THE ENDOCRINE SYSTEM OF THE GLASSY-WINGED SHARPSHOOTER, A VIABLE INSECTICIDE TARGET

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ABSTRACT
Death or dramatic changes in normal insect development can result from minor disruption of the insect endocrine system. Juvenile hormone (JH) is a key insect developmental hormone that has biological effects at low nanomolar levels in the hemolymph. JH analog (JHA) insecticides are green compounds that can mimic the action of JH and selectively disrupt the insect endocrine system. In this project we are testing the efficacy of JHAs against glassy-winged sharpshooter (GWSS) eggs, nymphs, and adults. We are also evaluating the potential of juvenile hormone esterase (JHE)- and JH epoxide hydrolase (JHEH)-encoding genes as a target for gene silencing-based control of GWSS. In terms of mode of action, the effects of JHA application, and JHE and/or JHEH knockdown are similar in that both approaches can enhance "JH action" during periods of developmental when endogenous JH levels are exceptionally low.

LAYPERSON SUMMARY
The insect endocrine system is a highly selective and highly sensitive target for insect control and for reducing vector competence. The overall goal of our project is to study and exploit targets within the endocrine system of glassy-winged sharpshooter (GWSS) that can be used to control GWSS or reduce its ability to spread Pierce's disease. We are taking two complementary approaches. Our direct approach is to determine the efficacy of various concentrations of juvenile hormone analog (JHA) insecticides against GWSS eggs, nymphs, and adults. A key objective of this approach is to quantify the minimum level of JHA insecticide that can efficiently reduce the emergence of nymphs from eggs and keep nymphal insects in the nymphal stage. The results of this direct approach will have near-term applicability since the JHA insecticides that we are testing are US-EPA registered and commercially available. Our indirect approach involves the identification and characterization of genes that are unique to GWSS endocrine system that metabolize a key insect hormone called JH. The objective of this approach is to evaluate these genes as potential targets for gene knockdown.

INTRODUCTION
Insect development is precisely regulated by the relative titers of juvenile hormone (JH) and molting hormones (i.e., ecdysteroids) (**Figure 1**). JHs form a family of sesquiterpenoids (**Figure 2A**) that regulate key biological events in insects including reproduction, behavior, polyphenisms, and development (reviewed in Riddiford, 2008). Minor disruption of an insect's hemolymph JH levels can result in insect death or dramatic alterations in insect development. Juvenile hormone analog (JHA) insecticides are green compounds that selectively target the insect endocrine system by mimicking the biological action of JH (reviewed in Dhadialla et al., 2005; Henrick, 2007). JHAs such as methoprene, fenoxycarb, and pyriproxyfen (**Figure 2B**) are US EPA-registered compounds that are commonly used to control mosquitoes, fleas, whiteflies, ants, and other insect pests. JHAs function as structural and/or biological mimics of JH. When pest insects are exposed to JHAs at a time during development when JH titer is normally undetectable, abnormal nymphal-pupal development and/or death is induced.

Abnormal developmental morphologies, similar to those induced by JHAs are also induced by the inhibition of a JH-selective esterase (JHE) with a chemical inhibitor such as OTFP (Abdel-Aal and Hammock, 1985). Inhibition of JHE putatively results in JH titers that are not below the threshold required for normal development. In this project we are attempting to clone and characterize the *jhe* gene and related JH epoxide hydrolase, *jheh*, gene as potential target genes for an RNAi-based strategy for the control of glassy-winged sharpshooter (GWSS).

OBJECTIVES
The current project is a continuation of our previous UC Pierce's Disease Research Grants Program (UC PDRGP)-funded project (#2010-259, 8/1/10-7/31/11). Objectives I, II, and III are from our previous project. Objectives IV and V are from our current UC PDRGP-funded grant. We are continuing work on all five of these objectives.

1. Evaluate the efficacy of JHA insecticides.
2. Characterize authentic JH esterase (JHE) activity in GWSS.
3. Isolate *jhe* gene and characterize recombinant JHE protein.
4. Investigate delayed effects of low dose JHA insecticide exposure.
5. Characterize recombinant JH epoxide hydrolase (JHEH) from GWSS.

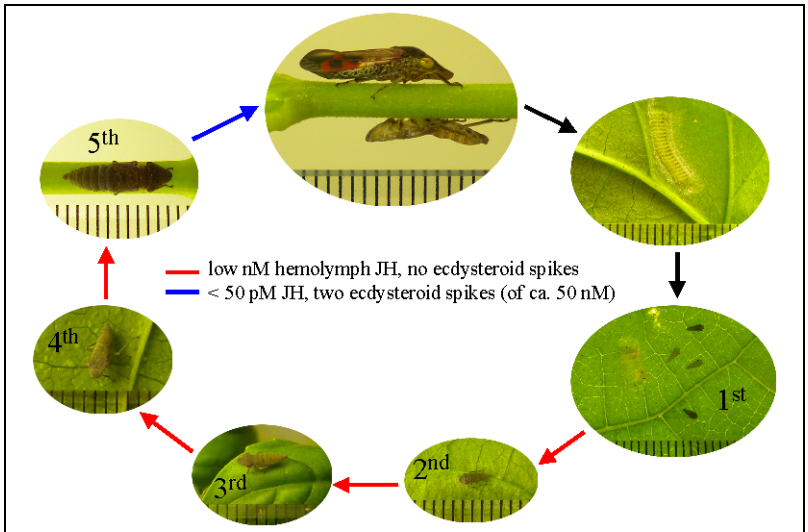

Figure 1. GWSS life cycle, and probable roles of JH and ecdysteroids during development. The size bars are 1 mm apart.

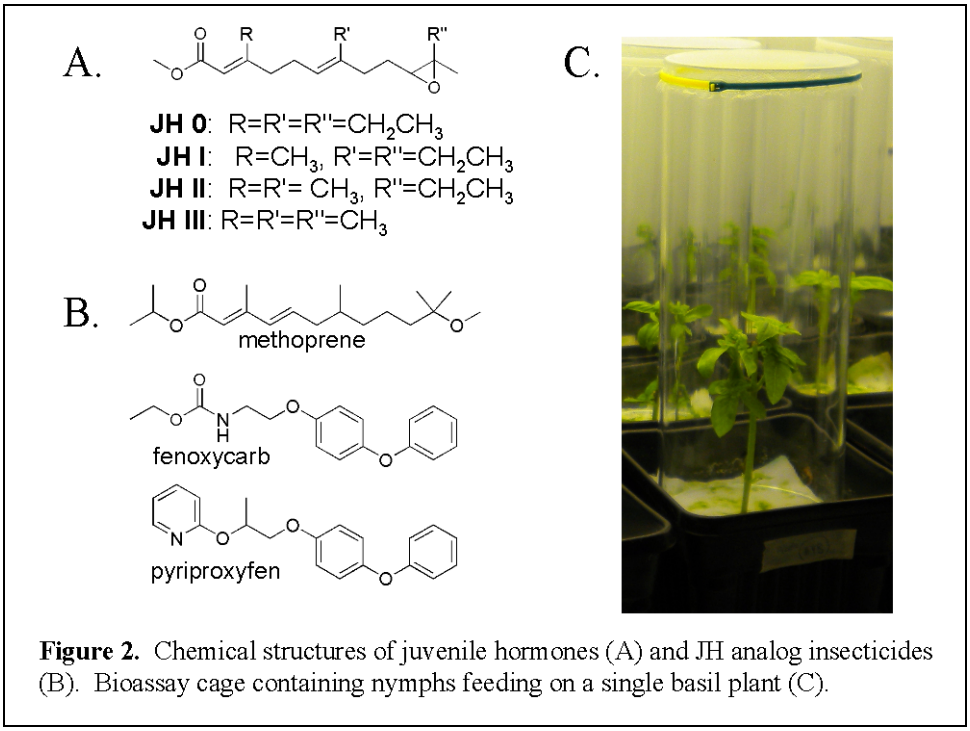

Figure 2. Chemical structures of juvenile hormones (A) and JH analog insecticides (B). Bioassay cage containing nymphs feeding on a single basil plant (C).

MATERIALS AND METHODS

Insect Rearing. Laboratory colonies of GWSS are maintained at the UC Davis Contained Research Facility in an environmental growth chambers set at 24ºC, 70% relative humidity, and a light:dark cycle of 14 h:10 h. Within the chambers the GWSS are grown in Bug Dorm insect cages each containing at least two cowpea, two cotton, and two basil plants.

Bioassays. The life cycle of GWSS was determined using freshly emerged nymphs placed on a single, caged, basil plant (ca. 10-15 cm in height) where the lower stems were removed (**Figure 1C**). The insects were observed daily for signs of molting (i.e., the presence of exuviae and increased head size).

Bioassays. First instar nymphs were exposed to the methoprene (0.2 ml of a 0.5 or 5.0 ppm solution applied to the surface of a 20- ml glass vial (32 cm^2 surface area) for 1 hour. Following exposure groups of five nymphs were placed on a single basil plant and observed daily as described above.

Gene cloning. Total RNAs were isolated from 5th instar nymphs at days 6, 7, 8, 9, and 10 post ecdysis. First strand cDNAs were generated from total RNA using a Creator SMART cDNA library construction kit (Clontech). Random amplification of the 3'-cDNA ends (3'RACE) was performed using an anchor primer and degenerate primers that recognized conserved sequences (reviewed in Kamita and Hammock, 2010) in known JHEs (e.g., GQSAG) and JHEHs (e.g., KPDTIG).

RESULTS AND DISCUSSION

A simple bioassay was developed to measure the development time of GWSS. The bioassay uses a single host basil plant for rearing the insects (**Figure 2C**). Using this bioassay, the duration of 1st, 2nd, 3rd, 4th, and 5th instar nymphs was 6.2±0.8, 4.9±0.8, 5.5±0.8, 7.0±1.1, and 10.7±1.0 days, respectively. The combined length of all of the nymphal stages was about 34 days. The developmental times were the same regardless of oviposition substrate (cotton or cowpea) on which the eggs were laid (**Table 1**).

Table 1. Mean development time of GWSS reared on basil.

Nymphal instar	Development time (days ± s.d.)[1]			
	Eggs from cotton	No. of insects	Eggs from cowpea	No. of Insects
1st	6.2 ± 0.9	110	6.1 ± 0.7	103
2nd	4.9 ± 0.6	68	4.9 ± 0.8	76
3rd	5.4 ± 0.8	57	5.5 ± 0.7	61
4th	7.0 ± 1.3	47	7.0 ± 0.9	56
5th	10.5 ± 0.9	43	10.7 ± 1.0	49
total days	34.0		34.2	

[1]No statistical differences were found in development times based on egg source (i.e., cotton or cowpea)

When first instar nymphs were exposed to the JHA methoprene at a dose 0.5 ppm for 1 h, the duration of the 1st instar was shorter by about one day, whereas that of 2nd, 3rd, 4th, and 5th instars was similar to nymphs that were not exposed to methoprene (**Table 2**). When the methoprene dose was increased 10-fold to 5.0 ppm, the length of the 5th instar was longer by about two days (**Table 2**). However, unlike insects that were not exposed to methoprene, there was tremendous variation in the length of this instar with some insects remaining as 5th instars for more than 20 days. We hypothesize that methoprene, a highly non-polar compound that easily penetrates the cuticle, is retained within the tissues of some insects and exerts a biological effect even after multiple molts. These finding suggest that exposure even at a dose as low as 5 ppm will have significant effects on the life history of GWSS. We are currently repeating these experiments with the JHA pyriproxyfen.

Table 2. Mean development time of GWSS following methoprene exposure.

Nymphal instar	Development time (days ± s.d.)/concentration	
	0.5 ppm	5.0 ppm
1st	4.8 ± 1.3	4.7 ± 0.8
2nd	5.7 ± 2.2	5.8 ± 0.9
3rd	4.7 ± 0.6	5.4 ± 0.9
4th	6.8 ± 0.5	7.2 ± 0.8
5th	10.7 ± 1.4	12.7 ± 8.8
total days	32.7	35.8

Both JHE and JHEH activities were found in the hemolymph of 5th instar GWSS nymphs. JHE activity was highest at days six,seven, and eight of the 5th instar with values of 24.4 ± 3.5, 21.8 ± 1.9, 22.7 ± 2.4 pmol of JH acid formed per min per ml of hemolymph. JHEH activity was consistently lower than JHE activity and showed a peak of 9.3 ± 1.7 pmol of JH diol formed per min per ml of hemolymph at day six of the 5th instar. Semiquantitative PCR analyses were consistent with the enzymatic activity assays, and identified putative *jhe* and *jheh* gene expression at days eight, nine, and 10 of the 5th nymphal instar (**Figure 3**). We are currently attempting to clone JHE- and JHEH-encoding sequences by 3'- and 5'-RACE approaches.

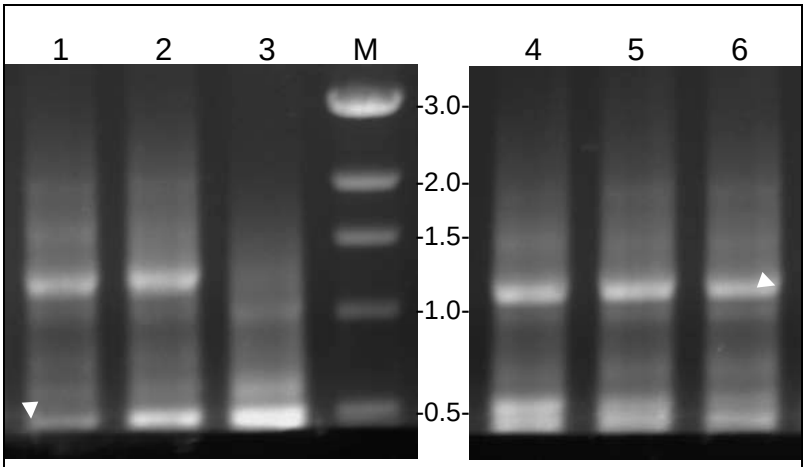

Figure 3. Expression of the putative *jheh* (left arrowhead) and *jhe* (right arrowhead) genes in 5th instar GWSS nymphs at day 8 (lanes 1 and 4), day 9 (lanes 2 and 5), and day 10 (lanes 3 and 6) of the instar. The size of molecular weight standards (lane M) is shown in kDa.

CONCLUSIONS

We have established a robust and easy to use bioassay for GWSS. Using this bioassay we have determined precise developmental times under standard rearing conditions. Knowledge of precise developmental times are critical for quantifying the efficacy and effects of the JHAs, effects of JHE-inhibitors, and enzyme activity levels of JHE/JHEH in GWSS. We have also used our bioassay to determine baseline lethal dose values with the JHA standard methoprene. We are continuing our bioassays at low doses and are in the process of isolating and characterizing JHE and JHEH encoding sequences as possible targets of RNAi.

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