Identification of juvenile hormone response genes in newly emerged female *Aedes aegypti*

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Abstract

Juvenile hormone (JH) plays pivotal roles in the development and reproduction of insects. Efforts to characterize the mechanisms of JH regulation are complicated due to JH pathways often being intertwined with those of 20-hydroxyecdysone (20E). Upon adult emergence, female *Aedes aegypti* enter a period of development during which they gain competence for mating, bloodfeeding, and egg production. JH levels rise dramatically and peak during the first 2-3 days post-emergence and remain relatively high until a bloodmeal is consumed, while 20E titers remain very low throughout the entire stage. Thus, post-emergence development offers a unique opportunity to study the effects of JH in the absence of 20E. In this study, four potential JH response genes were identified in newly emerged females. One such gene, *AaKr-h1*, is a homologue of *Kr-h1,* a zinc-finger transcription factor which has been characterized in *Manduca sexta, Drosophila melanogaster, Tribolium castaneum,* and *Apis mellifera*, and is involved in a diverse range of JH-regulated pathways. *AaKr-h1* demonstrated a dose-dependent transcriptional response to JHIII as well as two JH mimics in abdominal ligation assays. The findings of this study indicate that *Kr-h1* may be regulated by JH independently of any 20E regulation and suggests a fundamental, conserved role for *Kr-h1* in JH-regulated pathways.

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Introduction

Juvenile hormone

Juvenile hormone (JH) plays pivotal roles in the development and reproduction of holometabolous insects (i.e. those which undergo complete metamorphosis) [1, 2]. It is released from the corpora allata, a pair of glands commonly located posterior to the brain of the insect. Its most widely studied role is that of a regulator, which determines the specific developmental stage the insect will enter upon molting. During larval stages, a rise in the titer of the steroid hormone 20-hydroxyecdysone (20E) signals the occurrence of a molt. In the presence of JH, the molt will result in another larval stage. In the absence of JH, the rising 20E titer leads to a pupal molt, and the beginning of metamorphosis [3]. Thus, JH is commonly referred to as the "status quo" hormone. While many variations on this model do exist, it has been highly conserved throughout holometabolous insect evolution [4]. In addition to its role in development, JH has been shown to play a wide variety of roles in adult reproduction and behavior [1, 2].

Juvenile hormones are a group of related sesquiterpenoids [5]. The most common form of JH in insects is JHIII [6]. Figure 1 shows JHIII and several synthetic JH analogs (JHAs). Methoprene is a potent JHA used in mosquito population control by preventing larvae from entering or completing metamorphosis when it is added to standing water [7].

Met, br, and *Kr-h1*: genes in JH signaling

Although the effects of JH are widely studied and described, the mechanisms by which JH exerts its effects remain unclear. Efforts to determine JH modes of action have been hindered by difficulties in definitively identifying a receptor protein, due in large part to JH showing significant binding affinity with a vast array of proteins [8]. In addition to this, the regulatory effects of JH are enormously diverse and JH responses are usually intertwined with those of 20E, thus complicating the elucidation of the mechanisms. Further, JH can in some cases have seemingly contradictory effects. For example, at different developmental points during *Drosophila* metamorphosis, JH can either activate or suppress *broad* [9]. Three genes that have been identified in JH

signaling pathways are *broad* (*br*), *methoprene-tolerant* (*met*), and *Krüppel homolog 1* (*Kr-h1*).

***Broad* (*br*)**, also referred to as *Broad Complex*, encodes a C2H2-type zinc finger DNA-binding protein. *Br* plays a pivotal role in pupal development. During the final larval instar, *br* is activated by 20E in the absence of JH, and is a key initiator of metamorphosis. Consistent with this, amorphic *br Drosophila melanogaster* (fruit fly) mutants develop normally through the final larval instar, but cannot undergo metamorphosis [10]. In many holometabolous insects, *br* is first activated near the end of the last larval stage and declines prior to adult differentiation [11]. There is evidence that activation of *br* at the end of the final larval instar activates genes required for pupal development, while at the same time suppresses genes specifying adult development. For example, in *D. melanogaster* (order: Diptera) and the tobacco hornworm *Manduca sexta* (order: Lepidoptera), ectopic *br* expression during larval stages leads to premature pupal development, and during metamorphosis it causes prolonged pupal (and delayed adult) development [9]. Furthermore, it has been shown that it is through suppression of *br* that application of JH exerts it's known effect of delaying metamorphosis in *M. sexta*, and in certain tissues of *D. melanogaster* [9, 12]. Consistent with its role as a pupal specifier, there is evidence that the pupa-adult transition is the result of the suppression of *br*. In the red flour beetle *Tribolium castaneum* (order: Coleoptera) suppression of *br* has been shown to be critical for the timing of adult development, as knockdowns of *br* at the onset of metamorphosis lead to premature adult differentiation [13]. Interestingly, during the pupal-adult transition of *D. melanogaster* it is through a second pulse of 20E (in the absence of JH) that *br* is suppressed, and topical application of a JHA can block this repression, thus causing re-expression of *br* and a delay of adult development[9]. Thus, in larval-larval, larval-pupal, and pupal-adult molts, JH appears to exert its "status quo" action through suppression of 20E effects on *br* expression.

Br has roles in adult development as well. In the yellow fever mosquito *Aedes aegypti*, *br* encodes four isoforms (Z1-Z4) with different zinc-finger domains [14]. *Br* has been shown to play a complex role in the regulation of a 20E response gene, *Vitellogenin* (*Vg*) the major yolk protein precursor (YPP) gene: it appears that Z1 and Z4

repress, while Z2 and Z3 activate, *Vg* response to 20E [15]. Highly similar *br* isoforms are found in *D. melanogaster* [16, 17], and *M. sexta* [12].

M*ethoprene tolerant* (*met*) was first identified in *Drosophila* mutants demonstrating resistance to effects of methoprene or JHIII applied topically or incorporated in to the diet [18]. At this time, its product, a bHLH-PAS transcription regulator, is widely considered a strong candidate as a potential JH receptor [19]. MET can bind JHIII with high affinity (K_d = 5.3 ± 1.5 nm, mean ± SD), and a GAL4-DBD-MET fusion showed JH-dependent activation of a UAS reporter plasmid [20]. MET has been implicated in many JH-regulated processes. In *T. castaneum*, MET was shown to mediate both JH repression of metamorphosis [21] as well as repression of adult differentiation during pupal development [22]. In addition, MET is required for JH suppression of *br* at this stage[23].

Krüppel homolog-1 (Kr-h1) is a zinc-finger protein containing eight C_2H_2-type zinc fingers, and was first characterized in *Drosophila*. Its name is derived from its homology to *Krüppel*, a gene with a role in body segmentation during embryonic development [24]. In *Drosophila*, *Kr-h1* has several isoforms. *Kr-h1*β is expressed in neuronal cells during early development. It is essential for development, and is involved in the regulation of many 20E-regulated genes [25]. *Kr-h1*α is the predominant isoform expressed during the onset of metamorphosis (*Kr-h1*γ being expressed in a similar pattern at much lower levels) and essential for successful entry into metamorphosis, and, similar to *Kr-h1*β, is involved in 20E regulatory pathways [24].

Kr-h1 has been implicated in JH response during *Drosophila* metamorphosis [26]. Misexpression of *Kr-h1*α can mimic some phenotypes associated with JHA application at the onset of metamorphosis, and *Kr-h1*α is itself upregulated by such JHA application. Such JH responses are a result of *br* activation during pupal development, and *Kr-h1* appears to mediate this JH activation of *br* [26].

In *T. castaneum*, there is evidence to indicate that the *met*-mediated JH repression of adult differentiation during pupal development (mentioned earlier) involves activation of *Kr-h1* downstream of *met* [22]. In addition, *Kr-h1* has been shown to mediate the JH-dependent anti-metamorphic effects of *met* during larval development (Figure 2) [27].

In addition to its involvement with JH regulation of larval and pupal development, *Kr-h1* has been linked to JH-influenced adult behavior in the honeybee *Apis mellifera*. The transition of worker bees from nurses to foragers is effected by JH [28], vitellogenin [29], and queen mandibular pheromone [30]. It is hypothesized that these three factors interact with each other, and ultimately through *Kr-h1,* in order to regulate the transition to foraging behavior [31].

The *Aedes aegypti* mosquito

Ae. aegypti has been widely studied due to its role as an important vector for the diseases yellow fever, chikungunya, and dengue. Of these, dengue is the most serious health risk worldwide. According to the World Health Organization (WHO), dengue affects up to 50 million people per year, and the potentially lethal complication dengue hemorrhagic fever (DHF) affects 500,000 people per year, resulting in approximately 20,000 deaths, mostly among children. Approximately 2.5 billion people are at risk of dengue infection. The disease has spread consistently and dramatically over the past 50 years (an estimated 30-fold increase in average annual infections) and continues to do so in large part due to the geographic expansion of its primary vector, *Ae. aegypti*,. At this time, vector control is the only available and effective means of dengue and DHF prevention [32].

Upon adult eclosion from the pupa, both 20E and JH are at low levels in female *Ae. aegypti* [33, 34]. Immediately following emergence, JH titers rise rapidly, peak around 2 days post-emergence, and then slowly decline. Meanwhile, 20E titers remain very low throughout this time. After about 3 days, the female is competent to consume a blood meal. Upon blood feeding, juvenile hormone esterase activity sharply increases and JH titers drop precipitously, while 20E titers rise, ending the post-emergence phase of development [33, 34].

While JH functions in ways typical for holometabolous insects during larval and pupal development of *Ae. aegypti*, it plays additional roles during the first 2 to 3 days post-emergence, with several important developments under the control of JH [35]. For example, during this stage in the ovaries, the primary follicles grow to ~100µm, and the oocytes are distinguishable from the nurse cells. When females are allatectomized (CA

removed) at emergence, this development fails to occur. Delaying the allatectomy until 3 days post-emergence alleviates these effects, as does re-implanting the CA shortly after removal, or topical JHA application [36]. Ribosome proliferation in the fat body, normally observed at this stage and necessary for later yolk production, is also prevented by allatectomy at emergence, and restored by either re-implantation of the CA or JHA application[37]. While females can mate shortly after emergence, they are typically refractory to insemination for about 2 days. Allatectomy at emergence does not interfere with mating, but increases the duration of the refractory period [38]. Additionally, JHA application at or before emergence can reduce this refractory period [39].

During the post-emergence phase, several genes are regulated by JH. In abdominal ligation assays, in which the head and CA are removed from newly emerged females, differential mRNA expression has been demonstrated between groups treated with either JHA or an acetone control for the genes *Early Trypsin* [40] and *JHA15* [41]. Both genes code for female specific proteases expressed in the midgut during post-emergence development. There is also evidence that JH exerts posttranscriptional control over the 20E competence factor *βFTZ-F1* in the fat body during the post-emergence phase [42].

The period of development immediately following adult emergence of female *Ae. aegypti* offers a unique window to study the actions of JH. While many genes have been shown to be regulated by a combination of 20E and JH, with JH acting through modification of action of 20E, relatively few genes have thus far been shown to be directly regulated by JH alone [11]. The absence of 20E thus makes the post-emergence phase an opportune developmental stage in which to identify genes directly regulated by JH (the focus of the research in this report), as well as to study the mechanisms of such regulation.

Research goals

One goal of the Zhu lab is to study development in adult mosquitoes in order to develop strategies to control the spread of mosquito borne diseases. A particular area of focus is studying the role of JH in post-emergence development in *Ae. aegypti*

females. To this end, the series of experiments described in this report were performed in order to identify genes regulated by JH in newly emerged female *Ae. aegypti*.

As one of the world's most important disease vectors, information that may lead to novel control strategies for *Ae. aegypti* is invaluable. While this project specifically investigates gene regulation during post-emergence development, this in turn can affect every aspect of the biology of the adult mosquito, including reproductive development and behavior, host seeking behavior, and refractoriness to infection by parasites. All of these aspects represent potential points of intervention to either control *Ae. aegypti* populations or limit their competence as a vector of human disease.

Additionally, JH is a key hormone in nearly all insects, and a deeper knowledge of its modes of action will improve our understanding of insect development. As discussed earlier, the developmental stage being studied offers unique insight into JH regulation due to the absence of 20E. Further, since *Kr-h1* (a homolog of which is described in *Ae. aegypti* in this report), appears to be a key player in a variety of JH-regulated events across diverse insect species, knowledge gained of its role in JH regulation in this stage may contribute to an understanding of its broader, and possibly conserved, relationship to JH regulation.

Material and Methods

Mosquito rearing

Mosquitoes used in all experiments were *Aedes aegypti*, Rockefeller strain. Mosquito larvae were raised in distilled H_2O (~200 larvae per 1.5 liter tray) and fed pestle-ground TetraMin® fish food (Tetra Holding Inc.). Pupae were transferred to a 400 ml tray prior to adult emergence. Adults were fed 10% sucrose in H_2O. Both larvae and adults were raised at 28°C in a 16 hr light / 8 hr dark cycle.

Abdominal ligation assays

Female *Ae. aegypti* were collected and anesthetized using either CO_2, or by cold within 30 minutes of adult emergence. Ligations were performed by severing the head and adjacent 1/3 portion of the thorax, which includes the CA. 3M Vetbond™ tissue adhesive was used to seal the open wound, and affix the thorax/abdomen to a glass slide. Specimens were treated with hormone dissolved in acetone as specified in text. Sample groups (5-8 specimens each) were incubated at 28°C in moisture chambers for times specified in text, and then placed in 150 µl TRIzol® (Invitrogen) and stored at -80°C.

Tissue dissection

Tissues extracted and sample sizes are indicated in text with the following notes: Thorax included wings and legs. Ovaries included the two most posterior segments of the abdomen. Fat body included everything in the abdomen section minus the midgut and ovaries. All specimens were cold anesthetized prior to dissection. All tissue sample groups were placed in 150 µl TRIzol® and stored at -80°C.

RNAi

Template DNA for dsRNA synthesis: A 603 bp region of the *AaKr-h1* transcript (Figure 3) was PCR amplified using *Ae. aegypti* cDNA template and primers with added Pst1 and Xho1 restriction site sequences (see primers below). Reaction mixture: 50 µl PCR Supermix High Fidelity (Invitrogen); 0.5 µl each primer (see primers), 25 pmol/µl;

1.0 µl cDNA. PCR program: 3 min @ 94℃; 40 cycles of 30 sec @ 94℃, 30 sec @ 55℃, 1 min @ 72℃; 5 min @ 72℃. The resulting DNA fragment was cloned into a LITMUS 28i plasmid (New England Biolabs) between Pst1 and Xho1 restriction sites. The insert was flanked by two inverted T7 promoters on LITMUS 28i. Template DNA for *AaKr-h1* dsRNA, with T7 promoters at each end, was obtained by PCR using a minimal T7 primer. Template DNA for *Mal* dsRNA (described in text) was produced in the same fashion using 2.0 µl (50 ng/µl) of supplied LITMUS 28i*Mal* plasmid (New England Biolabs). The expected 768 bp (*Kr-h1* dsRNA template) and 962 bp (*Mal* dsRNA template) DNA fragments were confirmed on a 1% agarose gel.

The quantity of both templates was insufficient for dsRNA synthesis, so PCR products were purified using QIAquick PCR Purification (Qiagen), and the products were used as template for multiple PCR amplifications to generate needed quantities of template DNA. Reaction mixture and thermocycler program were the same as previous PCR, using 1.0 µl of each template (*Kr-h1* dsRNA template, 30 ng/µl; *Mal* dsRNA template, 60 ng/µl). A MiniElute PCR Purification Kit (Qiagen) was used to purify and concentrate products of multiple PCR reactions to concentrations and quantities sufficient for dsRNA synthesis.

Template DNA sequencing: The template DNA for *Kr-h1* dsRNA was cloned into a pCR®4-TOPO plasmid (TOPO TA Cloning® Kit for Sequencing, Invitrogen) and sent to Virginia Bioinformatics Institute for DNA sequencing using M13 forward and reverse primers. Chromas Lite 2.01 (Technelysium) was used to interpret sequences from the chromatogram result files. The sequence was compared with the putative transcript for *AaKr-h1* in Vectorbase (ID# AAEL002390) using Kalign and found to differ by two bases over the 603 bp region of homology.

dsRNA synthesis: MEGAscript® RNAi Kit (Ambion, #1626) was used for synthesis of dsRNA, using template DNA following the manufacturer's protocol. Product quality and size were confirmed on 1% agarose gel, and expected bands at ~730 bp (*Kr-h1*dsRNA) and ~930 bp (*Mal*dsRNA) were observed (Figure 4). Ethanol precipitation was use to concentrate final dsRNA products to ~3 µg/µl, in 0.1X PBS.

dsRNA injections: Newly emerged female *Ae. aegypti* were injected with 0.6 µg dsRNA in 200 nl 0.1X PBS between 2 hr and 4 hr post-emergence using a Nanoject II

nanoliter injector (Drummond Scientific Company). Mosquitoes were cold anesthetized, and injections were administered in the side of the thorax. Mosquitoes were then raised at 28℃ in a 16 hr light / 8 hr dark cycle for the specified analysis time.

RNA extraction / cDNA synthesis

All tissue and abdomen samples were stored in 150 µl TRIzol at -80℃ prior to RNA extraction. Samples were homogenized in 150 µl TRIzol in Eppendorf tubes using a plastic pestle. TRIzol was then added to 1.0 ml total volume, and manufacturer's protocol was followed for RNA extraction. The resulting purified RNA pellet was reconstituted in 40 µl nuclease-free H_2O. Concentration was determined by measuring the A_{260} with a spectrophotometer ($1A_{260}$ = 40 ng/µl). RNA quality was confirmed by running 1 µg per lane on a 1% agarose gel, and verifying two distinct ribosomal RNA bands. For all samples, 1 µg RNA was used for cDNA synthesis as follows: Residual genomic DNA was removed using Deoxyribonuclease I, Amplification Grade (Invitrogen) following the manufacturer's protocol. Reverse transcription was performed using the Omniscript RT Kit (QIAGEN) following manufacturer's protocol. Final reaction volume of 20 µl was diluted to 80 µl for subsequent qRT-PCR reactions.

qRT-PCR and analysis

All primers were checked for acceptable amplification efficiency ($m \leq 3.5$) and single amplification product (single melting curve peak) in serial cDNA dilutions and gene-specific DNA concentration standards. DNA concentration standards were serial 10-fold dilutions of PCR product, using *Ae. aegypti* cDNA template and gene-specific primer (see qRT-PCR primers) purified with PureLink™ PCR Purification Kit (Invitrogen). All qRT-PCR reactions used SYBR® GreenER qPCR Supermix for ABI PRISM (Invitrogen) per manufacturer's protocol, with 2 µl sample cDNA or gene-specific DNA standard. Reactions were performed on an ABI PRISM 7300 (Applied Biosystems) Thermal program for all reactions was 2 min @ 50℃; 10 min @ 95℃; 40 cycles of 15 sec @ 95℃, 1 min @ 60℃. Dissociation step was included and the melting curve checked for single peak in all reactions.

7300 System software (Applied Biosystems) was used to calculate Ct values, as well as standard curve slope and intercept values used to calculate relative quantities of mRNA copies in samples. All samples and standards were run in triplicate and results averaged. mRNA quantities for target genes were normalized against mRNA for ribosomal protein *AaS7*.

Primers

T7 minimal primer		dTAATACGACTCACTATAGG
Template DNA insert with added restriction sites		
	Forward (Pst1)	Reverse (Xho1)
	AA(CTGCAG)CGGTTAACACCACCGTCTTC	GC(CTCGAG)AGCCGCTTGCTAACCTTGTA
qRT-PCR Primers		
Target	Forward	Reverse
AaS7	CGGAACTAGGGTGTGCATAAATC	CCGTTTGGTGAGGGTCTTTATT
AaKr-h1	TGCGGAGAGCTTGGCAATA	AAACACCCTTTGCTTTCGTTCA
AaET	CCCACTGGTCTCTGGTGATAAACTG	TTCGAACACCTTAAACCTCGGAAAC
AaCBP	CACCACAGTGGCACCTACGA	GCAACACCACGGCAAAAAT
AaNaSS	GGACCTCTTCTGGGTGTCTTCA	AAGAGCCGCTCCCTTCCA
AaHairy	CCGCCGGCGTTTGTT	GATTGACGTGATTTTGGAAGATCA

(Primers are all indicated 5' – 3')

Results

Part 1. Identifying JH response genes in newly emerged female *Ae. aegypti*.

What genes are differentially expressed in response to a JH mimic in abdominal ligation assays?

Abdominal ligation assays, have been used to identify potential JH response genes in newly emerged adult female *Ae. aegypti*. In short, abdominal ligations (the removal of the head and adjacent one third of the thorax) are performed on newly emerged females. In doing so, the remaining thorax and abdomen are cut off from the corpora allata (source of JH) before JH titers begin to rise. Abdomens are treated with either JHA in acetone or acetone alone, and incubated in a humidity chamber at 28°C. Differential expression of a gene between the two groups implicates JH response.

In an experiment conducted previously in the Zhu lab, microarray analysis was used to generate a genome-wide transcription profile from cDNA from abdominal ligation assay samples. In the abdominal ligation assay, JHA groups were treated with 1.0 µg methoprene in 0.5 µl acetone, and control groups with 0.5 µl acetone alone. Samples were taken from both groups at 4 hr and 12 hr post-ligation. A list of potential JH-response genes was created consisting of genes which were significantly upregulated in the JHA-treated group relative to the control group in the abdominal ligation assay at either 4 hr or 12 hr post-ligation. qRT-PCR was used to confirm the microarray results. Table 1 shows the results from five genes for which differential mRNA expression was shown in both the microarray and qRT-PCR experiments. These five genes (described below) are the starting point for research described in this report.

***AaET* (AAEL007818)** has been well characterized in female *Ae. aegypti*. Its protein product, a midgut-specific protease, is expressed rapidly and abundantly shortly after a blood meal [43]. Interestingly, while *AaET* mRNA transcripts are detectable 24 hrs after emergence and reach a maximum level by 4 days post-emergence, the AaET protein is not detectable until after a bloodmeal is taken, indicating posttranscriptional

regulation [44]. *AaET* mRNA expression has been previously shown to be regulated by JH in newly emerged female *Ae. aegypti* in abdominal ligation assay experiments similar to those described in this report,[40].

***AaNaSS* (AAEL002576)** has a predicted function of a sodium/solute symporter (VectorBase) [45]. Analysis of the predicted amino acid sequence using PROSITE [46, 47] revealed a domain characteristic of membrane-bound sodium/solute symporters. Solutes include amino acids, glucose, and iodide.

***AaCBP* (AAEL002619)** is a putative chitin-binding protein. Analysis of the predicted amino acid sequence using PROSITE [46, 47] indicates that it contains 4 chitin-binding domains similar to domains found in Peritrophin-A, a protein associated with the peritrophic matrix found in the midgut of insects [48].

***AaHairy* (AAEL005480)** is a putative homolog of *hairy* (VectorBase) [45], a transcription repressor first described in *D. melanogaster* [49]. In *D. melanogaster*, *hairy* functions as a pair-rule gene establishing body segment boundaries during embryonic development, and in larval development it plays a role in the patterning of adult sensory bristles [50, 51].

***AaKr-h1* (AAEL002390)** The predicted product of *AaKr-h1* (VectorBase) [45] is a putative homolog of *Kr-h1* described earlier. Evidence for homology to *Kr-h1* is given in later in the results section. Analysis of the predicted amino acid sequence using PROSITE [46, 47] indicates *AaKr-h1* is a zinc finger protein with 8 putative C_2H_2 zinc fingers, similar to *Kr-h1* described in other species. *AaKr-h1* is described in more detail in part II of this project.

What are the temporal expression profiles of the identified genes in response to the JH mimic?

Results from the previously described abdominal ligation experiments suggest the five genes identified (Table 1) are differentially transcribed at 4 or 12 hours post-ligation in methoprene-treated vs. acetone control groups, and are therefore responding to methoprene. However, there is no data from the time of the ligations (0 hr post-ligation) to which the 4 or 12 hr post-ligation mRNA levels can be compared. Thus, it is unclear if what is being observed is, in fact, upregulation in the methoprene-treated

samples compared to unchanging expression in the acetone control samples. Additionally, with only two time points, it is possible that more complex expression patterns may exist. In order to address these issues, additional abdominal ligation assays were performed, with samples incubated for 0, 2, 4, 8, 12, or 16 hr. qRT-PCR was used to measure mRNA expression of the five genes identified (Figure 5).

All five genes tested demonstrated JH-dependent response over the 16 hr period following hormone treatment, evident by differential expression between methoprene-treated and acetone control groups. However, since the upregulation for *AaHairy* was relatively small (3 fold maximum), it was not considered to be worth further investigation and characterization in this study. *AaKr-h1* and *AaET* mRNA expression showed little or no increase in the acetone control groups over the 16 hrs sampled, indicating that the observed upregulation of these two genes in methoprene-treated groups may be attributed solely to the addition of methoprene. Results for *AaCBP* and *AaNaSS* do show upregulation in acetone control groups, indicating that some factor(s) other than methoprene treatment are likely responsible for some of the upregulation in the methoprene-treated groups. The upregulation of *AaNaSS* mRNA expression in acetone control groups was moderate (~4 fold max.) but quite dramatic in the case of *AaCBP* (~28 fold max.). In both cases, however, the upregulation in acetone control groups was minor compared to methoprene-treated groups, indicating methoprene had a significant influence in transcription activity of these genes.

Do the mRNA profiles of these genes in adult females correlate well with the endogenous JH titers?

If transcription of a gene is regulated by JH during post-emergence development, then the gene would likely have an mRNA profile which correlates well with JH titers; it would increase significantly shortly after emergence and, like JH, peak at 2 – 3 days post-emergence. In the two experiments described below, the question of whether the mRNA profiles of these genes in adult females correlate well with JH titers is addressed by generating a detailed mRNA profile in specific tissues and comparing them to published JH titers. Before this could be done, it was necessary to identify the tissues in which the genes are expressed.

1. In what tissues are the genes primarily expressed? The purpose of the first experiment was to identify the tissues in which each of the genes are expressed and upregulated during the first 5 days post-emergence. Samples of 5 tissue types (head, thorax, midgut, fat body, ovaries) from newly emerged females were collected at 2 hr, 2 day, and 5 day post-emergence. qRT-PCR was used to measure mRNA levels (Figures 6A – 9A). Results are discussed below.

2. Are mRNA expression profiles of the identified genes consistent with JH-regulation in adult females in specific tissues? The second experiment was designed to give a more detailed mRNA expression profile for each gene showing strong expression in midgut and/or fat body tissue, in order to compare it to JH titers. Samples of fat body and midgut tissue were collected at 2 hr, 12 hr, 1 day, 2 day, 3 day, 5 day, and 8 day post-emergence. qRT-PCR was used to measure mRNA levels. Figures 6B – 9B show mRNA expression for each gene along with published JH titers for the corresponding time points [34]. Results are discussed below.

***AaET*:** (Figure 6) When measured in all five tissues, *AaET* showed significant expression in midgut tissue only. The upregulation was quite dramatic, increasing 7,500 fold between 2 hr and 2 days post-emergence. While recorded mRNA levels in fat body and thorax tissue increased 21 fold and 82 fold respectively, the peak levels were only 0.02% and 0.54% of that recorded for peak midgut expression, and could be due to midgut contamination of fat body and thorax tissue during dissection. A detailed profile of *AaET* in the midgut shows a steep increase in mRNA over the first 2 days post-emergence, and then a leveling, with a very slight decrease over the next 6 days, which correlates well with JH titers.

AaNaSS: (Figure 7) mRNA expression of *AaNaSS* increased in all tissues, peaked at 2 days post-emergence, but with relatively small increases (2-3 fold) in tissues other than thorax (9.1 fold) and fat body (8.4 fold). A detailed profile of *AaNaSS* mRNA expression in fat body tissue confirmed a peak at 2 days post-emergence, and dropped to roughly one third of peak value for the remainder of the 8 days measured. While the drop in mRNA expression is more dramatic than that of JH after 2 days, the peak expression at 2 days post-emergence does correlate weel with JH titers.

AaKr-h1: (Figure 8) *AaKr-h1* showed upregualtion in all 5 tissue types, and in all cases peaked at 2 days. Peak expression for each tissue was between 2.8 and 4.9 fold increase, except for the midgut, which peaked with a 21 fold increase. Detailed expression profiles confirmed peak expression at 2 days post-emergence in both midgut and fat body tissues, which correlate well with JH titers. Overall midgut expression levels correlate well with JH titers throughout the entire 8 days of the experiment in both fat body and midgut tissue, though the amount of change in fat body tissue is relatively modest compared to midgut expression.

***AaCBP*:** (Figure 9) Significant expression and upreguation of *AaCBP* mRNA was recorded in midgut tissue only. Unlike expression of the other three genes, which showed peak expression at 2 days post-emergence, *AaCBP* expression increased steadily throughout the period measured to a maximum 43-fold increase at 8 days post-emergence, showing little correlation to the pattern of JH tites.

Part II: Further characterization of *AaKr-h1*

One of the genes identified in the first objective is a putative homolog of *Kr-h1* found in *D. melenogaster*, *T. castaneum*, and *A. mellifera* and described earlier. This led to the second objective, which was to more thoroughly characterize *AaKr-h1*. Three questions were addressed in the second part of this reseach:

1. Is *AaKr-h1* indeed a homolog of *Kr-h1* described in *D. melenogaster*, *T. castaneum*, and *A. mellifera*?
2. Does *AaKr-h1* demonstrate a dose-dependant response to JHIII and several JH mimics?*AaKr-h1*
3. What is the function of *AaKr-h1* in newly-emerged females in response to JH?

and RNAi was attempted in order to study the gene's function, particularly its relationship, if any, to *met* and *br* in *Ae. aegypti*.

Is *AaKr-h1* a homolog of *Kr-h1* discovered in D*. melenogaster, T. castaneum*, and *A. mellifera*?

There is strong evidence that *AaKr-h1* is indeed a homolog of *Kr-h1* described in other species. A BLAST-P query of using the predicted amino acid sequence of *AaKr-h1* (Vectorbase ID AAEL002390) produced single gene matches in species throughout holometabolous insect orders, all with a higher similarity to *AaKr-h1* than any other hits within the *Ae. aegypti* genome. BLAST-P results indicate a high degree of homology to *Kr-h1* identified in *D. melanogaster* (75% identity), *T. castaneum* (66% identity) and *A. mellifera* (67% identity). Figure 10 shows alignment data for these proteins. A region that includes 7 zinc fingers (ZF2 – ZF8) is highly conserved, including the 5-residue length linkers between the zinc fingers. Amino acid composition of ZF1 is less highly conserved among all four species, both in composition and distance from ZF2. However, ZF1 it is highly conserved between *AaKr-h1* and *DmKr-h1*. Similar to *DmKr-h1*, *Aakr-h1* contains flanking glutamine- and serine-rich regions.

Does *AaKr-h1* specifically respond to juvenile hormone in the abdominal ligation assays?

If *AaKr-h1* is indeed regulated by JH in abdominal ligation assays, then it should respond in a dose dependent manor. In addition, it should respond to JHIII as well as several JH mimics, indicting that it is the hormone's activity responsible for the response. *AaKr-h1* response to JHIII, methoprene, pyriproxifen, and farnesol (figure 1) was characterized in abdominal ligation assays (Figure 11). Methoprene and pyriproxifen are both JH mimics. Farnesol, a JH intermediate molecule with a similar structure to JHIII but with no known JHA activity in mosquitoes, was included as a negative control. Abdominal ligation assays were performed on newly emerged females, and triplicate sample groups of 6 abdomens were treated with either 0.5 µl acetone, or 0.5 µl acetone with one of the four described chemicals, doses ranging from 5 pg to 500 ng in tenfold increments. All samples were incubated for 12 hrs. Figure 11 indicates the dosages tested for each hormone, and compares the mean *AaKr-h1* mRNA level of triplicate samples for each hormone/dose.

AaKr-h1 showed a dose-dependent response to all three JHAs. Methoprene was the most sensitive with a 5-fold response to 0.5 ng/abdomen, and JHIII and pyriproxifen showing roughly equal sensitivity with roughly 6-fold and 4-fold responses, to 50 ng/abdomen. In the methoprene-treated samples, *AaKr-h1* mRNA expression increased notably between 0.5 and 500 pg/abdomen, with the largest increase response between 50 and 500 pg/abdomen. While mean mRNA expression continued to increase as methoprene dosage increased from 500 pg/abdomen to 500 ng/abdomen, the rate of increase declined. It is possible that as the methoprene dose was increased past 500 pg/abdomen, toxic effects became more prevalent. *AaKr-h1* response to pyriproxifen increased primarily between 500 pg and 50 ng per abdomen, and response to JHIII between 50 ng and 500 ng per abdomen. Maximum mRNA expression was greater in JHIII samples than in pyriproxifen samples, but it is unclear if this trend would continue with dosages greater than those tested. Farnesol produced no *AaKr-h1* response at any dosage relative to the acetone control group.

What is the function of *AaKr-h1* in newly-emerged females in response to JH?

RNAi was attempted via dsRNA injection in newly emerged females (figure 12). The primary goal in knocking down *AaKr-h1* was to determine its relationship to *met* and *br*. If a *AaKr-h1* knockdown could be achieved, then a drop in mRNA levels for *met* and/or *br* would indicate that they were likely downstream of *AaKr-h1* in the JH regulatory pathway. In a similar fashion, its relationship to the regulation of the other identified JH-regulation genes (*AaET*, *AaNaSS*, *AaCBP*) would be assessed. Additionally, obvious biological impacts, such as changes in post-emergence development or egg laying, would be noted for future studies.

dsRNA used for *AaKr-h1* knockdown (*AaKr-h1*dsRNA) targeted a 603 bp region of *AaKr-h1* mRNA (Figure 3). *Mal*dsRNA, targeting an 808 bp fragment of a non-functional portion of the bacterial *malE* gene was used as a negative control. Female *Ae. aegypti* were injected 2-4 hours after adult emergence with either *AaKr-h1*dsRNA (0.6 µg in 200 nl 0.1X PBS) or *Mal* dsRNA (0.6 µg in 200 nl 0.1X PBS). A third control group were not injected. Fat body and midgut tissue were collected at 3, 5, and 7 days, post-emergence. For each time point, 3-6 *AaKr-h1*dsRNA treated, 2-3 *Mal* dsRNA

treated, and 2-3 uninjected samples were collected. Each sample consisted of fat body or midgut tissue from 4 adult female mosquitoes. qRT-PCR was used to measure *AaKr-h1* mRNA levels. Figure 12 shows data from three experiments. No significant difference in *AaKr-h1* mRNA level was measured between *AaKr-h1*dsRNA samples and *Mal* dsRNA and uninjected control samples at any time point for midgut or fat body samples. The data for fat body tissue collected at 7 days post-emergence may indicate a modest (43%) *AaKr-h1* knockdown relative to the uninjected control, but only (22%) relative to the *Mal* dsRNA control.

These results did not indicate an effective *AaKr-h1* knockdown useful for a study of *AaKr-h1* function or its effect on transcription of other genes. While it is possible that some knockdown may have been achieved at 7 days in fat body tissue, *AaKr-h1* peak mRNA expression occurs at 2 days, and by 3 or 4 days post-emergence, females are competent to mate as well as take a blood meal, indicating that critical post-emergent developments have already been completed.

Conclusion and Discussion

JH target genes

The experiments described here have identified three genes (*AaET*, *AaNaSS*, *AaKr-h1*) which are likely regulated by JH in newly emerged female *Ae. aegypti*. These genes exhibit JH-dependent upregulation in abdominal ligation assays on newly emerged females, and mRNA expression patterns in adult females correlate well with published JHIII titers. Whether JH regulates *AaCBP* is less clear. *AaCBP* transcription is strongly influenced by a JHA in abdominal ligation assays, indicating that there is potentially some pathway available in newly emerged females which allows JH to regulate *AaCBP*.. Further, *AaCBP* transcription does begin to increase at the same time JH titers begin to rise. However, *AaCBP* transcription in midgut tissue does not reveal a pattern that correlates well with JH titers as expression steadily increases over the first 8 days rather than peaking at 2-3 days post-emergence. It is possible that transcription *AaCBP* is initially activated shortly after emergence by a relatively small increase in JH titers, and that further elevated JH titers do not further increase the rate of *AaCBP* transcription.

The results presented here for *AaET* are consistent with published studies showing *AaET* to be a female specific protease regulated by JH and expressed primarily in the midgut [40, 43, 52]. The upregulation in *AaET* transcription is a clear example of a JH-regulated preparation needed in order for the female to become competent for bloodfeeding. Interestingly, while *AaET* mRNA transcripts are abundant after 2 days post-emergence, translation of *AaET* does not begin until after a blood meal has been taken [44]. It is possible that the purpose of this “buildup” of stored mRNA is to shorten the response time needed to produce the first trypsin proteins when blood is ingested. The mechanism of this posttranscriptional regulation is at present unknown.

The role of *AaNaSS* in post-emergence development is unclear. *AaNaSS* transcription appears to be regulated by JH, and significant increases in mRNA levels are observed in all tissues sampled. However, its putative function as a sodium-solute

symporter, and its ubiquitous expression throughout the five samples tissues give few cues as to the developmental processes with which it may be associated.

Transcription of *AaCBP,* like that of *AaET,* may be in preparation for the female's first blood meal. The 4 predicted chitin-binding domains [46, 47], along with the observed localization of *AaCBP* transcripts to midgut tissue[53], are characteristic of proteins associated with peritrophic matrix (PM) in many insect species. In *Ae. aegypti*, the PM is formed in response to bloodfeeding, and encapsulates the blood meal, forming an extracellular membrane barrier between the ingested blood and the epithelial cells in the midgut [54, 55]. PM components are believed to be secreted by epithelial cells throughout the midgut. However, if *AaCBP* is in fact a component of the PM, it is unclear why *AaCBP* transcription is upregulated shortly after emergence, and continues to increase throughout the first 8 days post-emergence when no bloodmeal is taken. It is possible that *AaCBP* transcripts or proteins are sequestered until bloodfeeding in order to speed the formation of the PM, but in that case, a peak in transcripts would be expected no later than time the female was competent to bloodfeed (~3 days post-emergence).

AaKr-h1 role in JH regulation

Previous studies have identified *Kr-h1* as a component of JH regulation during metamorphosis in *D. melanogaster* [26] and during metamorphosis and larval development in *T. castaneum* [22, 27], when JH is believed to exert its effects through the suppression of 20E-activated pathways. JH-regulated events in female *Ae. aegypti* during the first few days post-emergence, when 20E titers are low, provide evidence that JH can exert its effects independent of 20E-regulated pathways. If *AaKr-h1* is shown to be a component of JH regulation during this period of development, then this may suggest a conserved role for *Kr-h1* in multiple JH-regulated pathways, as it would be involved in both 20E-dependent and 20E-independednt pathways.

In order to establish *AaKr-h1* as a component of JH regulation in newly emerged females, it was necessary to determine if *AaKr-h1* is regulated by JH, and to identify downstream effects of such regulation. The experiments described here provide evidence that *AaKr-h1* is upregulated by JH during post-emergence development.

However, without a successful knockdown of *AaKr-h1*, it was not possible to assess the function of *AaKr-h1*.

One possible target of *AaKr-h1* regulation is *br*. It has been shown that *Kr-h1* can upregulate *br* during metamorphosis in *D. melanogaster* [26] and *T. castaneum* [27]. In *Ae. aegypti,* two of the four *Aabr* isoforms are present in the midgut and fat body and all four are expressed in the ovaries during post emergent development [14]

In a similar RNAi experiment using dsRNA targeting *Aamet* a knockdown of *Aamet* did result in a 70% decrease in *AaKr-h1* mRNA expression at 3 days, post-emergence (Dr. Xing Zhang, Zhu lab, unpublished data). While these results are not conclusive, they do suggest that *Aamet* acts upstream of *AaKr*-h1, and is required for JH upregualtion of *AaKr-h1*. In this respect, the observed JH regulation of *Kr-h1* appears to follow a similar path to that described to take place during metamorphosis and larval development in *T. castaneum* [22, 27]

Future work

If there is to be further investigation of *Kr-h1* function in newly emerged female *Ae. aegypti*, the focus should be on determining its function (i.e., downstream effects). It would seem reasonable to continue to pursue RNAi further. The successful knockdown of *Aamet* using a nearly identical protocol to the one described in this study indicates that RNAi is, in theory, feasible during this period of development, and therefore a knockdown of *AaKr-h1* may be possible. A modification in the dsRNA target region of *AaKr-h1* may yield better results.

Figures / Tables

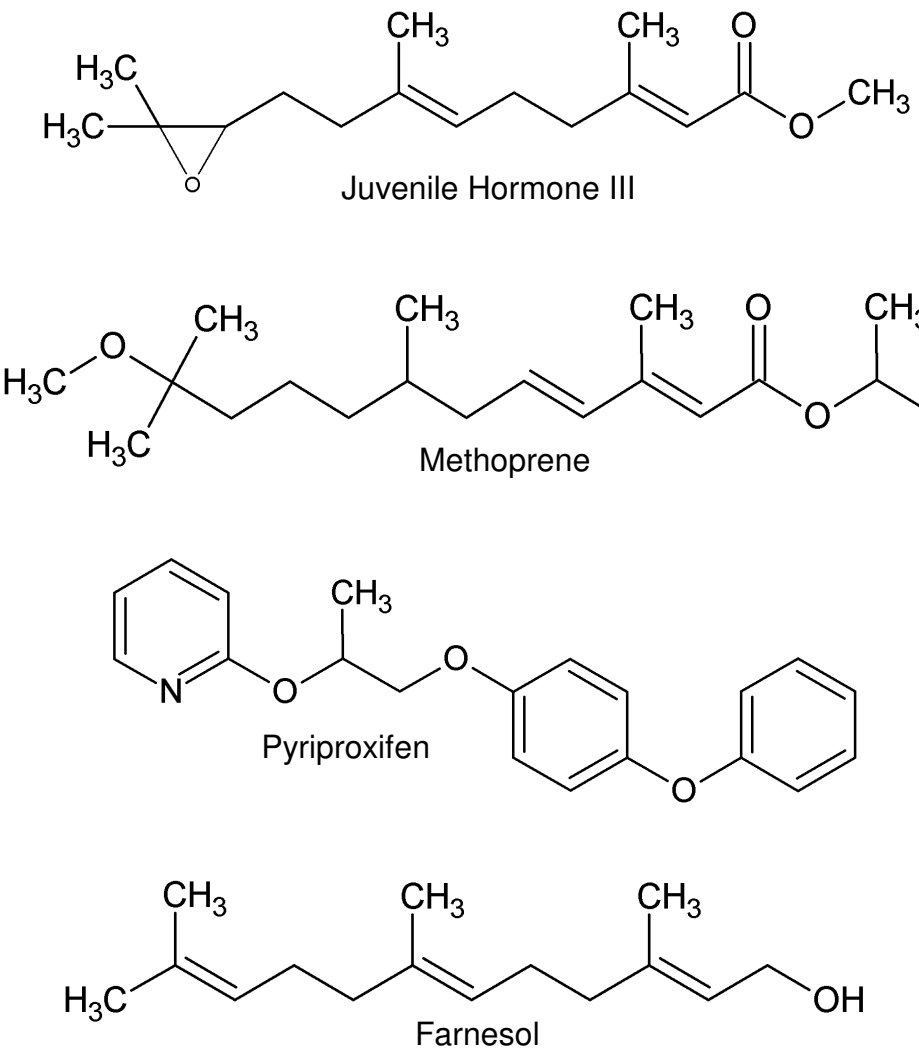

Figure 1. Structures of several JH analogs. JHIII is shown, followed by methoprene and pyriproxifen, both JH mimics. Also shown is farnesol, an intermediate molecule during JH synthesis with no known JH activity in *Ae. aegypti* and used as a negative control in experiments discussed in this report.

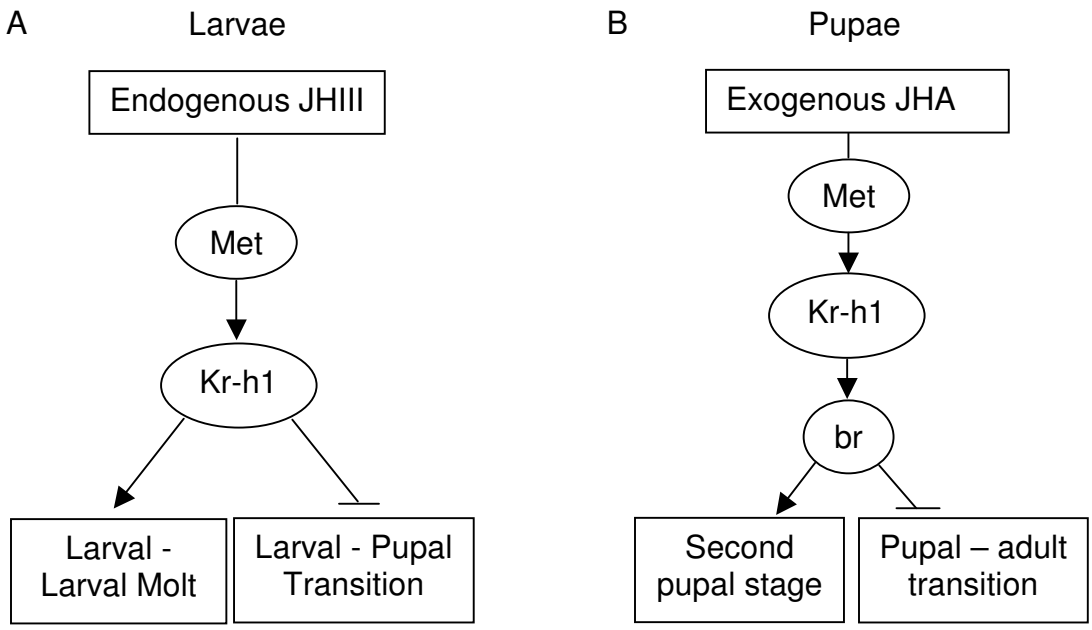

Figure 2. Models of JH signaling in *T. castaneum.* A) At the end of a larval stage, JHIII exerts a status quo effect through a met mediated upregulation of Kr-h1. A Knockdown of Met or Kr-h1 prior to larval-larval molts results in a precocious larval-pupal transition. B) Pupal-adult transition occurs after downregulation of br and in the absence of JH. Exogenous application of a JHA results in a met mediated upregulation of Kr-h1, which in turn upregulates br, resulting in a second pupal stage. Figure adapted from Minakuchi et al., 2009 [27].

			4hr MT/AC			12hr MT/AC		
Gene	**Genebank ID**	**Description**	**array**	**p-value**	**rtPCR**	**array**	**p-value**	**rtPCR**
AaKr-h1	AAEL002390	zinc finger protein (*Kr-h1*)	**3.08**	9.36E-03	**4.64**	**3.41**	6.32E-03	**4.56**
AaNaSS	AAEL002576	sodium/solute symporter	**2.11**	2.22E-02	**5.41**	**na**	na	**6.42**
AaCBP	AAEL002619	conserved hypothetical protein	**10.03**	1.49E-02	**15.49**	**7.65**	2.25E-02	**9.44**
AaHairy	AAEL005480	"hairy" protein (txn regulator)	**2.45**	2.46E-04	**2.27**	**2.85**	9.62E-04	**3.55**
AaET	AAEL007818	trypsin (*Early Trypsin*)	**4.16**	2.89E-04	**6.02**	**3.32**	1.73E-02	**158.39**

Table 1. Five potential JH response genes. Abdominal ligations were performed on newly emerged adult female *Ae. aegypti*, and treated with either 0.5 µg methoprene in 0.5 µl acetone (MT group), 0.5 µl or acetone alone (AC group), and incubated for either 4 or 12 hours. Microarray results from cDNA from these four groups (4 hr AC, 4 hr MT, 12 hr AC, 12 hr MT) normalized to *AaS7* ribosomal protein cDNA, were used to calculate a genome-wide profile of differential expression between the methoprene-treated and acetone control samples 4 hr and 12 hr post-ligation. A group of 13 genes showing an increase in expression in the methoprene-treated group relative to the acetone control group at either 4 hr or 12hr post-ligation were selected as potential JH response genes. qRT-PCR was used to validate the microarray results for these 13 genes, using *AaS7* for normalization. Table 1 shows 5 genes for which differential expression in the abdominal ligation assay samples was confirmed. For each gene, differential expression is expressed as MT / AC in both the microarray (array) and qRT-PCR results. The p-value shown is for the calculated fold change between the methoprene-treated and acetone control samples in the microarray results. Microarray results provided by Dr. Jinsong Zhu.

ATGGTGTACTATACGGGATTACCGCTGCTGATGCAACAAGCAGAGAACAACACGCTTACC
CAGCTATGCTTCAACGCAACGAACGACCATTCCCAGTCGCAGCCCCAACAGCAACAGCAA
TCGCTTGACCAATCCCTTCACCATCAGCAACAGCAACAGCATAGGTTCCAGCAGCAGGTC
GTCAATCTGAGCAACACCCAAGGCAACATGCAGTCACAGATGGGCCAGCAGCATCCCAGC
GCACCCTACACGAACTTCCACCAGACCAATAATGGTGGACTCGTGCCGACGACCATACCG
CAGACCAGTCCTCCCCCAGCCGGACCCGCCAAGTTCCGCTGCGAACAGTGCAACATCAAC
TTCGGTAGCAAAAGTGCCCACACCAGTCACATGAAATCTCACGCCAAACAGCAACAACAG
CAGCAAATGGTAGCGGAGAGCAAGGGTGGAGTCAGTGCCAAGGGATTGCTCAACGGACCG
GCCACTCCGGCCGACCCGTACCAGTGCGATGTGTGCAAGAAAACTTTTGCCGTTCCGGCC
CGGCTTGTGCGGCACTACCGGACACATACCGGTGAACGCCCGTTCGAGTGCGAGTTCTGC
CATAAGATGTTTAGCGTCAAGGAGAACCTGCAGGTTCACCGGCGGATCCACACCAAGGAA
CGGCCGTACAAGTGCGACATCTGCGAGCGAGCGTTTGAACACAGCGGAAAACTGCACAGG
CACATGCGGATCCATACGGGTGAACGACCACACAAGTGCAACGTGTGCGGAAAGACGTTC
ATCCAGTCGGGTCAACTAGTCATCCACATGCGGACCCATACCGGTGAAAAACCGTACAAA
TGTCCGGTAGAGGGTTGCGGAAAGGGCTTCACGTGCTCGAAACAGTTGAAAGTGCACTCG
CGGACTCACACCGGTGAGAAGCCGTACCATTGTGATATTTGCTTCCGGGACTTTGGCTAT
AACCATGTGCTGAAGTTGCACAGGGTACAGCACTACGGGTCGAAGTGCTACAAATGTACC
ATATGCGATGAAACGTTCAAGAGTAAGAAGGAAATGGAGGCCCACATCAAGGGCCATGCC
AACGAGATTCCGGATGAGGAAGAGACTGAAACCAAGCCCTTGGTGGCAGTGCCATCGAGT
AGCTCTAGTAGTCGAAGCAGTTTGGTAGAGTACTCGTCAAGCACGAGTAGTCTTGCAGCG
GCGGACAGTTTGTCCACGATGGAAGATGCCATGGAACTTGAAGAAGATCTCCACACTCCC
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AGCGGTAGCATAACAAACGAGAACAATGTCCATTATTACTCGCAATTTGAGCAGTCGGTT
TTCCGAAGCTACGGAGTCCAATCCGGAGTGAATCCAGCACTGTTGGCAGCGGCGTCAATC
GCTGCAGCTGCTACCGCTTCTTCAGCCGAACAAAATGAAAACATCCAAAGCAATAGGACA
CCATCACCGCCGCCATTGGTAGTGTTCTCGCAACAACAGCAACATCCGCAGCATCAGCAG
CTAATGACCTCAAATGGTGCAGAT**CGGTTAACACCACCGTCTTC**ACAGCAGCACCCAGCG
TCAATGGATCTGATGATTCACCATCTACCGACGCAACAACGCTTCAAGCGAGAAGAGTCG
CCGGTCAATGCTGATCCTTTCCTATACAAACCGTTAACAATCATGAAACATCACGGCTAC
TTTGCACCGGCTTCACAGGTTCCCGAATTCAGGTCATCCAGCGATCTCGTGCGACAAGTG
GAAGCGGCCATTGCCGGAACGGAAAATTTGCTGACACCCCCACGGTCCTCCCCGGAATCA
CCGGAACGGTCCTCGTCGCCTGAGTCCGATTCAGTATTAATGGCCGACCGGGACAACAAC
ACCTTACCGCCGAGAAAGCGAAAGCTTTACTTCAAGAACGAATCGCCGCAAGATCTGCAC
CATCACCTTCACCACCAGCATCTAACGGCGTCGTCAATGGACAACAGCTCAACGCCGCCG
TCGCCACCGTCTTCCTCGGCGGTCATGCGAATGAGCTCCGTCATTCAGTACGCCAAGGCA
TCCTAAGCGGACAATGCGGTGATATTTTTCTTCGAGGCGGCCATATTCGATGTAGAGTGC
GTATAGTTACAAGGTTAGCAAGCGGCTTAGTTGATAGAGGAAAGCAGACTTATTAGTGGA
TAGCTAAATTAATGGTAACGTTTTTAGAAGGTAATATTTATCTACAAGCGTAGAATGTAG
CGAGTCAGATTTAAGGAAAATCATACATACAAACACATACACACACATACATACATACAC
ACTAAATGCAAGAAGACTGTCTCAGGTTAATCAGTCATTACATTTGATAAGTTTATCGCT
TCTTCTGGAATGTGGATTGTTGACAGATGGAGAAGGTTAACATTGAAGTTGCGGAGAGCT
TGGCAATACTGCATATGGCGTCTTATCCGAATACAATGTGAGTGAACGAAAGCAAAGGGT
GTTTTGGACAAAGGAAATATTAAGACTGTTGAATTGTCAAAAAATTCTAGTAACCAAGAA
ATATTTTAAGGTGATTAGGTAACGACAAGGCAGAATCTAAATAAAGTTGGATCAAAATCG
GAATAGGGCGATATATTTTACTTACAAATCACATTTTTCTGGATAGGTATTAATTGCGCT
GGAAATCAAGAAGCAACTGAGAATAATCGAAATTTGAAGGCCATTTGAAAAGACTAGCTT
TTTTTATTGTAGCAAGAAACAAAACATCATTGAGCAAG

Figure 3. Putative transcript of *AaKr-h1*. Transcript length = 2798 bp. Exons are indicated in blue and black. Target region of RNAi using *Kr-h1* dsRNA is indicated in bold. Primer sites for template DNA are underlined. Yellow highlighting indicates UTR.

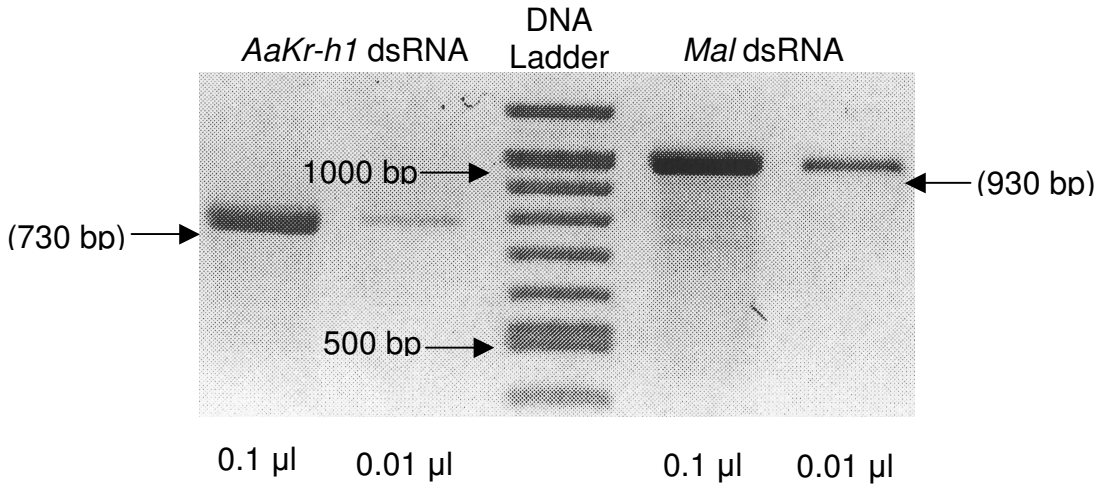


Figure 4. *Aakr-h1* and *Mal* dsRNA. Expected product sizes are indicated in parentheses. dsRNA bands appear slightly larger than expected, likely due to dsRNA migrating slower than DNA in the ladder. Amount of purified product per lane from dsRNA synthesis is indicated. Center lane is 1.0 µg 2-log DNA ladder (New England Biolabs).

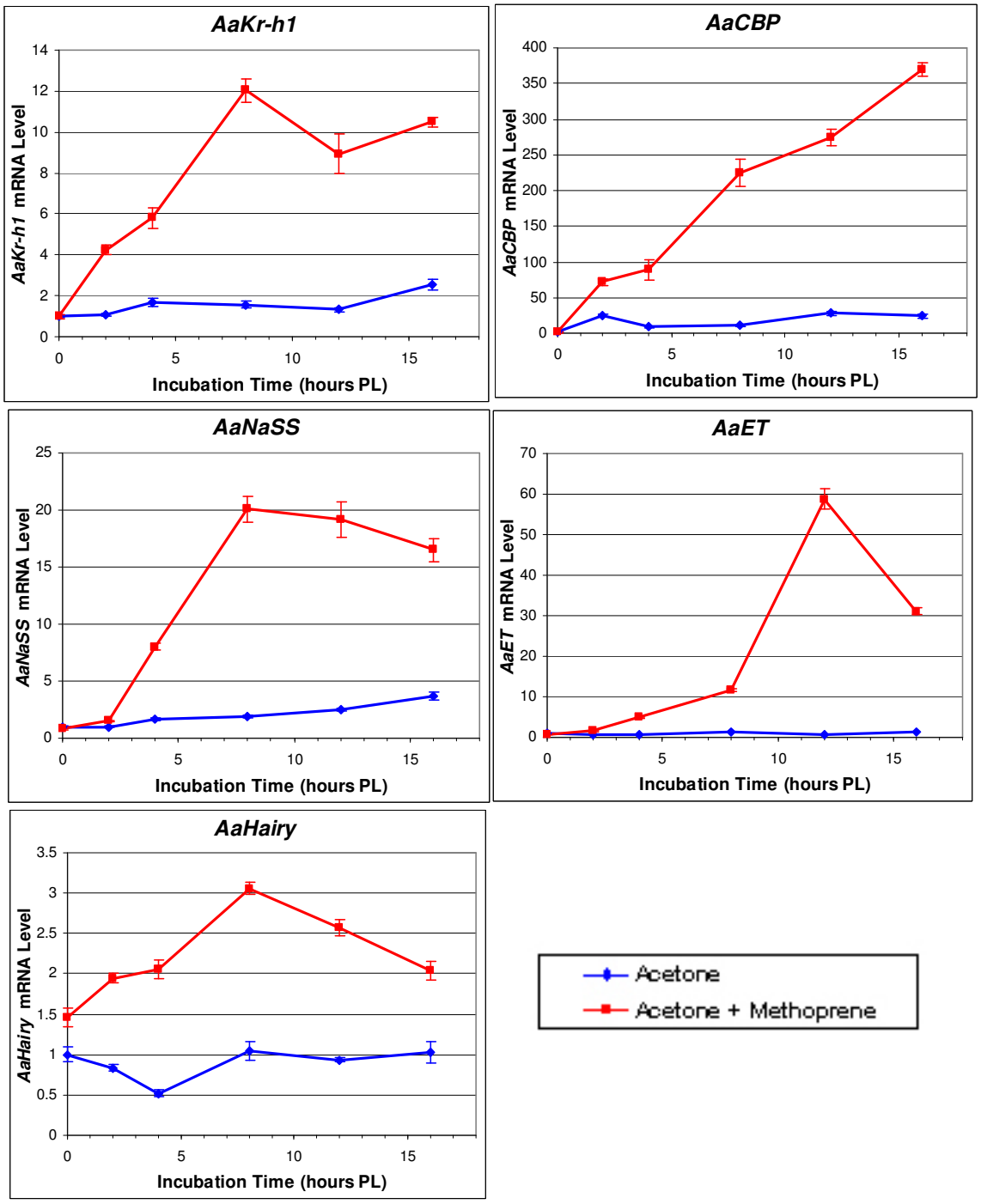
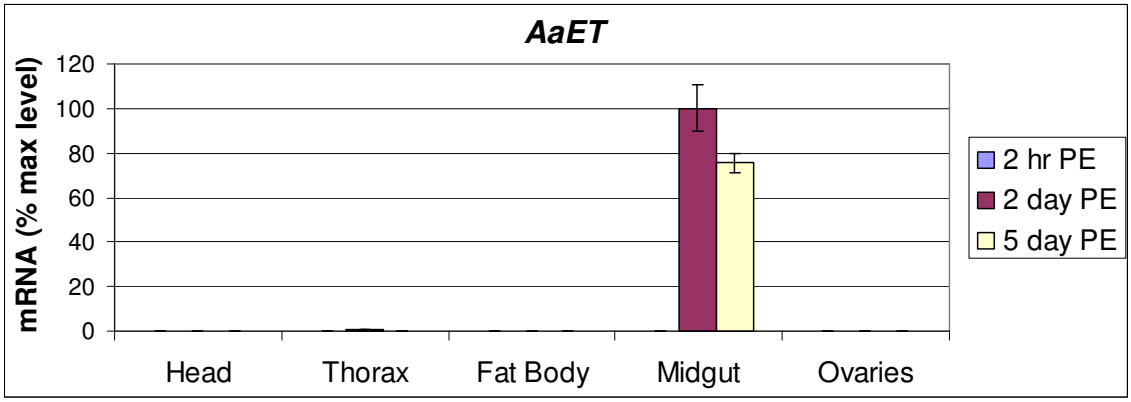


Figure 5. JHA-dependent upregulation in abdominal ligation assays. Abdominal ligations were performed on newly emerged female adult *Ae. aegypti*. Ligated abdomens were treated with either 0.5 µg methoprene in 0.5 µl acetone, or 0.5 µl acetone alone, and incubated for times indicated. Each sample group consisted of the

pooled RNA of 7-8 ligated abdomens. mRNA expression levels for each sample were measured using qRT-PCR, and normalized to *AaS7* ribosomal protein mRNA levels. Normalized mRNA levels are shown above as relative to the 0 hr acetone control group (0 hr acetone = 1). Error bars indicate standard error of triplicate measurements of each sample.

A)



B)

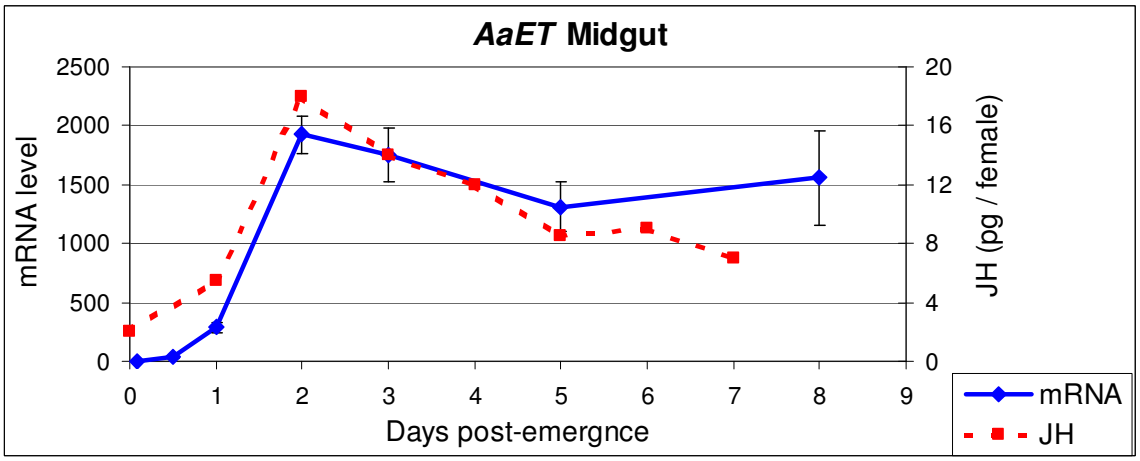
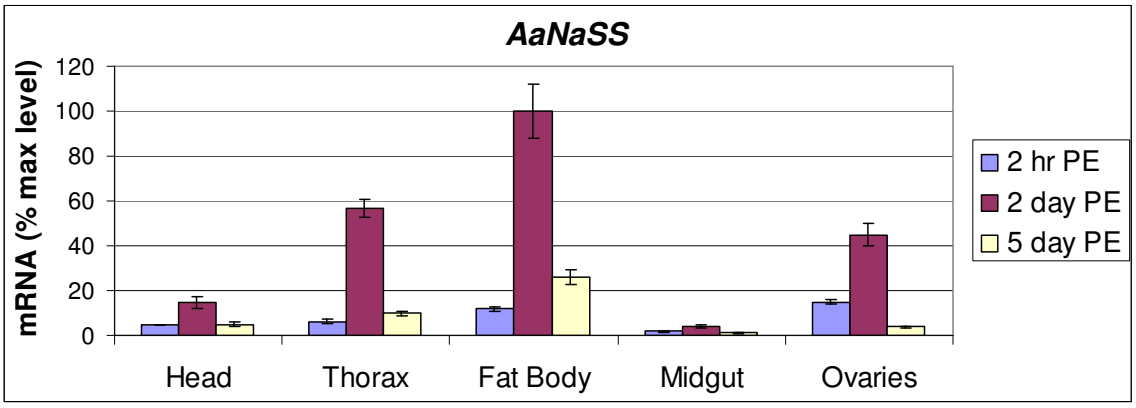


Figure 6. *AaET* mRNA expression in adult female *Ae. aegypti*. A) Five tissue types, collectively representing the whole adult female *Ae. aegypti* were obtained from adult female *Ae. aegypti* at 2 hours, 2 days, and 5 days post-emergence. For each collection time, 20 head, 8-10 thorax, 20 fat body, 28-33 midgut, 28-33 ovaries were each pooled for RNA extraction. *AaET* mRNA expression was quantified using qRT-PCR and normalized to *AaS7* ribosomal protein mRNA levels. Results above are expressed as percent of maximum levels recorded. B) Midgut *AaET* mRNA expression. Midgut tissue was collected from adult female *Ae. aegypti* at 2 , 12 hr, 1, 2, 3 , 5, 8 days post-emergence. For each time point, three samples, each consisting of midgut tissue from 6 mosquitoes, were collected for RNA extraction and mRNA quantification of indicated

genes using qRT-PCR. Results were normalized to *AaS7* ribosomal protein mRNA levels. The results shown average the three samples for each time point and standard error is indicated. Also shown for comparison are hemolymph JH titers (modified from Shapiro et al. [34])

A)



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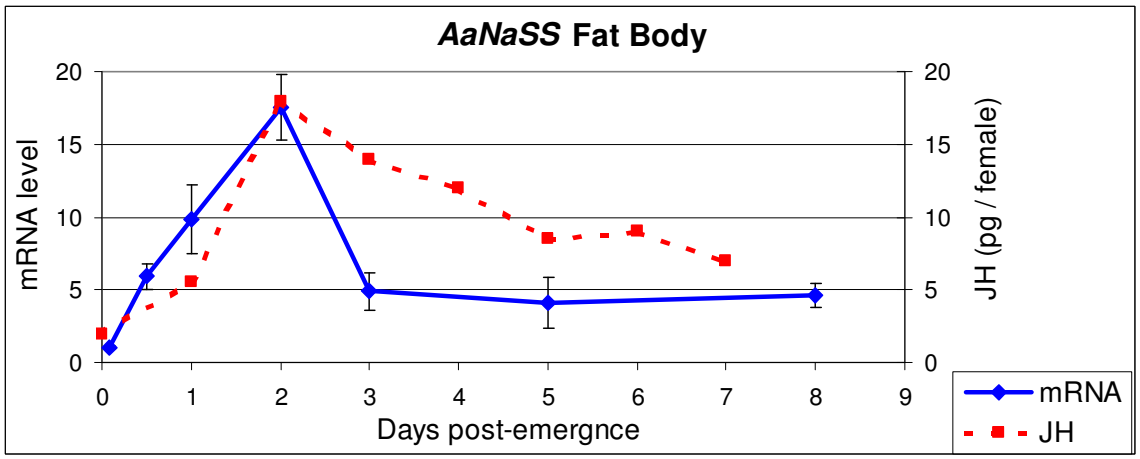
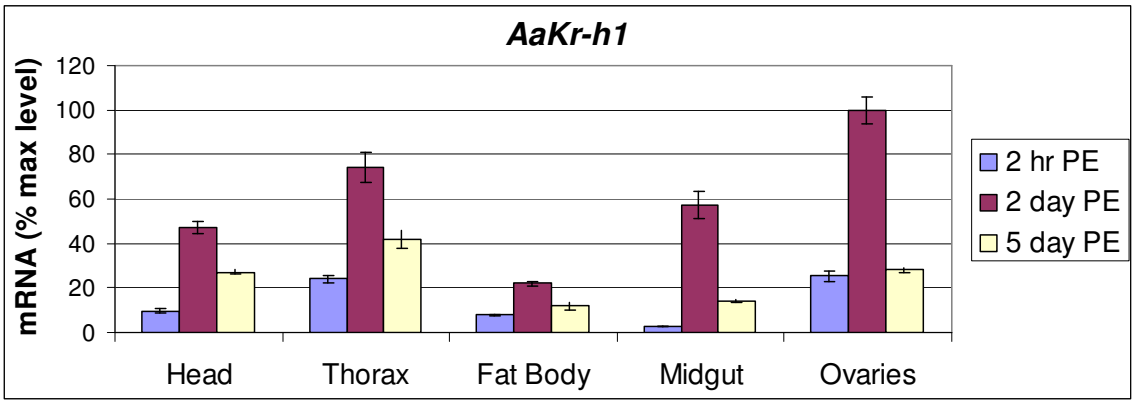


Figure 7. *AaNaSS* mRNA expression in adult female *Ae. aegypti*. A) Five tissue types, collectively representing the whole adult female *Ae. aegypti* were obtained from adult female *Ae. aegypti* at 2 hours, 2 days, and 5 days post-emergence. For each collection time, 20 head, 8-10 thorax, 20 fat body, 28-33 midgut, 28-33 ovaries were each pooled for RNA extraction. *AaNaSS* mRNA expression was quantified using qRT-PCR and normalized to *AaS7* ribosomal protein mRNA levels. Results above are expressed as percent of maximum levels recorded. B) Fat body *AaNaSS* mRNA expression. Fat body tissue was collected from adult female *Ae. aegypti* at 2 , 12 hr, 1, 2, 3 , 5, 8 days post-emergence. For each time point, three samples, each consisting of fat body tissue from 6 mosquitoes, were collected for RNA extraction and mRNA quantification of indicated genes using qRT-PCR. Results were normalized to *AaS7* ribosomal protein

mRNA levels. The results shown average the three samples for each time point and standard error is indicated. Also shown for comparison are hemolymph JH titers (modified from Shapiro et al. [34])

A)



B)

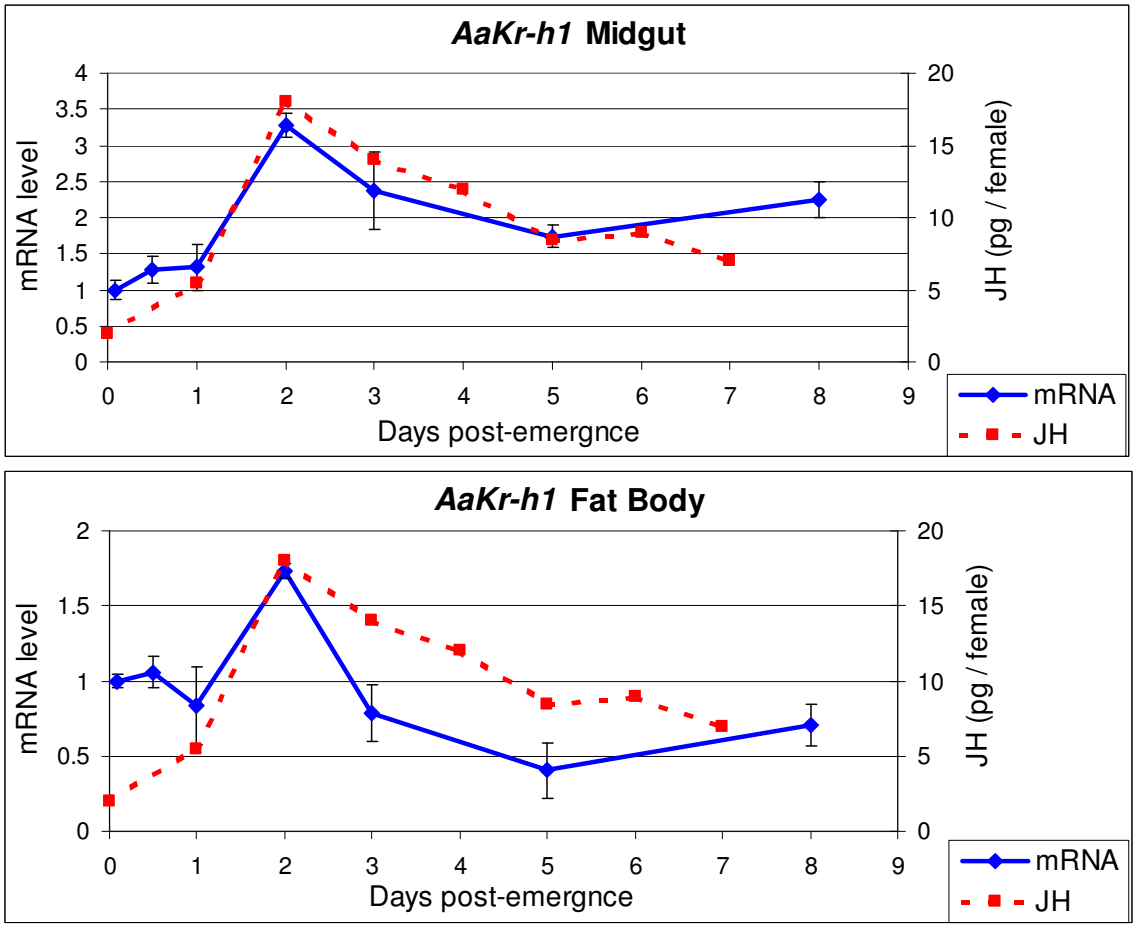
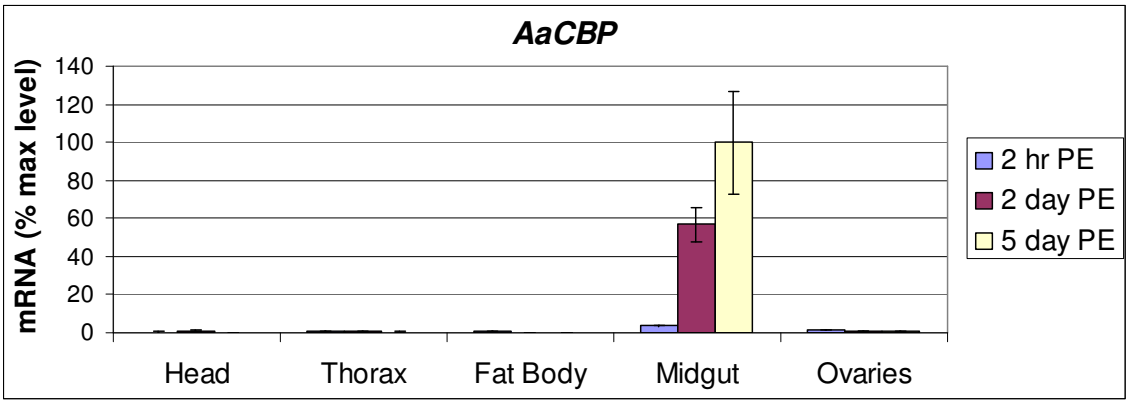


Figure 8. *AaKr-h1* mRNA expression in adult female *Ae. aegypti*. A) Five tissue types, collectively representing the whole adult female *Ae. aegypti* were obtained from adult female *Ae. aegypti* at 2 hours, 2 days, and 5 days post-emergence. For each collection time, 20 head, 8-10 thorax, 20 fat body, 28-33 midgut, 28-33 ovaries were each pooled

for RNA extraction. *AaKr-h1* mRNA expression was quantified using qRT-PCR and normalized to *AaS7* ribosomal protein mRNA levels. Results above are expressed as percent of maximum levels recorded. B) Midgut and fat body *AaKr-h1* mRNA expression. Midgut and fat body tissue was collected from adult female *Ae. aegypti* at 2, 12 hr, 1, 2, 3 , 5, 8 days post-emergence. For each time point, three samples, each consisting of midgut or fat body tissue from 6 mosquitoes, were collected for RNA extraction and mRNA quantification of indicated genes using qRT-PCR. Results were normalized to *AaS7* ribosomal protein mRNA levels. The results shown average the three samples for each time point and standard error is indicated. Also shown for comparison are hemolymph JH titers (modified from Shapiro et al. [34])

A)



B)

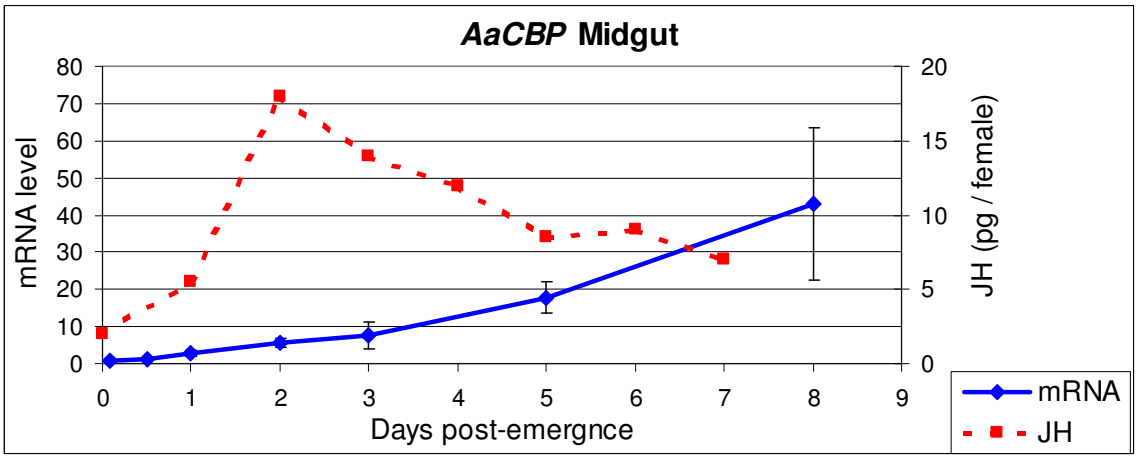
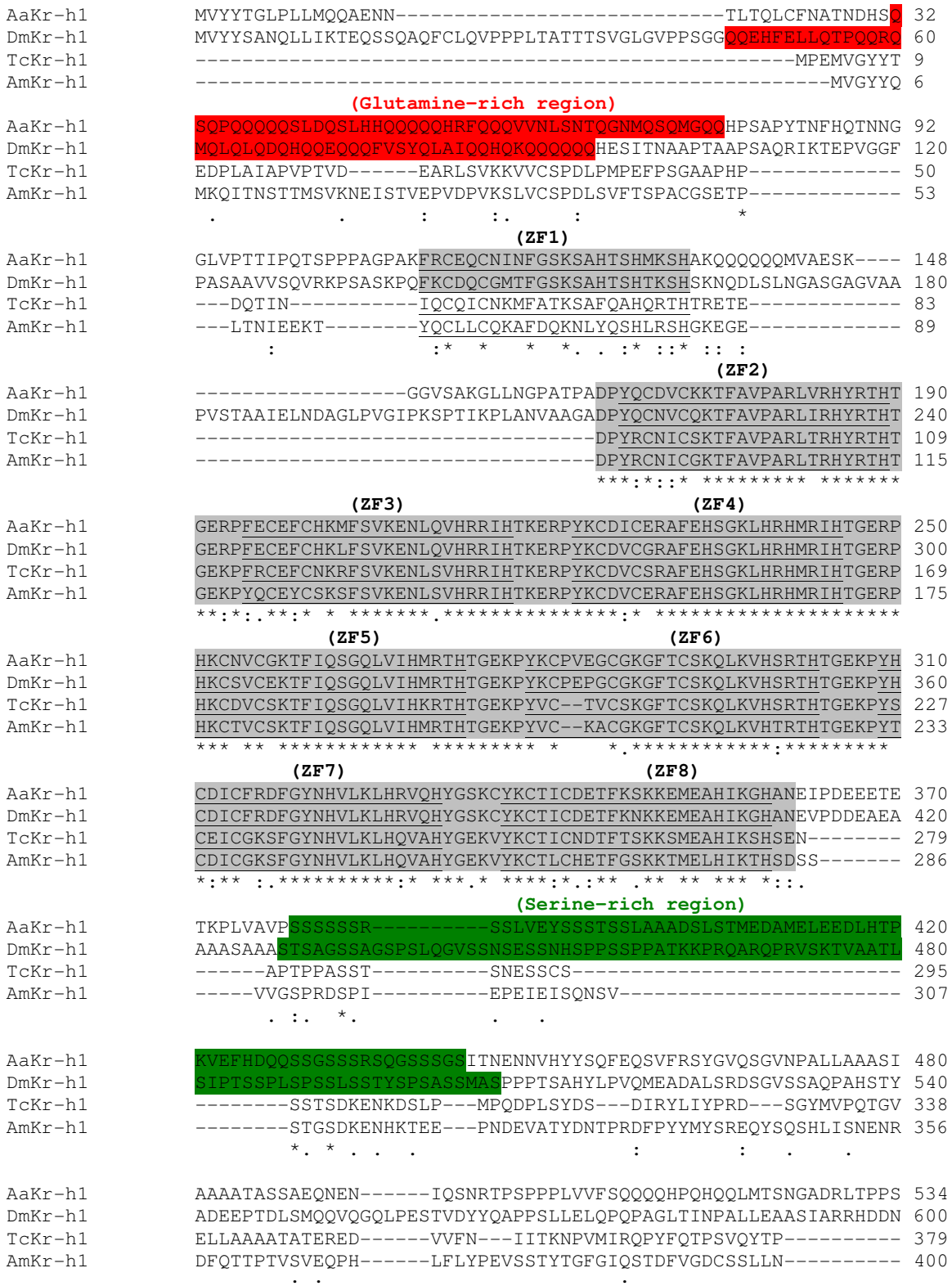


Figure 9. *AaCBP* mRNA expression in adult female *Ae. aegypti*. A) Five tissue types, collectively representing the whole adult female *Ae. aegypti* were obtained from adult female *Ae. aegypti* at 2 hours, 2 days, and 5 days post-emergence. For each collection time, 20 head, 8-10 thorax, 20 fat body, 28-33 midgut, 28-33 ovaries were each pooled for RNA extraction. *AaCBP* mRNA expression was quantified using qRT-PCR and normalized to *AaS7* ribosomal protein mRNA levels. Results above are expressed as percent of maximum levels recorded. B) Midgut *AaCBP* mRNA expression. Midgut tissue was collected from adult female *Ae. aegypti* at 2 , 12 hr, 1, 2, 3 , 5, 8 days post-emergence. For each time point, three samples, each consisting of midgut tissue from 6 mosquitoes, were collected for RNA extraction and mRNA quantification of indicated genes using qRT-PCR. Results were normalized to *AaS7* ribosomal protein mRNA levels. The results shown average the three samples for each time point and standard

error is indicated. Also shown for comparison are hemolymph JH titers (modified from Shapiro et al. [34])


(Glutamine-rich region)
(ZF1)
(ZF2)
(ZF3)
(ZF4)
(ZF5)
(ZF6)
(ZF7)
(ZF8)
(Serine-rich region)

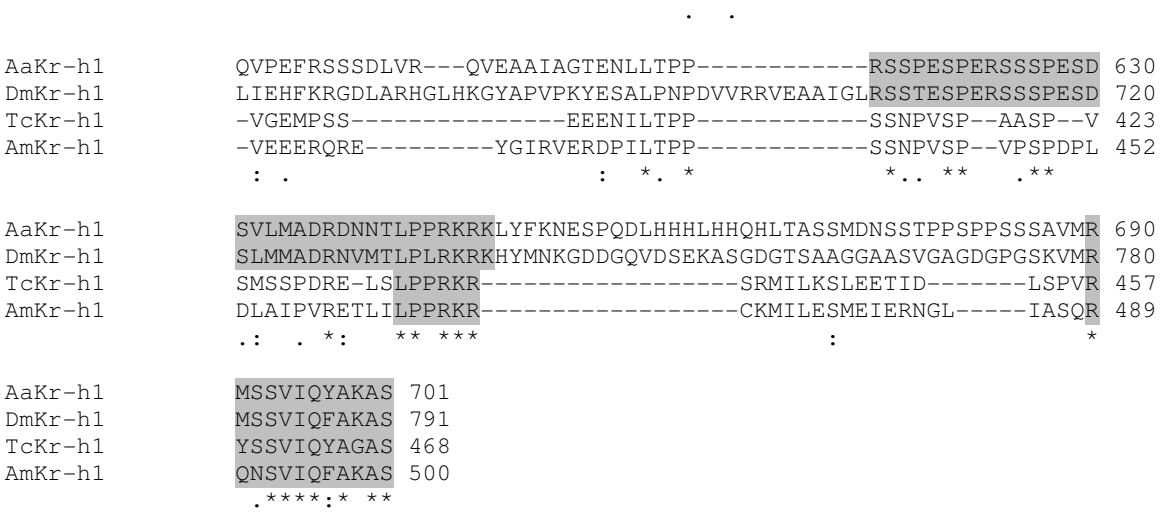


Figure 10. Alignment of predicted amino acid sequences of *Kr-h1* in *Ae. aegypti (AaKr-h1), D. melanogaster, (DmKr-h1), T. castaneum (TcKr-h1),* and *A. mellelifera (AmKr-h1).* Shading highlights highly conserved sequences. Also indicated are glutamine- and serine-rich regions in *AaKr-h1* and *DmKr-h1*. Predicted C_2H_2 zinc fingers ZF1 through ZF8 are underlined and numbered. DmKr-h1 sequence used here is the *α* isoform, which is the shortest of the three known isoforms, and conserved in its entirety in both *β* and *γ* isoforms. Alignments were performed using Clustal-W. Zinc finger and glutamine- and serine-rich region were identified using ScanProsite.

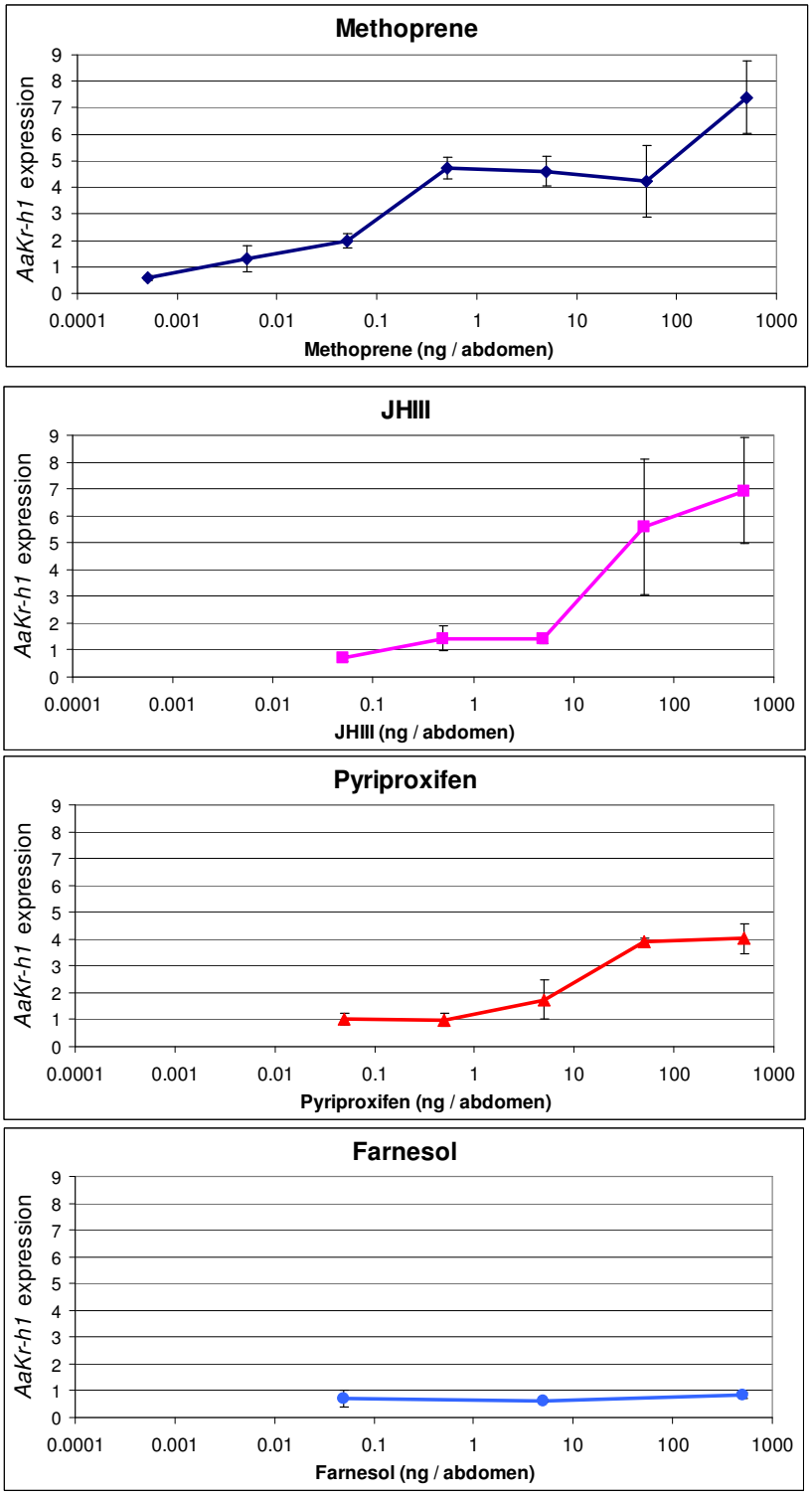


Figure 11. *AaKr-h1* response to JHAs. abdominal ligation assays were performed on newly emerged female *Ae. aegypti*. Abdomens were treated with 0.5 µl acetone alone (control) or 0.5 µl acetone with indicated amounts of methoprene, JHIII, pyriproxifen, or

farnesol. Abdomens were incubated at 28℃ for 12 hrs. Each sample group consisted of the pooled RNA of 5-6 ligated abdomens. mRNA expression levels in each sample were measured using qRT-PCR, and normalized to S7 ribosomal protein mRNA levels. Each data point shown represents the average normalized mRNA levels from three experiments. mRNA expression values are expressed as relative to the mean value of the control with no hormone (mean control = 1). Error bars indicate standard error.

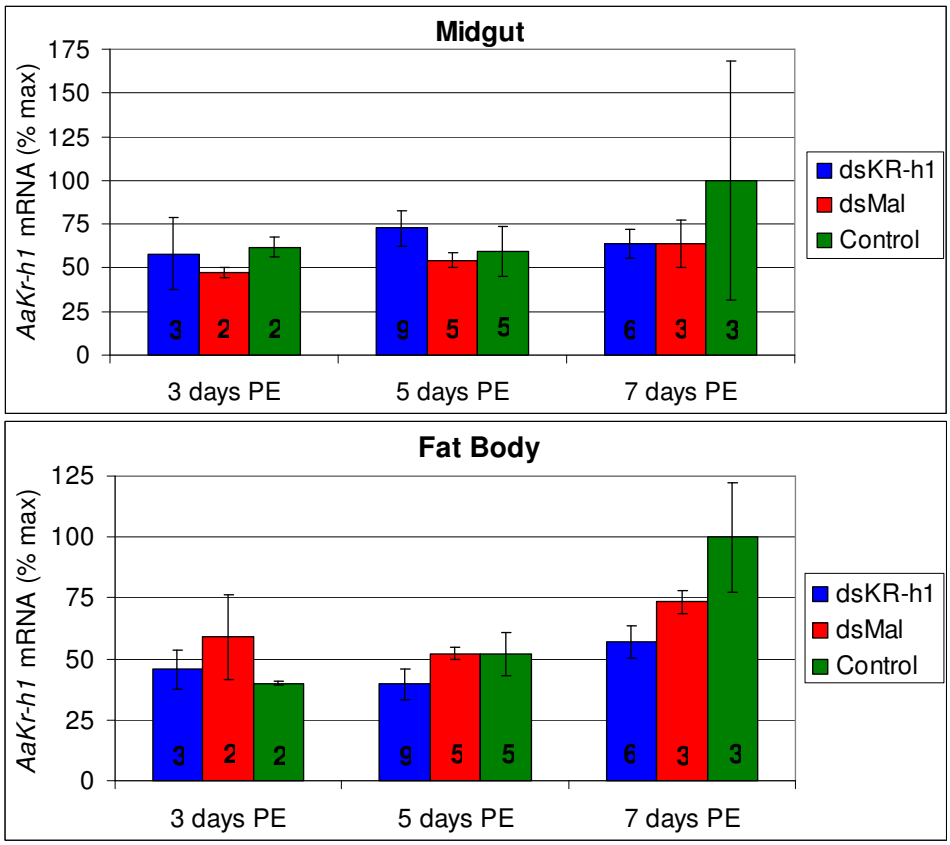


Figure 12. RNAi results. Newly emerged (2-4 hr post-emergence) adult female *Ae. aegypti* were injected with either *AaKr-h1* dsRNA (0.6 µg in 200 nl 0.1X PBS), *Mal* dsRNA (0.6 µg in 200 nl 0.1X PBS). A third group (control) received no injection. Midgut and fat body tissues were removed at 3, 5, and 7 days post-emergence. Individual samples consisted of 4 midguts or fat bodies pooled for RNA extraction and qRT-PCR quantification of *Aakr-h1* mRNA, and normalized to *AaS7* mRNA levels. Data shown are expressed as a percent of the maximum recorded value. Each data point is the mean value of replicate experiments. The number at the base of each bar indicates the number of replicate experiments. Error bars indicate standard error.

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