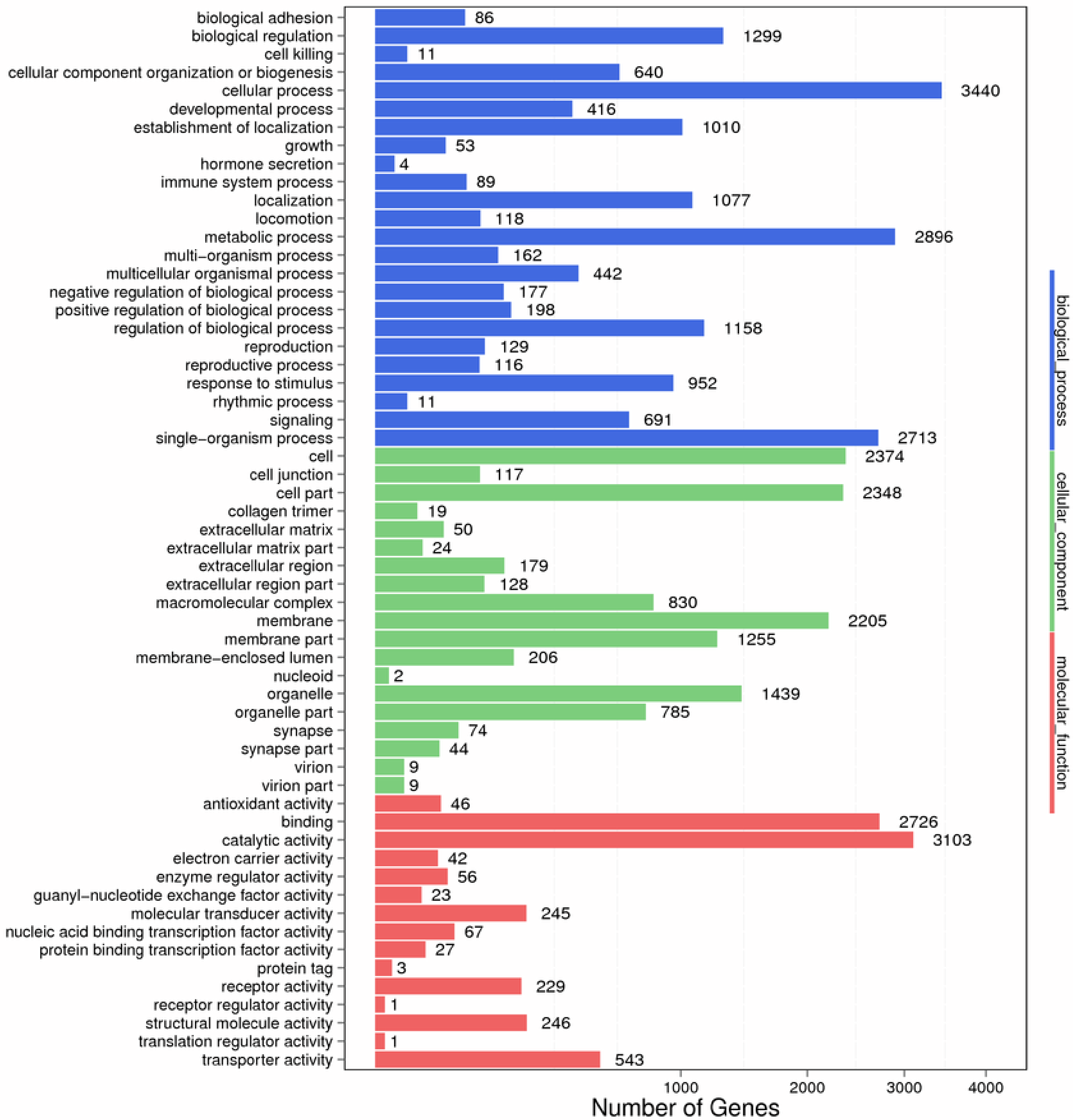

Figure 6. Blast2GO functional annotation of unique genes identified in the GWSS.

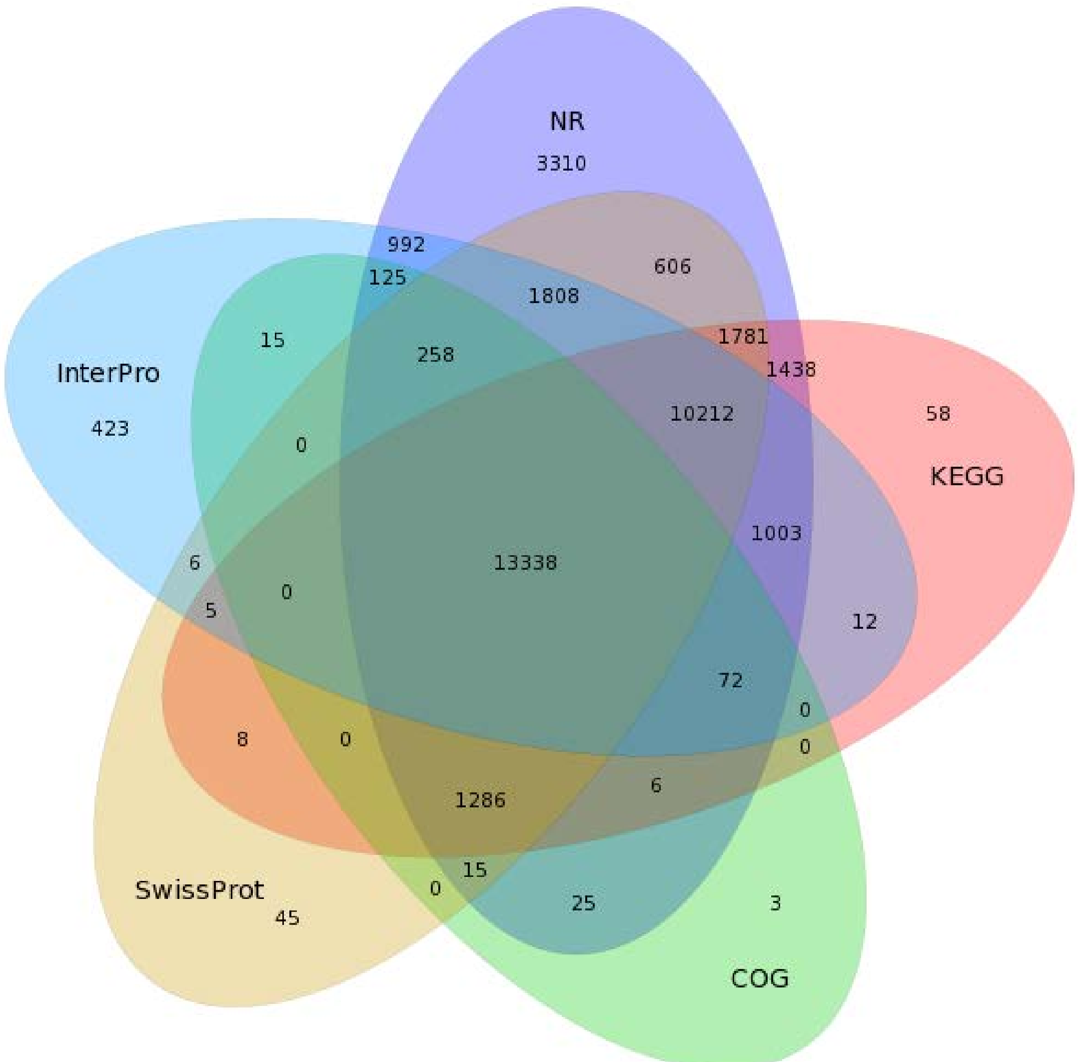

Figure 7. Venn diagram showing the results of GWSS gene annotation using 5 different database sources. Using these different procedures, 13,338 genes with common annotation were identified, and our goal will be to produce a consensus annotation for the GWSS genes.

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

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 total esterase activity and the sensitivity of the AChE to paraoxon in an individual insect.

We compared insects from the GBR (n=8), HWY65 (n=14), TEM (n=22) and Tulare (n=27) populations, and all the insects were sensitive to the diagnostic concentration of 30 µM paraoxon **(Figure 8)**. Insects were also tested from locations in Orange County and Tulare County, and these insects were also sensitive to the OP.

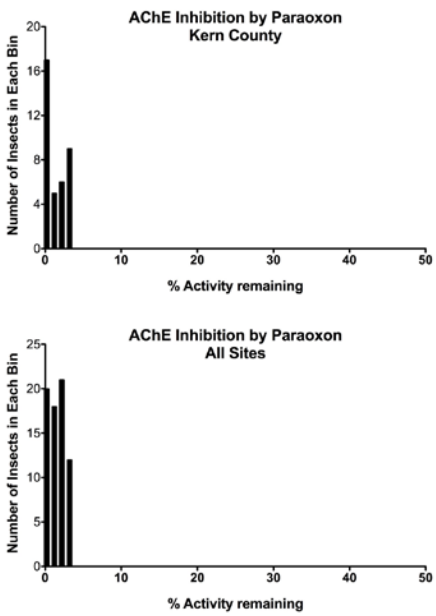

Figure 8. Frequency distribution of AChE inhibition in GWSS adults collected from multiple sites in California. AChE activity was measured in the absence and presence of 30 µM paraoxon in duplicate aliquots of GWSS homogenates over an assay time of 20 minutes. All assays conducted during the 2016 season resulted in complete inhibition of activity within the 20-minute assay time, 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 are 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 the Kern, Riverside, and Tulare County populations, and compared the new data with data from our studies in 2003 (Riverside County) and 2015 (Kern County) **(Figure 9)**.
We found no significant differences in esterase levels between the 5 populations, including the 2003 Ag-Ops population, and conclude that elevated levels of esterase activity cannot be used as a marker for resistance **(Figure 9)**.

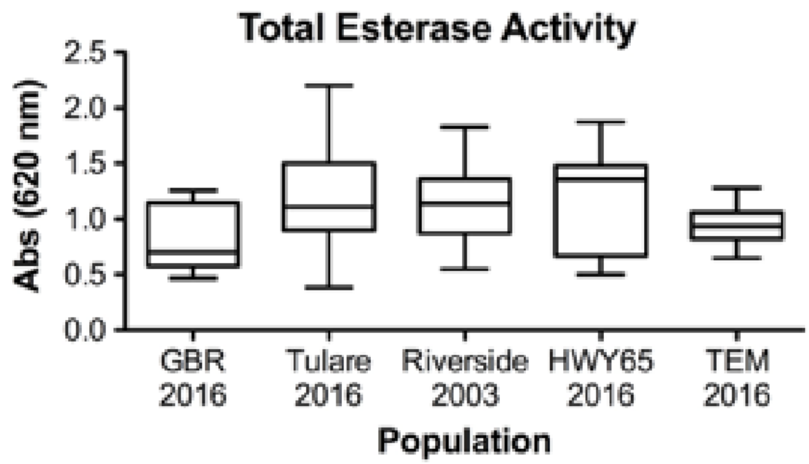

Figure 9. Total esterase activity measured in individual GWSS adults. Activity is represented as absorbance units (320 nm) measured after 30 min incubation with 0.3 mM 1-naphthyl acetate. Homogenates of individual heads were prepared in 0.1 M phosphate buffer, pH 7.5, and then an aliquot (equivalent to 0.01 head) used directly for assay.

PUBLICATIONS:

Other than the CDFA Proceedings, no publications to date. We are preparing a manuscript that will document the presence of resistance in GWSS.

RESEARCH RELEVANCE STATEMENT:

Bioassay techniques used in this project have identified high levels of resistance to imidacloprid, and moderate levels of resistance to the pyrethroid fenpropathrin. The data generated over the past two years confirms 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, 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. 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 2017 season, we will continue to monitor for resistance to imidacloprid and fenpropathrin in GWSS populations. We also plan to assess cross-resistance patterns within these two classes of insecticide, which will enable us to 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 lead 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. 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). Thus far, we see no evidence of resistance in Temecula. 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. Resistance has 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 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:

$311, 872.79 (direct) $33,299.18 (indirect) remain in the budget at this time.

SUMMARY AND STATUS OF INTELLECUAL PROPERTY:

Not relevant.

LITERATURE CITED:

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