Expression of juvenile hormone acid O-methyltransferase and juvenile hormone synthesis in Blattella germanica

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Keywords: Blattella germanica, corpora allata, JHAMT, juvenile hormone, nutritional signaling

Abstract: juvenile hormone (JH), a sesquiterpenoid synthetized by the insect corpora allata (CA), plays critical roles in metamorphosis and reproduction. Penultimate or last step of JH synthesis is catalyzed by juvenile hormone acid O-methyltransferase (JHAMT). Here we report the cloning and expression analysis of the JHAMT orthologue in the cockroach, Blattella germanica (L.) (BgJHAMT). BgJHAMT is mainly expressed in CA, with only expression traces in ovary. Three different isoforms, differing in the 3'-UTR sequence, were identified. Isoform A shows between 35 and 65 times higher expression than B and C in CA from penultimate nymphal instar and adult females. RNAi-triggered knock down of BgJHAMT produces a dramatic reduction of JH synthesis, concomitant with a decrease of fat body vitellogenin expression and basal follicle length. BgJHAMT mRNA levels in CA of females along the gonadotrophic cycle parallel, with a slight advancement, JH synthesis profile. BgJHAMT mRNA levels were reduced in starved females and in females in which we reduced nutritional signaling by knocking down insulin receptor and target of rapamycin (TOR). Results show that conditions that modify JH synthesis in adult B. germanica females show parallel changes of BgJHAMT mRNA levels and that the JH-specific branch of the JH synthesis pathway is regulated in the same way as the mevalonate branch. Furthermore, we demonstrate that nutrition and its signaling through the insulin receptor and TOR pathways are essential for activating BgJHAMT expression, which suggests that this enzyme can be a checkpoint for the regulation of JH production in relation to nutritional status.
Expression of juvenile hormone acid O-methyltransferase and juvenile hormone synthesis in *Blattella germanica*

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Short running title: JHAMT and JH synthesis in *B. germanica*
Abstract

Juvenile hormone (JH), a sesquiterpenoid synthetized by the insect corpora allata (CA), plays critical roles in metamorphosis and reproduction. Penultimate or last step of JH synthesis is catalyzed by juvenile hormone acid O-methyltransferase (JHAMT). Here we report the cloning and expression analysis of the JHAMT orthologue in the cockroach, *Blattella germanica* (L.) (BgJHAMT). BgJHAMT is mainly expressed in CA, with only expression traces in ovary. Three different isoforms, differing in the 3' UTR sequence, were identified. Isoform A shows between 35 and 65 times higher expression than B and C in CA from penultimate nymphal instar and adult females. RNAi-triggered knock down of BgJHAMT produces a dramatic reduction of JH synthesis, concomitant with a decrease of fat body vitellogenin expression and basal follicle length. BgJHAMT mRNA levels in CA of females along the gonadotrophic cycle parallel, with a slight advancement, JH synthesis profile. BgJHAMT mRNA levels were reduced in starved females and in females in which we reduced nutritional signaling by knocking down insulin receptor and target of rapamycin (TOR). Results show that conditions that modify JH synthesis in adult *B. germanica* females show parallel changes of BgJHAMT mRNA levels and that the JH-specific branch of the JH synthesis pathway is regulated in the same way as the mevalonate branch. Furthermore, we demonstrate that nutrition and its signaling through the insulin receptor and TOR pathways are essential for activating BgJHAMT expression, which suggests that this enzyme can be a checkpoint for the regulation of JH production in relation to nutritional status.

Keywords: *Blattella germanica*, corpora allata, JHAMT, juvenile hormone, nutritional signaling
Introduction

Juvenile hormones (JHs) are a family of sesquiterpenoid molecules specific of insects (or arthropods if we include methyl farnesoate (FM) into the family) (Goodman & Cusson, 2012). In insects, JHs are synthesized and secreted by the corpora allata (CA), endocrine glands that, together with the corpora cardiaca (CC), forms the retrocerebral complex, connected to the brain and to the suboesophageal ganglion by different neural connections (Goodman & Cusson, 2012). JH plays key roles in critical physiological processes, including diapause, pheromone production, polyphenisms, metamorphosis and reproduction. In particular, the presence of JH during the molt determines that the next stage will be a juvenile, whereas its absence triggers metamorphosis (Jindra et al., 2013). In addition, in the adult female of most insects, JH is the gonadotrophic hormone that induces the synthesis of yolk proteins in the fat body (Raikhel et al., 2004). Among JHs, JH III is the most common in insects and it is the only one found in cockroaches, including *Blattella germanica* (L.) (Camps et al., 1987).

Different factors have been involved in the regulation of JH synthesis (Goodman & Cusson, 2012). These include allatoregulatory neuropeptides (Bellés et al., 1994; Verlinden et al., 2015), biogenic amines (Pastor et al., 1991; Grunenko & Rauschenbach, 2008), nutritional signals (Maestro et al., 2009; Abrisqueta et al., 2014) and nuclear receptors (Borras-Castells et al., 2017).

JH synthesis pathway can be divided in two branches. The early steps, usually called the mevalonate branch, proceeds from acetyl CoA to farnesyl diphosphate. The mevalonate pathway is present in plants and animals, and in insects it is present in all cells (Bellés et al., 2005). This pathway has been extensively studied because one of its final products is cholesterol, a very important factor in human health. Nevertheless,
insect cannot synthetize cholesterol because they lack some enzymes of the sterol synthetic pathway (Bellés et al., 2005). The late steps, from farnesyl diphosphate to JH, are unique to arthropods and, in the case of insects, the enzymes that catalyze these reactions are specific, or mainly expressed, in CA cells (Bellés et al., 2005; Goodman & Cusson, 2012).

The order of two last steps in JH synthesis depends on the species. In cockroaches, and in most other groups, it is proposed that methylation of farnesoic acid (FA) to produce methyl farnesoate (MF) precedes epoxidation to JH (Hammock, 1975), whereas in Lepidoptera, the proposed way is epoxidation of FA and then methylation of juvenile hormone acid (JHA) (Goodman & Cusson, 2012). The enzyme that catalyzes the epoxidation is a cytochrome P450, CYP15A1 or JH epoxidase, that has already been characterized in B. germanica (Maestro et al., 2010). Independently of the order of the reactions, the enzyme that catalyzes the methylation is usually called JH acid O-methyltransferase (JHAMT). JHAMT is a S-adenosyl-methionine-dependent methyltransferase that was cloned for the first time in the silkworm Bombyx mori (Shinoda & Itoyama, 2003). Since then, activity for methylating both FA and JHA has been demonstrated in B. mori (Shinoda & Itoyama, 2003), the fruit fly Drosophila melanogaster (Niwa et al., 2008), the beetle Tribolium castaneum (Minakuchi et al., 2008), the moth Samia cynthia ricini (Sheng et al., 2008), the mosquito Aedes aegypti (Mayoral et al., 2009) and the psyllid Diaphorina citri (Van Ekert et al., 2015). A different protein, farnesoic acid O-methyltransferase (FAMeT), has been identified in the prawn, Metapenaeus ensis. Recombinant FAMeT produces MF from FA (Gunawardene et al., 2002). Nevertheless, the orthologue protein in insects does not appear to be related to JH synthesis (Burtenshaw et al., 2008; Marchal et al., 2011).
JHAMT depletion with RNAi in juvenile insects produces precocious metamorphosis in *T. castaneum* (Minakuchi *et al.*, 2008) and in the cricket *Gryllus bimaculatus* (Ishimaru *et al.*, 2016). In addition, similar treatments in adult females undergo a reduction of vitellogenin mRNA in *T. castaneum* (Sheng *et al.*, 2011) and in the planthopper *Nilaparvata lugens* (Lu *et al.*, 2016) and low JH synthesis measured in CA radiochemical assay in the locust *Schistocerca gregaria* (Marchal *et al.*, 2011).

In the CA of *B. germanica*, the expressions of the enzymes of the mevalonate pathway, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthases 1 and 2 and HMG-CoA reductase, have been previously examined because they were proposed as critical regulatory points of the mevalonate pathway in vertebrates (Goldstein & Brown, 1990). CA expression of HMG-CoA synthases 1 and 2 and HMG-CoA reductase during the first gonadotrophic cycle show a pattern paralleling that of JH synthesis (Maestro *et al.*, 2009). In addition, starved females and females treated with RNAi against target of rapamycin (TOR) or Insulin Receptor (InR), which show reduced JH synthesis, also showed reduced expression levels of these mevalonate pathway enzymes (Maestro *et al.*, 2009; Abrisqueta *et al.*, 2014). One interesting question is whether the JH-specific branch of the JH synthesis pathway is regulated in the same way as the mevalonate branch. In this context, JHAMT has been proposed as one of the critical enzymes in the JH-branch, thus in the regulation of JH synthesis (Kinjoh *et al.*, 2007). The aim of the present work has been to investigate the role of JHAMT in modulating JH synthesis in the hemimetabolous species *B. germanica* and to quantify its expression levels in relation to JH synthesis and to nutritional signaling.

**Material and methods**
Insects

*B. germanica* specimens were obtained from a colony reared on dry dog food and water, at 30 ± 1°C and 60-70 % relative humidity and in continuous dark. Dissections were carried out on carbon dioxide-anaesthetized animals, and tissues for analysis of mRNA levels were immediately frozen in liquid N₂ and stored at -80 °C until their use. For the study of animals during the first gonadotrophic cycle, virgin females were used. In the case of treatments during the oothecal transport period and analysis of gene expression in the second gonadotrophic cycle, females producing a proper ootheca after mating were used. For the starvation experiment, adult females received only water after the imaginal molt.

Cloning of *Bg*JHAMT cDNA

We used a degenerated primer (Table S1) based on a conserved region of JHAMT from different insect species and a polydT primer for obtaining a *B. germanica* homologue fragment. cDNA from CA of adult females was used as template. In this amplification, polydT bound to an A-rich sequence different to the polyA at the end of the mRNA, but it allowed the cloning of a 427 bp fragment JHAMT homologue. The amplified sequence was cloned in pSTBlue-1 vector (Novagen, Merck Millipore, Madison, WI, USA) and sequenced. 5’- and 3’-RACE methodologies (Invitrogen, Carlsbad, CA, USA) using specific primers were performed to complete the sequence.

Phylogenetic analysis

We used the JHAMT protein sequences from the following species: *A. aegypti* (GenBank Accession Number: XP_001651876), *B. mori* (NP_001036901), *D. citri*
(NP_001288290), Diploptera punctata (AHZ20738), D. melanogaster (NP_609793),
Lucilia cuprina (KNC28768), Nicrophorus vespilloides (XP_017772157), S. gregaria
(ADV17350), T. castaneum (NP_001120783) and Zootermopsis nevadensis
(BAU78328). In addition we use the FAMeT crustacean-type sequences from D.
melanogaster (AFA46666) and S. gregaria (HQ634704). Sequences were aligned using
the iterative refinement method G-INS-i from the program MAFFT v. 7 (Katoh &
Standley, 2016). The alignment was analyzed using the RAxML-HPC BlackBox tool of
the CIPRES Science Gateway v. 3.1 (Miller et al., 2010). The data sets were
bootstrapped for 100 replicates.

RNA extraction, cDNA synthesis and real-time PCR analyses

cDNAs were synthetized using as previously described (Abrisqueta et al.,
2017). The absence of genomic contamination was confirmed using a control without
reverse transcription. Gene expression was analyzed by quantitative real-time PCR
using iQ SYBR Green supermix in an iQ cycler and iQ single color detection system
and the iQ5 optical system software v. 2.0 (Bio-Rad Laboratories, Hercules, CA, USA),
as previously described (Maestro et al., 2010). Primers used to amplify total BgJHAMT
as well as the different 3'-UTR isoforms can be found in Table S1. Primers used to
amplify vitellogenin (BgVg) and BgActin 5C (used as a reference) have been already
reported (Süren-Castillo et al., 2012). The primers efficiencies were validated by
constructing serial cDNA dilutions standard curves. Results were expressed as copies of
a specific mRNA per copies of BgActin 5C.

RNA interference
Systemic RNAi in *B. germanica* females was performed as previously described (Maestro *et al.*, 2009). In the case of BgJHAMT, a 326-bp fragment spanning positions 392 to 717 of the BgJHAMT cDNA was used to synthesize the dsRNA (dsJHAMT). In the case of dsRNA against TOR (dsTOR) and InR (dsInR), their syntheses have been already reported (Maestro *et al.*, 2009; Abrisqueta *et al.*, 2014). A heterologous 307-bp fragment from the gene sequence of the polyhedrin of *Autographa californica* nucleopolyhedrovirus was used as control. Treatments were performed as already reported (Abrisqueta *et al.*, 2014).

Quantification of juvenile hormone synthesis

JH III synthesis by CC-CA incubated *in vitro* was quantified using a radiochemical assay as previously reported (Maestro *et al.*, 2009). Basically, individual pairs of CC-CA were incubated in 100 µl of 199 medium (Sigma-Aldrich, St. Louis, MO, USA) containing L-methionine (0.1 nM), Hank’s salts, Hepes buffer (20 mM) and Ficoll (20 mg/ml), to which L-[3 H-methyl] methionine (Perkin Elmer, Bonton, MA, USA) was added to achieve a final specific activity of 7.4 GBq/mmol. CC-CA were incubated for 3 h, after which JH III was quantified in the medium plus homogenized glands.

Results

Cloning of BgJHAMT and sequence analysis

Using a degenerate primer designed on conserved regions of JHAMT sequences from different insect species and cDNA from CA of adult *B. germanica* females as a template, a 427 bp fragment sequence of a putative JHAMT orthologue from *B.
germanica (BgJHAMT) was attained. Then, 5’-RACE methodologies were followed for completing the 5’ end. Using 3’-RACE we obtained three different 3’-UTR fragments (isoforms A, B and C), all of them finishing with the corresponding polyA tail. All three fragments shared the STOP codon and a contiguous 24-nt sequence. BgJHAMT isoform A (submitted to EMBL database under accession number LT716988) corresponds to a 43 nt sequence, isoform B (LT716989) to a different 187 nt sequence, and isoform C (LT716990) has a sequence identical to the one of isoform B but that continues with an extension of 93 nt (Fig. 1 and Fig. S1). Whereas isoform B shows a canonical polyadenylation signal (AAUAAA) (Retelska et al., 2006) (Fig. S1), isoform A and C do not show an already identified polyadenylation signal sequence. The occurrence of isoforms A and C were confirmed by cloning the sequence fragments obtained by PCR using specific reverse primers at the 3’ end of the respective sequence and a forward primer in the coding sequence. This assessment could not be obtained for the B isoform, because it shares its whole sequence with the C isoform. A recent draft version of the genome of B. germanica (https://www.hgsc.bcm.edu/arthropods/german-cockroach-genome-project), confirmed the sequence obtained by PCR, revealed that BgJHAMT is extended along 6 different exons and showed the different splicing sites that produce the different 3’-UTR isoforms (Fig. 1).

The unique coded protein consists in a 272 amino acid sequence which shows 48.2 %, 32.4 % and 25.0 % identity, respectively, with D. punctata, A. aegypti and D. melanogaster JHAMT sequences.

Using the BgJHAMT sequence and other representative JHAMT sequences available in databases, a maximum-likelihood analysis was performed. We also included into this analysis the proteins from S. gregaria and from D. melanogaster similar to crustacean FAMeT (Gunawardene et al., 2002) and one putative FAMeT
(LT716991) obtained from a _B. germanica_ ovary cDNA library made in our Institute (Irles _et al._, 2009). The topology of the tree indicates that _B. germanica_ JHAMT sequence corresponds to a JHAMT-like protein and clusters apart from the crustacean FAMeT molecules (Fig. S2).

_BgJHAMT_ has the typical organization of a JHAMT protein (Defelipe _et al._, 2011), showing the structure of the active site for binding SAM and the residues that will facilitate the binding and correct positioning of FA or JHA in order to be methylated at their carboxyl groups.

**RNAi of _BgJHAMT_**

In order to confirm the involvement of _BgJHAMT_ in JH synthesis, _BgJHAMT_ mRNA levels were reduced using systemic RNAi. Two micrograms of dsRNA encompassing 326 nt in the coding region of the mRNA (see Table S1 for primer sequences) were injected in adult females during the first day of oothecal transport (dsJHAMT). Treatment was repeated on day 7. Then, the ootheca was removed on day 12 for inducing a second gonadotrophic cycle. Dissections were performed on the fifth day of the second cycle. Control females were treated in an analogous way with a heterologous dsRNA. RNAi treatment produced a complete depletion of _BgJHAMT_ mRNA levels, concomitant with a complete reduction of JH synthesis (Fig. 2 A and B). In addition, JHAMT RNAi treatment triggered a 64 % decrease on vitellogenin expression and a severe reduction of the basal follicle length (Fig. 2 C and D).

**_BgJHAMT_ tissue expression**
We quantified the expression of BgJHAMT in different tissues, brain, CA, fat body, ovary and midgut, of 5-day old adult *B. germanica* females. Results showed that BgJHAMT was highly expressed in CA and only trace amounts were found in ovary (Fig. 3A).

We also analyzed the expression of the different BgJHAMT 3’-UTR isoforms in diverse tissues and developmental situations. We use a pair of primers for quantifying the isoform A, a second pair of primers for quantifying isoforms B plus C (isoform B could not be quantified independently because its sequence is shared by isoform C), and a third pair of primers for specifically quantifying isoform C (see Fig. S1 and Table S1).

The analyzed tissues were CC-CA from 4-day old penultimate instar nymphs (N5) and 4-day old adult females. Results showed that in both cases, the isoform A was the most expressed (around 95 % of the total expression corresponds to this isoform), and isoforms B and C were expressed at a similar very low level (Fig. 3B and C).

**BgJHAMT expression and JH production**

We studied BgJHAMT expression in different physiological and experimental conditions associated with different JH synthesis levels. First of all, we quantified BgJHAMT expression in CC-CA from adult females along the first gonadotrophic cycle. Results showed that BgJHAMT mRNA levels are low at the beginning of the cycle, then they increase in the vitellogenic period and decrease at the end of the cycle (day 7) coinciding with oocyte chorionation (Fig. 3D).

We also examined the relationship between nutrition and BgJHAMT expression. Thus, we quantified BgJHAMT mRNA levels in 5-day old adult females fed and starved since the imaginal molt. Results showed 81 % reduction of BgJHAMT mRNA
levels in CC-CA from starved compared to fed females (Fig. 4A). In addition, we
studied the role of InR and TOR nutritional signaling pathways on BgJHAMT
expression. Using RNAi methodologies, we depleted InR and TOR proteins and
quantified BgJHAMT mRNA levels. Treatment was done in the same way as for
depleting BgJHAMT except that a single injection on the first day of oothecal transport
was performed. Both treatments (dsInR and dsTOR) produced a significant decrease (73
% for dsInR and 40 % for dsTOR) in BgJHAMT mRNA levels in CC-CA of treated vs.
control females (Fig. 4B and C).

Discussion

The involvement of JH in crucial physiological processes implies that the
regulation of its titer must be tightly controlled. Since hemolymph JH titers in B.
germanica nymphs and adult females generally correlate with JH synthesis levels, it is
postulated that the regulation of circulating JH results from the regulation of its
synthesis (Treiblmayr et al., 2006). JHAMT has been pointed out as one of the most
determinant enzymes in the regulation of JH synthesis (Kinjoh et al., 2007; Sheng et
al., 2008; Li et al., 2013). In the present work we report the cloning and expression
studies of the B. germanica orthologue of JHAMT (BgJHAMT). BgJHAMT displays
the characteristic sequence of a SAM-dependent methyltransferase, showing the
structure of the active site for binding SAM and transferring the methyl group to FA or
JHA (Defelipe et al., 2011). It has been proposed that the order in which the two lasts
enzymes of the JH synthesis, JHAMT and JH epoxidase (CYP15A1), act in a given
insect species depends on the JH epoxidase substrate specificity and affinity (Defelipe et
al., 2011). In the cockroach Blaberus giganteus, it has been reported that epoxidase is
more active on methyl farnesoate than in FA, thus suggesting that the order of the
enzymatic activities would first be the methylation and then the epoxidation (Hammock, 1975). We presume that this would also be the case in *B. germanica*.

The topology of a phylogenetic tree constructed using the alignment of the BgJHAMT protein sequence with sequences from other insects, showed that BgJHAMT clusters with known JHAMT proteins and separated from FAMeT crustacean-type proteins, indicating that the identified sequence is the genuine JHAMT orthologue of *B. germanica*.

The involvement of BgJHAMT in JH III synthesis in *B. germanica* is demonstrated by the dramatic reduction of JH III synthesis observed in CA from JHAMT-depleted females. This reduction was concomitant with a decrease in fat body vitellogenin production and basal follicle growth. An experiment showing a reduction of JH synthesis after JHAMT RNAi treatment has only been reported in the locust *S. gregaria* (Marchal *et al.*, 2011). In the cockroach *D. punctata*, a double RNAi treatment, for HMG-CoA reductase and JHAMT, also produced a reduction on JH synthesis (Huang *et al.*, 2015).

In *B. germanica* adult females, BgJHAMT mRNA was detected in CA, with only traces in ovaries. JHAMT has been also reported to be exclusively or very predominantly expressed in CA of all the insects where tissue expression has been tested (Bomtorin *et al.*, 2014; Huang *et al.*, 2015; Lu *et al.*, 2016; Niwa *et al.*, 2008; Nouzova *et al.*, 2011; Marchal *et al.*, 2011; Sheng *et al.*, 2008; Shinoda & Itoyama, 2003). With respect to the presence of JHAMT in ovaries, in *A. aegypti* it has been reported that ovaries can synthesize JH III from JHA III, which points to the occurrence of an operative JHAMT in this tissue (Van Ekert *et al.*, 2014).

We have identified three different BgJHAMT mRNA isoforms (A, B and C) in *B. germanica*, with the differences located in the 3′-UTR. To our knowledge, this is the
For the first time that different JHAMT mRNA isoforms have been reported. The expression of the three isoforms in the CA of adult and penultimate instar nymph females was examined. In both cases, isoform A was the one which showed the highest expression, between 35 and 65 times higher than isoforms B and C. The functional implications of the differential expression of the three isoforms in the stages and tissues in which the enzyme is expressed is beyond the scope of the present work, although we can point to, among other possibilities, a differential regulation of mRNAs stability by miRNAs (Ylla et al., 2016) or the putative occurrence of short open reading frames in the 3′-UTR sequences (Landry et al., 2015).

mRNA levels of BgJHAMT measured in CCL-CA along the gonadotrophic cycle showed low levels in the previtellogenic period, an increase during vitellogenesis, and a posterior reduction in the moment of oviposition. This profile parallels, although with a slight advancement, that of JH synthesis (Maestro et al., 1994). A parallelism between JH synthesis and JHAMT mRNA levels during the juvenile and adult stages has been reported in D. punctata (Huang et al., 2015), A. aegypti (Nouzova et al., 2011), M. sexta (Shinoda & Itoyama, 2003) and S. cynthia ricini (Sheng et al., 2008). In the case of the locust S. gregaria, low JHAMT mRNA levels during the last nymphal instar correlate with the low JH levels necessary for the imaginal molt, whereas higher and relatively constant JHAMT mRNA in CA of adult females do not seem to correlate with the presumable JH synthesis changes produced during the gonadotrophic cycle (Marchal et al., 2011).

Starvation produces a strong reduction of JH synthesis in B. germanica adult females (Maestro et al., 2009). A similar reduction was observed when we knocked down InR or TOR proteins (Maestro et al., 2009; Abrisqueta et al., 2014), what led us to conclude that nutritional status signals to JH synthesis through InR and TOR
pathways. In this context, we decided to quantify BgJHAMT in the three conditions.

BgJHAMT mRNA expression was reduced in CC-CA from starved B. germanica females, as well as from InR or TOR-depleted females, although at different levels. The fact that starvation produces the highest reduction (81%) may be due to the likely effect of starvation on both InR and TOR pathways. The differences between dsInR and dsTOR treatment (73% and 40% of reduction of BgJHAMT mRNA levels, respectively) may be explained by a different depletion of the RNAi treatments of InR and TOR mRNAs, by differences in the protein turnover, but also by a differential action of the two pathways on the regulators of JHAMT expression. Also in the mosquito, A. aegypti, starved and InR and TOR knockdown adult females show reduced JH synthesis and low JHAMT mRNA levels (Pérez-Hedo et al., 2013; 2014). All these results suggest that nutrition activates InR and TOR pathways and that this activation directly or indirectly stimulates the expression of JHAMT, which will result in an increase of JH synthesis.

Taking together, our results show that conditions that modify JH synthesis in adult B. germanica females, as during the gonadotrophic cycle, starvation or reduction of nutritional signaling, show parallel changes of BgJHAMT mRNA levels. This indicates that the JH-specific branch of the synthesis pathway is regulated in the same way as HMG-CoA synthases and reductase in the mevalonate branch (Maestro et al., 2009; Abrisqueta et al., 2014), thus leading to changes in JH production. In the mosquito A. aegypti and the moth B. mori, mRNA of the enzymes of the JH synthetic pathway change their levels in a coordinated way that matches JH synthesis profile (Kinjoh et al., 2007; Nouzova et al., 2011; Rivera-Perez et al., 2014). This is particularly clear in the case of JHAMT, which expression, at least in the case of the moth, has been postulated to be essential to the shutdown of JH synthesis after the
penultimate larval instar and its resume by CA of adult females (Kinjoh et al., 2007; Nouzova et al., 2011). In addition, we have demonstrated that nutrition and its signaling through the InR and TOR pathways are essential for activating JHAMT expression and JH synthesis. This suggests that JHAMT can be a checkpoint for the regulation of JH production in relation to the nutritional status.

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Disclosure

Authors have no potential conflicts of interest to disclose.

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Figure legends

Fig. 1. Structure of the *B. germanica* JHAMT gene. The positions of the codons corresponding to the putative first methionine (Met) and STOP are shown as dotted lines. The common sequence is shown as clear rectangles. The sequences corresponding to the different isoforms are shown as striped rectangles.

Fig. 2. Effect of BgJHAMT RNAi. dsRNAs targeting BgJHAMT (dsJHAMT) or a non-homologous dsRNA (Control) were administered in the first day of oothecal transport and again at day 7. The ootheca was removed on day 12 of oothecal transport and a second gonadotrophic cycle was triggered. Dissections of CC-CA were performed 5 days later. (A) mRNA levels of BgJHAMT (n = 9-10). (B) Rates of JH synthesis by CA incubated in vitro (n = 5-6). (C) mRNA levels of vitellogenin (BgVg) (n = 8-10). (D) Basal follicle lengths (n = 14-16). In the case of mRNA levels the y-axis indicates copies per copy of BgActin 5C. The results are expressed as the mean ± S.E. Asterisks represent significant differences between Control and dsJHAMT subjects (Student’s *t*-test, **P < 0.005; ***P < 0.0001).

Fig. 3. BgJHAMT expression in tissues, isoforms mRNA levels and CA expression during the gonadotrophic cycle. (A) Analysis of BgJHAMT mRNA levels in different *B. germanica* female tissues. (n = 3). Brain, fat body (FB) and midgut values were under the level of detection. CA = corpora allata. (B and C) Differential expression of the BgJHAMT 3’-UTR isoforms in CC-CA from N5 and adult females. Samples were obtained from 4-day old penultimate (5th) nymphal instar (N5) (B) and 4-day old adult females (C). Primers for quantifying isoform A, isoforms B + C and isoform C were used. Results for isoform B were calculated by subtracting values for C from values for
B + C. (n = 4). (C) Expression pattern of BgJHAMT in CC-CA of *B. germanica* females during the first gonadotrophic cycle. (n = 3-4). The different letters (a-b) represent groups with significant differences according ANOVA test (Tukey, P < 0.05). In all cases the y-axis indicates copies per copy of BgActin 5C. The results are expressed as the mean ± S.E.

**Fig. 4.** BgJHAMT expression in CC-CA from starved and TOR and InR RNAi-treated females. (A) BgJHAMT mRNA levels of starved compared to fed females. Starved females received only water after the imaginal molt. Dissections were performed at day 5 of the first gonadotrophic cycle; (n = 5-6). (B) and (C) dsRNAs targeting BgInR (dsInR), BgTOR (dsTOR) or a non-homologous dsRNA (Control) were administered in the first day of oothecal transport. The ootheca was removed on day 12 of oothecal transport and a second gonadotrophic cycle was triggered. Dissections were performed 5 days later. (B) Effect of dsInR treatment (n = 5-7). (C) Effect of dsTOR treatment (n = 6-7). The y-axis indicates copies per copy of BgActin 5C. The results are expressed as the mean ± S.E. Asterisks represent significant differences between Control and treated subjects (Student’s *t*-test, **P < 0.005; ***P < 0.001).
A) Relative Bg-JHAMT mRNA levels

B) JH synthesis (pmol/hxCA pair)

C) Relative Bg-Vg mRNA levels

D) Basal follicle length (mm)
Relative BgJHAMT mRNA levels

A

brain CA FB ovary midgut

B

3'-UTR A 3'-UTR B 3'-UTR C

N5

C

Adult

D

Relative BgJHAMT mRNA levels

Age (days)

0 1 3 5 6 7

a a a a

b
Fig. S1. Sequences of the different BgJHAMT 3'-UTR isoforms. Sequences are shown from position 824. STOP codon, common to the three sequences, is shown in italics. Specific isoform sequences are shown with different colors: purple for isoform A, blue for isoform B and blue (common to B) + green (specific) for isoform C. Canonical polyadenylation signal (AAUAAA) for isoform B is shown in bold letters. Positions of the primers forward and reverse used for quantifying the isoforms are underlined and double-underlined, respectively.
Fig. S2. Phylogenetic relationships of *B. germanica* JHAMT. The tree was constructed using protein sequences and maximum-likelihood approach. Branch lengths are proportional to sequence divergence. The bar represents 0.5 substitutions per site. Bootstrap values (100 replicates) are shown in the nodes. The root of the tree is placed at the divergence between JHAMTs and FAMeTs crustacean-type sequences. GenBank Accession Numbers are shown after the species name.
### Table S1. Primer sequences

#### Degenerated primer used for BgJHAMT cloning

| Forward | 5’-TT(C/T)GAC(A/C)ANAT(C/T)TT(C/T)TCNTT-3’ |

#### Primers used for synthesizing dsJHAMT

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#### Primers used for qPCR

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<th>JHAMT isoform C</th>
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<td>Forward</td>
<td>5’-TGAAATACTGGTGCCACCTTA-3’</td>
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<tr>
<td>Reverse</td>
<td>5’-TGACTCCATTTCGATTTTTTACTCTG-3’</td>
<td>Reverse</td>
<td>5’-GCCAACATAGCTGGTGTTAATA-3’</td>
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