**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*

17 **Abstract**

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

39

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

42 Introduction

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

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

61 JH synthesis pathway can be divided in two branches. The early steps, usually
62 called the mevalonate branch, proceeds from acetyl CoA to farnesyl diphosphate. The
63 mevalonate pathway is present in plants and animals, and in insects it is present in all
64 cells (Bellés *et al.*, 2005). This pathway has been extensively studied because one of its
65 final products is cholesterol, a very important factor in human health. Nevertheless,

66 insect cannot synthesize cholesterol because they lack some enzymes of the sterol
67 synthetic pathway (Bellés *et al.*, 2005). The late steps, from farnesyl diphosphate to JH,
68 are unique to arthropods and, in the case of insects, the enzymes that catalyze these
69 reactions are specific, or mainly expressed, in CA cells (Bellés *et al.*, 2005; Goodman &
70 Cusson, 2012).

71 The order of two last steps in JH synthesis depends on the species. In
72 cockroaches, and in most other groups, it is proposed that methylation of farnesoic acid
73 (FA) to produce methyl farnesoate (MF) precedes epoxidation to JH (Hammock, 1975),
74 whereas in Lepidoptera, the proposed way is epoxidation of FA and then methylation
75 of juvenile hormone acid (JHA) (Goodman & Cusson, 2012). The enzyme that
76 catalyzes the epoxidation is a cytochrome P450, CYP15A1 or JH epoxidase, that has
77 already been characterized in *B. germanica* (Maestro *et al.*, 2010). Independently of the
78 order of the reactions, the enzyme that catalyzes the methylation is usually called JH
79 acid *O*-methyltransferase (JHAMT). JHAMT is a S-adenosyl-methionine-dependent
80 methyltransferase that was cloned for the first time in the silkworm *Bombyx mori*
81 (Shinoda & Itoyama, 2003). Since then, activity for methylating both FA and JHA has
82 been demonstrated in *B. mori* (Shinoda & Itoyama, 2003), the fruit fly *Drosophila*
83 *melanogaster* (Niwa *et al.*, 2008), the beetle *Tribolium castaneum* (Minakuchi *et al.*,
84 2008), the moth *Samia cynthia ricini* (Sheng *et al.*, 2008), the mosquito *Aedes aegypti*
85 (Mayoral *et al.*, 2009) and the psyllid *Diaphorina citri* (Van Ekert *et al.*, 2015). A
86 different protein, farnesoic acid *O*-methyltransferase (FAMeT), has been identified in
87 the prawn, *Metapenaeus ensis*. Recombinant FAMeT produces MF from FA
88 (Gunawardene *et al.*, 2002). Nevertheless, the orthologue protein in insects does not
89 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 HMGC_oA 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.

112

113 Material and methods

114 *Insects*

115 *B. germanica* specimens were obtained from a colony reared on dry dog food
 116 and water, at $30 \pm 1^{\circ}\text{C}$ and 60-70 % relative humidity and in continuous dark.
 117 Dissections were carried out on carbon dioxide-anaesthetized animals, and tissues for
 118 analysis of mRNA levels were immediately frozen in liquid N_2 and stored at -80°C
 119 until their use. For the study of animals during the first gonadotrophic cycle, virgin
 120 females were used. In the case of treatments during the oothecal transport period and
 121 analysis of gene expression in the second gonadotrophic cycle, females producing a
 122 proper ootheca after mating were used. For the starvation experiment, adult females
 123 received only water after the imaginal molt.

124

125 *Cloning of BgJHAMT cDNA*

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

134

135 *Phylogenetic analysis*

136 We used the JHAMT protein sequences from the following species: *A. aegypti*
 137 (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 (AAF46666) and *S. gregaria* (HQ634704). Sequences were aligned using
the iterative refinement method G-INS-i from the program MAFFT v. 7 (Kato &
Standley, 2016). The alignment was analyzed using the RAXML-HPG BlackBox tool of
the CIPRES Science Gateway v. 3.1 (Miller *et al.*, 2010). The data sets were
bootstrapped for 100 replicates.

147

148 *RNA extraction, cDNA synthesis and real-time PCR analyses*

149 cDNAs were synthesized using as previously described (Abrisqueta *et al.*,
150 2017). The absence of genomic contamination was confirmed using a control without
151 reverse transcription. Gene expression was analyzed by quantitative real-time PCR
152 using iQ SYBR Green supermix in an iQ cycler and iQ single color detection system
153 and the iQ5 optical system software v. 2.0 (Bio-Rad Laboratories, Hercules, CA, USA),
154 as previously described (Maestro *et al.*, 2010). Primers used to amplify total BgJHAMT
155 as well as the different 3'-UTR isoforms can be found in Table S1. Primers used to
156 amplify vitellogenin (BgVg) and BgActin 5C (used as a reference) have been already
157 reported (Süren-Castillo *et al.*, 2012). The primers efficiencies were validated by
158 constructing serial cDNA dilutions standard curves. Results were expressed as copies of
159 a specific mRNA per copies of BgActin 5C.

160

161 *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).

170

171 *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.

180

181 **Results**

182 *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.*

186 *germanica* (BgJHAMT) was attained. Then, 5'-RACE methodologies were followed for
187 completing the 5' end. Using 3'-RACE we obtained three different 3'-UTR fragments
188 (isoforms A, B and C), all of them finishing with the corresponding polyA tail. All three
189 fragments shared the STOP codon and a contiguous 24-nt sequence. BgJHAMT isoform
190 A (submitted to EMBL database under accession number LT716988) corresponds to a
191 43 nt sequence, isoform B (LT716989) to a different 187 nt sequence, and isoform C
192 (LT716990) has a sequence identical to the one of isoform B but that continues with an
193 extension of 93 nt (Fig. 1 and Fig. S1). Whereas isoform B shows a canonical
194 polyadenylation signal (AAUAAA) (Retelska *et al.*, 2006) (Fig. S1), isoform A and C
195 do not show an already identified polyadenylation signal sequence. The occurrence of
196 isoforms A and C were confirmed by cloning the sequence fragments obtained by PCR
197 using specific reverse primers at the 3' end of the respective sequence and a forward
198 primer in the coding sequence. This assessment could not be obtained for the B isoform,
199 because it shares its whole sequence with the C isoform. A recent draft version of the
200 genome of *B. germanica* ([https://www.hgsc.bcm.edu/arthropods/german-cockroach-](https://www.hgsc.bcm.edu/arthropods/german-cockroach-genome-project)
201 [genome-project](https://www.hgsc.bcm.edu/arthropods/german-cockroach-genome-project)), confirmed the sequence obtained by PCR, revealed that BgJHAMT is
202 extended along 6 different exons and showed the different splicing sites that produce the
203 different 3'-UTR isoforms (Fig. 1).

204 The unique coded protein consists in a 272 amino acid sequence which shows
205 48.2 %, 32.4 % and 25.0 % identity, respectively, with *D. punctata*, *A. aegypti* and *D.*
206 *melanogaster* JHAMT sequences.

207 Using the BgJHAMT sequence and other representative JHAMT sequences
208 available in databases, a maximum-likelihood analysis was performed. We also
209 included into this analysis the proteins from *S. gregaria* and from *D. melanogaster*
210 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

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

238 We also analyzed the expression of the different BgJHAMT 3'-UTR isoforms in
239 diverse tissues and developmental situations. We use a pair of primers for quantifying
240 the isoform A, a second pair of primers for quantifying isoforms B plus C (isoform B
241 could not be quantified independently because its sequence is shared by isoform C), and
242 a third pair of primers for specifically quantifying isoform C (see Fig. S1 and Table S1).
243 The analyzed tissues were CC-CA from 4-day old penultimate instar nymphs (N5) and
244 4-day old adult females. Results showed that in both cases, the isoform A was the most
245 expressed (around 95 % of the total expression corresponds to this isoform), and
246 isoforms B and C were expressed at a similar very low level (Fig. 3B and C).

247

248 *BgJHAMT expression and JH production*

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

255 We also examined the relationship between nutrition and BgJHAMT expression.
256 Thus, we quantified BgJHAMT mRNA levels in 5-day old adult females fed and
257 starved since the imaginal molt. Results showed 81 % reduction of BgJHAMT mRNA

258 levels in CC-CA from starved compared to fed females (Fig. 4A). In addition, we
 259 studied the role of InR and TOR nutritional signaling pathways on BgJHAMT
 260 expression. Using RNAi methodologies, we depleted InR and TOR proteins and
 261 quantified BgJHAMT mRNA levels. Treatment was done in the same way as for
 262 depleting BgJHAMT except that a single injection on the first day of oothecal transport
 263 was performed. Both treatments (dsInR and dsTOR) produced a significant decrease (73
 264 % for dsInR and 40 % for dsTOR) in BgJHAMT mRNA levels in CC-CA of treated vs.
 265 control females (Fig. 4B and C).

266

267 Discussion

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

283 enzymatic activities would first be the methylation and then the epoxidation (Hammock,
284 1975). We presume that this would also be the case in *B. germanica*.

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

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

298 In *B. germanica* adult females, BgJHAMT mRNA was detected in CA, with
299 only traces in ovaries. JHAMT has been also reported to be exclusively or very
300 predominantly expressed in CA of all the insects where tissue expression has been
301 tested (Bomtorin *et al.*, 2014; Huang *et al.*, 2015; Lu *et al.*, 2016; Niwa *et al.*, 2008;
302 Nouzova *et al.*, 2011; Marchal *et al.*, 2011; Sheng *et al.*, 2008; Shinoda & Itoyama,
303 2003). With respect to the presence of JHAMT in ovaries, in *A. aegypti* it has been
304 reported that ovaries can synthesize JH III from JHA III, which points to the occurrence
305 of an operative JHAMT in this tissue (Van Ekert *et al.*, 2014).

306 We have identified three different BgJHAMT mRNA isoforms (A, B and C) in
307 *B. germanica*, with the differences located in the 3'-UTR. To our knowledge, this is the

308 first time that different JHAMT mRNA isoforms have been reported. The expression of
309 the three isoforms in the CA of adult and penultimate instar nymph females was
310 examined. In both cases, isoform A was the one which showed the highest expression,
311 between 35 and 65 times higher than isoforms B and C. The functional implications of
312 the differential expression of the three isoforms in the stages and tissues in which the
313 enzyme is expressed is beyond the scope of the present work, although we can point to,
314 among other possibilities, a differential regulation of mRNAs stability by miRNAs
315 (Ylla *et al.*, 2016) or the putative occurrence of short open reading frames in the 3'-
316 UTR sequences (Landry *et al.*, 2015).

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

329 Starvation produces a strong reduction of JH synthesis in *B. germanica* adult
330 females (Maestro *et al.*, 2009). A similar reduction was observed when we knocked
331 down InR or TOR proteins (Maestro *et al.*, 2009; Abrisqueta *et al.*, 2014), what led us
332 to conclude that nutritional status signals to JH synthesis through InR and TOR

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

347 Taking together, our results show that conditions that modify JH synthesis in
348 adult *B. germanica* females, as during the gonadotrophic cycle, starvation or reduction
349 of nutritional signaling, show parallel changes of BgJHAMT mRNA levels. This
350 indicates that the JH-specific branch of the synthesis pathway is regulated in the same
351 way as HMG-CoA synthases and reductase in the mevalonate branch (Maestro *et al.*,
352 2009; Abrisqueta *et al.*, 2014), thus leading to changes in JH production. In the
353 mosquito *A. aegypti* and the moth *B. mori*, mRNA of the enzymes of the JH synthetic
354 pathway change their levels in a coordinated way that matches JH synthesis profile
355 (Kinjoh *et al.*, 2007; Nouzova *et al.*, 2011; Rivera-Perez *et al.*, 2014). This is
356 particularly clear in the case of JHAMT, which expression, at least in the case of the
357 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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532

533 **Figure legends**

534

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

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

549 **Fig. 3.** BgJHAMT expression in tissues, isoforms mRNA levels and CA expression
550 during the gonadotrophic cycle. (A) Analysis of BgJHAMT mRNA levels in different
551 *B. germanica* female tissues. (n = 3). Brain, fat body (FB) and midgut values were
552 under the level of detection. CA = corpora allata. (B and C) Differential expression of
553 the BgJHAMT 3'-UTR isoforms in CC-CA from N5 and adult females. Samples were
554 obtained from 4-day old penultimate (5th) nymphal instar (N5) (B) and 4-day old adult
555 females (C). Primers for quantifying isoform A, isoforms B + C and isoform C were
556 used. Results for isoform B were calculated by subtracting values for C from values for

557 B + C. (n = 4). (C) Expression pattern of BgJHAMT in CC-CA of *B. germanica* females
558 during the first gonadotrophic cycle. (n = 3-4). The different letters (a-b) represent
559 groups with significant differences according ANOVA test (Tukey, $P < 0.05$). In all
560 cases the y-axis indicates copies per copy of BgActin 5C. The results are expressed as
561 the mean $\pm$ S.E.

562 **Fig. 4.** BgJHAMT expression in CC-CA from starved and TOR and InR RNAi-treated
563 females. (A) BgJHAMT mRNA levels of starved compared to fed females. Starved
564 females received only water after the imaginal molt. Dissections were performed at day
565 5 of the first gonadotrophic cycle; (n = 5-6). (B) and (C) dsRNAs targeting BgInR
566 (dsInR), BgTOR (dsTOR) or a non-homologous dsRNA (Control) were administered in
567 the first day of oothecal transport. The ootheca was removed on day 12 of oothecal
568 transport and a second gonadotrophic cycle was triggered. Dissections were performed
569 5 days later. (B) Effect of dsInR treatment (n = 5-7). (C) Effect of dsTOR treatment (n =
570 6-7). The y-axis indicates copies per copy of BgActin 5C. The results are expressed as
571 the mean $\pm$ S.E. Asterisks represent significant differences between Control and treated
572 subjects (Student's *t*-test, $**P < 0.005$; $***P < 0.001$).

573

Isoform A

AAAATATAGTTTCAGCTATGAAATACTGGTGGCGCACTTACGGAAGTCATAACTCACTA
CCGCCATCTATAGGATAACTACAGAAGGTATTAACACCAGCTATGTTGGCTCAATCGGT
TGACAAGTATGAAAAAAAAAAAAAAAAAAAAA

Isoform B

AAAATATAGTTTCAGCTATGAAATACTGGTGGCGCACTTACGGAAGTCATAACTCACTA
CCGCCATCTATAGGATAACTACAGAAGACCAATGATGGGAAAAGCTGGAGCCATGGTAC
GTTTGGCAACGCTCAGCGGACGCTTACTTACTTGTGGTTGAATTGCCATTACTTTAAAT
GGTTTTTACGCAATTTCTACGTATATAGAGAGTTTCTTAAGTAACTTTTTATTAATTCC
AAAATTATAATATAAT**AAATAA**TTAATATCCTATACCAAAAAAAAAAAAAAAAAAAAA

Isoform C

AAAATATAGTTTCAGCTATGAAATACTGGTGGCGCACTTACGGAAGTCATAACTCACTA
CCGCCATCTATAGGATAACTACAGAAGACCAATGATGGGAAAAGCTGGAGCCATGGTAC
GTTTGGCAACGCTCAGCGGACGCTTACTTACTTGTGGTTGAATTGCCATTACTTTAAAT
GGTTTTTACGCAATTTCTACGTATATAGAGAGTTTCTTAAGTAACTTTTTATTAATTCC
AAAATTATAATATAATAATAAATAAATTAATATCCTATACCTATTTTGTGTATTTTCTGTAA
CGTGAAAAGTGTGGTATCAGAAAATATCCTGTAATTATTTATAATTCTTACTTAACAT
TTACTCAGTTTCAAAAAAAAAAAAAAAAAAAAA

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'
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Primers used for synthesizing dsJHAMT

Forward	5' -CTTCTGTCTGCATTTTGTAGTGGAT-3'
Reverse	5' -TGGCTTGTAGTGAATCTTGAAGAA-3'

Primers used for qPCR

Total JHAMT	
Forward	5' -GACCTGGTGGTGAAGTCTTGG-3'
Reverse	5' -TGACTCCATTTTCGATTTTTTACTCTG-3'
JHAMT isoform A	
Forward	5' -TGAAATACTGGTGGCGCACTTA-3'
Reverse	5' -GCCAACATAGCTGGTGTTAATA-3'
JHAMT isoforms B + C	
Forward	5' -TGAAATACTGGTGGCGCACTTA-3'
Reverse	5' -CTCCAGCTTTTCCCATCATTG-3'
JHAMT isoform C	
Forward	5' -CGCAATTTCTACGTATATAGAGAGTT-3'
Reverse	5' -TCACGTTACAGAAAATACAACAAAATAGG-3'