Interim Progress Report for CDFA Agreement Number 14-0138-SA

Selective Disruption of GWSS Maturation and Reproduction by RNAi

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Time Period Covered: March 2015 to July 2015

Introduction

In California, the control of GWSS relies primarily on the use of neonicotinoid insecticides such as imidacloprid and to a lesser extent on biological control using parasitic wasps and on other classes of chemical insecticides. Both metabolic and target site resistance to neonicotinoids are found in hemipterans and other insects [1]. The effectiveness of imidacloprid treatment against GWSS also appears to be on the decline in California [2]. Furthermore, neonicotinoids have been linked to negative off-target effects such as colony collapse disorder in honeybee resulting in restrictions in their use in the European Union. The registration of several neonicotinoids is also under re-review by the US EPA. Because of the potential loss of imidacloprid both in terms of its efficacy and availability (due to regulatory restrictions), alternative technologies to control GWSS should be considered.

RNA interference (RNAi)-based technologies [3,4] that selectively target the GWSS endocrine system is a potential alternative tactic for controlling GWSS and the diseases that it transmits. RNAi is a natural process that is found in a wide range of organisms that regulates gene function and protects against viruses. The natural RNAi process can be artificially induced in insects by the introduction of an RNAi effector, i.e., double-stranded RNA (dsRNA) or small interfering RNA (siRNA) that targets a specific messenger RNA. This technology has been shown to work in insects that feed on artificial diet infused with dsRNA or siRNA as well as on transgenic plants that express dsRNA. Two critical keys for developing an RNAi-based control tactic are (1) the identification of a highly selective and effective gene target, and (2) the availability of a system to produce and deliver the RNAi effector in whole insects. In this project, genes that are found in the GWSS endocrine system are being developed as targets for RNAi. Genes in the insect endocrine are ideal targets for knockdown because they are part of an essential and highly sensitive developmental pathway that is only found in arthropods. In the second year of the project, a production and delivery system for RNAi effectors that is based on GWSS pathogenic viruses will be developed.

List of Objectives

I. Develop *jheh* as a model target for RNAi-based control of GWSS maturation
II. Mine the GWSS transcriptome for other RNAi targets
III. Develop virus-based dsRNA production and delivery systems for controlling GWSS

Description of Activities and Summary of Accomplishments

I. Develop *jheh* as a model target for RNAi-based control of GWSS maturation

Juvenile hormones (JHs) and molting hormones (ecdysones) are key components of the insect endocrine system that help to regulate insect development. JHs also regulate other important biological actions such reproduction, mating behavior, feeding induction, and diapause (reviewed in [5]). The level of JH within an insect is determined by a combination of its biosynthesis and degradation. JH acid methyl transferase (JHAMT) is the enzyme that catalyzes the final step of JH biosynthesis. On the other hand, JH degradation occurs through the action two hydrolytic enzymes called JH epoxide hydrolase (JHEH) and JH esterase (JHE). JHEH and JHE metabolize the epoxide and ester moieties that are found on all JH molecules resulting in the formation of JH diol and JH acid, respectively (Figure 1). Minor changes in normal JH levels through alteration in the action (or lack of action) of JHEH, JHEH and/or JHAMT are hypothesized to cause dramatic changes in insect development and/or death. The sensitivity of the insect endocrine system to minor changes is a critical factor in the success of JH analog insecticides such as pyriproxyfen and methoprene.

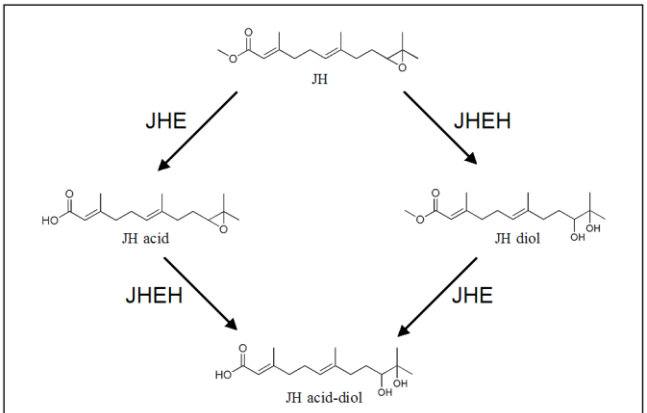
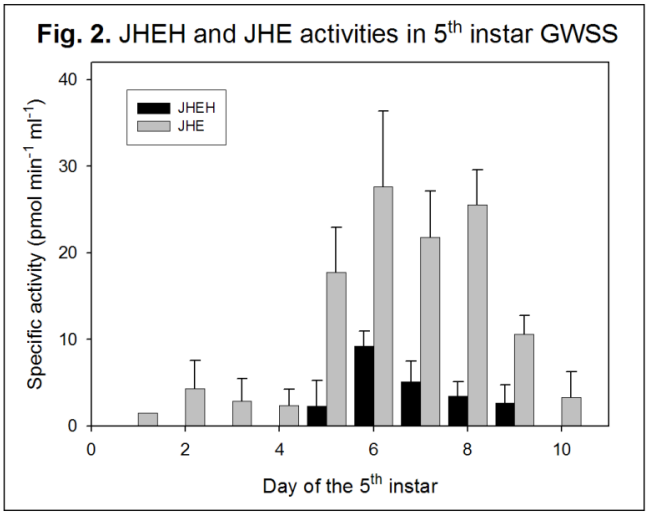


Fig. 1. Structure of juvenile hormone III (JH) and metabolism of JH III by JH esterase (JHE) and JH epoxide hydrolase (JHEH).

The coding sequence of the *jheh* gene of GWSS has been identified and confirmed to encode a biologically active JHEH in a previous project [6]. This gene is now being developed as a target for RNAi in GWSS. Plasmid constructs for the expression of full-length dsRNAs corresponding to *jheh* of GWSS have been designed and are in the construction process. The baseline levels of JHEH and JHE activities in control 5th instar GWSS has been quantified (Figure 2). Detailed information of these enzyme activities is needed to quantify the efficacy and selectivity of the RNAi against the *jheh* and *jhe* genes. During the first four days of



the 5th instar, JHE activity was relatively low (1.5 to 4.4 pmol of JH acid formed min^{-1} ml^{-1} of hemolymph) and found at relatively constant levels. JHE activity dramatically increased (by about 7-fold) on the 5th day of the 5th instar. JHE activity remained high (9- to11-fold higher than that found on the 4th day of the 5th instar) on the 6th, 7th, and 8th days of the 5th instar, then started to decline on the 9th day of the 5th instar. The dramatic increase in JHE activity during the second half of the 5th instar is predicted to remove residual JH from the hemolymph so that (in conjunction with small spikes of ecdysteroids) the juvenile insect undergoes a nymph-to-adult molt instead of a nymph-to-nymph molt. JHEH activity was lower than JHE activity during all of the time points tested. JHEH activity increased by about 4-fold on the 6th day of the 5th instar, a delay of about 1 day in comparison to the spike in JHE activity. These findings suggested that JHE may play a more predominant role than JHEH in JH metabolism in GWSS.

II. Mine the GWSS transcriptome for other RNAi targets

A transcriptome is defined a set of all of the RNA molecules that are found in a specific set of cells at particular moment in time. The cooperator Prof. Bryce Falk's laboratory has recently determined the sequence of the transcriptome of 5th instar GWSS [7]. By computer software-based screening of the GWSS transcriptome, multiple *jhe*-like coding sequences were identified. These potential JHE encoding sequences were manually analyzed (24 deduced amino acid sequences during the initial screening) for the presence of conserved motifs (see [8]) that are found in biologically active JHEs. A rank order of the potential of these sequences to encode a

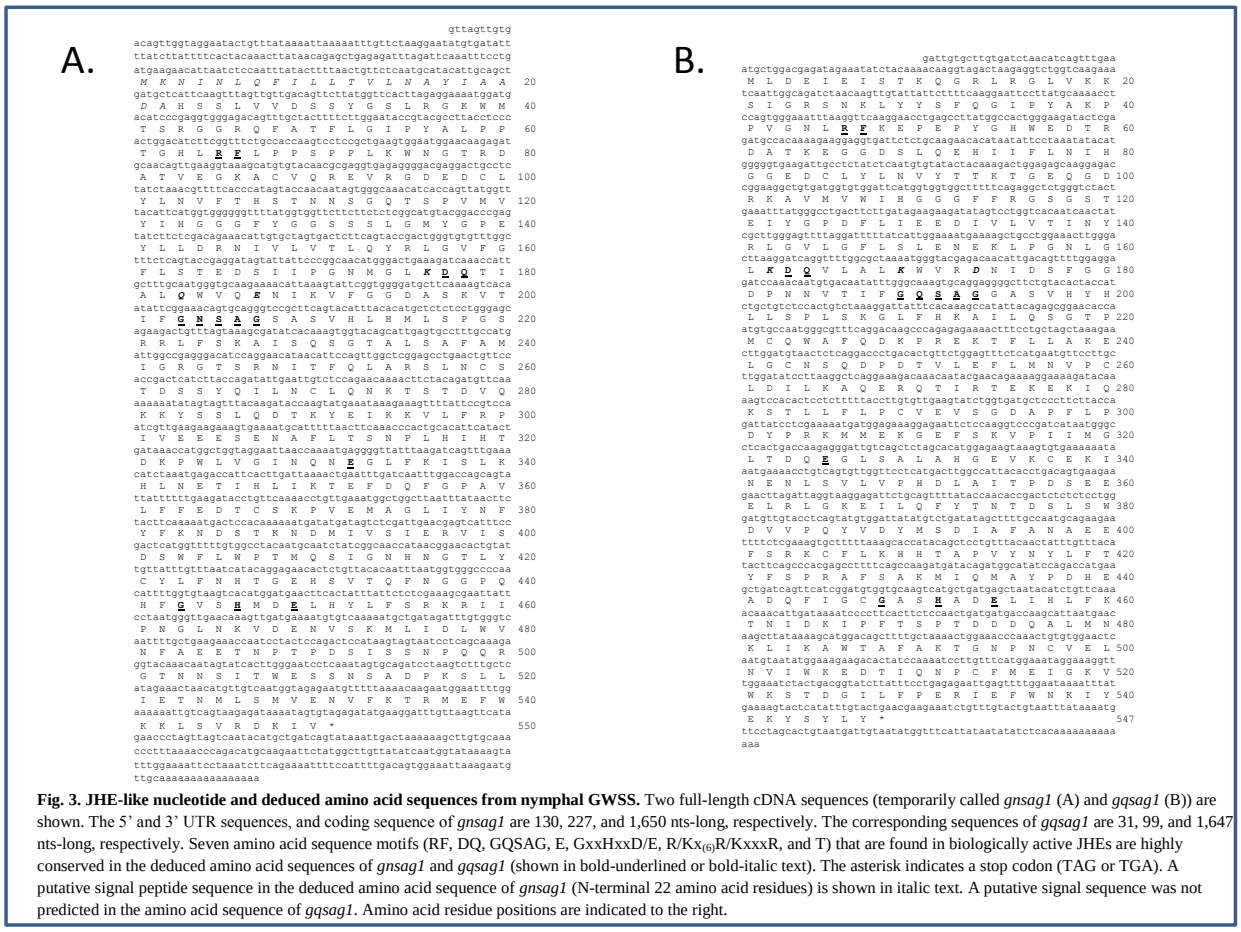


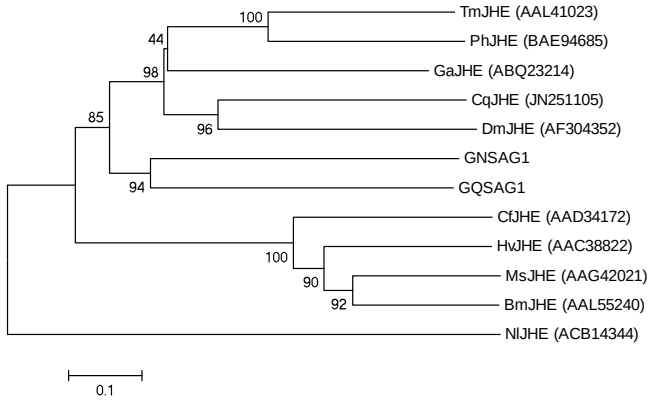
Fig. 3. JHE-like nucleotide and deduced amino acid sequences from nymphal GWSS. Two full-length cDNA sequences (temporarily called *gnsag1* (A) and *gqsag1* (B)) are shown. The 5' and 3' UTR sequences, and coding sequence of *gnsag1* are 130, 227, and 1,650 nts-long, respectively. The corresponding sequences of *gqsag1* are 31, 99, and 1,647 nts-long, respectively. Seven amino acid sequence motifs (RF, DQ, GQSAG, E, GxxHxxD/E, R/Kx$_{(6)}$R/KxxxR, and T) that are found in biologically active JHEs are highly conserved in the deduced amino acid sequences of *gnsag1* and *gqsag1* (shown in bold-underlined or bold-italic text). The asterisk indicates a stop codon (TAG or TGA). A putative signal peptide sequence in the deduced amino acid sequence of *gnsag1* (N-terminal 22 amino acid residues) is shown in italic text. A putative signal sequence was not predicted in the amino acid sequence of *gqsag1*. Amino acid residue positions are indicated to the right.

biologically active JHE was determined and primer sequences were designed for the amplification of the full-length sequences of the top three candidates. In order to mine the full-length JHE sequence from GWSS, double-stranded cloned DNA (ds cDNA) libraries were generated from a developmentally mixed population of 5th instar GWSS (30 individuals) as well as individual GWSS at day 7, 8, and 9 of the 5th instar. The ds cDNAs were used as template sequences for 3'- and 5'-random amplification of cDNA ends (RACE) procedures to generate full-length gene coding sequences.

The RACE procedures identified two full-length JHE coding sequence (*gnsag1* and *gqsag1*, Figure 3) from the ds cDNA library generated from a mixed population of 5th instar GWSS. The *gqsag1* sequence was identified and characterized during the current reporting period. *Gnsag1* and *gqsag1* encode open reading frames of 550 and 547 amino acid residues respectively. Seven amino acid sequence motifs that are found in known biologically active JHEs were highly conserved in the deduced amino acid sequences of *gnsag1* and gqsag1, i.e., GNSAG1 and GQSAG1 (Figure 3). GQSAG1, however, was not predicted to encode a signal peptide sequence as is found in biologically active JHEs. Phylogenetic analysis placed GNSAG1 and GQSAG1 in the same clade (Figure 4A). This clade, however, was different than that of NlJHE from the hemipteran *Nilaparvata lugens*. Additionally, GNSAG1 and GQSG1 were in a clade that was separate from the lepidopteran JHEs and other JHEs (excluding NlJHE).

In order to determine if GNSAG1 and GQSAG1 are able to

A.



B.

Specific activity of GNSAG1, GQSAG1, and CqJHE for JH III

Protein[1]	Specific Activity (nmol JH III acid/min/mg)
GNSAG1	not detected[2]
GQSAG1	not detected[2]
CqJHE	600 ± 26

[1]GNSAG1 and GQSAG1 were crude enzyme preparations, whereas CqJHE was >90% pure.
[2]No activity was detected under assay conditions that could detect 3 nmol of JH III acid formed per minute per mg of enzyme.

Fig. 4. Phylogenetic relatedness and JH III hydrolytic activity of GNSAG1 and GQSAG. A. Phylogenetic analysis was performed using MEGA version 6. The tree was generated by the Neighbor-Joining method using a ClustalW generated alignment of 10 known JHE sequences (GenBank accession numbers are shown within the parentheses). The percentage of replicate trees in which the sequences clustered together in the bootstrap analysis (1000 replicates) is shown at the branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The five insect orders from which the sequences are derived are: Coleoptera: TmJHE and PhJHE; Diptera: CqJHE and DmJHE; Hemiptera: GNSAG1, GQSAG1, and NlJHE; Lepidoptera: CfJHE, HvJHE, MsJHE, and BmJHE; and Orthoptera: GaJHE. B. The specific activity of GNSAG1 and GQSAG1 was determined in 100 mM sodium phosphate buffer, pH 8, containing 1 mg/ml BSA, and 5 µM JH III. The reaction was allowed to proceed at 30ºC for 15 minutes. The ability of CqJHE, a known JHE from the mosquito *Culex quinquefasciatus*, to hydrolyze JH III under the same conditions was used as a positive control.

hydrolyze JH at a rate that is consistent with known JHEs, the sequences encoding these proteins were subcloned into a baculovirus transfer vector, and the resulting constructs were used to generate recombinant baculovirus expression vectors for recombinant protein expression. Both GNSAG1 and GQSAG1 were unable to hydrolyze JH III at an appreciable rate (Figure 4B). Experiments to identify and characterize other putative GWSS JHE coding sequences as well as other target sequences are ongoing.

III. Develop virus-based dsRNA production and delivery systems for controlling GWSS

Insect viruses are used as highly effective biological insecticides to protect against pest insect of forests and agricultural planting such as soybean. Insect viruses have been genetically modified to further improve their efficacy for crop protection. For example, leaf damage caused by the tobacco budworm in tomato plants can be reduced by up to 45% when they are infected with a genetically modified virus that expresses a *jhe* gene [9]. Two viruses from GWSS, *Homalodisca coagulata* virus-1 (HoCV-1) [10] and *Ho. vitripennis* reovirus (HoVRV) [11], are well-characterized. HoCV-1 and HoVRV are naturally found in GWSS populations in the field but they are not severely pathogenic against GWSS. Recently, an in vitro system (e.g., a continuous cell line) that appears to support the replication of HoCV-1 has been identified [12]. The availability of an in vitro system (e.g., [12,13]) is a critical tool for the genetic modification of a virus that is highly pathogenic in GWS. The primary goal of Objective III is to identify new, highly pathogenic GWSS viruses that are supported by a robust in vitro system that can be used in the genetic modification of these viruses. The experiments to accomplish these goals are planned for the second year of the project.

Publications Produced and Pending, and Presentations Made

No publications (produced or pending) have been generated from the reporting period.

Research Relevance Statement

The overall goal of this project is to study and exploit targets within the endocrine system of GWSS that can be used to control GWSS or reduce its ability to spread Pierce's Disease. The approach involves the identification and characterization of genes that are unique to the GWSS endocrine system that metabolize a key insect hormone called JH. Once characterized the genes will be targeted for gene knockdown using a process called RNAi. A potential outcome of this project is the development of an alternative control strategy for GWSS.

Layperson Summary of Project Accomplishments

A natural process called RNAi is used by a wide range of organisms to regulate normal gene function and defend against viruses. This process can be artificially manipulated and potentially used as a "gene based" insect control tactic. Two critical keys for developing an RNAi-based control tactic are (1) the identification of a selective target gene, and (2) the development of a system to produce and deliver RNAi effectors in whole insects. In this project,

we are identifying genes that are found in endocrine system of GWSS as targets for RNAi. A field-applicable delivery system for inducing RNAi against these targets will also be developed.

Status of Funds

The budget for the grant's first year has been expended as of the end of June 2015.

Summary and Status of Intellectual Property

At least one peer-reviewed publication is expected from the research results that are generated from this project. Any reagent, technology, nucleotide sequence or other material that is generated from this project will be made freely available to other researchers.

Literature Cited

1. Casida JE, Durkin KA (2013) Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annu Rev Entomol 58: 99-117.
2. Porter T (2013) Area-wide management programs for controlling the glassy-winged sharpshooter in grape production areas. Sacramento, CA: California Department of Food and Agriculture.
3. Burand JP, Hunter WB (2013) RNAi: future in insect management. J Invertebr Pathol 112: S68-S74.
4. Gu LQ, Knipple DC (2013) Recent advances in RNA interference research in insects: Implications for future insect pest management strategies. Crop Prot 45: 36-40.
5. Riddiford LM (2008) Juvenile hormone action: A 2007 perspective. J Insect Physiol 54: 895-901.
6. Kamita SG, Oshita GH, Wang P, Morisseau C, Hammock BD, et al. (2013) Characterization of Hovi-mEH1, a microsomal epoxide hydrolase from the glassy-winged sharpshooter *Homalodisca vitripennis*. Archives of Insect Biochemistry and Physiology 83: 171-179.
7. Nandety RS, Kamita SG, Hammock BD, Falk BW (2013) Sequencing and *de novo* assembly of the transcriptome of the glassy-winged sharpshooter (*Homalodisca vitripennis*). PLoS ONE 8: e81681.
8. Kamita SG, Hammock BD (2010) Juvenile hormone esterase: biochemistry and structure. Journal of Pesticide Science 35: 265-274.
9. El-Sheikh ESA, Kamita SG, Vu K, Hammock BD (2011) Improved insecticidal efficacy of a recombinant baculovirus expressing mutated JH esterase from *Manduca sexta*. Biological Control 58: 354-361.
10. Hunnicutt LE, Hunter WB, Cave RD, Powell CA, Mozoruk JJ (2006) Genome sequence and molecular characterization of *Homalodisca coagulata* virus-1, a novel virus discovered in the glassy-winged sharpshooter (Hemiptera : Cicadellidae). Virology 350: 67-78.
11. Stenger DC, Sisterson MS, Krugner R, Backus EA, Hunter WB (2009) A new phytoreovirus infecting the glassy-winged sharpshooter (*Homalodisca vitripennis*). Virology 386: 469-477.
12. Biesbrock AM, Powell CM, Hunter WB, Bextine BR (2014) Propagation of *Homalodisca coagulata* Virus-01 via *Homalodisca vitripennis* cell culture. J Vis Exp e51953.
13. Kamita SG, Do ZN, Samra AI, Hagler JR, Hammock BD (2005) Characterization of cell lines developed from the glassy-winged sharpshooter, *Homalodisca coagulata*

(Hemiptera: Cicadellidae). In Vitro Cellular & Developmental Biology - Animal 41: 149-153.