Juvenile hormone acid O-methyltransferase in Drosophila melanogaster

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1

1 **Abstract (162 words)**

2 Juvenile hormone (JH) acid *O*-methyltransferase (JHAMT) is the enzyme that transfers a

3 methyl group from S-adenosyl-L-methionine (SAM) to the carboxyl group of JH acids to

4 produce active JHs in the corpora allata. While the *JHAMT* gene was originally identified

5 and characterized in the silkworm *Bombyx mori*, no orthologs from other insects have

6 been studied until now. Here we report on the functional characterization of the

7 *CG17330/DmJHAMT* gene in the fruit fly *Drosophila melanogaster*. Recombinant

8 DmJHAMT protein expressed in *E. coli* catalyzes the conversion of farnesoic acid and JH

9 III acid to their cognate methyl esters in the presence of SAM. *DmJHAMT* is

10 predominantly expressed in corpora allata, and its developmental expression profile

11 correlates with changes in the JH titer. While a transgenic RNA interference against

12 *DmJHAMT* has no visible effect, overexpression of *DmJHAMT* results in a pharate adult

13 lethal phenotype, similar to that obtained with application of JH analogs, suggesting that

14 the temporal regulation of *DmJHAMT* is critical for *Drosophila* development.

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Introduction

Juvenile hormones (JHs) are a family of sesquiterpenoid hormones that play a central role in the control of many of biological processes in insects, including development, growth and reproduction (Gilbert et al., 2000). JHs are synthesized *de novo* in specialized endocrine glands, the corpora allata (CA). A strict regulation of JH titer throughout insect's life is critical to its successful development and reproduction, and the biosynthetic activity in the CA is generally considered to be a major factor in the regulation of JH titer (Gilbert et al., 2000).

A number of genes responsible for the biosynthetic activity in the CA have been characterized (Shinoda and Itoyama 2003; Helvig et al., 2004; Noriega et al., 2006; Kinjoh et al., 2007). Among them, the *JHAMT* gene encodes the *S*-adenosyl-L-methionine (SAM)-dependent JH acid *O*-methyltransferase, which is the enzyme catalyzing the final step of the JH biosynthesis pathway in Lepidoptera (Shinoda and Itoyama 2003). It has been shown that JHAMT of the silkworm *Bombyx mori* (BmJHAMT) methylates the carboxyl group of JH I, II, and III acids (JHAs) to generate hormonally active JHs (Shinoda and Itoyama 2003). BmJHAMT also catalyzes the methylation of farnesoic acid (FA) to methylfarnesoate (MF), the putative JH in crustaceans and in some insect species, including the fruit fly *Drosophila melanogaster* (Jones and Jones 2007). *BmJHAMT* is specifically expressed in the CA, and the temporal gene expression profile of *BmJHAMT* strongly correlates to the JH biosynthetic activity of the CA, suggesting that the transcriptional control of the *BmJHAMT* gene is critical for the regulation of JH biosynthesis in the CA (Shinoda and Itoyama 2003; Kinjoh et al., 2007). As putative orthologs of *JHAMT* have been found in several insects (Shinoda and Itoyama 2003), the *JHAMT* genes appear to have a conserved role among species.

1 However, direct evidence for the significance of *JHAMT* in the regulation of JH

2 biosynthesis in insects other than *B. mori* is still missing.

3   Toward understanding the roles of JHAMT in insect development via the

4 regulation of JH biosynthesis *in vivo*, here we report on the identification and the

5 functional characterization of the *Drosophila melanogaster* ortholog of *JHAMT*. We

6 show that purified recombinant DmJHAMT, like BmJHAMT, catalyzes the conversion of

7 JHA III and FA to JH III and MF, respectively, in the presence of SAM. *DmJHAMT* is

8 predominantly expressed in the CA, and its developmental expression profile correlates

9 well with changes in the JH titer during *Drosophila* development. Furthermore,

10 overexpression of *DmJHAMT* leads to pupal lethality and a misorientation of male

11 genitalia, which are similar to phenotypes obtained following application of JH analogs.

12 These results suggest that the temporal control of *DmJHAMT* activity is critical for

13 *Drosophila* development.

14

15

16 **Materials and methods**

17

18 **Animal strains and culture**

19 All *Drosophila melanogaster* flies were reared on a standard medium at 25 ºC under a

20 12-h light/12-h dark photoperiod. The *D. melanogaster* strains, *AUG21-GAL4*

21 (Siegmund and Korge 2001) and *Actin5c-GAL4* (originally established by Dr. Y. Hiromi),

22 were provided from Dr. G. Korge and the Bloomington stock center, respectively.

23

24 **Chemicals**

25 Racemic JH III was purchased from Sigma and purified by HPLC before use as described

4

1 (Shinoda and Itoyama 2003). JH III acid (JHA III) was prepared from the purified

2 racemic JH III as described (Goodman and Adams 1984). Farnesoic acid (FA;

3 (*2E,6E,10E*)-3,7,11-trimethyldodecatri-2,6,10-eneoic acid) and methyl farnesoate (MF;

4 Methyl-(*2E,6E,10E*)-3,7,11-trimethyldecatri-2,6,10-eneoate) were purchased from

5 Echeron Biosciences. A synthetic JH analog, methoprene, was a gift from Dr.

6 Ehrenstorfer-Schafers (Augsburg, Germany). JH I was purchased from SciTech (Prague,

7 Czech Republic). Other reagents were purchased from commercial suppliers as described

8 (Shinoda and Itoyama 2003).

9

10 **Molecular cloning**

11 *Drosophila JHAMT* (*DmJHAMT*) was identified from the *Drosophila* genome sequence

12 by a BLAST search and corresponds to the gene annotated as *CG17330*

13 (www.FlyBase.org). The cDNA containing the entire open reading frame (ORF) for

14 *DmJHAMT* was amplified by RT-PCR. RNA was extracted from the Oregon R strain

15 using the RNAeasy Mini kit (Qiagen). After a reverse-transcription reaction using

16 First-strand cDNA synthesis kit (Amersham-Pharmacia) with an oligo dT primer, PCR

17 was performed using the following primers: DmJHAMT-F,

18 5´-AAACATATGAATCAGGCCTCTCTATATCAG-3´; DmJHAMT-R, 5´-

19 AACTCGAGGACTCTGTTAACAAATGCAATTACTG-3´. The PCR product was

20 cloned into a TA-cloning vector, pDrive (Qiagen). The DNA sequence of *DmJHAMT* was

21 deposited in GenBank (Accession no. **AB113579**).

22

23 **Protein expression**

24 The fragment containing the *DmJHAMT* ORF was excised from a *Nde*I/*Xho*I-digested

25 *DmJHAMT*-pDrive and then cloned into a *Nde*I/*Xho*I-digested pET28a(+) (Novagen).

1   BL21(DE3) *E. coli* cells (Novagen) were then transformed with *DmJHAMT*-pET28a(+)

2   for protein expression. Preparation and purification of recombinant 6xHis-tagged protein

3   was performed essentially as described (Shinoda and Itoyama 2003). After the protein

4   purification with a HiTrap Chelating column HP (GE Healthcare), glycerol was added to

5   the enzyme solution (final concentration 25%), and the sample was frozen immediately in

6   liquid N_2 and stored at -80 ºC until use.

7

8   **Antiserum and western blot**

9   A polyclonal antiserum against the DmJHAMT was prepared by immunizing a rabbit

10 with 0.2 mg of the purified recombinant DmJHAMT protein by 6 injections. Western blot

11 analysis was performed with the anti-DmJHAMT antiserum (1:5000 dilution) and

12 peroxidase-conjugated anti-Rabbit IgG (Pierce; 1:10,000 dilution), followed by detection

13 using the TMB Membrane Peroxidase Substrate System (KPL). Precision plus protein

14 standards (BIO-RAD) were used as molecular markers.

15

16 **Enzyme assay and analysis**

17 Enzyme assays were performed essentially as described with some modifications

18 (Shinoda and Itoyama 2003). Briefly, JHA III (50 μM), FA (50 μM), lauric acid (LA; 100

19 μM), or palmitic acid (PA; 100 μM) were dissolved individually in 500 μl of Tris-Cl

20 buffer (50 mM, pH 7.5), with or without 500 μM SAM in a siliconized glass tube (12 x 75

21 mm). The enzymatic reactions were started by the addition of 2 μg (for JHA III and FA)

22 or 10 μg (for LA and PA) of the purified DmJHAMT protein. After incubation at 25 ºC for

23 5 min (for JHA III and FA) or 60 min (for LA and PA), the reactions were stopped by the

24 addition of 500 μl of CH$_3$CN. Under these conditions, the rate of product formation was

25 linear during the assays (data not shown). The reaction mixture was centrifuged at 4,200

6

1   *g* for 5 min, and the supernatant was subjected to qualitative and quantitative analyses of

2   the products by HPLC and GC-MS.

3   JH III and MF generated from JHA III and FA, respectively, were analyzed by

4   reversed-phase (RP)-HPLC with a Shimadzu LC10 apparatus and a Shiseido ODS UG80

5   column (3 x 150 mm). The conditions were: mobile phase, 60% CH_3CN for JH III (Fig. 2,

6   A-C) and 80% CH_3CN for MF (Fig. 2, D-F); flow, 0.5 ml/min; detector, UV at 219 nm.

7   Stereospecificity of the enzymatically produced JH III was analyzed by a chiral-HPLC

8   (Ichikawa et al., 2007) using a HP1100 series HPLC system (Hewlett-Packard) and a

9   chiral-column, Chiralpack IA (4.6 x 250 mm, DAICEL). Supernatant from the reaction

10   with JHA III was extracted with hexane and concentrated under N_2 gas stream and

11   subjected to chiral-HPLC. The HPLC conditions were: mobile phase, hexane:EtOH,

12   99:1; flow, 0.5 ml/min; detector, UV at 219 nm.

13   Products from LA and PA were analyzed by GC-MS. Supernatants of the

14   reactions with LA and PA were extracted with hexane containing 5 μg/ml

15   methyltridecanoate as an internal standard, concentrated under N_2 gas stream and

16   analyzed with GCMS-QP2010 (Shimadzu) equipped with a GC column DB-35MS (30 m

17   x 0.25 mm I.D., 0.25 μm film, J&W Scientific). The carrier gas was helium (1.2 ml/min),

18   and the injector port temperature was 280 ºC. The samples were introduced by split

19   injection. The column oven temperature was programmed at 120 ºC for 2 min before

20   being elevated to 240 ºC at 12 ºC increase per min and then held for 10 min. EI-MS

21   detector was set at 70-eV ionization with selective ion monitoring (SIM) mode on m/z at

22   74.

23

24   **Reverse transcription (RT)-PCR analysis**

25   Total RNA preparation derived from whole fly bodies and single-stranded cDNA

1   synthesis were performed as previously described (Niwa et al., 2004). Specific primers

2   for *DmJHAMT* (forward, 5´-GACCATGTCACCTCGTTCTACTGC-3´;   reverse,

3   5´-GAAGTCATCCAGGAACTGTTCATGC-3´) and *juvenile hormone esterase* (*jhe*)

4   (forward,   5´-GGTGAACATTCTGGGCAATGAGACG-3´;   reverse,

5   5´-GTGACTGGAGCACCTCAATGGAG-3´) were used. *rp49* was used as a loading

6   control (Niwa et al., 2004). Quantitative RT-PCR was performed as previously described

7   (Shinoda and Itoyama 2003). PCR conditions for data shown in Fig. 3B, 3I and 4A were

8   94 ºC for 5 min, followed by 34 cycles of 94 ºC for 30 sec, 57 ºC for 30 sec, and 72 ºC for

9   1 min.

10

11   **Transgenic flies**

12   Overexpression and RNA interference (RNAi) studies were performed using the

13   GAL4/UAS system (Brand and Perrimon 1993). The construct for *DmJHAMT*

14   overexpression (*UAS-DmJHAMT*) was generated by first amplifying an ORF from

15   *DmJHAMT*-pDrive   by   PCR   using   the   primers   DmJHAMT-F/BglII

16   (5'-AAAAGATCTATGAATCAGGCCTCTCTATATCAG-3') and DmJHAMT-R, and

17   ligating the product into the *Bgl*II/*Xho*I site of the pUAST vector (Brand and Perrimon

18   1993). The construct for transgenic RNAi (Kennerdell and Carthew 2000) against

19   *DmJHAMT* was generated from the 4-677 bp region of exon and intron of the *DmJHAMT*

20   gene (nucleotide numbering as in **AB113579**). The cDNA region was amplified by PCR

21   using   the   primers   DmJHAMT[655-635]/XhoI

22   (5´-AAACTCGAGCTTTCAGAGTCCTTACACCT-3´)   and   DmJHAMT[7-26]/XbaI

23   (5´-AAATCTAGAAATCAGGCCTCTCTATATCA-3´). The genomic region, containing

24   a part of the 3´ end of the first intron, was amplified by PCR using primers:

25   DmJHAMT[7-26]/BglII   (5´-AAAAGATCAATCAGGCCTCTCTATATCA-3´)   and

1   DmJHAMT[744-725]/XhoI   (5´-AAACTCGAGAAAGGACAAATGGCCTTTAC-3´).

2   The cDNA and genomic fragments were digested by *Xho*I/*Xba*I and *Bgl*II/*Xho*I,

3   respectively, and then ligated into pUAST digested with *Bgl*II/*Xba*I. *Drosophila*

4   transformants were obtained using standard protocols (Spradling 1986).

5

6   **Topical application of JH and its analog**

7   Methoprene (5 ng) or JH I (25 ng) diluted in 100% ethanol was applied to wandering

8   Oregon R 3rd instar larvae, as the only sensitive period for application is during late

9   larval-early pupal development (Postlethwait and Weiser 1973).

10

11   **Histological analyses**

12   *In situ* RNA hybridization and immunostainng were performed as described (Niwa et al.,

13   2004). Digoxigenin (DIG)-labeled RNA probes were synthesized from

14   *DmJHAMT*-pDrive using a DIG RNA labeling kit (Roche) and T3 or SP6 RNA

15   polymerase (Invitrogen). Under our conditions, no signal was detected using a sense

16   RNA probe (data not shown). For immunostaining, the rabbit anti-JHAMT antiserum was

17   applied at a 1:200 dilution in PBS at 4 ℃ and incubated overnight. The signal was

18   visualized using the Alexa488-coupled secondary antibody (Molecular Probes) at a 1:200

19   dilution. Alexa568-conjugated phalloidin (Molecular Probes) was also used. Specimens

20   were observed under a laser scanning confocal microscope LSM510 (Zeiss). For

21   observations using the scanning electron microscope (SEM), pharate adults were

22   dissected from pupara, glued on a SEM stage with a regular bond, and then observed with

23   a S-3000N SEM (Hitachi) under a low vacuum condition.

24

25

Results and Discussion

CG17330 encodes functional SAM-dependent JH acid *O*-methyltransferase (DmJHAMT)

A tBlastn search has revealed that *CG17330* in *Drosophila melanogaster* is the only gene encoding a predicted protein with substantial similarity to the BmJHAMT protein (41%) (Shinoda and Itoyama 2003). The predicted protein CG17330 contains a SAM binding motif (LLDIGCGSG) that is commonly found in members of SAM-dependent methyltrasnferases (Wu et al., 1992). We hereafter refer to this gene as *DmJHAMT* (*Drosophila melanogaster homolog of JHAMT*).

The *D. melanogaster* CA produces JH III, JH III bisepoxide (JHB$_3$), and MF as major products (Jones and Jones 2007). We investigated whether DmJHAMT introduced the methyl group to the immediate precursors of JH III and MF, namely JHA III and FA, respectively, *in vitro*. We expressed His-tagged recombinant DmJHAMT protein in *E. coli* and prepared a purified protein using a Ni-column (Fig. 1A). When JHA III and FA were incubated with the purified DmJHAMT protein in the presence of SAM, a major peak was observed in the reactions by reverse-phase (RP)-HPLC (Fig. 2 B and E). The retention times of the major metabolites of JHA III and FA corresponded to the standard JH III and MF, respectively (Fig. 2C and F). These peaks were further confirmed to be JH III and MF, respectively, by GC-MS (data not shown). In contrast, these peaks were not observed in the same reactions but lacking SAM (Fig. 2A and D). The catalytic activity of DmJHAMT against JHA III and FA were nearly equal (Table I). Preliminary kinetic analyses revealed that *Km* (μM) and *Vmax* (mol product/mol enzyme/min) values were 3.32±2.28 and 5.58±1.66 against racemic JHA III, and 0.18±0.03 and 5.77±0.90 against FA (mean±SD, n=3). When the DmJHAMT protein was incubated with saturated

1   long-chain fatty acids, laurate acid (LA) and palmitate acid (PA), in the presence of SAM,

2   the production of low levels of methyl laurate and methyl palmitate was detected by

3   GC-MS, respectively. However, the catalytic activities of DmJHAMT for LA and PA

4   were less than 1% that for FA (Table I). These results suggest that, like BmJHAMT

5   (Shinoda and Itoyama 2003), DmJHAMT is highly specific to farnesoid acids, such as FA

6   and JHA III, but not to non-branched fatty acids.

7   We also examined whether the DmJHAMT protein was enantioselective. Such

8   enantioselectivity has been documented in a study on an JHAMT activity in *Cecropia*

9   (Peter et al., 1981), whereas it has been found absent in *Locusta* has been reported

10   (Hamnett et al., 1981). In our experiment, we used racemic JHA III prepared by an

11   alkaline hydrolysis method (Goodman and Adams 1984) from racemic JH III (Fig. 2G).

12   However, chiral-HPLC analysis revealed that the metabolite from the JHA III racemate

13   (25 μM for each *R* and *S* isomer) by DmJHAMT contained *(10S)-* and *(10R)-*JH III

14   isomers at a ratio of 20:80 (Fig. 2H). Under the same conditions, recombinant

15   BmJHAMT generated *(10S)-* and *(10R)-*JH III isomers at a ratio of 2:98 (Shinoda,

16   unpublished data). This result indicates that DmJHAMT has moderate enantioselectivity

17   to *(10R)-*JHA III. Although the absolute configuration of JH III in *D. melanogaster*

18   remains to be determined, JHB_3 produced by another Dipteran, *Lucilia cuprina*, has the

19   absolute configuration 10*R* (Herlt et al., 1993). The stereospecificity of DmJHAMT is

20   consistent with an idea that the *(10R)-*isomer is also the natural form of JH III in *D.*

21   *melanogaster*.

22   These results demonstrate that *DmJHAMT/CG17330* encodes a functional

23   JHAMT involved in the production of JH III and MF from JHA III and FA, respectively.

24   JHB_3, the major JH in *Drosophila*, is expected to be synthesized by the methylation of

25   6,7;10,11-bisepoxy farnesoic acid (JHB_3 acid) to JHB_3, rather than by the epoxidation of

MF and JH III (Moshitzky and Applebaum 1995). DmJHAMT is thus likely to also catalyze the methylation of JHB_3 acid to JHB_3.

Tissue- and stage-specific expression of *DmJHAMT*

The temporal expression profile of *DmJHAMT* was examined by quantitative real-time RT-PCR analysis. The expression level of *DmJHAMT* was high in the 1st instar larvae and then gradually decreased during larval development, while the significant re-increase of the *DmJHAMT* expression was observed in the wandering 3rd larval stage (Fig. 3A). The lowest amount of expression was observed in the early- and mid-pupal stages (Fig. 3A). After this, the expression increased in the late pupal stage in both male and female adults (Fig. 3A). This overall change in the mRNA expression level of *DmJHAMT* positively correlated with changes in hemolymph JH titers during *Drosophila* development (Riddiford 1993). RT-PCR analysis also revealed that *DmJHAMT* was predominantly expressed in larval tissues containing CA, and a trace amount was also detected in the testis (Fig. 3B).

To further analyze spatial localization of *DmJHAMT* during development, *in situ* RNA hybridization and immunostaining were performed. In embryogenesis, no *DmJHAMT* expression was detected in the blastoderm, gastrulation and germ band elongation stages (data not shown). *DmJHAMT* expression was first seen at the germ band retraction stage (stage 13) in somatic muscles (Fig. 3C). Then *DmJHAMT* disappeared in all tissues and reappeared in primordial CA at stage 17 (Fig. 3D). In addition to being expressed in late embryogenesis, *DmJHAMT* mRNA was expressed specifically in larval CA cells but not in the prothoracic gland or corpora cardiaca cells of the ring gland, or other tissues examined except the testis (Fig. 3B and E). For immunostaining, we generated a specific antiserum against DmJHAMT protein (Fig. 1B).

1   Using the antiserum, DmJHAMT was also observed predominantly in the CA in both the

2   larval and adult stages (Fig. 3F and H). These results indicate that the expression of

3   *DmJHAMT* coincides with JH biosynthesis in both a spatial and temporal manner, which

4   is similar to the characteristics of *BmJHAMT* (Shinoda and Itoyama 2003). It is also of

5   note that, to our knowledge, this is the first identification of a gene expressed

6   predominantly in the CA of *D. melanogaster*.

7

8   **Overexpression of *DmJHAMT* causes a pupal lethality and a rotation defect on male**

9   **genitalia**

10 To assess the importance of *DmJHAMT* during *Drosophila* development, we examined

11 gain-of-function phenotypes in developing flies. We found that the overexpression of

12 *DmJHAMT* specifically in the CA using flies carrying *UAS-DmJHAMT* and

13 *AUG21-GAL4*, which is active in the CA cells (Siegmund and Korge 2001; Adám et al.,

14 2003), showed no irregular phenotype. In contrast, we found that individuals

15 overexpressing *DmJHAMT* using a ubiquitous *Actin5c* promoter failed to develop into

16 adults. The flies carrying *Actin5c-GAL4* and *UAS-DmJHAMT* (*Actin5c>DmJHAMT*)

17 consistently exhibited higher expression of *DmJHAMT* through the wandering larval and

18 pupal stages, as compared to wild type (Fig. 4A). Under our experimental conditions,

19 wild type flies took 4 days from puparium formation to eclosion, and there was no

20 significant mortality at the pharate adult stage (P4) in wild type animals (<1%; n=99). In

21 contrast, the *Actin5c>DmJHAMT* animals completed embryogenesis and larval

22 development normally (data not shown), but showed a prolonged pupal development.

23 The majority (97%; n=110) of the *Actin5c>DmJHAMT* flies became pharate adults 9

24 days after puparium formation (P9) and all died before eclosion (100%; n=107). The

25 phenotype of the *Actin5c>DmJHAMT* animals were reminiscent of wild type animals

13

1   topically treated with JH analogs, which also exhibit no effect during the larval stage but

2   show lethality at the pharate adult stage (Riddiford and Ashburner 1991). To address the

3   question whether the *Actin5c>DmJHAMT* animals exhibited another class of phenotypes

4   that is related to higher doses of JH, male genitalia of the *Actin5c>DmJHAMT* animals

5   were also examined. It has been shown that either the application of high doses of JH

6   analogs or the presence of mutation presumed to result in higher JH titer *in vivo* could

7   cause a rotation defect of male genitalia (Riddiford and Ashburner 1991; Adám et al.,

8   2003). Interestingly, the *Actin5c>DmJHAMT* animals (70%; n=10) also displayed

9   rotation defects (Fig. 4, B and C) that are very similar to those observed in flies with high

10   doses of JH I (Fig. 4D) or methoprene (data not shown). These observations support the

11   idea that the forcible expression of *DmJHAMT* causes the overproduction of active JH

12   and results in abnormalities during pupal-adult development, a time when JH must be

13   absent.

14   Our data demonstrate that overproduction of DmJHAMT in the whole body, but

15   not CA, affects *Drosophila* pupal development. The expression of hemolymph JH

16   esterase (JHE), which catalyzes the catabolism of active JHs into JHAs, significantly

17   increases during the pupal stage (Fig. 3I) (Kethidi et al., 2005). Therefore, one possible

18   interpretation is that, even if the CA of the *AUG-21>DmJHAMT* flies continue to

19   synthesize active JHs in the wandering and pupal stages, the hemolymph JHE is sufficient

20   to inactivate JHs before they reach to the target tissues. On the other hand, the

21   *Actin5c>DmJHMAT* animals express *DmJHAMT* in almost all somatic cells. Therefore,

22   even after JHE inactivates JHs and produces JHAs in hemolymph, DmJHAMT in the

23   peripheral target tissues can convert JHAs to active JHs, resulting in a phenotype that is

24   similar to phenotypes caused by JH application. In addition, our results suggest another

25   possibility that JHE plays a more important role than JH epoxide hydrolase (JHEH) in the

1 control of JH titer at the pupal stage as previously reported (Khlebodarova et al., 1996),

2 because JH acids generated by JHE, but not JH diols metabolized by JHEH, can=be

3 converted to active JHs.

4

5 **Knock-down of *DmJHAMT* by transgenic RNAi**

6 Since a *DmJHAMT* genetic mutant is not currently available, we examined the effects of

7 the knock-down of *DmJHAMT* using a transgenic RNAi technique that is known to be

8 effective in degrading endogenous target mRNA in *Drosophila* (Kennerdell and Carthew

9 2000). We established transgenic lines in which double-stranded RNA molecules

10 corresponding to *DmJHAMT* were generated using an inverted repeat construct under the

11 control of the *UAS* promoter. To knock down *DmJHAMT* specifically in the CA, the *UAS*

12 lines were crossed with *GAL4* lines, *AUG21-GAL4*, in which the *GAL4* transgene is

13 active in the CA cells (Siegmund and Korge 2001; Adám et al., 2003). The DmJHAMT

14 protein level in the CA of the RNAi larvae significantly decreased and was undetectable

15 by immunostaining with anti-DmJHAMT antibody (Fig. 3G). The *DmJHAMT* RNAi

16 animals, however, exhibited no visible effect on *Drosophila* development (data not

17 shown). We did not find any irregular phenotype in *Actin5c-GAL4* and *UAS-RNAi*

18 constructs either (data not shown). The absence of a phenotypic effect from the RNAi

19 treatment was puzzling given that the experimental removal of JH causes premature

20 metamorphosis in insects (Riddiford 1996). It is possible that this is due to a peculiarity of

21 the cyclorrhaphous Diptera, including *Drosophila*, where exogenous JH does not show

22 the typical "status quo" effect on larval development that normally occurs in other insects

23 (Gilbert et al., 2000). Alternatively, it is important to point out that RNAi, in general,

24 results in partial, but not complete, loss-of-function animals. Therefore, even though

25 DmJHAMT protein was undetectable by immunostaining in the CA of the RNAi animals,

1 it is still possible that the residual DmJHAMT activity is enough to produce small but

2 sufficient amount of JH to maintain larval status in *Drosophila*. This hypothesis is

3 supported by the fact that flies overexpressing JHE under the *DmJHAMT* RNAi

4 background cause premature wandering behavior, which may indicate early

5 metamorphosis (E. Gervasio and J.-P. Charles, unpublished data). Isolation and study of

6 genetic null mutants of *DmJHAMT* are necessary in order to determine the function of

7 *DmJHAMT* in more detail.

8

9 **Conclusion**

10 Our study demonstrated that the *CG17330/DmJHAMT* gene encodes a JH acid

11 *O*-methyltransferase that is specifically expressed in the CA and functions to produce MF

12 and JH III, and most likely JHB$_3$. This is the first *JHAMT* gene to be functionally

13 characterized in Diptera. Furthermore, transgenic flies overexpressing *DmJHAMT*

14 suggest that the proper temporal regulation of this gene is critical for *Drosophila*

15 development. Transgenic flies overproducing or reducing *DmJHAMT* have promise to

16 reveal hidden functions of JH in this species and greatly contribute to the dissection of the

17 molecular mode of JH action.

18

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9

10

1 **Figure legends**

2

3 **Fig. 1. Preparation of recombinant DmJHAMT protein expressed in *E. coli***

4 (A) Coomassie brilliant blue (CBB) staining of recombinant DmJHAMT expressed in *E.*

5 *coli*. Samples were: lane 1, crude supernatant from BL21(DE3)/pET28a (empty vector);

6 lane 2, crude supernatant from BL21(DE3)/pET28a/DmJHAMT; and lane 3, DmJHAMT

7 (marked by arrowhead) purified from the product shown in lane 2 by Ni-column. The

8 amounts loaded were equivalent to 100 μl of *E. coli* culture in lane 1 and 2; and 0.3 μg of

9 the purified DmJHAMT in lane 3. M, molecular size marker. (B) Western blot using

10 anti-DmJHAMT antiserum. 1/10 volume of the amounts shown in A were loaded. The

11 specific signal of DmJHAMT is marked with an arrowhead.

12

13 **Fig. 2. Recombinant DmJHAMT methylates JHA III and FA *in vitro***

14 (A-F) RP-HPLC analyses were performed after the incubation of the purified

15 recombinant DmJHAMT protein and substrates. Vertical axes represent UV absorption at

16 219 nm. Metabolites generated from JHA III and FA by recombinant DmJHAMT, in the

17 absence (A and D, respectively) and presence of SAM (B and E, respectively) are shown.

18 Arrows and arrowheads indicate JH III and MF peaks, respectively. The retention times

19 of enzymatically produced JHs (11.5 min for JH III and 11.7 min for MF) were identical

20 to those of standards (C and F). (G, H) Chiral-HPLC analysis was performed with racemic

21 JH III (G) and metabolites obtained from incubation of racemic JHA III with purified

22 DmJHAMT protein and SAM after a 4 min reaction (H). It should be noted that the *S:R*

23 ratio of the racemic JHA III used as substrate was ~50:50 (data not shown), as the JHA III

24 was synthesized by alkaline hydrolysis from the racemic JH III shown in (G) (Goodman

1 and Adams 1984). Arrows and arrowheads indicate (*10S*)-JH III (retention time: 14.0

2 min) and (*10R*)-JH III (retention time: 16.4 min), respectively.

3

4 **Fig. 3. Spatiotemporal expression pattern of *DmJHAMT* mRNA and protein**

5 (A) Quantitative RT-PCR analysis showing temporal expression profile of *DmJHAMT*

6 and a reference gene, *rp49*. *DmJHAMT/rp49* indicates the levels of *DmJHAMT* mRNA

7 normalized to the levels of internal *rp49* mRNA. Total RNAs were extracted from whole

8 fly bodies in each stage. Embryos (E) and 1st (L1), 2nd (L2) and 3rd (L3) instar larvae

9 were collected at 12 h intervals from 0 hours after egg laying (AEL) to 144 h AEL. Pupae

10 (P) were also collected at 12 h intervals from 0 h after puparium formation (APF) to 96 h

11 APF. Adults (Ad) were collected 0-12 h after eclosion. (B) RT-PCR analysis showing

12 tissue expression profile in wandering 3rd instar larvae. (C-E) *in situ* RNA hybridization

13 of *DmJHAMT*. (C, D) Embryonic expression. Signals were detected in somatic muscles

14 at stage 13 (C) and in embryonic CA at stage 17 (D, arrowhead). (E) Brain-ventral nerve

15 cord-ring gland complex of the wandering stage of 3rd instar larvae. Expression was

16 detected only in the region of the CA (arrowhead). (F-H) Imunostaining with

17 anti-DmJHAMT antibody (green). Overall morphology of tissues was visualized with

18 fluorescence-phalloidin (purple). Arrowheads indicate CA. (F, G) 3rd instar larval ring

19 gland. (F) DmJHAMT protein was localized in the CA of a control animal

20 (*yw;AUG21-GAL4/+*). (G) No signal was detected in the CA of *DmJHAMT* RNAi

21 animals (*yw; AUG21-GAL4; UAS-DmJHAMT-IR*). (H) CA in wild type adult males. es,

22 esophagus; pv, proventriculus. (I) RT-PCR analysis showing the expression profile of *JH*

23 *esterase (jhe)* and *rp49*. The samples are the same as shown in A.

24

25 **Fig. 4. Overexpression of *DmJHAMT* causes rotation defect of male genitalia**

1   *yw; UAS-DmJHAMT/+; Actin5c-GAL4/+* was used as the overexpressor of *DmJHMAT*

2   (*DmJHAMT* o/e). (A) RT-PCR analysis showing the expression of *DmJHAMT* and a

3   reference gene *rp49* in control animals, *yw; UAS-DmJHAMT/+* (left), and *DmJHAMT* o/e

4   (right). (B-D) Scanning electron micrographs of male abdomen (ventral view; posterior is

5   downwards). The direction of genitalia rotation in each sample is marked by an arrow,

6   whose starting point is at the position of the penis. (B) A control animal (*yw;*

7   *UAS-DmJHAMT*). (C) The *DmJHAMT* o/e animal. (D) A wild type (Oregon R) with

8   topical application of 25 ng JH I at wandering 3rd instar larval stage.
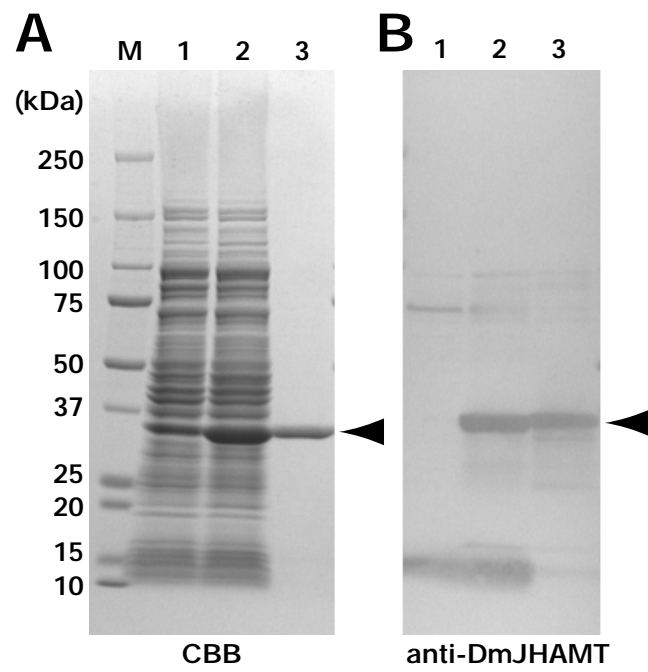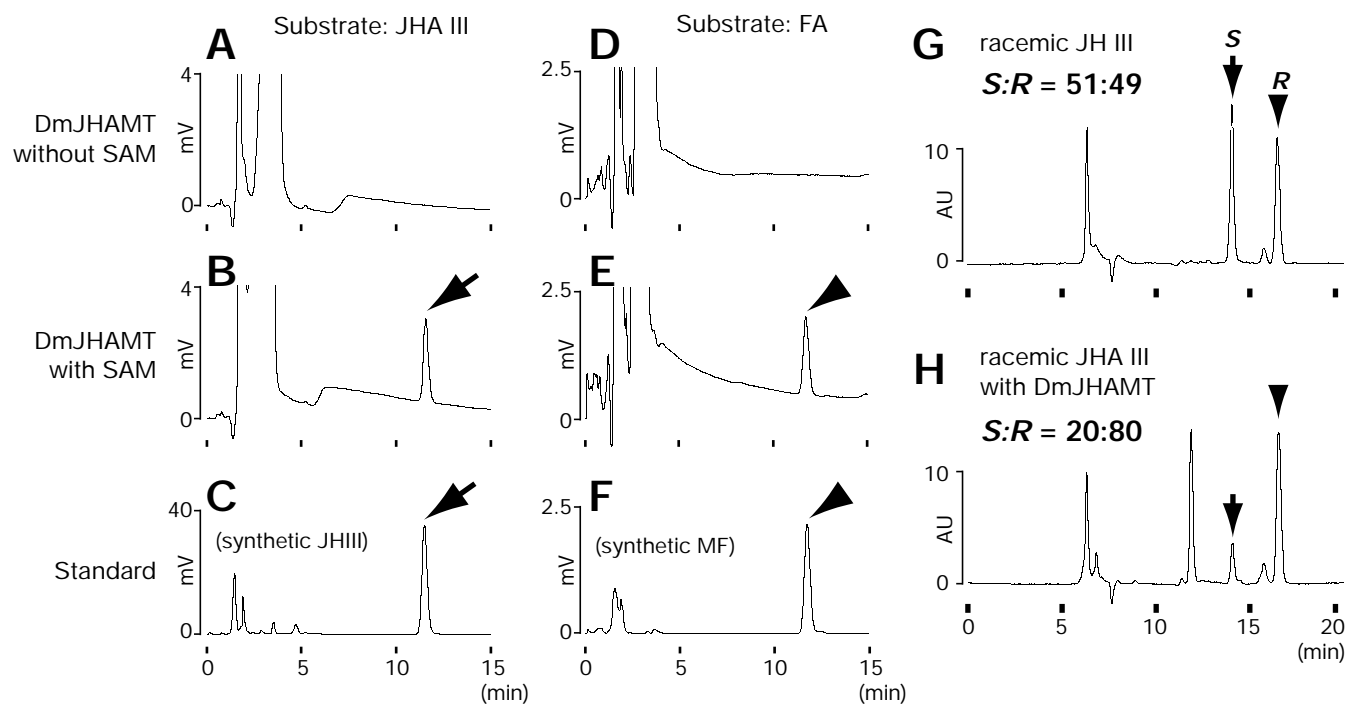
9

Fig. 1
Niwa et al.

A

M   1   2   3

(kDa)

250

150

100
75

50

37

25
20

15
10

CBB

B   1   2   3

anti-DmJHAMT

Figure 2

Fig. 2
Niwa et al.

A Substrate: JHA III

DmJHAMT without SAM

B DmJHAMT with SAM

C Standard (synthetic JHIII)

D Substrate: FA

DmJHAMT without SAM

E DmJHAMT with SAM

F (synthetic MF)

G racemic JH III
S:R = 51:49

H racemic JHA III with DmJHAMT
S:R = 20:80

Figure 3

Fig. 3
Niwa et al.

Figure 4

Fig. 4
Niwa et al.

A

| | L3 | P1 | P2 | P3 | P4 | | L3 | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |

DmJHAMT

rp49

wild type   *DmJHAMT* o/e

B   **C**   **D**
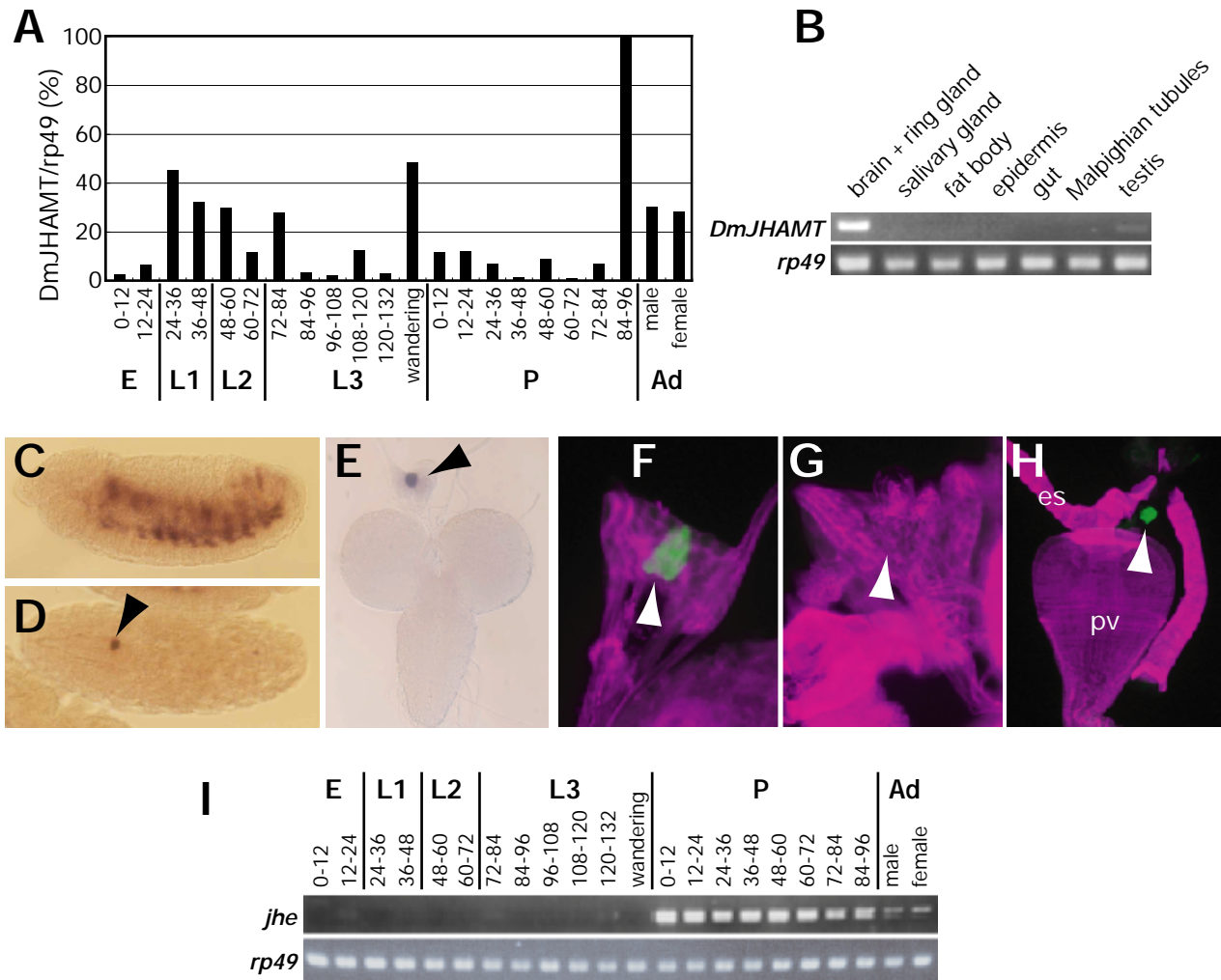
wild type   *DmJHAMT* o/e   JH I
application

Table 1

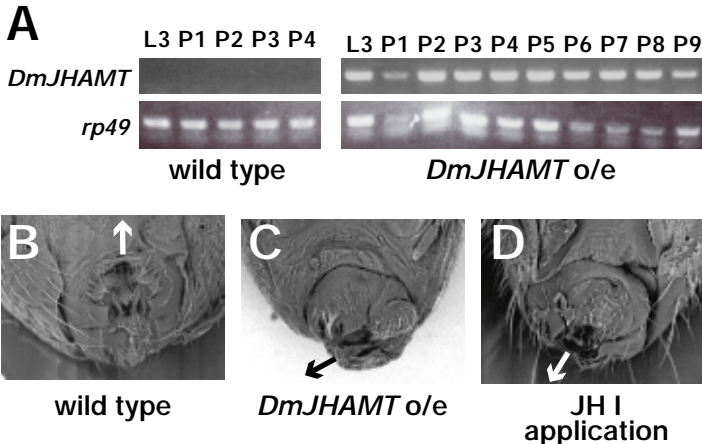**Table I. Enzymatic activity of recombinant DmJHAMT on JH III, FA and fatty acids**

Substrates	k_{cat} (min^{-1}) (mean±SD, n=3)
JH III acid (racemate)	7.7 ± 0.4
Farnesoic acid	10.1 ± 0.5
Laurate acid	0.103 ± 0.007
Palmitate acid	0.041 ± 0.008