



Final steps in juvenile hormone biosynthesis in the desert locust, *Schistocerca gregaria*

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ABSTRACT

Two genes coding for enzymes previously reported to be involved in the final steps of juvenile hormone (JH) biosynthesis in different insect species, were characterised in the desert locust, *Schistocerca gregaria*. Juvenile hormone acid *O*-methyltransferase (JHAMT) was previously described to catalyse the conversion of farnesoic acid (FA) and JH acid to their methyl esters, methyl farnesoate (MF) and JH respectively. A second gene, *CYP15A1* was reported to encode a cytochrome P450 enzyme responsible for the epoxidation of MF to JH. Additionally, a third gene, *FAMeT* (originally reported to encode a farnesoic acid methyltransferase) was included in this study. Using q-RT-PCR, all three genes (*JHAMT*, *CYP15A1* and *FAMeT*) were found to be primarily expressed in the CA of the desert locust, the main biosynthetic tissue of JH. An RNA interference approach was used to verify the orthologous function of these genes in *S. gregaria*. Knockdown of the three genes in adult animals followed by the radiochemical assay (RCA) for JH biosynthesis and release showed that SgJHAMT and SgCYP15A1 are responsible for synthesis of MF and JH respectively. Our experiments did not show any involvement of SgFAMeT in JH biosynthesis in the desert locust. Effective and selective inhibitors of SgJHAMT and SgCYP15A1 would likely represent selective biorational locust control agents.

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1. Introduction

Juvenile hormones (JHs) represent a family of sesquiterpenoid hormones unique to insects. JHs are involved in several key processes during insect life. They play a central role in metamorphosis, moulting, ageing, diapause, reproduction, behaviour, polyphenism, as well as caste differentiation in social insects (Applebaum et al., 1997; Goodman and Granger, 2005; Hartfelder, 2000; Verma, 2007). JHs are synthesised and secreted from the corpora allata (CA), a pair of small specialised endocrine organs. Several JH homologues have been found – MF, JH acid, JH O, JH I, 4-methyl-JH I, JH II, JH III, bisepoxy-JH III, skipped bisepoxide JH III and hydroxy JHs – of which JH III is the most widespread and predominant JH in insects (Darrouzet et al., 1997; Goodman and Granger, 2005; Kotaki et al., 2009). In *Schistocerca gregaria*, only JH III was reported as circulating in the haemolymph (Tawfik et al., 2000). The early steps in the biosynthetic pathway of JH III include

the mevalonate pathway from acetyl-CoA to farnesyl pyrophosphate (FPP), a conserved pathway in both vertebrates and invertebrates. The late steps involve the hydrolysis of FPP to farnesol followed by oxidation to farnesal and farnesoic acid (FA). FA is finally converted to the active JH III by means of an epoxidation (C10,11) and a methyl transfer. The order in which these two final steps in JH III biosynthesis occurs, appears to be insect order dependent. In orthopteran and dictyopteran insects, FA is first methylated to methyl farnesoate (MF), which in turn undergoes a C10,C11 epoxidation to JH III. In Lepidoptera however, the converse appears to be the case: epoxidation precedes methylation (Fig. 1). Two genes (*JHAMT* and *CYP15A1*) involved in these last steps were recently identified in several insect species. *JHAMT* (juvenile hormone acid methyltransferase) was first functionally characterised in the silkworm, *Bombyx mori* (Kinjoh et al., 2007; Shinoda and Itoyama, 2003). *BmJHAMT* is specifically expressed in the CA. Its expression correlates well with the JH biosynthetic activity of the CA. *BmJHAMT* was shown to methylate the carboxyl group of JH I, II and III acids to produce the active JHs in the presence of *S*-adenosyl-L-methionine (AdoMet). Additionally, the enzyme is also able to catalyse the methylation of FA to MF (Shinoda and Itoyama, 2003).

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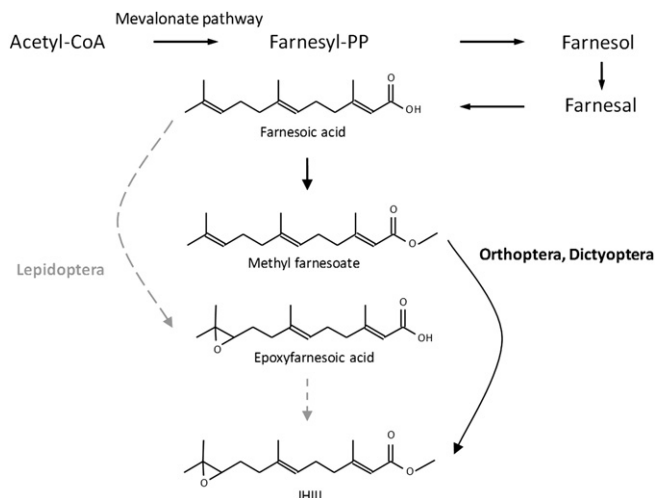


Fig. 1. Divergent final steps in JH III biosynthesis in different insect orders. The grey dashed arrows indicate the final two reactions as described previously in Lepidoptera. The final steps in Orthoptera and Dictyoptera are represented by black arrows.

There have been reports on orthologs of *JHAMT* in several other insect species, confirming its role in JH biosynthesis. Niwa et al. (2008) identified and functionally characterised *JHAMT* in the fruitfly, *Drosophila melanogaster*, and also in another dipteran, the mosquito *Aedes aegypti*, *JHAMT* was functionally characterised (Mayoral et al., 2009). Representatives of functional lepidopteran and coleopteran *JHAMT* were described in the eri silkworm, *Samia cynthia ricini* and the red flour beetle, *Tribolium castaneum* (Minakuchi et al., 2008; Sheng et al., 2008). Moreover, comparing gene expression and JH production in insects of wildtype, knock-down or overexpressed *JHAMT*, several of these studies have suggested that *JHAMT* is required for normal development (Kinjoh et al., 2007; Minakuchi et al., 2008; Niwa et al., 2008). *CYP15A1* was first functionally characterised in the cockroach *Diploptera punctata*. This gene was also found to be specifically expressed in the CA and was shown to encode a microsomal cytochrome P450 enzyme catalysing the epoxidation of MF to JH III (Helvig et al., 2004). To date, there have been no reports on the functionality of orthologs in any other insect species. But a partial *CYP15A1* was recently cloned from another cockroach species, the German cockroach, *Blattella germanica* (Maestro et al., 2010).

FAMEt (FA methyltransferase) – first reported in a crustacean, the shrimp *Metapenaeus ensis* (Gunawardene et al., 2001, 2002) – was initially suggested to be able to convert FA to MF, an active juvenoid end product in crustaceans. In this study, the production of MF was shown to increase in relation to increasing amounts of recombinant FAMEt in a radiochemical assay (Gunawardene et al., 2002). However, subsequent studies in other Crustacea (*Litopenaeus vannamei*, *Homarus americanus*, *Cancer pagurus*) and insect species (*Ceratitis capitata*, *Nilaparvata lugens* and *Melipona scutellaris*) did not verify this activity (Holford et al., 2004; Hui et al., 2008; Liu et al., 2010; Ruddell et al., 2003; Vannini et al., 2010; Vieira et al., 2008). Moreover, two recent studies in *D. melanogaster* have shown that DmFAMEt is not involved in JH biosynthesis (Burtenshaw et al., 2008; Zhang et al., 2010).

This report focuses on the cloning and characterisation of the orthologs of *JHAMT* and *CYP15A1* in a major pest insect, the desert locust, *S. gregaria*. Desert locust swarms can destroy agricultural production in some of the world's poorest countries and threaten the livelihood of a tenth of the world's population. These enzymes (SgJHAMT and SgCYP15A1) may constitute possible targets for selective pest control. Using q-RT-PCR, the tissue distribution and

expression of these genes were examined throughout the 5th larval and adult development of *S. gregaria*. The conversion of FA to MF in Orthoptera is thought to occur through the action of a farnesoic acid methyltransferase. An RNAi-based approach was used to examine whether *SgJHAMT* encodes this enzyme and whether *SgCYP15A1* encodes a functional MF epoxidase. Additionally, the possible role of SgFAMEt in JH biosynthesis was studied using this same technique.

2. Material and methods

2.1. Animals

Desert locusts were reared under crowded conditions in large cages (38 × 38 × 38 cm), in which temperature (32 ± 1 °C), ambient relative humidity (40–60%) and light (13 h photoperiod) were kept constant. The animals were fed daily with dry oat flakes and fresh cabbage *ad libitum*. Following mating, mature females deposited their eggs in pots filled with damp sand. Each week, these pots were collected and set in empty cages, where eggs were allowed to hatch into first instar larvae. In the described experiments, 5th larval and adult locusts were collected at the time of ecdysis to obtain pools of synchronised animals.

2.2. Tissue collection

Tissues were dissected in locust ringer solution (1 L: 8.766 g NaCl; 0.188 g CaCl₂; 0.746 g KCl; 0.407 g MgCl₂; 0.336 g NaHCO₃; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) under a binocular microscope and immediately transferred to liquid nitrogen to prevent RNA degradation. Tissues were stored at –80 °C until further processing.

2.3. Characterisation and phylogenetic analysis of *SgJHAMT*, *SgCYP15A1* and *SgFAMEt*

Degenerate primers for *SgJHAMT* and *SgCYP15A1* were designed, based on conserved amino acid sequences found in a multiple sequence alignment of several Arthropod orthologs. (*SgCYP15A1* F: GTNYTNAAYWSNYMTNTGGCCNATG, based on VLNS/RLWAM; *SgCYP15A1* R: CCNGCCATRAANARRTCNARRCA, based on CLDL/FFMAG *SgJHAMT* F: TTYWSNTTYTAYTGYYTNCAYTGG, based on FSYCLHW and *SgJHAMT* R: RTVRTGRTANGGNSWDATRWA, based on F/YISPYHH/D/Y). Partial sequences of *SgJHAMT* and *SgCYP15A1* were found using these primers in a T-gradient polymerase chain reaction (PCR) using REDTaq[®] DNA polymerase (Sigma–Aldrich Co.). CA cDNA was used in this amplification reaction with the following thermocycling profile: 3 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 2 min at 55 °C (with an 18 °C gradient) and 3 min at 72 °C. PCR products were loaded on a 1.2% agarose gel, separated during a 1 h gel electrophoresis and finally visualised using UV. Bands of the expected size were cut out and extracted with a GenElute[™] Gel extraction Kit (Sigma–Aldrich Co.). Resulting DNA fragments were subcloned into a pCR4-TOPO vector using the TOPO[®] TA Cloning Kit (Invitrogen). DNA sequences were determined using the ABI PRISM 3130 Genetic Analyser (Applied Biosystems) following the protocol outlined in the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). At the same time, an in-house EST database of the central nervous system of *S. gregaria* became available and will soon become publicly accessible (Badisco et al., unpublished results). Hits were found for *CYP15A1* (Contig: LC.2139.C1.Contig2300) and *JHAMT* (singlet: LC01004X1B01) that allowed us to further complete their sequences. Finally, specific primers were designed to be used in a RACE-PCR (Rapid Amplification of cDNA Ends) using

Table 1
Primer sequences for cloning of the full-length *SgJHAMT*, *SgCYP15A1*, *SgFAMeT*.

Full-length sequence	F-primer	R-primer
<i>SgJHAMT</i>	5'-ATGGACAAGCGGAGCTGTACT-3'	5'-TCAACGTACAGTTCGAACTCCGTT-3'
<i>SgCYP15A1</i>	5'-ATGTACATAATTTACTTGGGGA-3'	5'-CTAATCTTGGTGTGAGGGT-3'
<i>SgFAMeT</i>	5'-ATGGCAGTCGAACTCCAG-3'	5'-TCACTTGGCAACGAGAACTT-3'

the SMARTer™ RACE cDNA Amplification Kit (Clontech). *SgJHAMT* 3': GCCGCGCCGGTTTCGAGTCACG; *SgCYP15A1* 5': CAGCCACCAGACATATCAGTAGCAGC; *SgCYP15A1* 3': GGATTAGGTAGACGCAGGTGTC TGGGAG; *SgCYP15A1* 3' nested: GGCTTGCAGGAGCACTATGTTT CCG. Gel electrophoresis, gel extraction, subcloning and sequencing were performed as described above.

A full-length sequence for *SgFAMeT* (Contig: LC.757.C1.Contig882) was found in the EST database.

Finally, the full-length sequences of *SgJHAMT*, *SgCYP15A1* and *SgFAMeT* were amplified in a PCR reaction using CA cDNA as a template, REDTaq® DNA polymerase (Sigma–Aldrich Co.) and the primers described in Table 1. Further sequence analysis was performed as described above.

2.4. Quantitative real-time PCR (q-RT-PCR)

The pooled dissected tissues were transferred to reaction tubes containing “green beads” (Roche) and homogenised using a MagNA Lysor instrument (Roche). Total RNA was subsequently extracted from the tissue homogenate with the RNeasy Lipid Tissue Kit (Qiagen) according to the manufacturer's instructions. An additional DNase treatment (RNase-free DNase set, Qiagen) was performed to remove contaminating genomic DNA. Because of the relative small size of the CA, RNA from this tissue was extracted using the RNAqueous-Micro Kit (Ambion), followed by the recommended DNase step. Quality and concentration of the resulting RNA samples were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc.). An equal amount of RNA was transcribed in subsequent cDNA synthesis utilising Superscript III and random hexamers following the manufacturer's protocol (Invitrogen Life Technologies). Prior to q-RT-PCR transcript profiling, several previously described housekeeping genes (Van Hiel et al., 2009) were tested for their stability in the designed experiment (Table 2). Optimal housekeeping genes were selected using geNorm software (Vandesompele et al., 2002). β -actin and EF1 α appeared to be most stable in the CA and the other tissues measured to obtain a tissue distribution profile (Fig. A provided in supplementary data). q-RT-PCR primers for reference genes and target genes were designed using Primer Express software (Applied Biosystems).

Primer sets were validated by designing relative standard curves for gene transcripts with serial (5 \times) dilutions of a CA cDNA sample. Efficiency of q-RT-PCR and correlation coefficient (R^2) was measured for each primer pair. All PCR reactions were performed in duplicate in 96-well plates on a StepOne System (ABI Prism, Applied Biosystems). Each reaction contained 10 μ l fast Sybr Green, 1 μ l Forward and Reverse primer (10 μ M), 3 μ l MQ water and 5 μ l cDNA. For all q-RT-PCR reactions, the following thermal cycling profile was used: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Finally, a melt curve analysis was performed to check for primer dimers. For all transcripts, only a single melting peak was found during the dissociation protocol. Additionally, PCR products were run on a 1.2% agarose gel containing GelRed™ (Biotium). After electrophoresis only a single band could be seen which was further cloned and sequenced (TOPO® TA cloning kit for sequencing, Invitrogen) to confirm target specificity. For each tested cDNA sample, the normalisation factor for the housekeeping genes relative to a calibrator sample was calculated and used to determine the normalised expression levels of the target genes relative to the calibrator, as previously described (Vandesompele et al., 2002).

Three biologically independent pools of adult *S. gregaria* tissues (a first pool containing 40 animals, the second and third pool containing 10 animals) were used to obtain the tissue distribution. Three biologically independent pools (7 animals each) were used to obtain the developmental profile and to check the effect of dsRNA injection.

2.5. RNA interference (RNAi)

dsRNA constructs for *SgJHAMT*, *SgFAMeT* and *SgCYP15A1* were prepared using the MEGAscript® RNAi Kit (Ambion) which is designed for the construction of dsRNA larger than 200 bp. The procedure is based on the high-yield transcription reaction of a user-provided linear transcript with a T7 promoter sequence. dsRNA for *SgJHAMT* and *SgCYP15A1* was made by cloning the coding sequence into a pCR4-TOPO vector in a sense and antisense direction using the primers given in Table 3; insuring that the restriction site GTTAAC was on the 3' end of both sense and antisense strand.

Table 2

Oligonucleotide sequences for primers used in q-RT-PCR for reference and target genes. EF1 α : elongation factor 1 alpha; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Rp49: ribosomal factor 49; Sg: *Schistocerca gregaria*.

	F-primer	R-primer
<i>Reference genes</i>		
β -actin	5'-AATTACCATTGGTAACGAGCGATT-3'	5'-TGCTCCATACCCAGGAATGA-3'
CG13220	5'-TGTTTCAGTTTTGGCTCTGTCTGA-3'	5'-ACTGTTCTCCGGCAGAATGC-3'
EF1 α	5'-GATGCTCCAGGCCACAGAGA-3'	5'-TGCACAGTCGGCCTGTGAT-3'
GAPDH	5'-GTCTGATGACAACAGTGCAT-3'	5'-GTCCATCAGCCCAACTTTC-3'
Rp49	5'-CGTACAAGAAGCTTAAGAGGTCAT-3'	5'-CCTACGGCCGCACTCTGTTG-3'
Tubulin	5'-TGACAATGAGGCATCTATG-3'	5'-CGCAAAGATGCTGTGATTGA-3'
Ubiquitin	5'-GACTTTGAGGTGTGGCTAG-3'	5'-GGATCACAACACAGAACGA-3'
<i>Target genes</i>		
<i>SgJHAMT</i>	5'-CGGAGCAAAGGCAAGCA-3'	5'-CCACTTCACCCCTGGTTT-3'
<i>SgFAMeT</i>	5'-GGAGGTCAAGAATCGCAAA-3'	5'-ACCCAGCAGCCTCACA-3'
<i>SgCYP15A1</i>	5'-AAAGCAACTTCATTCACAGATG-3'	5'-CAGAGCCAGCCATGAACAAA-3'

Table 3
Nucleotide sequences of primers used in preparation of dsRNA constructs.

RNAi constructs	F-primer	R-primer
<i>SgJHAMT</i> sense	5'-TCTACCAGCTGGCGCCATTC-3'	5'- GTTAAC GGGTGCAGAGCGTGACTCGA-3'
<i>SgJHAMT</i> antisense	5'-GGGTGCAGAGCGTGACTCGA-3'	5'- GTTAACT CTACCAGCTGGCGCCATTC-3'
<i>SgCYP15A1</i> sense	5'-CGTCTCTGAGCTACTAAACATTG-3'	5'- GTTAAC GGCATCCTGCTGTTAATGTTGT-3'
<i>SgCYP15A1</i> antisense	5'-GGCATCCTGCTGTTAATGTTGT-3'	5'- GTTAAC CGTCTCTGAGCTACTAAACATTG-3'
<i>SgFAMeT</i>	5'- TAATACGACTCACTATAGGGAGAT GGCAGTCGAAGTCCAGACTCCG-3'	5'- TAATACGACTCACTATAGGGAGCACT GCACCCATCCCGTACAA-3'

The bold values in the primer sequences of *SgJHAMT* and *SgCYP15A1* represent the restriction site for KspAI(GTTAAC).

The bold value in the sequence of *SgFAMeT* represents the T7 promoter site.

Using CA cDNA as a template, the target region for *SgJHAMT* and *SgCYP15A1* was first amplified in a simple PCR reaction using REDTaq[®] DNA polymerase (Sigma–Aldrich). Next, PCR fragments were cloned into a pCR4-TOPO vector holding a T7 promoter site (Invitrogen) and sequenced. Plasmids, in which sequence and orientation of the transcript were correct, were first linearised with KspAI, and used in a high-yield transcription reaction resulting in two complementary RNA transcripts for *SgJHAMT* and *SgCYP15A1*. dsRNA was formed after annealing. Remaining ssRNA and DNA were removed in a nuclease digestion. The dsRNA was further purified according to the manufacturer's instructions (Ambion). A different strategy was employed when constructing *SgFAMeT* dsRNA. A single PCR reaction with CA cDNA as a template with T7 promoter sites appended to both PCR primers was performed. This PCR product could then directly be used in a single high-yield *in vitro* transcription reaction. Resulting dsRNA was then purified further as described above. Quality and concentration of the dsRNA were checked with a nanodrop instrument (Thermo Fisher Scientific Inc.). Diluted dsRNA was run on a 1.2% agarose gel to examine integrity of the construct and efficiency of duplex formation.

Adult locusts were injected with 5 µg of dsRNA dissolved in 10 µl of the MEGAscript[®] RNAi Kit's elution solution for a first time right after ecdysis (day 0). Two more injections followed (5 µg dsRNA/animal) on days 4 and 8 after adult moult. Control animals were injected with 10 µl of the elution solution (10 mM Tris–HCl pH 7, 1 mM EDTA). CA were dissected on day 12 and immediately stored in liquid nitrogen for RNA extraction or put in TC199 medium (GIBCO; 1.3 mM Ca²⁺, 2% Ficoll, methionine-free) for further use in the radiochemical assay. During the dissections of the day 12 female locusts, the lengths of their developing oocytes were measured using a binocular microscope and millimetre paper to check the effect of RNAi-mediated silencing on female reproductive physiology. Observation of our in-house *S. gregaria* colony has shown that, under optimal growth conditions, female adults are in full vitellogenesis 12 days after their final moult.

2.6. Radiochemical assay (RCA)

Rates of JH release and JH content were measured by the *in vitro* radiochemical assay originally described by Tobe and Pratt (1974) and Pratt and Tobe (1974) and further discussed (Feyereisen and Tobe, 1981; Yagi and Tobe, 2001). In this experiment, the RCA measures the rate of incorporation of the methyl group from [Methyl-¹⁴C] methionine (50 µM, 2.11 GBq/mmol, New England Nuclear Co.) into JH in isolated CA.

In a sterile environment, CA from control and dsRNA injected animals were dissected directly out of the locust's head and transferred to TC199 medium. After dissection, individual CA were transferred to conical glass vials holding 50 µl of radioactive TC199 medium (3 µCi/ml medium) and incubated for 3 h at 30 °C with shaking. Next, CA were transferred to fresh radioactive TC199 medium supplemented with 30 µM farnesic acid (FA) to stimulate JH synthesis (Tobe and Pratt, 1974). These were incubated for

another 3 h during which the first incubation medium was extracted using 300 µl of iso-octane. The samples were vortexed and centrifuged for 10 min at 2000 rpm. The top 200 µl of iso-octane layer was removed and put into scintillation vials containing 3 ml of scintillant (ICN) and measured in a liquid scintillation counter (Beckman, LS-6500). After the 2nd 3 h incubation, CA were transferred to fresh non-radioactive TC199 medium. JH release into the 2nd 3 h incubation medium was determined as described above. Thin layer chromatography was used to examine the gland content. TLC plates (20 cm × 20 cm, Merck Silica gel 60 F₂₅₄) were washed three times in 100% methanol (MeOH), air-dried and marked to obtain individual lanes. Gland incubation was terminated by adding 100 µl NaEDTA, 200 µl MeOH, 20 µl of stock (5 µl pure chemical in 3 ml iso-octane) cold carrier for MF and JH III and finally 1 ml of chloroform. Samples were vortexed and centrifuged for 10 min at 2000 rpm. The bottom chloroform layer was removed using a pulled glass pipette and loaded onto a Na₂SO₄ filter. A second extraction was performed with 500 µl of chloroform following the procedure described above. The samples were dried under N₂, redissolved in 100 µl of diethyl ether and finally spotted on individual lanes of a previously washed TLC plate. Another 50 µl of diethyl ether was added to the samples, vortexed and spotted in the corresponding lane. The plates were then focused twice with 100% MeOH (~4 cm), allowing to air dry before the 2nd focussing. Plates were next developed in a solvent system (85% toluene, 15% ethyl acetate, four drops of glacial acetic acid) and air-dried. MF and JH III bands were visualised and marked under short UV. Bands were cut out and put into scintillation vials containing 3 ml of scintillant (ICN). Gland content of MF and JH III was then quantified using the liquid scintillation counter and reported as pmol. Prior to the actual experiment, we checked whether other intermediate compounds besides JH were released into the medium. Individual CA were transferred to radioactive medium (3 µCi/ml medium) and incubated for 3 h. After incubation, CA were removed from the medium, which was extracted with chloroform. Products were then separated by thin layer chromatography (TLC). Only JH and not MF was found to be released into the medium (data not shown).

2.7. Statistical analysis

All statistical analysis was performed using GraphPad Prism 4. For RCA data, the non-parametric Mann–Whitney test was carried out. When comparing controls to treated animals in RNAi q-RT-PCR and oocyte length measurements, a two-tailed unpaired *t* test was used to check for significant silencing effects.

3. Results

3.1. Cloning and sequence analysis of *SgJHAMT*, *SgCYP15A1* and *SgFAMeT*

Orthologous sequences were cloned for *JHAMT*, *CYP15A1* and *FAMeT* in *S. gregaria*. Upon blastx National Center for Biotechnology

Information (NCBI) database searches, the orthologs showed substantial similarity to the previously described *JHAMTs*, *CYP15A1s* and *FAMeTs* in other insect and crustacean species. Multiple sequence alignments of *SgJHAMT*, *SgCYP15A1* and *SgFAMeT* with orthologs from other insect and crustacean species are provided as supplementary data (Fig. B–D).

The full-length *SgJHAMT* sequence was cloned, encoding a protein of 308 amino acids which was found to be 35% identical to the functionally characterised *B. mori* *JHAMT*. A conserved domain search in the NCBI database revealed that the *SgJHAMT* contains a sequence similar to conserved motifs found in several AdoMet-dependent methyltransferases. They are characterised by a very conserved motif I, involved in AdoMet binding, hh(D/E)hGxGxG, where h represents a hydrophobic residue. The AdoMet-binding motif of *SgJHAMT* (VLDVGCAG) is given in Fig. B in supplementary data.

The full-length *SgCYP15A1* encodes a cytochrome P450 enzyme of 484 amino acids in length which shares 58% identity with the functionally characterised *D. punctata* *CYP15A1*. *SgCYP15A1* is clearly homologous to microsomal cytochrome P450 enzymes, with a hydrophobic N-terminal anchor preceding a proline/glycine hinge region (Feyereisen, 2005; Werck-Reichhart and Feyereisen, 2000). Other structural attributes of CYPs are present, i.e. helix-C (WxxxR) of which the arginine is thought to form a charge pair with the propionate of the haem; helix-I (AGxxT) which corresponds to a proton transfer groove on the distal side of the haem; helix-K (ExxR) which stabilises the core structure of the enzyme through a set of salt bridge interactions; the aromatic region or “PERF” motif (PxxFxxPxxRF) and finally the haem-binding loop (PFxxGxxRxxCxG) with a very conserved cysteine that serves as 5th ligand to the haem iron (Fig. C in supplementary data).

Additionally, the full-length sequence of *SgFAMeT* was recovered from the *S. gregaria* EST database, encoding a protein 292 amino acids in length (Fig. D in supplementary data). In comparison to several other insect and crustacean species, only one isoform for *SgFAMeT* was found. A conserved domain search in the NCBI database revealed features in *SgFAMeT* common to other insect *FAMeT* sequences: The N-terminal part of *SgFAMeT* contains a Methyltransf_FA domain (residues 34–134) corresponding to the region of the CF-domain (Holford et al., 2004; Vieira et al., 2008) and the C-terminal portion contains two consecutive DM9 domains (residues 153–221 and residues 222–292) No AdoMet-binding motif was found.

The three full-length sequences *SgJHAMT*, *SgCYP15A1* and *SgFAMeT* were uploaded on NCBI's GenBank and received accession numbers HQ634702, HQ634704 and HQ634703, respectively.

3.2. Quantitative real-time PCR – tissue specific and developmental expression of *SgJHAMT*, *SgCYP15A1* and *SgFAMeT*

The tissue specificity and temporal expression profiles of *SgJHAMT*, *SgCYP15A1* and *SgFAMeT* were examined by quantitative real-time PCR analysis. *SgJHAMT* and *SgCYP15A1* mRNA expression is mainly located in the CA of day 10 adult locusts (Fig. 2). Trace amounts were present in the brain, thoracic ganglia, and midgut as well as the male accessory glands and testes. The *SgFAMeT* tissue distribution is much wider, as described for orthologs in other insect and crustacean species (Gunawardene et al., 2003; Liu et al., 2010; Ruddell et al., 2003; Vannini et al., 2010). In addition to a relatively high *SgFAMeT* transcript level in the CA, expression was also found in various parts of the central nervous system such as brain, CC, TG and SOG (Fig. 2). Since, for all three genes, a relatively high level of expression was found in the CA, we used this tissue to assess the changes in *SgJHAMT*, *SgCYP15A1* and *SgFAMeT* transcript levels during 5th larval and adult development (Fig. 3). Transcript

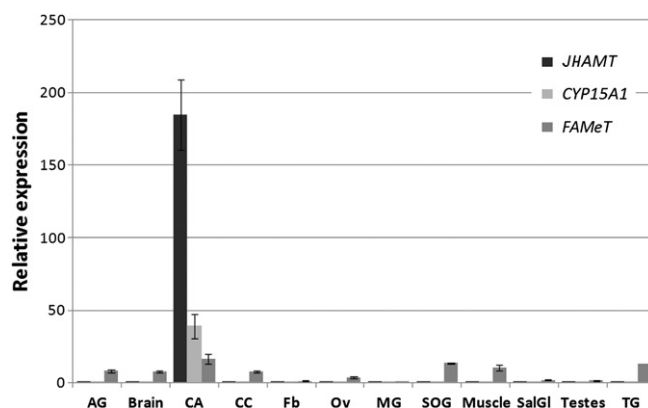


Fig. 2. Graphic representation of the relative *SgJHAMT*, *SgCYP15A1* and *SgFAMeT* transcript levels measured in different *S. gregaria* tissues using q-RT-PCR. All tissues were dissected from 10-day old adult female locusts, except for the male accessory glands (AG) and testes. Abbreviations on the X-axis: CA: corpora allata; CC: corpora cardiaca; Fb: fat body; SOG: suboesophageal ganglion; SalGI: salivary gland; TG: thoracic ganglia. The data represent means of 3 independent pools of day 10 adult animals (40, 10 and 10 animals/pool; CA: 3 times 7 animals/pool), run in duplicate and normalised to β -actin and EF1 α expression levels. The vertical bars indicate S.E.M.

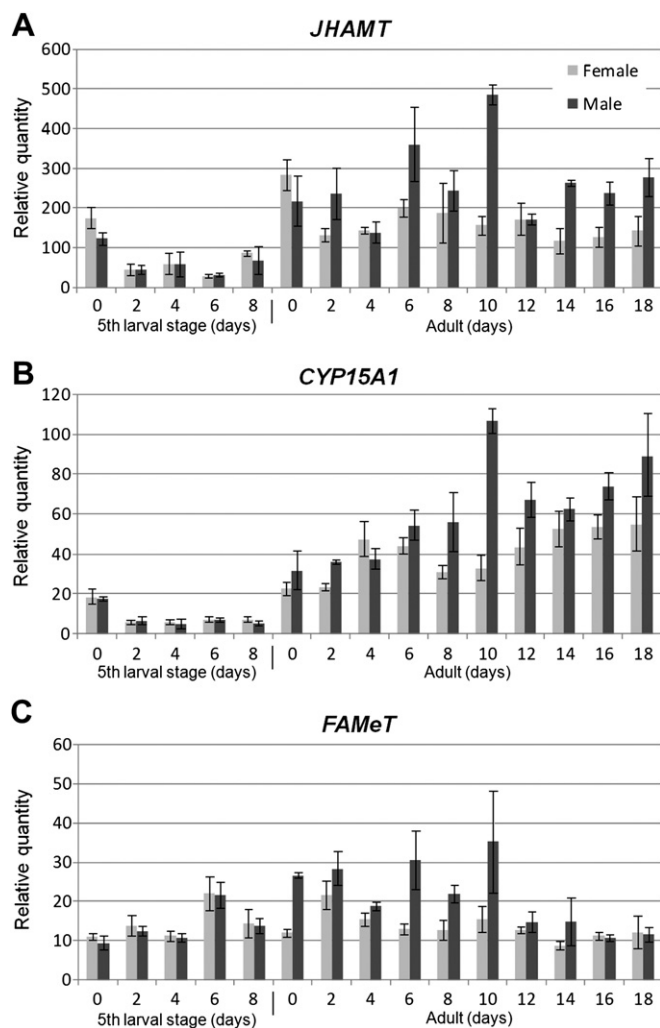


Fig. 3. Graphic representation of the relative (A) *SgJHAMT*, (B) *SgCYP15A1* and (C) *SgFAMeT* transcript levels measured in the CA of *S. gregaria* during 5th larval and adult development. For every time point, 3 independent pools of 7 animals were measured in duplicate using q-RT-PCR and normalised to β -actin and EF1 α expression levels. Measurements were taken every other day during locust development. The columns represent averages with vertical bars indicating S.E.M.

levels were measured every other day during both developmental stages.

The relative levels of *SgJHMT* and *SgCYP15A1* transcripts were high at the beginning of the 5th larval stage (day 0, the day on which the 4th instars moulted to the 5th) in both females and males. Lower levels were found during the rest of the last larval stage in both sexes. The transcript level rises again at the beginning of adult development and was found throughout the period of sexual maturation in both female and male locusts. For *SgFAMeT*, no decrease during the 5th larval stage was observed. In fact, the gene appeared to be expressed throughout the 5th larval stadium and adult development in the CA.

3.3. RNAi experiments

To examine the role of *SgJHMT*, *SgCYP15A1* and *SgFAMeT* in female adult locusts, RNAi-mediated knockdown of all three genes was performed by injecting dsRNA into the haemocoel of day 0 adult females. Control animals were injected with Tris–EDTA buffer (MEGAscript® RNAi Kit). Two more injections were done on days 4 and 8. Finally, to confirm the efficiency of RNAi-mediated knockdown, the CA were assayed on day 12 of adult development. As shown in Fig. 4, injection of *SgJHMT*, *SgCYP15A1* and *SgFAMeT* dsRNA all significantly suppressed the transcript level of the respective genes in the CA of treated animals compared to control animals. Transcript levels dropped 88.4%, 98.7% and 92.7% for *SgJHMT*, *SgCYP15A1* and *SgFAMeT* respectively. The effect of dsRNA treatment on female reproduction was examined by measuring basal oocyte lengths. When comparing oocyte lengths for control and dsRNA-treated female locusts, significantly smaller basal oocytes were found in animals treated with *SgJHMT* dsRNA (Fig. 5).

3.4. RCA

3.4.1. JH release from isolated CA

Changes in JH release from CA in different treatment groups were analysed using an *in vitro* radiochemical assay. Comparison of controls from the 1st and 2nd incubations revealed that the addition of exogenous FA during the 2nd incubation dramatically enhanced the release of JH (Fig. 6). From the data of the 1st

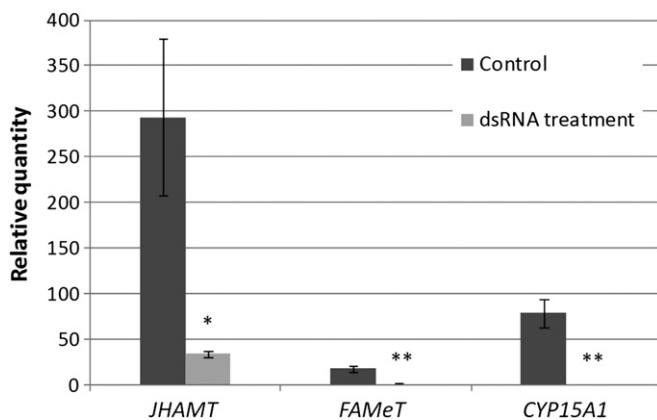


Fig. 4. Efficiency of RNAi-mediated knockdown in female adult *S. gregaria*. Relative quantity of *SgJHMT*, *SgFAMeT* and *SgCYP15A1* expression levels in CA dissected from control animals compared to CA from locusts injected with dsRNA for the 3 genes respectively. Both control and treated animals were sacrificed on day 12 of the adult stage. The data represent averages of 3 independent biological replicates (3 pools of seven 12 day old adult females), run in duplicate using q-RT-PCR and normalised to β -actin and EF1 α expression levels. Vertical bars indicate S.E.M. Significant differences ($p < 0.05$; $p < 0.01$) are indicated by (an) asterisk(s) (* and ** resp.).

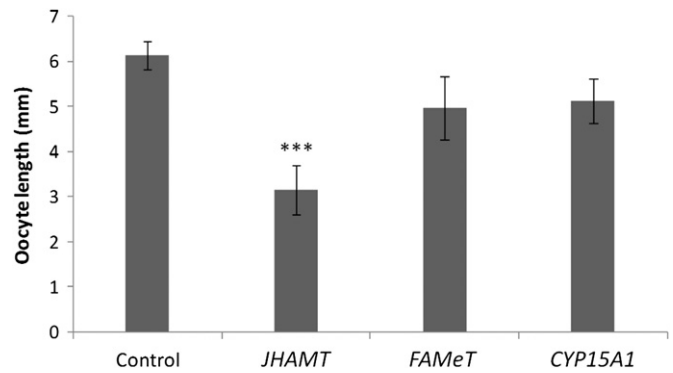


Fig. 5. The length (mm) of basal oocytes dissected from control and *SgJHMT*, *SgCYP15A1* and *SgFAMeT* dsRNA-treated day 12 adult females. The data represent averages of 21 individual animals. Vertical bars indicate S.E.M. Significant differences ($p < 0.001$) are indicated by asterisks (***).

incubation, JH release from *SgJHMT* dsRNA-treated animals was significantly lower than from control CA in both females and males. For *SgCYP15A1* dsRNA-treated animals, this difference was only observed in female CA. No significant inhibition of JH release was found between control CA and CA treated with *SgFAMeT* dsRNA. Adding FA not only stimulated JH release from control CA during the 2nd incubation, but also from *SgFAMeT* dsRNA-treated CA. In contrast, only a very limited stimulation was observed in CA treated with *SgJHMT* or *SgCYP15A1* dsRNA in both sexes.

3.4.2. MF content in isolated CA

The amount of MF present in the CA was measured after the 2nd incubation with FA. Medium and glands were extracted separately. For results of extracted medium, see §3.4.1. Using TLC, MF and JH were separated to measure possible build-up of MF in the CA. Fig. 7 shows a very low amount of MF present in the CA of control animals whereas for *SgCYP15A1* dsRNA-treated CA, a very high level was found.

4. Discussion

This study analysed the expression and function of two *S. gregaria* genes (*SgJHMT* and *SgCYP15A1*), for which orthologs have been previously described to be involved in juvenoid biosynthesis in other insect species. Based on the following observations, it can be concluded that *SgJHMT* and *SgCYP15A1* encode a functional farnesoic acid methyltransferase and an MF epoxidase respectively, key enzymes in the final two steps in JH III biosynthesis in the desert locust.

First, *SgJHMT* and *SgCYP15A1* show the typical protein domains associated with the functional enzymes. *SgJHMT* contains the typical domains for an AdoMet-dependent MTase, with a very conserved motif I, as previously described for *JHMT* orthologs (Kinjoh et al., 2007; Mayoral et al., 2009; Minakuchi et al., 2008; Niwa et al., 2008; Sheng et al., 2008). *SgCYP15A1* is a microsomal P450 enzyme. The microsomal nature of the MF epoxidase was previously demonstrated in *Blaberus giganteus*, *D. punctata* and *Locusta migratoria* (Feyereisen et al., 1981; Hammock, 1975; Helvig et al., 2004).

Second, expression of *SgJHMT* and *SgCYP15A1* was tissue- and stage-specific. For both genes, transcripts were almost exclusively detected in the CA, the primary site of JH biosynthesis. Both genes showed stage-specific expression in the CA during locust development in both females and males. Transcripts were abundant at the beginning of the final larval stage, decreased during the remainder of this stage, but increased again during adult development. Similar expression profiles were described previously for

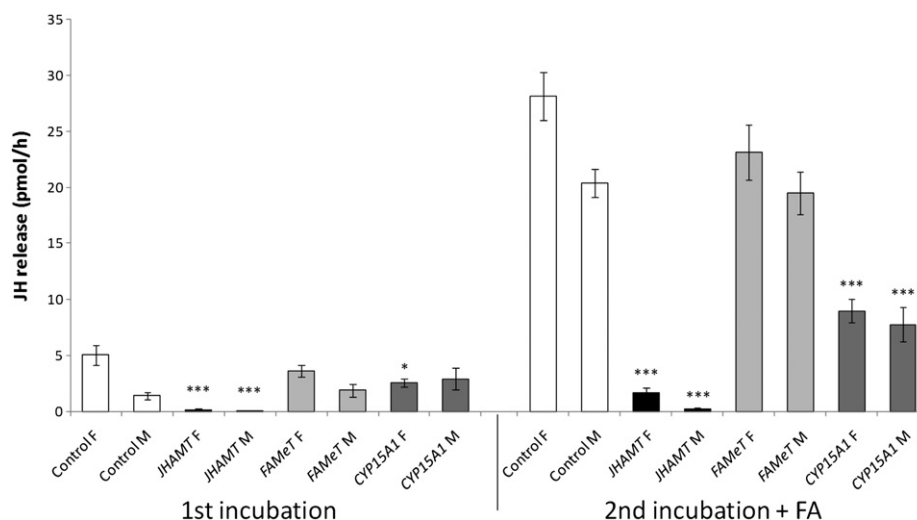


Fig. 6. JH release from *S. gregaria* CA in pmol/h/gland. Both female and male locusts were treated with *SgJHAMT*, *SgFAMEt* or *SgCYP15A1* dsRNA. Rates of JH release were determined using RCA in two 3 h incubations. FA was added to the 2nd 3 h incubation to stimulate JH release. The data represent means of 15–25 individual CA. Bars indicate S.E.M. Significant differences ($p < 0.05$ and $p < 0.001$) are indicated by (an) asterisk(s) (* and *** resp.).

several other insect *JHAMTs* (Kinjoh et al., 2007; Mayoral et al., 2009; Minakuchi et al., 2008; Niwa et al., 2008; Sheng et al., 2008). In these holometabolous insects, *JHAMT* transcript levels are lower at the beginning of the final larval stage, prior to metamorphosis, and increase sharply when the insect reaches the adult stage. Based on these results, *JHAMT* was demonstrated to be the key enzyme determining the timing of larval–pupal metamorphosis, through the control of JH biosynthesis. It is therefore reasonable to suggest that, in the hemimetabolous insect, *S. gregaria*, suppression of JH synthetic enzymes is needed to guarantee successful completion of the final larval–adult moult. Although the developmental profile of JH titre has not yet been fully examined during the transition of the last larval stage to the adult stage, the temporal expression profile of *SgJHAMT* and *SgCYP15A1* may correlate well with the JH biosynthetic activity in the CA, as observed previously for *JHAMT* in *B. mori*, *A. aegypti* and *D. melanogaster* and for *CYP15A1* in *D. punctata* (Helvig et al., 2004; Kinjoh et al., 2007; Mayoral et al., 2009; Niwa et al., 2008; Shinoda and Itoyama, 2003). Moreover, FA-stimulated JH production was shown to decline in final stage *D. punctata*, suggesting that the activity of the final enzymes is reduced (Yagi et al., 1991). Additionally, a previous study reported a high JH titre in the haemolymph during sexual maturation of both female and male *S. gregaria*

(Tawfik et al., 2000). JH has been reported to play multiple roles in the reproductive physiology of locusts. In females, it has been implicated in patency and induction of vitellogenin biosynthesis by the fat body (Sevala et al., 1995; Verlinden et al., 2009; Wyatt et al., 1996). In males, JH enhances spermatogenesis and pheromone biosynthesis and appears to be involved in the induction of yellow colouration of sexually mature crowded locusts (Jones, 1978; Pener, 1991; Tawfik et al., 2000; Wybrandt and Andersen, 2001). A peak level of *SgJHAMT* and *SgCYP15A1* was found in day 10 male adults. This peak may coincide with an initial rise in yellow protein transcription in male locusts of this age, as was measured by Sas et al. (2007). The correlation between adult transcript levels and the female gonadotropic cycle may seem unclear. Female adult locusts were found to start vitellogenesis around day 8, to commence mating on day 12 and finally deposited their eggs around day 18. The relative quantity of *SgCYP15A1* does show a rising trend during female development, which cannot be seen in the *SgJHAMT* transcript levels measured. It must be kept in mind that neither of these enzymes may be the rate-limiting factor to the JH pathway and circulating JH titres not only depend on the level of JH synthesis alone, but also on the level of JH binding proteins and on JH catabolism. A high level of expression of genes involved in the final steps of JH biosynthesis may be needed to maintain a high circulating JH titre and associated stimulation of vitellogenesis.

Third, RNAi-mediated knockdown of *SgJHAMT* and *SgCYP15A1* resulted in effects on JH release and MF content in an *in vitro* RCA. Knockdown of *SgJHAMT* not only resulted in a lower JH release, but also appeared to suppress FA-stimulated JH release. Very little MF was found in control CA, whereas in the *SgJHAMT* dsRNA-treated glands, MF quantity was lowered to a non-detectable level, suggesting that silencing this gene blocks the conversion of FA to MF. Moreover, the knockdown of *SgJHAMT* in adult female locusts apparently resulted in a delay in sexual maturation, as we observed with basal oocyte lengths. The silencing of *SgCYP15A1* also resulted in reduced JH release and an accumulation of MF within the CA. Through RNAi of *SgCYP15A1*, it was possible to effectively block the conversion of MF to JH III. Nevertheless, Fig. 6 shows an obvious continued production of JH, albeit at a much lower rate in the *SgJHAMT* and *SgCYP15A1* dsRNA-treated CA. It must be kept in mind that even though RNAi knocks down the transcript level significantly, some translation will still take place resulting in functional proteins and thus JH production. In the same context, the levels of

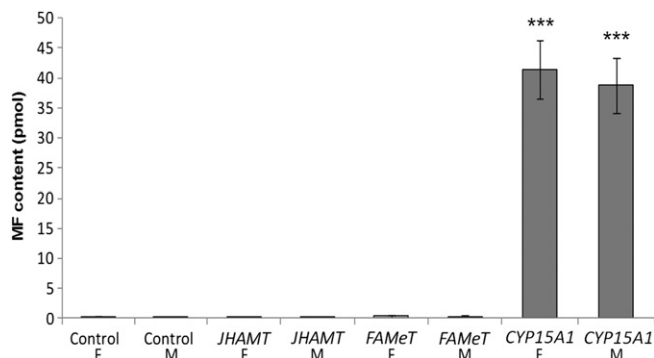


Fig. 7. MF content of *S. gregaria* CA (in pmol). Both female and male locusts were treated with *SgJHAMT*, *SgFAMEt* or *SgCYP15A1*. MF contents were measured after the 2nd incubation. The data represent means of 15–25 individual CA. Bars indicate S.E.M. Significant differences ($p < 0.001$) are indicated by asterisks (***).

silencing of *SgJHAMT* and *SgCYP15A1* are not proportional to the phenotypic effect on oocyte length and on JH biosynthetic activity as measured *ex vivo*. It is possible that *SgCYP15A1* has a longer half life relative to *SgJHAMT*.

Additionally, a *S. gregaria* orthologue for FAMEt (originally reported to be a farnesoic acid methyltransferase in Crustacea) was included in this study; however no evidence was found for a role of SgFAMEt in the final steps of JH biosynthesis in the locust. Clear differences appear to exist between crustacean and insect FAMEt sequences. Crustacean FAMEt enzymes contain two CF domains, whereas in insects there is only one, together with other domains such as two DM9 domains of unknown function (Hui et al., 2010). As previously described for other FAMEt sequences, no AdoMet-binding motif I could be found in SgFAMEt. SgFAMEt mRNA was not only detected in the CA but also in several other tissues associated with the central nervous system. No stage-specific expression related to JH biosynthesis was observed in the CA. Although the silencing of the *SgFAMEt* was successful, no effects of this RNAi were seen on either JH release or MF content of the CA. The data indicate that FAMEt does not have an FA methyltransferase function in *S. gregaria*. Moreover, together with the previous studies on FAMEt in *D. melanogaster* (Burtenshaw et al., 2008; Zhang et al., 2010), there is reasonable evidence from different insect species showing that FAMEt is not what its name indicates.

Functional JHAMTs have been found in several insect species and one JHAMT sequence was recently reported from a crustacean, *Daphnia pulex* by Hui et al. (2010). The occurrence of crustacean JHAMTs, specifically expressed in the mandibular organ was already predicted by Shinoda and Itoyama (2003). This suggestion was based on the wide distribution pattern of the crustacean FAMEts and the lack of an AdoMet-binding motif in these sequences. It will be of interest to determine whether the *D. pulex* JHAMT can act as a functional methyltransferase.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ibmb.2010.12.007.

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