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## MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF A JUVENILE HORMONE ACID METHYLTRANSFERASE EXPRESSED IN THE CORPORA ALLATA OF MOSQUITOES

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### Abstract

A juvenile hormone acid methyltransferase (JHAMT) was isolated as an abundant EST in a library of the corpora allata of the adult female mosquito *Aedes aegypti*. Its full-length cDNA encodes a 278-aa protein that has 43 % amino acid identity with *BmJHAMT*, a juvenile hormone acid methyltransferase previously cloned from *Bombyx mori*. Heterologous expression produced a recombinant protein that metabolizes farnesoic acid (FA) into methyl farnesoate, as well as juvenile hormone acid into juvenile hormone III (JH III) with exquisite stereo specificity. Real time PCR experiments showed that JHAMT mRNA levels are not an unequivocal indicator of JH III synthesis rates; the *A. aegypti* JHAMT gene, silent in female pupae, was transcriptionally activated just 4–6 hours before adult eclosion. Radiochemical methyltransferase assays using active and inactive corpora allata glands (CA) dissected from sugar and blood-fed females respectively, clearly indicated that significant levels of JHAMT enzymatic activity are present when the CA shows very low spontaneous rates of JH III synthesis. Having the last enzymes of the JH synthetic pathway readily available all the time might be critical for the adult female mosquito to sustain rapid dynamic changes in JH III synthesis in response to nutritional changes or peripheral influences, such as mating or feeding. These results suggest that this gene has different roles in the regulation of JH synthesis in pupal and adult female mosquitoes, and support the hypothesis that the rate-limiting steps in JH III synthesis in adult female mosquitoes are located before entrance of FA into the synthetic pathway.

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## Keywords

*Aedes aegypti*; mosquito; juvenile hormone; farnesoic acid; methyltransferase

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## INTRODUCTION

The addition of a methyl group to biologically active molecules such as hormones, neurotransmitters, lipids, proteins and nucleic acids causes a change in the physicochemical properties of these molecules. The biological functions of methylation are wide ranging and include biosynthesis, metabolism, detoxification, signal transduction, protein sorting and repair, and nucleic acid processing (Martin and McMillan, 2002). The diversity in the roles of methylation is matched by the vast number of methyltransferase (MT) enzymes (EC 2.1.1). Although several classes of MT enzymes are known, the great majority of methylation reactions are catalyzed by the S-adenosylmethionine-dependent MTs (SAM-MTs). This class of enzyme utilizes the methyl donor S-adenosylmethionine (SAM or AdoMet) to methylate substrates. Around 120 members of the SAM-MT family have been classified (EC 2.1.1.X) based on their substrate (Martin and McMillan, 2002).

Juvenile hormone III (JH) is the key hormone regulating metamorphosis and previtellogenic ovarian development in mosquitoes (Klowden, 1997). JH is synthesized and released from the corpora allata (CA), a pair of endocrine glands with nervous connections to the brain (Li et al., 2003a).

The biosynthetic pathway of JHs is divided conventionally into two steps, the early steps and the late steps (Goodman and Granger, 2005). The early steps of JH III biosynthesis follow the mevalonate pathway, with the formation of five-carbon (5C) isoprenoid units from acetate via mevalonic acid, with the sequential head to tail condensation of three 5C units to form farnesyl pyrophosphate (FPP) (Bellés et al., 2005). In the late steps FPP is hydrolyzed by a pyrophosphatase to farnesol, then oxidized successively to farnesal and farnesoic acid (FA) by an alcohol dehydrogenase and an aldehyde dehydrogenase, respectively (Goodman and Granger, 2005). The last two steps diverge depending on the insect order. In Lepidoptera, a C-10, 11 epoxidation by a P450 monooxygenase converts the FA to the epoxy acid (JH acid or JHA), that is afterward methylated by an O-(SAM) dependent methyltransferase (JHAMT) to form the methyl ester. In Orthoptera and Dictyoptera epoxidation follows methylation (Schooley and Baker, 1985; Goodman and Granger, 2005); this is also believed to be the case in mosquitoes (Li et al., 2003a, 2003b).

Because the late-step enzymes are highly specific to insects, they could be excellent targets for selective insect growth regulators; thus, characterization of these molecules is of great importance. Here we describe the molecular and functional characterization of an *A. aegypti* juvenile hormone acid methyltransferase (or *AaJHAMT*) that is highly expressed in the CA. It is a member of the same protein family involved in the methylation of JHA in the lepidopteran *Bombyx mori* (Shinoda and Itoyama, 2003) and in *Drosophila melanogaster* (Niwa et al., 2008). In addition, we describe the developmental stage-dependent expression of this gene in correlation to methyl farneosate and JH synthesis in the CA, as well as the expression in normal and nutritional deficient female mosquitoes. These studies suggest that *AaJHAMT* plays different roles in JH synthesis regulation during metamorphosis and reproduction.

## MATERIALS AND METHODS

### Chemicals

(*E,E*)-farnesoic acid and (*E,E*)-methyl farnesate (MF) were purchased from Echelon (Salt Lake City, UT) JH III (79% purity) was purchased from Scitech (Prague, Czech Republic). Methylaurate, methylpalmitate, lauric acid and palmitic acid were purchased from DOOSAN Serdary Research Laboratories (Toronto, Canada). Linolenic acid, linoleic acid and tridecanoic acid were purchased from Acros Organics (Geel, Belgium).

### Insects

*Aedes aegypti* of the Rockefeller strain were reared at 28°C and 80 % relative humidity under a photoperiod of 16 h light: 8 h dark. Mated adults were offered a cotton pad soaked in 3% sucrose solution. We will refer to the cotton pad sucrose-fed adults as sugar fed. Three-day-old female mosquitoes were fed pig blood equilibrated to 37 °C, and ATP was added to the blood meal to a final concentration of 1mM immediately before use as previously described (Noriega et al., 1999).

### Nutrient-deficient mosquitoes

small nutrient-deficient mosquitoes were produced as previously described (Caroci et al., 2004); briefly, larvae were reared in pans (23 cm x 35 cm x 13 cm) containing 1 liter of distilled water, with the following amounts of a 10 % solution of bovine liver powder (ICN, Aurora, OH) diet: 0.75 ml on day 1, 3, 5 and 7. Under these rearing conditions, most larvae pupate at day 8. Only those females with wing length under 2.5 mm were used.

### AaJHAMT cloning

Eleven full length AaJHAMT sequences were obtained from an *A. aegypti* corpora-allata + corpora cardiaca library, constructed and sequenced as previously described (Noriega et al., 2006). The AaJHAMT EST sequence was queried against the *A. aegypti* database at VectorBase (<http://www.vectorbase.org/index.php>); it revealed an identical sequence with the accession number AAEL006280-RA.

### Secondary structure analysis

Related sequences from other insect JHAMTs were identified by using BLAST (Altschul et al., 1990). Sequence identities with *A. aegypti* are as follows: *Culex pipiens* 68% (accession number: XM\_001853694), *Anopheles gambiae* 60% (accession number: AGAP005256-PA), *Drosophila melanogaster* 48% (accession number: AB113579) and *Bombyx mori* 43% (accession number: NP\_001036901). Multiple sequence alignment was performed with the T-COFFEE program (Notredame et al., 2000). Secondary structure analysis was performed using the PSIPRED (Jones, 1999) and Jnet programs (Cuff and Barton, 2000). DISOPRED, a disorder prediction program, was utilized to confirm loop regions (Ward et al., 2004). A consensus within the different programs was employed to select the secondary structure elements.

### Expression of recombinant protein

The coding region of the AaJHAMT cDNA was cloned into the expression vector pET28a(+) (Novagen). *E. coli* BL21(DE3) strain cells were transformed with the construct and expressed as previously described (Shinoda and Itoyama, 2003).

Recombinant His-tagged protein was purified from the supernatant by using a HiTrap chelating column (Amersham Pharmacia). Elution buffer containing 300 mM imidazole was exchanged to Tris-Cl (50 mM, pH 7.5) with a PD-10 desalting column (Amersham Pharmacia). Glycerol was added to the enzyme solution (final concentration 50%), and the sample was stored at -80°

C until use. Quantification and SDS-PAGE analysis of the purified protein were performed as described (Shinoda and Itoyama, 2003).

### Enzyme assays

Recombinant *AaJHAMT* crude extracts were used to test the enzymatic properties against several juvenoid acids and fatty acids following the protocol previously described by Shinoda and Itoyama (2003) with small modifications. Farnesoic acid (FA; 85  $\mu$ M), racemic JH III acid (JHA III; 700  $\mu$ M), linoleic acid (288  $\mu$ M), linolenic acid (285  $\mu$ M), palmitic acid (PA; 78  $\mu$ M), lauric acid (LA; 100  $\mu$ M), and tridecanoic acid (93  $\mu$ M) were assayed in 500  $\mu$ l of Tris-Cl buffer (50 mM, pH 7.4) containing 200  $\mu$ M SAM and 250  $\mu$ l of crude extracts for 2 hours at 25°C. For negative control, bacterial crude extracts without the expression vector were added to the same reaction mixtures. The reaction mixtures were extracted and the production of methyl esters were analyzed by RP-HPLC and GC-MS as previously described (Shinoda and Itoyama, 2003). In an other series of experiments, purified recombinant *AaJHAMT* was incubated in 500  $\mu$ l of Tris-Cl (50 mM, pH 7.5) buffer containing SAM (500  $\mu$ M) and either one of the following substrate: FA (50  $\mu$ M), racemic JHA III (50  $\mu$ M), LA (100  $\mu$ M), or PA (100  $\mu$ M). Amount of *AaJHAMT* and incubation times at 25°C were adjusted to consume less than 15% of the initial substrates during the assay: 2.6  $\mu$ g and 10 min for FA and JHA III; 10  $\mu$ g and 60 min for LA and PA. Reactions were stopped by adding 500  $\mu$ l of CH<sub>3</sub>CN and vortexing. To analyze the methylated products of FA (MF) and racemic JHA III (JH III), the samples were cleared by centrifugation and the supernatants were analyzed directly by reversed-phase HPLC (Niwa et al., 2008). To analyze the methylated LA (methylaurate) and PA (methylpalmitate), the supernatants were extracted with hexane containing 5  $\mu$ g/ml methyltridecanoate as an internal standard and analyzed by GC-MS. Enzyme activities were measured by at least three independent reactions for each substrate. To analyze the stereo specificity of the JH III produced, purified *AaJHAMT* (2.6  $\mu$ g) was incubated in 500  $\mu$ l of Tris-Cl (50 mM, pH 7.5) buffer containing SAM (500  $\mu$ M) and racemic JHA III (5  $\mu$ g) for 60 min at 25°C, and the reaction was stopped with 500  $\mu$ l of CH<sub>3</sub>CN as described above. After centrifugation the supernatant was extracted with hexane and analyzed by chiral-HPLC as previously described (Ichikawa et al., 2007).

### Quantitative real-time PCR (qPCR)

Total RNA was isolated using RNA-binding glass powder as previously described (Noriega and Wells, 1993). Contaminating genomic DNA was removed using the DNA-free™ kit (Ambion, Austin, TX). Reverse transcription was carried out using the Reverse-iT™ First Strand Synthesis Kit (ABgene, Epsom, UK) by an oligo dT priming method according to the manufacturer's recommendations, using 300 ng of total RNA in 20  $\mu$ l reactions. Real-time PCR was performed with the 7300 Real Time PCR System using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The primer probes for house keeping gene 60S ribosomal protein L32 (*rpL32*; AAEL003396 from VectorBase) and for juvenile hormone acid methyltransferase (*AaJHAMT*) genes were as follows:

*rpL32* Forward: 5' CCATCAGTCCGATCGCTATGA 3';

*rpL32* Reverse: 5'GTTGTCAATACCTTTTCGGCTTACG 3';

*rpL32* Probe: 5'CAAGCTTGCCCCCAACTG 3';

*AaJHAMT* Forward: 5' CCAAGCCAGATTCCTAACATTCAAA 3';

*AaJHAMT* Reverse: 5' TGCCCTGTCCGCAACC 3';

*AaJHAMT* Probe: 5' CTTGCAATCATTACCACAGCAC3'.

Primer/probes were synthesized by Applied Biosystems and reactions were carried in a 20  $\mu$ l volume according to manufacturer recommendations for Custom TaqMan® Gene Expression Assays. Reactions were run in duplicate using 1  $\mu$ l of cDNA per reaction. Standard curves to quantify relative gene copy number were made from serial dilutions of plasmids containing rpL32 or AaJHAMT gene (300,000; 30,000; 3,000; 300; 30 copies of a plasmid per reaction). Real-time data were collected by 7300 System SDS Software and analyzed in Microsoft Excel. AaJHAMT transcript levels were normalized with rpL32 transcript levels in the same sample. Relative AaJHAMT transcript levels are expressed as a number of copies of AaJHAMT transcript per 10,000 copies of rpL32 transcript. Each RT-PCR data point is average of three independent biological replicates.

### Corpora allata in vitro radiochemical assay for AaJHAMT activity

The CA complexes were isolated as previously described (Li et al., 2003a). Rates of MF and JH III biosynthesis were estimated using an *in vitro* radiochemical assay (Feyereisen and Tobe, 1981; Feyereisen, 1985a), as previously modified (Li et al., 2003a; 2003b). Briefly, CA complexes were incubated for 4h in fresh medium containing  $^3$ H-labeled methionine. After extraction and separation by thin-layer chromatography, the MF and JH III bands were removed, placed into scintillation cocktail and assayed for  $^3$ H. The quantity of MF and JH III produced was calculated from the specific activity of the  $^3$ H-labeled methionine in the medium. The effect of FA on MF and JH III synthesis was tested by adding the precursor directly into the CA incubation medium.

### Statistical analysis

Statistical analysis of the data was performed by *t*-test using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA). The results were expressed as mean  $\pm$  SEM, and considered significantly different at  $P < 0.05$ .

## RESULTS

### A juvenile hormone acid methyltransferase sequence from a corpora allata EST collection

A cDNA library was prepared using corpora-allata + corpora cardiaca from two- and three-day-old *A. aegypti* adult females (80 pair) as previously described (Noriega et al., 2006). Sequencing revealed 11 ESTs encoding full length ORFs coding for a protein of 278 amino acids (Fig. 1A), that had 43 % amino acid identity with *BmJHAMT*, a juvenile hormone acid methyltransferase previously cloned from *B. mori* (Shinoda and Itoyama, 2003). Analysis of the protein sequence showed the presence of a typical SAM-MT fold composed of alternating 6 stranded  $\beta$  sheets with 9  $\alpha$  helices (Fig. 1A). The SAM-binding region, localized close to the N-terminal, was very well conserved among *B. mori*, *D. melanogaster* and 3 different species of mosquitoes (Fig. 1A). The expected topology of the fold of *AaJHAMT* was based on comparisons with previous studies (Martin and McMillan, 2002) (Fig. 1B).

### Functional characterization of AaJHAMT

Using crude *E. coli* extracts expressing recombinant AaJHAMT, we detected high catalytic activity against FA and JHA III in the presence of SAM (data not shown). However, such activity was not detected in the reaction lacking SAM or with crude extract of *E. coli* transformed with an empty vector in the presence of SAM. No significant activity was detected against linoleic acid, linolenic acid, palmitic acid, lauric acid and tridecanoid acid (data not shown). We used purified rAaJHAMT to further characterize the enzyme specificity; rAaJHAMT showed high activity against racemic JHA III ( $k_{cat}$  ( $\text{min}^{-1}$ ) = 1.033) and FA ( $k_{cat}$  ( $\text{min}^{-1}$ ) = 0.260) (Table 1). In contrast, rAaJHAMT showed trace activity against lauric acid and palmitic acid even at a higher concentration (100  $\mu$ M). When the JH III enzymatically

produced from racemic JHA III by rAaJHAMT was analyzed by chiral-HPLC, it contained almost only (*10R*)-JH III (>99.5%), showing that this enzyme has high stereo specificity against (*10R*)-JHA III (Fig. 2). These results revealed that AaJHAMT is a member of the previously described juvenile hormone acid *O*-methyltransferase family (Shinoda and Itoyama, 2003); because AaJHAMT has the ability to efficiently use two juvenoid acid substrates (FA and JHA), it could functionally be defined as a juvenoid acid methyltransferase.

### Tissue-specific and developmental expression of AaJHAMT

Real time PCR was used to analyze the transcript tissue specificity. AaJHAMT mRNA expression was very high in the CA, with trace amounts present in the thoracic ganglia, head, heart, and other female tissues, as well as in the testis and accessory glands of the male (Fig. 3). Transcripts for AaJHAMT in thorax are most likely produced in the corpora allata; there was a 500 fold difference in AaJHAMT mRNA expression between the first thoracic segment containing the CA and the rest of the thorax (results not shown). Therefore, the whole thorax was used to assess changes of AaJHAMT mRNA during larvae, female pupal and adult development. Transcript levels were high in early 4<sup>th</sup> instar larvae, undetectable in early and one-day old pupae, and rapidly increased 44–46 h after pupation to relatively high levels at adult eclosion (approximately 48–50 h after pupation). With sugar-fed females, the highest transcript level was observed on day 1 after adult eclosion (Fig. 4A), at the time of maximum biosynthetic activity of the CA (Li et al., 2003a). On the other hand, the messenger level significantly decreased 24 h after blood feeding, when JH synthesis is low, to increase again 48 h after a blood meal, just before the reactivation of JH synthesis observed 3 days after blood feeding (Fig. 4B). Expression of AaJHAMT transcript was also studied in nutrient-deficient mosquitoes; at 24 h after eclosion, the relative levels of AaJHAMT mRNA were similar in large and small mosquitoes (Fig. 5).

### Farnesoic acid methyltransferase activity in isolated corpora allata

Changes in the activity of AaJHAMT in mosquito corpora allata were analyzed using an *in vitro* radiochemical assay. Methylation of FA results in the formation of radioactive MF, which is further transformed by a P450 epoxidase into radioactive JH III; therefore the sum of the two juvenoids (MF and JH III) represents the endogenous catalytic activity of AaJHAMT. The addition of exogenous FA dramatically enhanced the production of MF and JH III in the active glands of the 24 h old sugar-fed females, as well as in the inactive gland of the 24 h blood-fed female (Fig. 6).

## DISCUSSION

### AaJHAMT is structurally and functionally a juvenile hormone acid methyltransferase

We have structurally and functionally characterized a novel *A. aegypti* juvenile hormone acid methyltransferase involved in the synthesis of JH in the mosquito corpora allata. AaJHAMT was isolated as a fairly abundant EST from the CA of female *A. aegypti* (Noriega et al., 2006). Analysis of the secondary structure of AaJHAMT revealed the presence of a typical SAM-MT fold, composed by a 6-stranded  $\beta$  sheet with 9  $\alpha$  helices. In all SAM-MT studied, the SAM-binding region is located close to the N-terminal part of the protein (Martin and McMillan, 2002). The substrate-binding region is located in the C-terminal part of the polypeptide, and varies entirely in structure and topology among different groups of SAM-MTs (Martin and McMillan, 2002; Boissier et al., 2006). When lipids are methyl acceptor substrates, they are expected to bind internal cavities of a mostly non-polar character, as observed in *Mycobacterium tuberculosis* mycolic acid cyclopropane synthase (Boissier et al., 2006; Huang et al., 2002) and *Mycobacterium leprae* lipid methyltransferase (Graña et al., 2007). These lipid binding pockets structurally are hydrophobic tunnels extending from the surface to the SAM binding site; the entrance of the hydrophobic tunnel is often defined by the

$\alpha 2$  and  $\alpha 3$  domains (Graña et al., 2007). Common additional features of lipid MTs are: a poorly formed  $\alpha C$ , a short helix inserted between  $\beta 4$  and  $\alpha D$ , 1 or 2  $\alpha$  helices inserted between  $\beta 5$  and  $\alpha E$  and 2 or 3  $\alpha$  helices inserted between  $\beta 6$  and  $\beta 7$ . With the exception of the absence of  $\beta 4$  and  $\alpha C$  these structural features can be recognized in the predicted AaJHAMT secondary structure (Fig. 1A), suggesting that it is a member of the lipid transfer SAM-MT family. This fact was confirmed studying the activity of the recombinant AaJHAMT. Our enzymatic assays showed that rAaJHAMT efficiently recognized the 15-carbon isoprenoid chain of FA or JHA. It had very weak activity against palmitic acid (16C), and did not methylate shorter fatty acids such as lauric (12C) or tridecanoic acid (13C) or the longer linoleic or linolenic acids (18C). It is remarkable that when the JH III generated from the JHA III racemate was analyzed by chiral HPLC it contained almost only (*R*)-JH III (*R*:*S*=99.5:0.5). Whether JHA is present in CA of mosquitoes remains to be seen. However, if it plays any role on JH synthesis, the chirality of (10*R*)-JH III would be ensured by the stereospecificity of this enzyme.

### Transcription of AaJHAMT is suppressed during metamorphosis

Metamorphosis is a developmentally regulated process that is tightly controlled by hormones. Once the insect larva has reached its species-specific size, JH concentrations decrease, allowing pupation and metamorphosis to proceed (Riddiford, 2008). There are no reports on JH titers or synthesis in mosquito pupae; presumably the CA is inactive before adult eclosion. Supporting this statement is the observation that JH-stimulated adult-specific genes are not expressed in pupae, but can be induced by raising them in the presence of JH analogues (Noriega and Wells, 1999). To guarantee a successful completion of metamorphosis, it is reasonable that transcription of genes coding for JH synthetic enzymes is suppressed during pupa development; similar low levels of JHAMT mRNA were described in pupa of *D. melanogaster* (Niwa et al., 2008) and *B. mori* (Kinjoh et al., 2007), suppression of JHAMT transcription was recently described as critical for induction of larval-pupal metamorphosis in *Tribolium castaneum* (Minakuchi et al., 2008). Transcripts levels were high in early 4<sup>th</sup> instar *A. aegypti* larvae. Conversely, AaJHAMT mRNA was undetectable in CA of newly pupated mosquitoes or 1 day-old pupae; this is the only time in which we did not detect AaJHAMT transcripts. AaJHAMT mRNA steady state levels sharply increased during the last 4–6 hours before adult eclosion, and newly eclosed females had significantly higher levels of AaJHAMT mRNA. At the same time, JH titers (Shapiro et al., 1986) and spontaneous CA synthetic activity (Li et al., 2003a) are very low in newly eclosed females. Remarkably, the synthesis of juvenoids can be rapidly activated if the CA of the newly eclosed females is stimulated with allatotropin and FA (Li et al., 2003b), suggesting that JHAMT activity is already present.

### AaJHAMT plays a different role in JH synthesis during reproduction

Sexual maturation and reproduction in adult mosquitoes are nutrient-dependent processes (Briegel, 1990). JH is part of the signal transduction pathway that connects changes in the nutritional status with activation of specific physiological events during reproduction (Noriega, 2004). The JH level increase during the first day after adult emergence, remains high in sugar-fed females, and rapidly falls after a blood meal (Shapiro et al., 1986). The initial rise in JH after eclosion is essential for female's reproductive maturation, it signals that ecdysis of the adult has finished and reproductive processes should begin (Klowden, 1997). Teneral reserves are utilized to initiate previtellogenic ovarian development in mosquitoes. Only when nutrients are appropriate can factors released from the brain induce the CA to synthesize enough JH to activate reproductive maturation (Caroci et al., 2004). Allocation of these nutritional reserves for ovarian development is life-threatening if the resources are not sufficient (Martinez-Hernandez et al., 2007). Nutritional deficient females having emerged with low teneral reserves have reduced JH synthesis and previtellogenic development (Caroci et al., 2004), however, they show JHAMT mRNA levels equivalent to those of normal mosquitoes with high JH synthesis. For the female mosquito a tight regulation of JH titers is vital. Having the last

enzymes of the JH synthetic pathway readily available all the time might be critical for rapid dynamic changes in JH synthesis in response to nutritional changes or peripheral influences, such as mating, feeding, ovarian development, etc. Evidence of the dynamic regulation comes from the observation that spontaneous and FA stimulated rates of *AaJHAMT* activity are not correlated. For example, after blood meal, the spontaneous rates are very low, but the stimulated rates are among the highest detected. The rate of spontaneous juvenoid biosynthesis is a direct estimate of the overall activity of the CA and is clearly linked with JH titers (Li et al., 2003a). The FA stimulated rates indicate the relative importance of rate-limitation at early steps in JH biosynthesis; exogenous FA is efficiently utilized by the CA to nullify the effect of rate-limiting factors at an early step(s) in JH synthesis (Feyereisen, 1985b; Li et al., 2003b). The spontaneous synthesis of juvenoids in CA dissected from both sugar-fed and blood-fed females is always markedly stimulated by the addition of FA to the medium, demonstrating that the supply of this precursor is a rate limiting factor in these glands, and not the activity of *AaJHAMT*.

## Conclusions

In summary, these studies demonstrate that the *AaJHAMT* isolated from a mosquito CA EST library is structurally and functionally a juvenile hormone acid methyltransferase that catalyzes the methylation of farnesoic acid into methyl farnesoate, as well as juvenile hormone acid into juvenile hormone with exquisite stereo specificity. The combination of transcriptional studies and biosynthetic assays showed that the gene has different roles in the regulation of JH synthesis in pupa and adult mosquitoes. Our studies using inactive CA glands from nutritional deficient mosquitoes as well as CA dissected from blood-fed females, clearly suggest that significant levels of the enzyme are always available after adult eclosion, and the rate-limiting factors in JH synthesis, as well as the targets for allatoregulatory peptides (Li et al., 2006), are located before the entrance of FA into the synthetic pathway.

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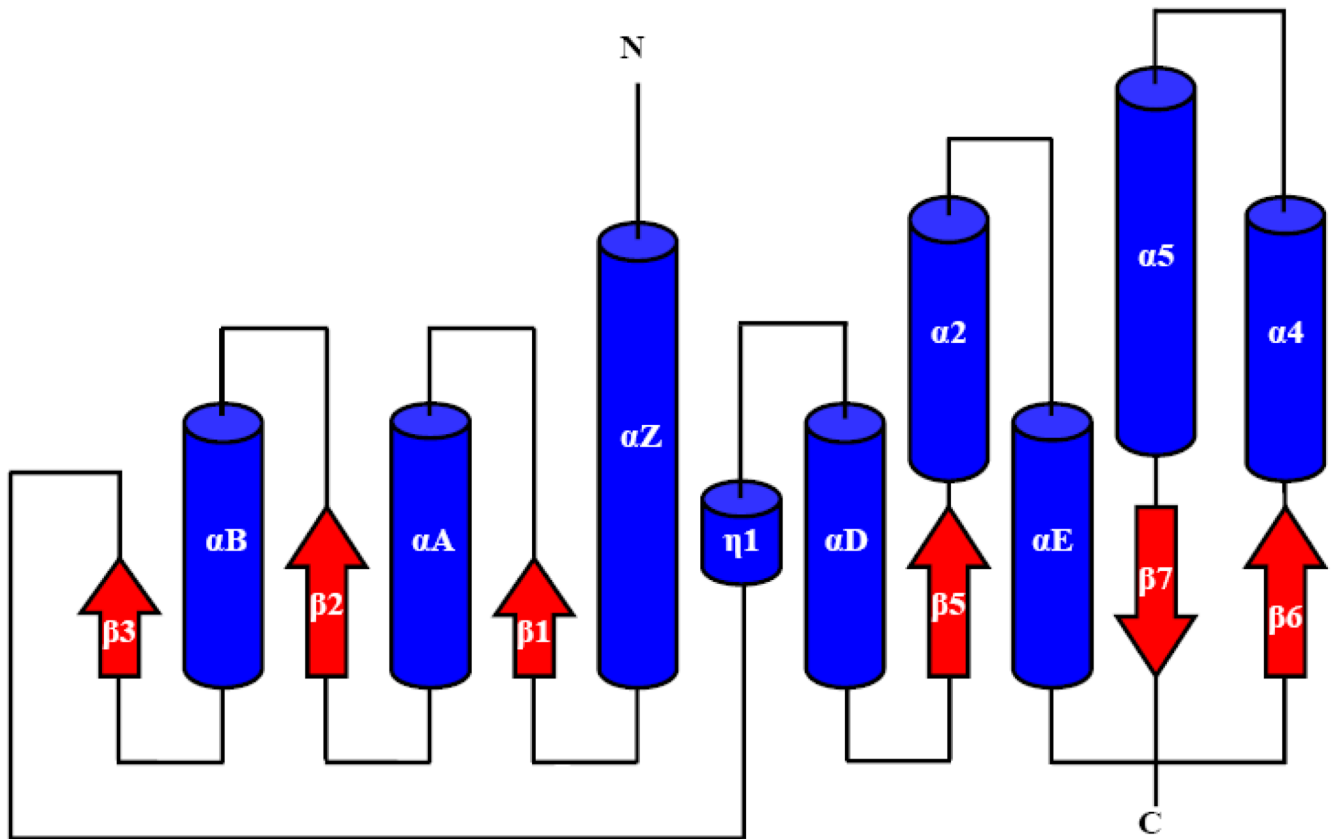


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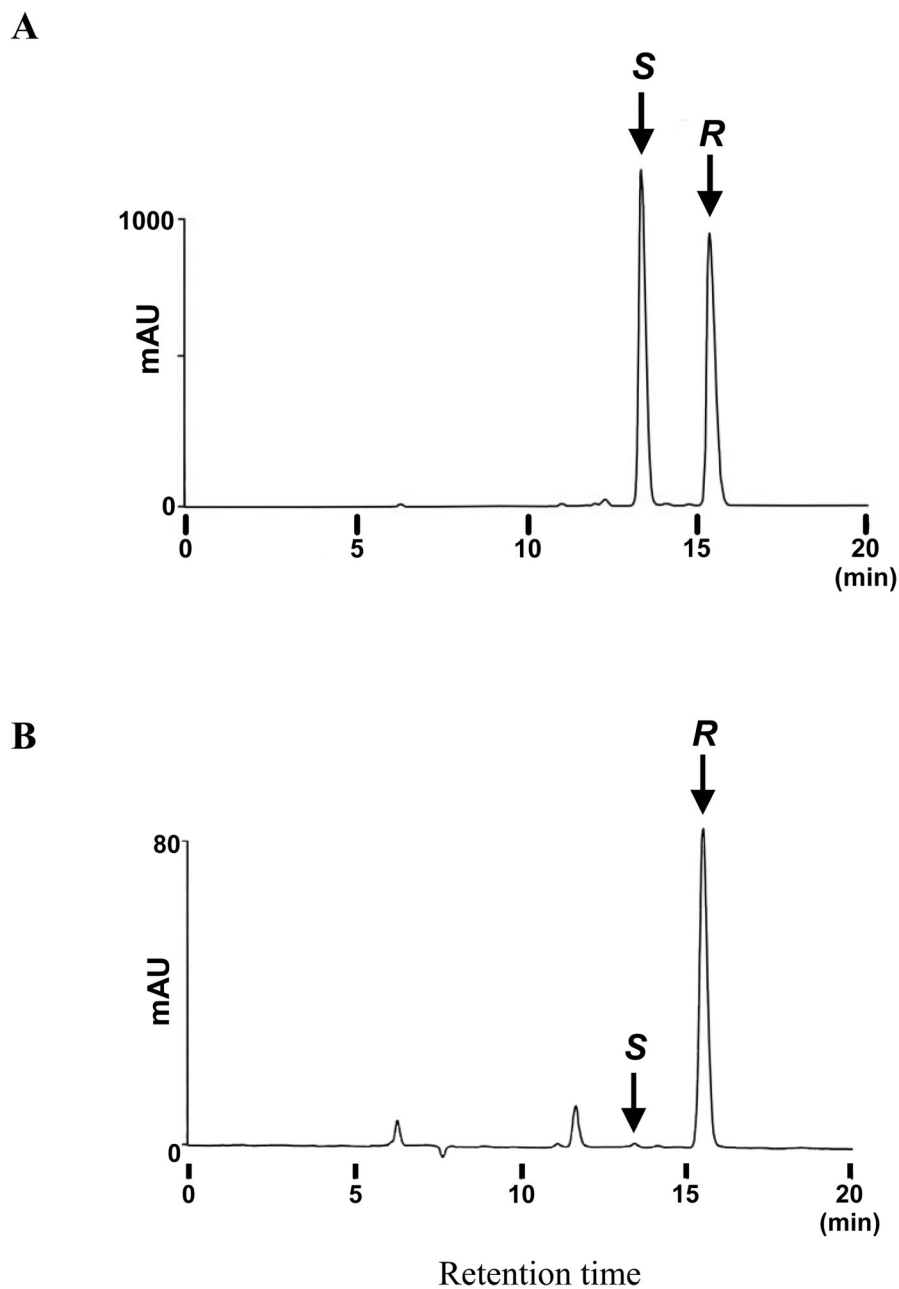
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Figure 1A

	<b><math>\alpha Z</math></b>	<b><math>\beta 1</math></b>	<b><math>\alpha A</math></b>	
<i>A. aegypti</i>	MNKP <u>NLYHR</u> ANGVQRRDAKEILDEHGHLRLRWKEENED-S <u>LLDI</u> GCGSGI <u>VLIDFVI</u> PMVP	59		
<i>C. pipiens</i>	MNKP <u>NLYHR</u> ANGLQRRDAEEILDEFHGHLRLRWREDGKD-S <u>LLDV</u> GSGCGI <u>VLVDFVI</u> PLIP	59		
<i>A. gambiae</i>	MNKANLYQ <u>Q</u> ANGVQRRDAMEILKEYAPLISCPRDGEEVA <u>LLDI</u> GCGSGI <u>VLVDYIL</u> PVLS	60		
<i>B. mori</i>	MNNADLYRKSNSLQRRDALRCLLEEHANKIKWKKIG-D-R <u>VIDI</u> G <u>CADG</u> V-TDILK <u>VY</u> MP	57		
<i>D. melanogaster</i>	MNQASLYQH <u>AN</u> QVQRHDAKILDEFAS <u>T</u> MQWRSDGED-A <u>LLDV</u> GSGSGI <u>VLMDFV</u> KPLLP	59		
	**:.***:.* : **::** *.*. : : : : **:*..*.* * : * : .:			
	<b><math>\beta 2</math></b>	<b><math>\alpha B</math></b>	<b><math>\beta 3</math></b>	
<i>A. aegypti</i>	PK--R <u>ARVL</u> GTDVSE <u>Q</u> MVRFARKVHSDVENLFF <u>FETL</u> DIEGDIS-SFLNKWGCDFHITS	115		
<i>C. pipiens</i>	QN--F <u>VIL</u> GTDISE <u>Q</u> MVRFARKIYSGRKNVS <u>FDRL</u> DIGGDVQ-QFMSKWGRFNHITS	115		
<i>A. gambiae</i>	RGSTPVA <u>RAL</u> ATDISE <u>Q</u> MVRHARESYRHVKTIE <u>FDTL</u> DIGIKLDSAKLSRWGFSSHVTS	120		
<i>B. mori</i>	KN--Y <u>GLV</u> GCDISE <u>E</u> EMVKYANKHH-GFGRTS <u>FRVL</u> DIEGDL-TADLKQ--GFDHVFS	110		
<i>D. melanogaster</i>	IR---G <u>QLV</u> GTDISS <u>Q</u> MVHYASKHYQREERTR <u>FQVL</u> DIGCERLPEELS--GRFDHVTS	113		
	:. *.*:***:.* : : * ** . :. *.*: **			
	<b><math>\eta 1</math></b>	<b><math>\alpha D</math></b>	<b><math>\beta 5</math></b>	<b><math>\alpha 2</math></b>
<i>A. aegypti</i>	<u>YCLH</u> WVRS <u>QRS</u> AFSNIYNLMAPN-G <u>DCLL</u> GF <u>LARNP</u> I <u>FDIYD</u> QLSNSAKWSMYMTDVKY	174		
<i>C. pipiens</i>	<u>YCLH</u> WIRN <u>QSI</u> AFANIFNLLRAE-G <u>DCLL</u> TF <u>LARNP</u> I <u>FDIYY</u> QLSKTDKWKYMSDVRD	174		
<i>A. gambiae</i>	<u>YCLH</u> WVQN <u>QHV</u> AFSNIYNLLQHG-G <u>DCLL</u> VF <u>LANNP</u> I <u>FDIYN</u> QLSRSPKWSKYMYDVEKY	179		
<i>B. mori</i>	<u>YTLH</u> WIRD <u>Q</u> ERAFRNI FNLLGDE-G <u>DCLL</u> FLGHPTI <u>FDVYR</u> TLSSHTEKWHSWLEHVDRF	169		
<i>D. melanogaster</i>	<u>YCLH</u> WVQN <u>LKG</u> ALGNIYNLLKPEGG <u>DCLL</u> AF <u>LASN</u> PVVEYVKILKTNDKWSITYMQDVENF	173		
	* ***:.. * : **:*: ***** ** . *:::* * . . * * : : *:::			
	<b><math>\alpha E</math></b>	<b><math>\beta 6</math></b>	<b><math>\alpha 4</math></b>	
<i>A. aegypti</i>	ISPYQYCENPVGEIEEILSSVGF <u>T</u> KYKIHIA <u>DKI</u> YVYEG <u>IDS</u> LKKAV <u>Q</u> AVNPFSE <u>RMPLD</u>	234		
<i>C. pipiens</i>	ISPYQYCENP <u>SEE</u> IGKILSSVGFN <u>KYKI</u> QIM <u>DRI</u> YKYK <u>IDS</u> LK-PVLA <u>VN</u> PFCE <u>RMPLD</u>	233		
<i>A. gambiae</i>	ISPYQYCENP <u>ASE</u> IEDLLCTVGF <u>Q</u> YQIQVR <u>DKLY</u> VYEGLDNLKRAVLA <u>VN</u> PFSE <u>RMPP</u> E	239		
<i>B. mori</i>	ISPYHDNEDPE <u>KEV</u> KKIM <u>ER</u> VGFSN <u>IEV</u> QCKTLFYVYDDLDVLKKSVA <u>AIN</u> PF--NIPK <u>D</u>	227		
<i>D. melanogaster</i>	ISPLHYSLNP <u>GEE</u> FS <u>QLL</u> NVGFV <u>QHN</u> VE <u>IR</u> NEVFVYEG <u>VRTL</u> KDNVKA <u>IC</u> PFLE <u>RM</u> PA <u>D</u>	233		
	** * : : * * * . : : * * : : : . : * . : : * * * * : * * : * * : * * : :			
	<b><math>\alpha 5</math></b>	<b><math>\beta 7</math></b>		
<i>A. aegypti</i>	<u>LQED</u> FLNDYIAVVRMSLSE <u>NC</u> CG-NENDYKFIT-PY <u>KL</u> VVVYA <u>VK</u> -----	278		
<i>C. pipiens</i>	<u>LQED</u> FMDDYIDIVRKMAYHKNGHE-DANDYKFIT-PY <u>KL</u> VIVYA <u>SK</u> -----	277		
<i>A. gambiae</i>	<u>LQDR</u> FLLDYIAVVRQMYLTKTGSEENDCNLQFIS-PY <u>KL</u> VVVYA <u>KK</u> -----	284		
<i>B. mori</i>	<u>ILED</u> FLEDYIDVVRERMLLDRCNN-NVGESVSIKFN <u>YK</u> VISVY <u>ARK</u> -----	272		
<i>D. melanogaster</i>	<u>LHEQ</u> FLDDFIDIVISMNLQ <u>QG</u> --E-NNEDQKFLS-PY <u>KL</u> VVAY <u>ARK</u> TPEFVNNVFLE <u>PTH</u>	289		
	: : * : * : * * * * . : : : . : * * : : * * * : * * * : * * : :			
<i>A. aegypti</i>	-----			
<i>C. pipiens</i>	-----			
<i>A. gambiae</i>	-----			
<i>B. mori</i>	--LCLSLM 278			
<i>D. melanogaster</i>	QNLVKGIN 297			

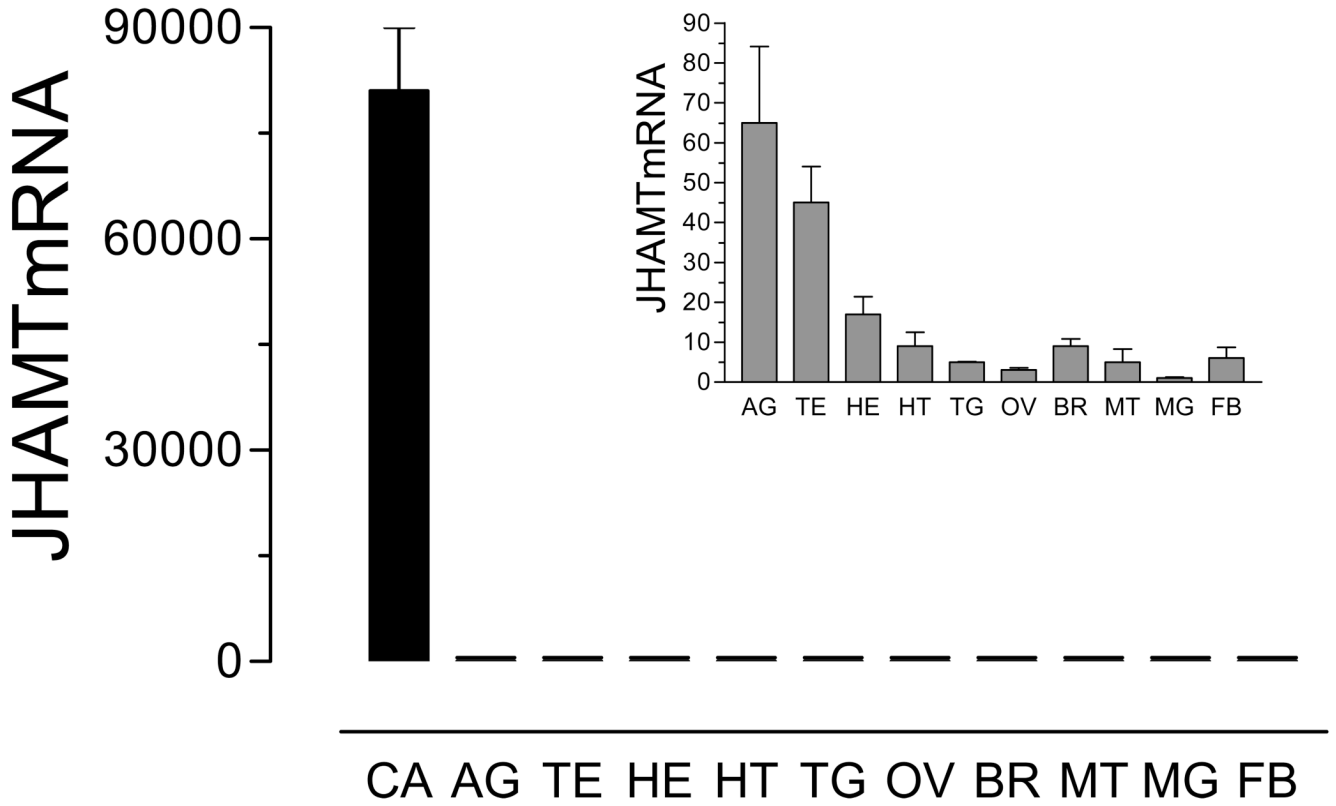


**Fig. 1. Juvenile hormone acid methyltransferase sequences and secondary structure comparison**  
**(A)** Multiple alignments of several insect juvenile hormone acid methyltransferases. Secondary structure elements are named according to the literature,  $\alpha$ -helices and  $\beta$ -sheets are blue and red colored respectively. The conserved SAM-binding region is highlighted by a brown box.  
**(B)** Schematic showing the expected topology of the fold of the *A. aegypti* juvenile hormone acid methyltransferase. Helices are shown as blue cylinders and  $\beta$ -sheets as red arrows.



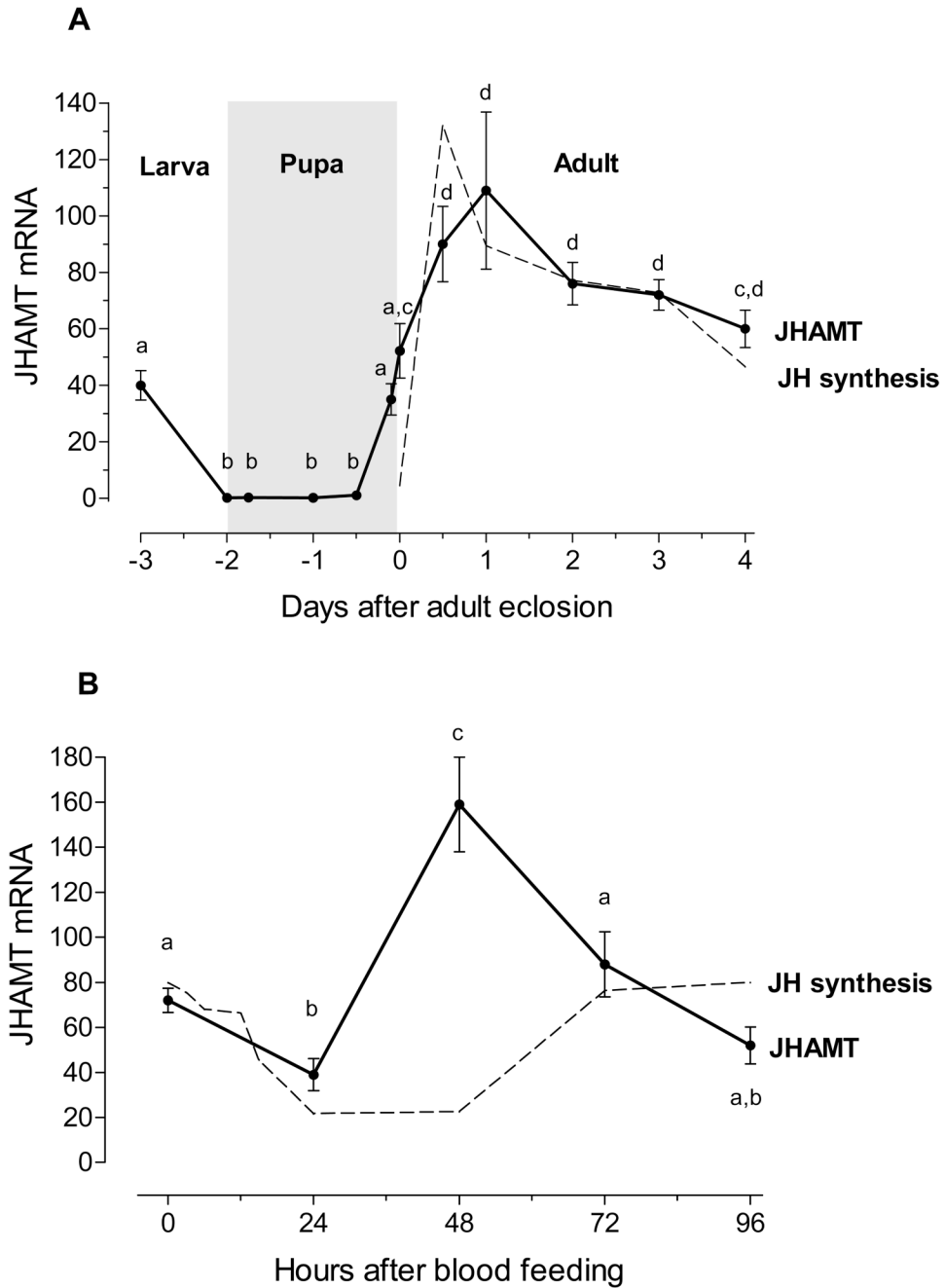
**Fig. 2. Enantioselectivity of *rAaJHAMT***

Chiral-HPLC analysis was performed with (A) racemic JH III, and (B) metabolites from racemic JHA III incubated with the purified *rAaJHAMT* protein. Arrows indicate (10*S*)-JH III (*S*: retention time 13.4 min) and (10*R*)-JH III (*R*: retention time 15.5 min), respectively. The *S*:*R* ratio of the racemic JHA III used as the substrate should have been ~50:50 (data not shown), as the JHA III was synthesized by alkaline hydrolysis from the racemic JH III showing 50:50 *S*:*R* ratio in (A). The JH III (0.83  $\mu$ g) produced from racemic JHA III (5  $\mu$ g) contained almost only (10*R*)-JH III (>99.5%) and trace amount of (10*S*)-JH III (B).



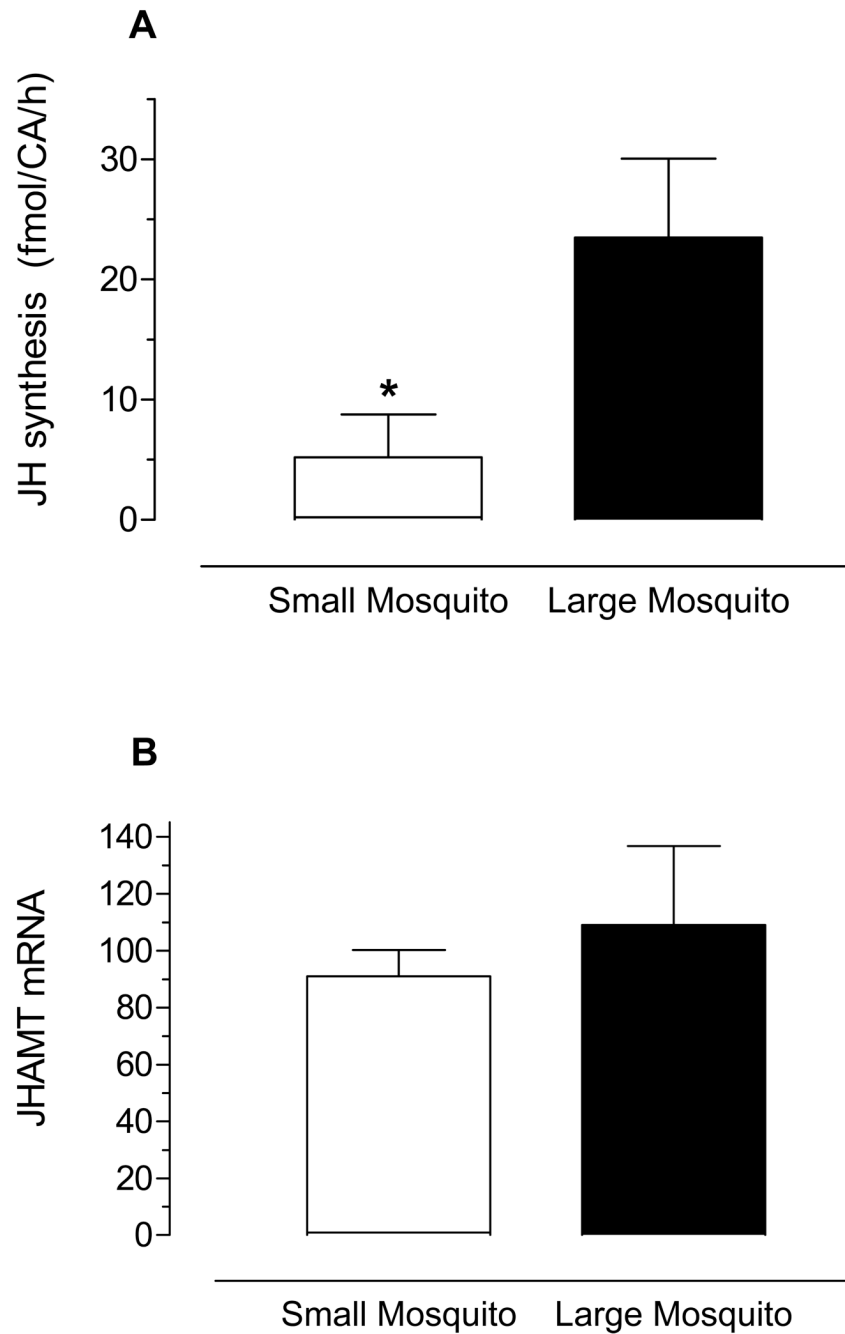
**Fig. 3. Tissue specific expression of *Aa*JHAMT**

All tissues were dissected from three-day old sugar-fed females, except for testis (TE) and accessory glands (AG) dissected from three-day old sugar-fed males. CA: corpura allata; HE: head; HT: heart; TG: thoracic ganglia; OV: ovaries; BR: brain; MT: Malpighian tubules; MG: midgut, and FB: fat body. The insert shows a detail of values of tissues with less expression. *Aa*JHAMT mRNA is expressed as copy number of *Aa*JHAMT mRNA/ 10,000 copies of rpL32 mRNA. Each RT-PCR data point is average of three independent biological replicates of 10–20 tissue samples.



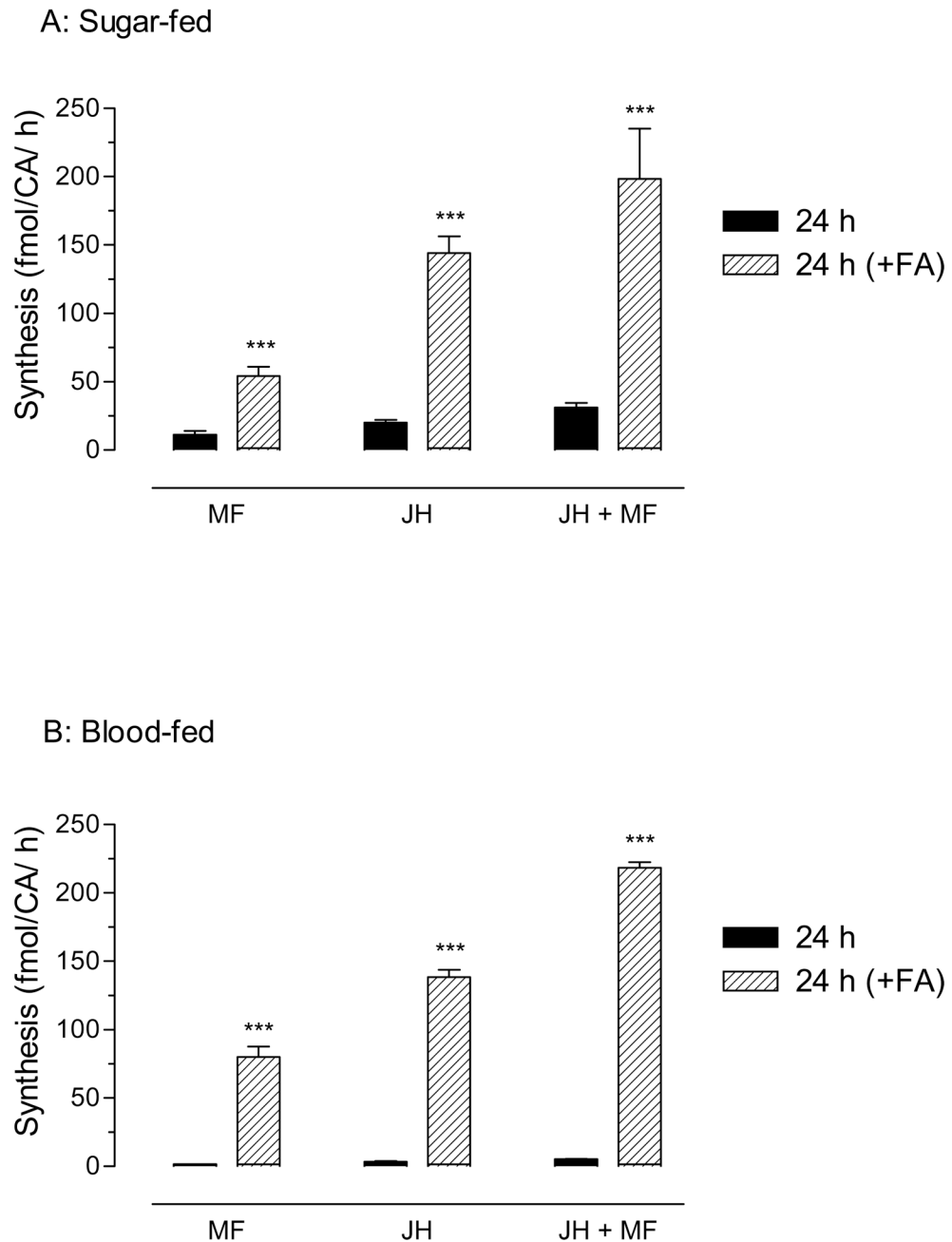
**Fig. 4. Developmental expression of *AaJHAMT***

**A)** Expression of *AaJHAMT* mRNA in thorax of newly-molted last instar larvae, pupa and sugar-fed females. **B)** Expression of *AaJHAMT* mRNA in thorax of blood-fed females. *AaJHAMT* mRNA (solid lane) is expressed as copy number of *AaJHAMT* mRNA/ 10,000 copies of *rpL32* mRNA. Each RT-PCR data point is average of three independent biological replicates of three insects. Values labeled with the different letters denote significant differences (unpaired *t*-test  $P \leq 0.05$ ). JH biosynthesis values (dotted lane) are based on Li et al., 2003a, and are expressed as relative levels.



**Fig. 5. Expression of *AaJHAMT* mRNA and JH synthesis in nutrient-deficient mosquitoes**  
**A)** JH biosynthesis was evaluated *in vitro* in CA dissected 24h after emergence, and it is expressed as femtomols per pair of glands per hour. Each bar represents the means  $\pm$  SEM of 5–10 independent determinations of individual CA (unpaired *t*-test;  $*P \leq 0.05$ ). **B)** *AaJHAMT* mRNA levels are expressed as copy number of *AaJHAMT* mRNA/ 10,000 copies of rpL32 mRNA. Each RT-PCR data point is average of three independent biological replicates of three insects. Large are control mosquitoes (dark bar) and small are nutrient-deficient mosquitoes (white bar).





**Fig. 6. Farnesoic acid methyltransferase activity in isolated corpora allata**

CA were incubated *in vitro* in culture medium alone (solid bars) or with 40  $\mu$ M FA (stripped bars). A) Sugar-fed: CA from 3-day-old sugar-fed females. B) Blood-fed: CA from females 24h after blood feeding. MF: methyl farneosate, JH: Juvenile hormone III. Bars represent the means  $\pm$  S.E.M. of 5–10 independent replicates of individual CA. (unpaired *t*-test; \*\*\* $P \leq 0.001$ ).

**Table 1**Enzymatic activity of purified recombinant *Aa*JHAMT on juvenile hormone acid III, farnesoic acid and two fatty acids

Substrate	Activity (mol /mol enzyme <sup>-1</sup> / min <sup>-1</sup> )
JHA III	1.033 ± 0.008
FA	0.260 ± 0.009
Palmitic acid	0.00089 ± 0.00012
Lauric acid	0.00010 ± 0.00017