Progress Report for CDFA agreement number 11-0145-SA

Title of Project: The endocrine system of GWSS, a viable insecticide target

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Time period covered: August 2011 to March 2012

Objectives:

I. Investigate the delayed effects of low dose JHA insecticide exposure
   A. Determine sublethal dose in eggs and 1st instar nymphs
   B. Evaluate delayed effects of sublethal exposure on egg development
   C. Evaluate delayed effects of sublethal exposure on nymph development
II. Characterize recombinant JHEH from GWSS
   A. Clone full-length *jheh* gene of GWSS
   B. Biochemically characterize recombinant JHEH
   C. Screen JHA insecticides for JHEH inhibitory activity

Introduction

Insect development is precisely regulated by the relative titers of juvenile hormone (JH) and molting hormones (i.e., ecdysteroids). JHs form a family of sesquiterpenoids (Fig. 1A) 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 (Fig. 1B) are US EPA-registered compounds that are commonly used to control mosquitoes, fleas, whiteflies, ants, and other insect pests. 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.

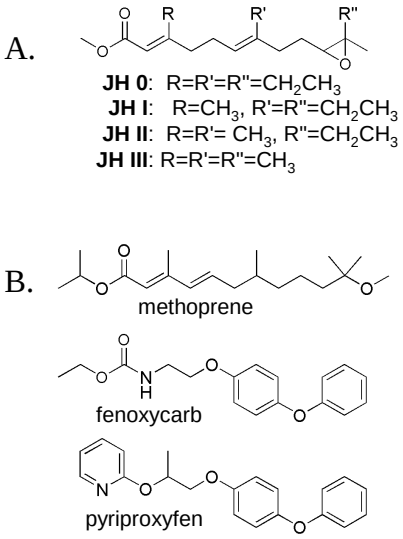

Figure 1. Chemical structures of the major forms of juvenile hormone (A) and commercial juvenile hormone analog insecticides (B).

Abnormal developmental morphologies, similar to those induced by JHAs are also induced by inhibiting an esterase that is selective for JH (Abdel-Aal and Hammock, 1985). Inhibition of the JH esterase (JHE) putatively results in JH titers that are not below the threshold required for normal development. Similarly, we hypothesize that inhibition of another JH-metabolizing enzyme called JH epoxide hydrolase (JHEH) will also result in the induction of abnormal nymphal-pupal development and/or death of GWSS.

In this project we are testing the efficacy of commercially available JHAs against GWSS eggs and nymphs. In particular, we are focusing on the delayed effects of exposure to JHAs. We are also attempting to characterize GWSS JHEH, an enzyme that metabolizes the epoxide moiety that is found on all known JHs. The gene that encodes this enzyme, *jheh*, could have potential as a target for gene silencing-based control of GWSS. Similarly, we are attempting to characterize a JH-selective esterase (JHE) that metabolizes an ester moiety that is also found on all known JHs. In terms of mode of action, the effects of JHA application, and JHEH and/or JHE knockdown by gene silencing are similar in that both approaches can enhance "JH action".

Objective I. Investigate the delayed effects of low dose JHA insecticide exposure.

We have developed a simple bioassay to quantify the length of each developmental stage of GWSS under standard rearing conditions. Our bioassay uses a single host basil plant for rearing the insects. 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 (Fig. 2). 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).

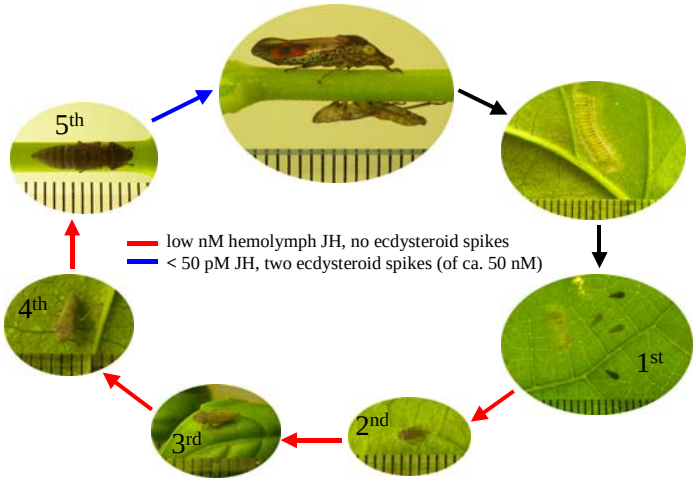

Figure 2. GWSS life cycle, and probable roles of JH and ecdysteroids during development. The size bars are 1 mm apart.

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)

We are now using our optimized bioassay to quantify GWSS developmental times following exposure to various concentrations of methoprene. Methoprene was the first commercially available JHA and, although not commonly used for the control of non-dipteran insects, serves as an excellent "bridge" compound for comparing efficacy between various insect orders. 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 1 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 2 days (Table 2). However, unlike insects that were not exposed to methoprene, we found 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

Objective II. Characterize recombinant JHEH from GWSS.

Both JHEH and JHE activities were found in the hemolymph of 5th instar GWSS nymphs. JHE activity was highest at days 6, 7, and 8 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 6 of the 5th instar. Semiquantitative PCR analyses were consistent with the enzymatic activity assays, and identified putative *jheh* and *jhe* gene expression at days 8, 9, and 10 of the 5th nymphal instar (Fig. 3). We are currently attempting to clone JHE- and JHEH-encoding sequences by 3'- and 5'-RACE approaches.

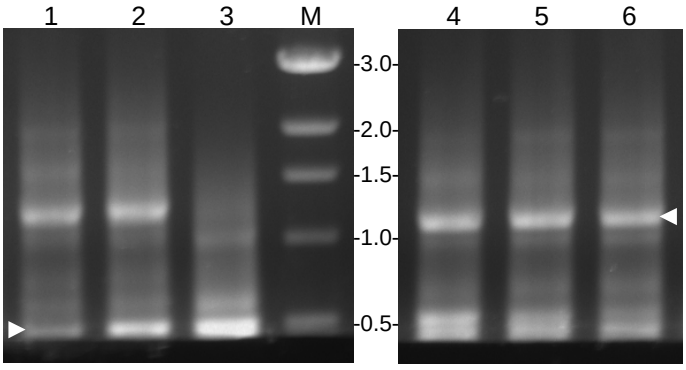

Figure 3. Expression of the putative *jheh* (left arrowhead) and *jhe* (right arrowhead) genes in 5th instar GWSS nymphs at day 8 (lanes 1 and 4), day 9 (lanes 2 and 5), and day 10 (lanes 3 and 6) of the instar. The size of molecular weight standards (lane M) is shown in kDa.

Discussion

We have established a robust and easy to use bioassay for determining the length of each development stage of 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 JHAs, effects of JHE-inhibitors, and enzyme activity levels of JHEH/JHE 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 JHEH and JHE encoding sequences as possible targets of RNAi.

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.

Life cycle. 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. 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 5^{th} 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).

Intellectual Property

No issues associated with intellectual property have been generated with the project. At present, there are no pending peer-reviewed publications resulting from this project.

References

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