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

SELECTIVE DISRUPTION OF GWSS MATURATION AND REPRODUCTION BY RNAI

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Reporting Period: The results reported here are from work conducted July 1, 2014 through June 30, 2016.

ABSTRACT

The overall goal of this project is to develop an RNAi-mediated system to inhibit maturation and reproduction of GWSS. The initial target for RNAi will be GWSS *jheh* (also known as *hovi-meh1*), the gene that encodes JH epoxide hydrolase (JHEH). GWSS *jheh* will be used as a model gene target to establish an efficient expression and screening system for characterizing RNAi effectors. This system will then be used to evaluate other JH metabolic genes including those that encode JH esterase, JH acid methyl transferase, and other identified genes as targets for RNAi. These gene sequences will be mined from the recently determined transcriptome sequence of GWSS. Finally, plant virus- or insect virus-based systems for expression and delivery of the RNAi effectors in insects will be developed.

LAYPERSON SUMMARY

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.

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.

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

RESULTS AND DISCUSSION

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. In many insects, 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).

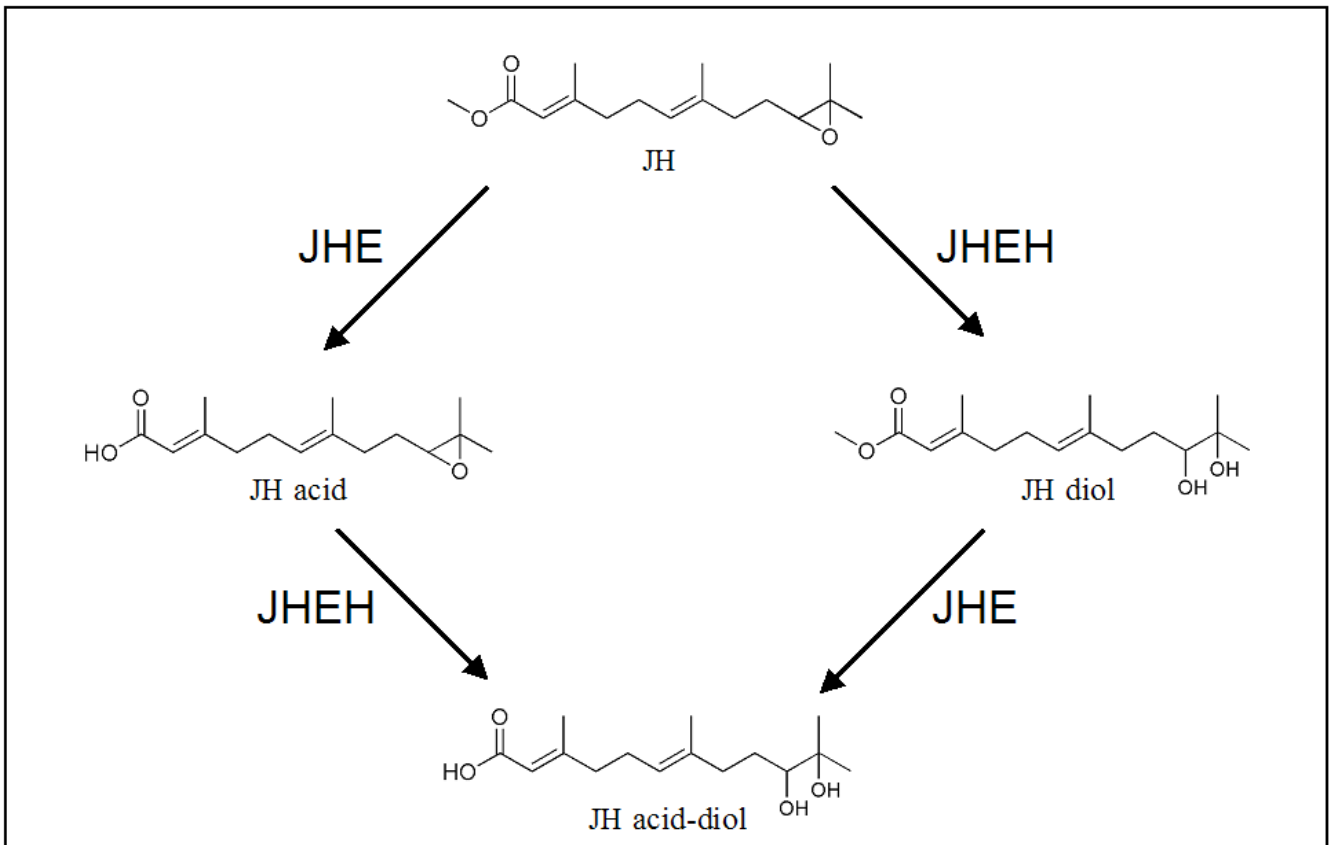


Fig. 1. Structure of juvenile hormone III (JH) and metabolism of JH III by JH esterase (JHE) and JH epoxide hydrolase (JHEH).

Minor changes in normal JH levels through alteration in the action (or lack of action) of JHEH, JHE, and/or JHAMT have been shown 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.

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).

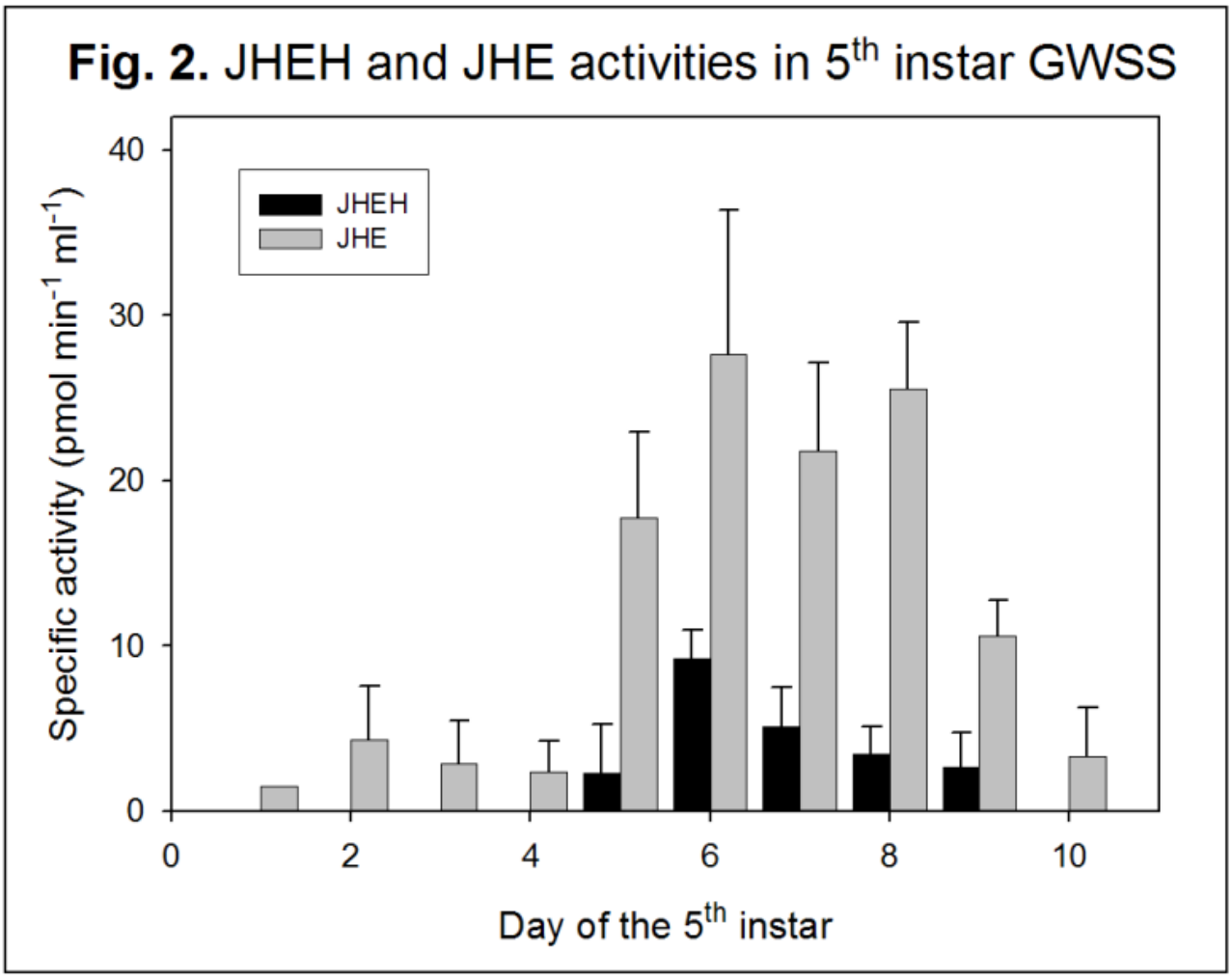


Fig. 2. JHEH and JHE activities in 5th instar GWSS

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 of GWSS, 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

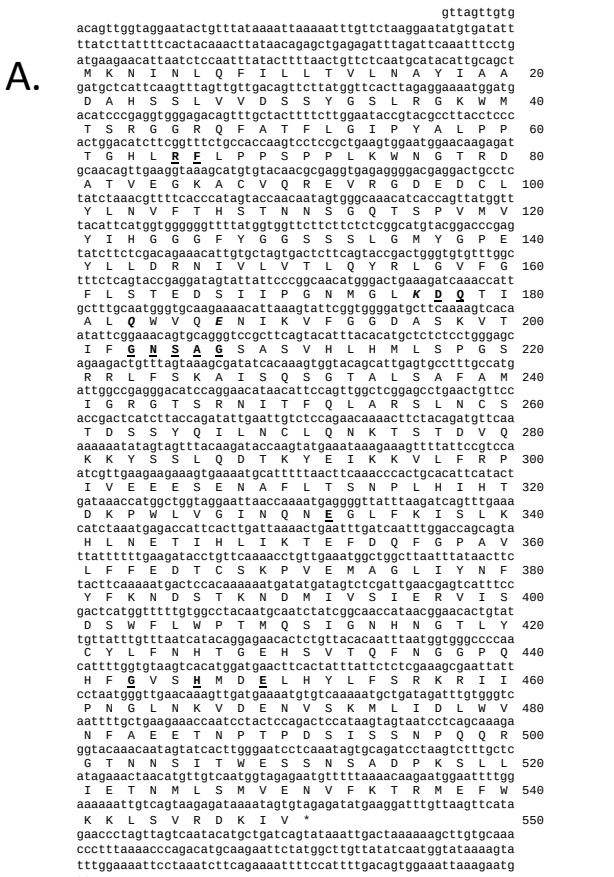
on the 6th day of the 5th instar, a delay of about 1 day in comparison to the spike in JHE activity. These findings suggested that JHE may play a more predominant role than JHEH in JH metabolism in GWSS.

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 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 three full-length JHE coding sequence (*gnsag1*, *gqsag1*, and *gqsag2*, Figure 3) from the ds cDNA library generated from a mixed population of 5th instar GWSS. *Gnsag1*, *gqsag1*,and *gqsag2* encode open reading frames of 550, 547, and 580 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*, *gqsag1*, and *gqsag2*, i.e., GNSAG1, GQSAG1, and GQSAG2 (Figure 3). A signal peptide sequence that is found in all known biologically active JHEs was predicted in GNSAG1 but not GQSAG1 or GQSAG2. However, two additional methionine codons are found within the N-terminal 12 amino acid residues of GQSAG2. Should translation initiation start from either of these ATG codons, a signal peptide sequence is predicted. Phylogenetic analysis placed GNSAG1 and GQSAG1 in the same clade (Figure 4). GQSAG2, however, was found in a clade that was separate from that of GNSAG1 and GQSAG1, and that of known JHEs from lepidopteran insects. Surprisingly, GNSAG1, GQSAG1, and GQSAG2 did not align with NlJHE, a JHE from the hemipteran *Nilaparvata lugens*.

A.



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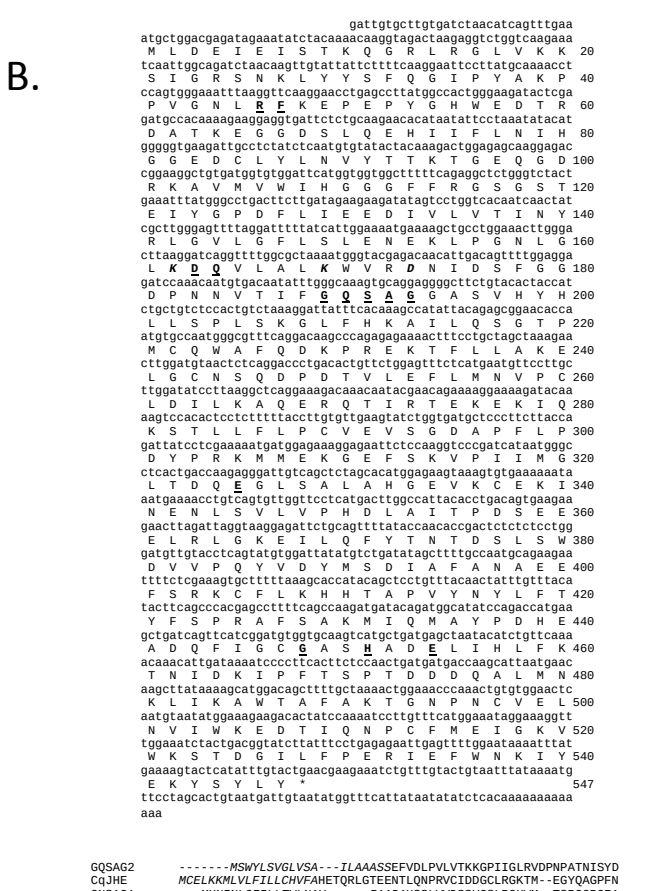
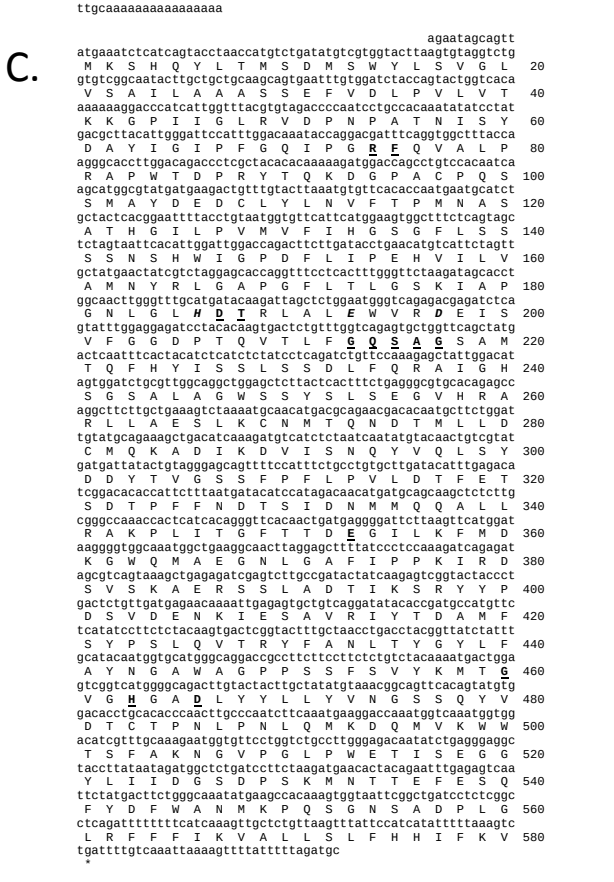


C.



D.

Figure 3 legend. JHE-like nucleotide and deduced amino acid sequences from nymphal GWSS. Three full-length cDNA sequences (named *gnsag1* (A), *gqsag1* (B), and *gqsag2* (C)) are shown. The open reading frames of *gnsag1*, *gqsag1*, and *gqsag2* encode putative proteins of 550, 547, and 580 amino acid residues, respectiviely. The asterisk indicates a stop codon (TAG or TGA). 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*, *gqsag1*, and *gqsag2* (shown in bold-underlined or bold-italic text). A comparison of these conserved motifs with those found in a known JHE (CqJHE) is shown in panel D (putative catalytic site residues are shown within the boxes). Putative signal peptide sequences in the deduced amino acid sequence of *gnsag1* (N-terminal 22 amino acid residues) and *gqsag2* (amino acid residues 12-30, assuming translation begins at the third ATG) are 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.

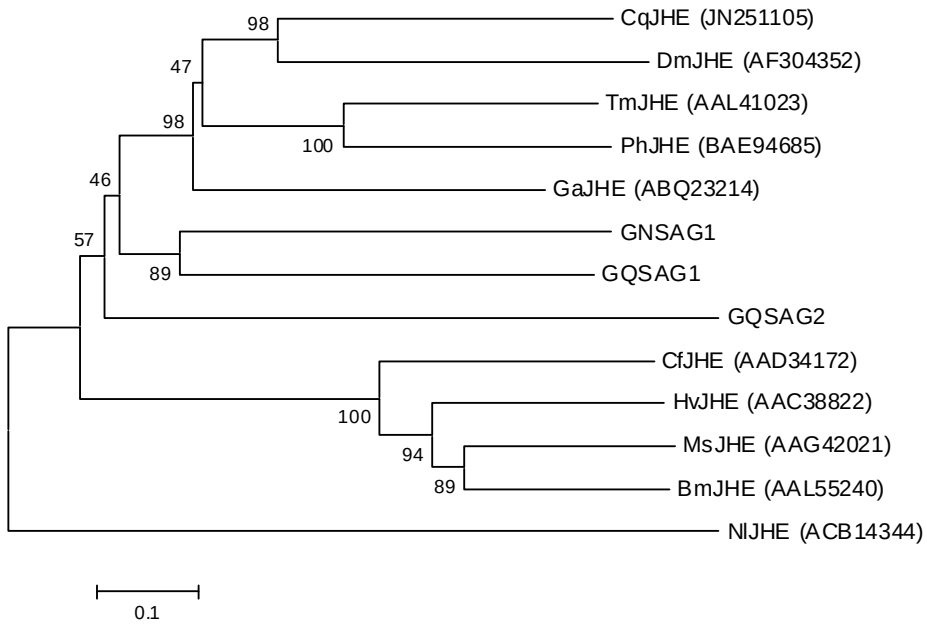


Figure 4. Phylogenetic relatedness GNSAG1, GQSAG1, and GQSAG2 with known JHEs and their hydrolytic activity for JH III. A. Phylogenetic analysis was performed using MEGA version 6. The tree was generated by the Neighbor-Joining method using a ClustalW generated alignment of 10 known JHE sequences (GenBank 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, GQSAG2, and NlJHE; Lepidoptera: CfJHE, HvJHE, MsJHE, and BmJHE; and Orthoptera: GaJHE.

In order to determine if GNSAG1, GQSAG1, and GQSAG2 are able to hydrolyze JH at a rate that is consistent with known JHEs, recombinant baculoviruses expressing these proteins were generated. Initially, four constructs were generated that expressed GNSAG1, GQSAG2, and two forms of GQSAG2. Namely, constructs

expressing the full-length GQSAG2 (i.e., GQSAG2L) and a slightly (11 amino acid residues) shorter version of GQSAG2 (i.e., GQSAG2S) were produced. The GQSAG2S protein initiates from the third methionine codon (see Figure 2C) resulting in a protein (unlike GQSAG2L) that encodes a predicted signal peptide for secretion. Unfortunately, these constructs produced recombinant proteins that showed approximately 3000-fold or lower specific activity for JH III in comparison to a known JHE that was expressed and assayed under identical conditions (Table 1). In order to confirm that the cDNA insert of these recombinant baculoviruses was correct, new recombinant baculoviruses were isolated and the recombinant protein expressed by these new baculoviruses was tested for JH hydrolytic activity. These newly expressed proteins showed the same pattern of specific activity for JH III as the original constructs (Table 1).

Table 1. Specific activity of recombinant GNSAG1, GQSAG1, GQSAG2L, and GQSAG2S for JH III

Protein Source[1]	Specific Activity[2] (nmol JH III acid/min/ml)	Total Activity (nmol JH III acid/min)
GNSAG1-A supernatant	<0.007	<0.7
GNSAG1-B supernatant	<0.003	<0.3
GQSAG1-A supernatant	0.007 ± 0.001	0.7
GQSAG1-B supernatant	0.005 ± 0.002	0.5
GQSAG2L-A supernatant	<0.007	<0.7
GQSAG2L-B supernatant	<0.003	<0.3
GQSAG2S-A supernatant	<0.007	<0.7
GQSAG2S-B supernatant	0.003 ± 0.001	0.3
CqJHE supernatant	22.1 ± 3.5	2,210
CqJHE cell lysate	1.3 ± 0.2	130

[1]The culture supernatant of recombinant baculovirus-infect High Five cells was diluted 1:10 for the recombinant GWSS proteins or 1:1000 for CqJHE. The cell pellet of the CqJHE baculovirus-infected High Five cells was resuspended in the same volume of buffer (100 mM sodium phosphate buffer, pH 8) as was used for cell culture. The baculovirus-infected High Five cells and supernatant was harvested at 65 h post inoculation. The "A" and "B" notations indicate supernatant from cells that were inoculated with independently isolated recombinant baculovirus clones.
[2]Specific activity was determined in 100 mM sodium phosphate buffer, pH 8, containing 1 mg/ml BSA, and 5 µM JH III. The reactions were allowed to proceed at 30ºC for 15 or 150 minutes. The hydrolytic activity of CqJHE, a known juvenile hormone estersase from the mosquito *Culex quinquefasciatus*, was determined under the same conditions.

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 ongoing.

CONCLUSIONS

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. <<<Thus far we have determined the baseline levels of JHE and JHEH activities in 5th instar GWSS nymphs. We have cloned and sequenced the complete coding sequence of three esterase-encoding cDNAs from 5th instar nymphs. We have expressed recombinant proteins from the major open reading frame of each of these cDNAs. We have shown that two of the cDNAs do not encode a protein with JHE activity. We are in the process of confirming the biological activity of the third cDNA. Experiments to develop a production and delivery system for RNAi effectors that target the *jheh* or *jhe* gene are ongoing.>>>

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FUNDING AGENCY

Funding for this project was provided by the California Department of Food and Agriculture's Pierce's Disease and Glassy-winged Sharpshooter Board