1 The potential role of juvenile hormone acid methyltransferase in
2 methyl farnesoate (MF) biosynthesis in the swimming crab,

3 *Portunus trituberculatus*

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Abstract: Juvenile hormone (JH) and methyl farnesoate (MF) play essential roles in the development and reproduction of insects and crustaceans respectively. Juvenile hormone acid methyltransferase (JHAMT) catalyzes the methyl esterification in insect JH biosynthesis, while the corresponding step in crustacean MF biosynthesis was long thought to be catalyzed by farnesoic acid *O*-methyltransferase (FAMeT). However, the new discovery of JHAMT orthologs in crustaceans indicates that JHAMT may also play essential role in the MF biosynthesis in crustaceans. Here we cloned and characterized the full-length cDNA encoding JHAMT in the swimming crab *Portunus trituberculatus* (*PtJHAMT*). Sequence and structure analysis of *PtJHAMT* revealed that it was composed of a 6-stranded β sheet with 9 α helices, and contained a signature Sadenosyl-L-methionine (SAM) binding motif, which is the hallmark in all SAM dependent methyltransferases (SAM-MTs). Several active sites that are critical for the interaction of SAM and JH/FA substrate were also conserved in *PtJHAMT*. The gene expression of *PtJHAMT* was highly specific to the mandibular organ, which is the sole site of MF synthesis. *PtJHAMT* expression significantly increased in the late-vitellogenic stage and mature stage, which suggests a possible role of *PtJHAMT* in modulating ovarian development. The role of *PtJHAMT* and *PtFAMeT* in MF biosynthesis was further investigated by RNA interfering (RNAi). Injection of *PtJHAMT* and *PtFAMeT* dsRNA both led to a decrease in hemolymph MF titers. Injection of *PtHMGR* dsRNA caused the decrease in *PtJHAMT* expression, but had no effect on mRNA level of *PtFAMeT*. Together these results suggested that JHAMT and FAMeT are both involved in the MF biosynthesis in crustaceans, while the

45 JHAMT is highly specific to FA substrate, and FAMeT may have more catalytic
46 functions.

47 **Keywords:** JHAMT; MF biosynthesis; ovarian maturation; RNAi; *Portunus*
48 *trituberculatus*

49 **1. Introduction**

50 Juvenile hormone (JH) is known to regulate many aspects of insect physiology,
51 including development, reproduction, diapause, and polyphenisms(Nijhout, 1998;
52 Riddiford, 1994; Wyatt and Davey, 1996). As insufficient evidence for the existence
53 of JH in crustaceans, the methyl farnesoate (MF), an unepoxidized form of insect JH
54 III, is commonly considered as an equivalent of JH in crustaceans(Miyakawa et al.,
55 2014). The physiological functions of MF in crustaceans have been investigated
56 extensively, including regulation of morphogenesis (Laufer et al., 2005; Rotllant et al.,
57 2000), osmoregulation (Lovett et al., 2006, 2001), reproduction (Chan et al., 2005;
58 Laufer et al., 1998, 1992; Mak et al., 2005), and molting (Chang et al., 1993; Tamone
59 and Chang, 1993).

60 Both JH and MF are sesquiterpenoids, that are synthesized in the corpora allata
61 (CA) in insects and mandibular organ (MO) in crustaceans, respectively. Biosynthesis
62 of insect JH and crustacean MF is similar, which conventionally can be divided into
63 two phases. The early phase involves the production of farnesyl pyrophosphate (FPP)
64 from simple acetate molecules via the conserved mevalonate biosynthetic pathway
65 (Bellés et al., 2005). 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), the
66 rate-limiting enzyme for mevalonic acid biosynthesis in mammals, is also an

67 important enzyme in the early steps of JH and MF biosynthesis (Bellés et al., 2005; Li
68 et al., 2010). In the later phase, FPP is sequentially transformed to farnesol, farnesal
69 and farnesoic acid (FA) (Wen et al., 2015). Then FA is methyl esterified and epoxied
70 to produce JH in insects, whereas FA is only methyl esterified to produce MF in
71 crustaceans (Miyakawa et al., 2014).

72 The methyl esterification in the final steps of the insects JH biosynthesis is
73 catalyzed by the Juvenile hormone methyltransferase (JHAMT), which was thought to
74 be highly specific in insects (Li et al., 2013). The JHAMT gene was first cloned from
75 the CA of the silkworm, *Bombyx mori* (Shinoda and Itoyama, 2003) and was found to
76 belong to the Sadenosyl-L-methionine-dependent methyltransferase (SAM-MT)
77 superfamily. The expression levels of *BmJHAMT* exhibited a strong correlation with
78 the rates of JH biosynthesis, and transcriptional suppression of *BmJHAMT* was found
79 to be critical for the initiation of metamorphosis (Kinjoh et al., 2007; Shinoda and
80 Itoyama, 2003). Subsequently, several JHAMT gene were cloned and characterized
81 from other insects in different orders, which showed the spatial and catalytic
82 properties were similar to BmJHAMT (Minakuchi et al., 2008; Mayoral et al., 2009;
83 Marchal et al., 2011; Niwa et al., 2008; Li et al., 2013). All these studies have
84 displayed essential roles of JHAMT in insect metamorphosis and reproduction.

85 In crustaceans, the methyl esterification of FA to MF was thought to be
86 catalyzed by another MTase, farnesoic acid *O*-methyltransferase (FAMeT), which was
87 well identified in several crustaceans (Gunawardene et al., 2002, 2001; Holford et al.,
88 2004; Hui et al., 2008; Kuballa et al., 2007; Xie et al., 2013; Yang et al., 2012;),

89 FAMEt lacks the signature SAM-binding motif which is present in insects JHAMT,
90 thus it may represent a novel SAM-dependent MTase (Gunawardene et al., 2001).
91 Protein and mRNA of FAMEt are widespread among tissues, but its catalytic activity
92 has only been detected in the MO of crustaceans. FAMEt has been demonstrated to
93 catalyze the conversion of FA to MF in the shrimp *Metapenaeus ensis* (Gunawardene
94 et al., 2002), and the lobster *Homarus americanus* (Holford et al., 2004). RNA
95 interference (RNAi) of FAMEt mRNA expression has been found to affect molting in
96 the shrimp *Litopenaeus vannamei* (Hui et al., 2008).

97 Recently, orthologs of JHAMT have been identified in three crustacean species,
98 including the water flea *Daphnia pulex* (Hui et al., 2010), *Daphnia magna* (NCBI
99 protein id: JAN67028.1) and the shrimp *Neocaridina denticulata* (Sin et al., 2015),
100 which altered our earlier opinion that JHAMT is unique to insect. Presence of
101 JHAMT in crustaceans raises the question about the potential interaction with MF
102 biosynthesis and crustacean endocrinology. Although *JHAMT* is known to be one of
103 the most up-regulated genes in the presence of kairomone signals in water fleas
104 *Daphnia pulex* (Miyakawa et al., 2010), the functional role of newly discovered
105 JHAMT in crustaceans is poorly understood.

106 The swimming crab, *Portunus trituberculatus* is a commercially important
107 crustacean species and has been extensively artificially propagated and cultivated. The
108 ovarian development of *P. trituberculatus* may be positively regulated by MF, and the
109 two MF synthesis enzymes HMGR and FAMEt are closely involved (Xie et al., 2015).
110 In this study, the full-length cDNA encoding a novel crustacean JHAMT was cloned

111 from the swimming crab *Portunus trituberculatus*, and expression pattern of this gene
112 suggested it may also be involved in the ovarian maturation of *P. trituberculatus*. In
113 addition, RNAi-mediated gene knockdown was utilized to investigate the functional
114 role of JHAMT in the MF biosynthesis of this species.

115 **2. Materials and methods**

116 **2.1 Experimental animals**

117 Female swimming crabs (body weight: 300-350 g) were collected before their
118 first spawning and temporarily reared at the Ninghai Deshui Aquaculture Farm. Crabs
119 were maintained in large tanks with through-flowing sea water and fed with live razor
120 clams once a day. Based on the gonadosomatic index (GSI%, $\text{ovary weight} \times 100 /$
121 body weight) and external features of the ovary, the second ovarian development of *P.*
122 *trituberculatus* can be divided into stage I (pre-vitellogenic stage), stage II (early-
123 vitellogenic stage), stage III (late-vitellogenic stage), and stage IV (mature stage) (Yao
124 et al., 2007). Intact crabs from different ovarian developmental stages were selected
125 for sampling. Mandibular organs of each staged crab were removed, and placed in
126 RNA preservation fluid (Shanghai Sangon), and stored at -20°C. For the crabs at stage
127 I, tissues sampled included eyestalk, Y-organ, thoracic ganglia, muscle,
128 hepatopancreas, ovary, and heart for detecting the tissues distribution of JHAMT.
129 Experiment of the dsRNA injection was carried on the 25th day after the first spawning
130 of swimming crabs, a time most crabs are at stage III and crabs in each injection group
131 were sacrificed at 0, 12, 24, 48, and 96 h post injection. Hemolymph was then
132 collected for detecting MF titer, and MOs were removed for further expression

133 analysis. All crab investigations were carried out according to Animal Care and Use of
134 Science and Technology guidelines.

135 2.2 RNA isolation and cDNA preparation

136 Total RNA of the sampled tissues was extracted using Trizol RNA isolation
137 reagent (Sangon) according to the manufacturer's instructions, and genomic DNA was
138 removed by DNase I (Takara). RNA concentrations was determined using a
139 NanoDrop 2000 UV Spectrophotometer (Thermo Fisher), and RNA integrity was
140 determined by visualization of clear bands of the 18S and 28S ribosomal RNA on
141 agarose gel. cDNA was then prepared with 1 µg RNA using Perfect Real Time
142 PrimeScript® RT reagent Kit (Takara), and stored in -80 °C.

143 2.3 Cloning of the full-length cDNAs encoding PtJHAMT

144 All Gene-specific primers in cDNA cloning were designed from an ortholog of
145 JHAMT in our *P. trituberculatus* transcriptome database (Table 1). The cDNA of MO
146 was used as templates for PCR validation of the sequence information. To obtain the
147 full-length cDNA, 5' RACE and 3' RACE were performed according to the
148 manufacturer's instructions of SMARTer™ RACE cDNA Amplification kit (Clontech,
149 USA). The following program was used for each PCR amplification: initial
150 denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C (58°C in
151 3' RACE) for 30 s, and 72°C for 90 s, with a final elongation at 72°C for 10 min. All
152 PCR products were analyzed by 1% agarose gel electrophoresis, and the PCR
153 products were gel-purified using the PCR purification kit (Sangon), ligated with the
154 pMD19-T vector (Takara), and transformed into competent *Escherichia colicells*.

155 After transformation, three positive clones were picked for sequencing (Invitrogen).

156 2.4 Sequence alignment and analysis

157 Sequence splicing and open reading frame (ORF) identification were performed
158 using Vector NTI 10.0 software. The sequences were compared with representative
159 JHAMT sequences selected in the NCBI database using the BLAST program
160 (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequence analysis was performed using the
161 ExPASy Molecular Biology server (<http://www.us.expasy.org/>) and SignalP 4.0 Server
162 (<http://www.cbs.dtu.dk/services/SignalP>). The secondary structure of AmJHAMT was
163 predicted using PredicProtein (<https://www.predictprotein.org/>), PRIPRED
164 (<http://bioinf.cs.ucl.ac.uk/psipred/>), and Jpred 3
165 (<http://www.compbio.dundee.ac.uk/jpred/>). Consistent structural predictions from
166 these different methods were considered to be strong evidence for secondary structure
167 elements. The phylogenetic tree was generated based on the representative JHAMT
168 and FAMeT sequences using MEGA 4.0.

169 2.5 RNA interference

170 Amplicons of *PtHMGR* (GenBank accession no.KF280756), *PtFAMeT*
171 (GenBank accession no.KC192659), *PtJHAMT*, and *green fluorescent protein (GFP)*
172 were cloned into pMD19-T vector (Takara) and amplified by PCR using a T7 promoter
173 linked primers (Table 1). The resultant DNA was used as templates to synthesize
174 dsRNA. The dsRNA was synthesized and purified according to the manufacturer's
175 instructions of the MEGAscript RNAi Kit (Ambion). Integrity of the dsRNA was
176 checked on agarose gel, and the concentration of dsRNA were checked with a

177 NanoDrop 2000 UV Spectrophotometer (Thermo Fisher). Crabs receiving injection
178 were divided into five groups, and the injection was carried via the base of the last
179 walking foot with 1 mL syringe. The control group were injected with 150 μ L crab
180 saline (Duan and Cooke, 1999), while the remaining four groups were respectively
181 injected with 300 μ g (dissolved in 150 μ L crab saline) dsRNA of *PtHMGR*, *PtFAMeT*,
182 *PtJHAMT*, and *GFP*.

183 2.6 Detection of hemolymph MF titers

184 Hemolymph levels of MF in the post-injection crabs was detected by gas
185 chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM).
186 The GC-MS sample preparation and the GC-MS detection were conducted as
187 previously described (Xie et al., 2015).

188 2.7 Analysis of gene expression

189 Gene expression in this study were all determined using quantitative real time
190 PCR (qPCR). The cDNAs prepared above were amplified with gene specific primers
191 and normalized relative to the beta-actin gene of *P. trituberculatus* (Table 1). PCR
192 was carried out using the SYBR[®] Premix Ex Taq[™] II kit (Dalian Takara) according
193 to the manufacturer's instructions. PCR conditions were as follows: 95°C for 2 min,
194 followed by 40 cycles of 95°C for 15 s, and 58°C for 20 s. To confirm product
195 specificity, a melting curve analysis of the amplified DNA was performed following
196 amplification, at temperatures between 55 and 95°C, with the temperature increasing
197 at a rate of 0.2°C /s. Each cDNA sample was assayed three times and data expressed
198 as mean $\pm$ SEM. Results were analyzed by the $2^{-\Delta\Delta C_t}$ method. Transcript abundance in

199 a representative replicate was set as calibrator. All other quantities were expressed as
200 an n-fold difference relative to the calibrator. The statistical significance of
201 differences among means were determined by student's t test using SPSS 17.0.

202 **3. Results**

203 3.1 Cloning and characterization of *PtJHAMT*

204 The full length cDNA sequence of *PtJHAMT* is 1307 bp long, including a 211 bp
205 5' untranslated region (UTR) and a 280 bp 3' UTR that contains a polyA signal
206 (AATAAA) and a 19 bp polyA tail (Fig. 1). The *PtJHAMT* ORF encodes a protein of
207 271 amino acids with a predicted molecular weight of 31.13 kDa and a theoretical
208 isoelectric point of 5.09. No signal peptide cleavage sites were detected in the amino
209 acid sequence, indicating that it is a non-secretory protein. Sequence alignment
210 showed that *PtJHAMT* shared 72, 43, 34, 33, 31 and 30% amino acid identity with the
211 reported JHAMTs from *Neocaridina denticulate*, *Daphnia pulex*, *Schistocerca*
212 *gregaria*, *Drosophila melanogaster*, *Bombyx mori*, and *Apis mellifera* respectively
213 (Fig. 2). The typical SAM binding motif “hh(D/E) hGxGxG” which is present in
214 insects JHAMT, was also found in *PtJHAMT* as VLDIGCGSG. Analysis of the
215 secondary structure of *PtJHAMT* revealed the presence of a typical SAM-MT fold,
216 composed by a 6-stranded β sheet with 9 α helices. In addition, several active sites
217 that are critical for interaction with SAM and FA/JHA substrates are also conserved in
218 *PtJHAMT* (Fig. 2). A phylogenetic tree was constructed based on representative
219 JHAMT and FAMeT amino acid sequences selected in the NCBI database. The
220 topologic phylogenetic tree revealed that the JHAMTs and FAMeTs were each grouped

221 together. The *PtJHAMT* belonged to the JHAMT group, and was more closely related
222 to other crustacean JHAMTs (Fig. 3). The full length sequence of *PtJHAMT* was
223 submitted to GenBank with the accession number KU159280.

224 3.2 Tissue distribution of *PtJHAMT* and its expression during ovarian maturation

225 By using qPCR, the transcript level of *PtJHAMT* was detected in several tissues
226 including MO, eyestalk (ES), Y-organ (YO), muscle (MS), thoracic ganglion (TG),
227 hepatopancreas (HP), ovary (OV), and heart (HT). *PtJHAMT* was highly expressed in
228 the MO, while its expression in other tissues was at very low levels (Fig. 4). To test
229 the correlation of *PtJHAMT* expression level with ovarian maturation, the variation of
230 *PtJHAMT* mRNA level was determined during the second ovarian development. The
231 expression of *PtJHAMT* was at low level in pre-vitellogenic stage (Stage I) and
232 early-vitellogenic stage (stage II), and then increased significantly in late-vitellogenic
233 stage (stage III) and mature stage (stage IV) (Fig. 5).

234 3.3 Efficiency of RNAi

235 The dsRNA treatments in this study all exhibited acceptable efficiency in
236 interfering the mRNA level of target genes. A clear decrease in *PtHMGR* and
237 *PtJHAMT* mRNA level was detected at 12 and 24 h post injection, when respectively
238 compared to the control group (Fig. 6A and 6C). The significant reduction of
239 *PtFAMeT* expression occurred only at 24 h post injection (Fig. 6 B).

240 3.4 Effect of RNAi on hemolymph MF titers

241 MF titers in the hemolymph samples obtained from dsRNA treatment were tested
242 by GC-MS. As showed in Fig. 7, the hemolymph MF titers maintained above 20

243 ng/mL in the control group and GFP control group. The *HMGR* dsRNA reduced the
244 hemolymph MF titers to about 10 and 6 ng/mL at 24 h and 48h respectively. Level of
245 MF respectively dropped to 10 and 12 ng/mL at 12 h and 24 h post the injection of
246 *JHAMT* dsRNA. In the *FAMeT* dsRNA treatment group, a significantly reduction of
247 hemolymph MF titers was only found at 24 h post injection, when the corresponding
248 MF concentration was about 13 ng/mL.

249 3.5 Expression of *PtFAMeT* and *PtJHAMT* in respond to *PtHMGR* dsRNA treatment

250 To further explore the role of *PtFAMeT*, and *PtJHAMT* in MF biosynthesis, the
251 mRNA transcript levels of *PtFAMeT* and *PtJHAMT* were respectively determined
252 within the *PtHMGR* dsRNA injection group. The expression of *PtFAMeT* was not
253 affected by the RNAi of *PtHMGR*, while the expression of *PtJHAMT* was
254 significantly down-regulated by *PtHMGR* dsRNA at 24 h and 48 h post injection (Fig.
255 8).

256 4. Discussion

257 JHAMT has been mostly described in insects with the catalytic activity in the
258 methylation of FA/JHA to MF/JH III (Li et al., 2013; Marchal et al., 2011; Mayoral et
259 al., 2009; Shinoda and Itoyama, 2003; Van Ekert et al., 2014). However, in
260 crustaceans, JHAMT has only been reported in the water flea *D. pulex* (Hui et al.,
261 2010), *D. magna* and the shrimp *N. denticulata* (Sin et al., 2015). This study cloned
262 and characterized a full-length cDNA encoding JHAMT in the swimming crab *P.*
263 *trituberculatus*, which suggests that JHAMT may be more widely presented in
264 crustaceans and play an important role in the biosynthesis of MF in crustaceans.

265 The PtJHAMT protein contains a conserved SAM-binding motif (VLDIGCGSG)
266 located close to the N-terminal part of the protein, indicating it belongs to the
267 SAM-MT superfamily, which use SAM as the methyl donor to methylate substrates
268 (Martin and McMillan, 2002). Analysis of the secondary structure of PtJHAMT
269 revealed the presence of a typical SAM-MT fold, composed by a 6-stranded β sheet
270 with 9 α helices. The expected topology of the fold of PtJHAMT was close to that
271 known from the lipid SAM-MT fold (Grana et al., 2007). This suggests lipids are the
272 source of the substrates of PtJHAMT. In fact, all insect JHAMTs have a relatively
273 high substrate specificity to JHA or FA than to other fatty acids (Mayoral et al., 2009;
274 Minakuchi et al., 2008; Niwa et al., 2008; Shinoda and Itoyama, 2003). To better
275 understand the substrate specificity, several amino acid residues critical for
276 interactions with SAM and substrates were identified in insects through modeling
277 tertiary structure of JHAMTs visually (Defelipe et al., 2011). In *Aedes aegypti* JHAMT,
278 for instance, Asp-69, Asp-41, Val-70 and Ile-95 are responsible for SAM binding and
279 locating, Gln-14, Trp-120, Ile-151, Ile-154, Tyr 155, Leu-158, Val-221, and Val-224
280 are responsible for FA or JHA binding and locating, and Ser-176 and Tyr-178 can bind
281 the epoxide group of (10R)-JHA (Defelipe et al., 2011). All active sites of AaJHAMT
282 are also conserved in PtJHAMT, indicating the enzyme may have potential for
283 catalyzing JHA or FA.

284 This is signified by the finding that transcripts of *PtJHAMT* are localized
285 primarily in the MO, which is the sole site of MF synthesis in crustaceans (Nagaraju,
286 2007). This result is consistent with the tissue distribution of insect JHAMT, whose

expression is highly specific to the CA (Mayoral et al., 2009; Shinoda and Itoyama, 2003). The specific expression of *PtJHAMT* in MO would be reminiscent the distribution features of HMGR, whose gene expression and enzyme activity are both specific to MO (Li et al., 2003; Qiu et al., 2014). HMGR is involved in the production of mevalonate, and has been proved to be essential for the MF or JH synthesis in crustaceans and insects (Bellés et al., 2005; Li et al., 2010; Wen et al., 2015). Expression pattern of *PtJHAMT* and *PtHMGR* are also similar in the process of ovarian maturation and variation of *PtJHAMT* expression is well correlated with that of *PtHMGR* expression and hemolymph MF titers previously reported during the ovarian maturation of *P. trituberculatus* (Xie et al., 2015).

To explore the role of *PtJHAMT* in MF biosynthesis, we applied a RNAi-mediated gene knockdown by the injection of *PtHMGR* and *PtJHAMT* dsRNA respectively into the late-vitellogenic crabs. In the *PtJHAMT* dsRNA group, the hemolymph MF titers significantly decreased at 12 h and 24 h post injection, the same as the time-points when *PtJHAMT* expression was down-regulated. This result may show a close relation of *PtJHAMT* to the MF biosynthesis. As expected, the expression of *PtJHAMT* was significantly down-regulated by the *PtHMGR* dsRNA injection, although in a delayed manner. This delay may be due to the earlier appearance of HMGR in MF biosynthesis. The *PtHMGR* dsRNA can also lead to the significantly decline in hemolymph MF titers. The MF reduction by *PtHMGR* dsRNA may be partly through the inhibition of *PtJHAMT* expression, for the decrease of *PtJHAMT* mRNA level and hemolymph MF titers were synchronized. For the closely

309 relation to PtHMGR and MF synthesis, together with the highly structural similarity
310 to insect JHAMT, we suggested that the PtJHAMT is probably involved in MF
311 biosynthesis. It may be specifically responsible for the conversion of FA to MF, just
312 like the insect JHAMT.

313 As the conversion of FA to MF in MO has long been thought to be catalyzed by
314 FAMEt in the past decades (Gunawardene et al., 2002, Holford et al., 2004, Hui et al.,
315 2008), we further investigated the role of PtFAMEt in MF biosynthesis. A
316 significantly loss of hemolymph MF titers was detected as shown by the decrease in
317 *PtFAMEt* expression level caused by *PtFAMEt* dsRNA treatment. This result
318 indicated that PtFAMEt is also essential for MF biosynthesis, which is consistent with
319 early finding that MF production increased in relation to the increasing amounts of
320 rFAMEt purified from the shrimp *Metapenaeus ensis* (Gunawardene et al., 2002).
321 However, the mRNA level of *PtFAMEt* was not affected by the *PtHMGR* dsRNA
322 injection, indicating the expression of FAMEt was not mediated by HMGR. As the
323 HMGR-mediated mevalonate pathway is the only identified pathway in producing FA,
324 FAMEt may not be specific to FA substrate, and it may also catalyze other fatty acids.
325 This hypothesis could further explain why the FAMEt activity on producing MF has
326 only been detected in MO when its protein and mRNA are widespread in crustaceans
327 (Gunawardene et al., 2002; Holford et al., 2004; Hui et al., 2010, 2008).

328 To conclude, we have identified and characterized the full-length cDNA of
329 JHAMT in *P. trituberculatus*, and this is the first report of JHAMT in brachyurans.
330 The PtJHAMT is structurally similar to the insect JHAMTs, and is highly specific to

the MO. The expression pattern of *PtJHAMT* suggested it is closely involved in the ovarian maturation of *P. trituberculatus*. It may catalyze the methyl esterification of FA to MF, thereby regulating ovarian development. FAMEt is also involved in MF biosynthesis and ovarian maturation, but its catalytic function may not be restricted to MF production. Our findings not only shed new light on the sesquiterpenoid hormone biosynthesis in *P. trituberculatus*, but also contribute to a better understanding of the endocrinological basis underlying the regulation of reproduction and other MF-mediated physiological processes in crustaceans.

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486 **Table 1.** Primers used in this study

Primer	Sequence(5' - 3')	Purpose
JHAMTF	TTCCGCCAGCTAGACATT	RT-PCR
JHAMTR	CTCGCAAGACACCACCTC	RT-PCR
GSPF	TTCCTGAAGCGGGTGCCT	3'-RACE
GSPR	CGGCTCGCTCAATGTCTA	5'-RACE
JHAMTQF	TTCCTGAAGCGGGTGCCT	qPCR
JHAMTQR	GCCGTCGTTCTCTTGGTCT	qPCR
FAMeTQF	GAGTTCTGGATTGCCGTGG	qPCR
FAMeTQR	GCCCCAGCCAGTGGAGTA	qPCR
HMGRQF	ATTGCTGGTCGCCTGTTGT	qPCR
HMGRQR	ATCCTGCCTCAGTCCCCTTA	qPCR
Actin-F	CGAAACCTTCAACACTCCCG	qPCR
Actin-R	GATAGCGTGAGGAAGGGCATA	qPCR
JHAMTIF	<u>TAATACGACTCACTATAGGGG</u> TGCTGGACATTGGCTGTG	RNAi
JHAMTIR	<u>TAATACGACTCACTATAGGGG</u> TCTTGCCGTCGTTCTCTT	RNAi
FAMeTIF	<u>TAATACGACTCACTATAGGGG</u> CCTCTGCCATCAGGTTCA	RNAi
FAMeTIR	<u>TAATACGACTCACTATAGGGG</u> AACGAAGACAAGGATGTAAGC	RNAi
HMGRIF	<u>TAATACGACTCACTATAGGGG</u> GCTATCATCAAGGAAGGGTTT	RNAi
HMGRIR	<u>TAATACGACTCACTATAGGGG</u> CCAGCAGCTAAGGCAGACAT	RNAi
GFPIF	<u>TAATACGACTCACTATAGGGG</u> CGACGTAAACGGCCACAAGT	RNAi
GFPIR	<u>TAATACGACTCACTATAGGGG</u> CTTGACAGCTCGTCCATGC	RNAi

487 T7 promoter sequence was underlined.

488 **Figure captions**

489 **Fig. 1 The nucleotide and deduced amino acid sequence of PtJHAMT from the**
490 **swimming crab, *P. trituberculatus*.** The open reading frame (ORF) is shown in bold
491 letters; the deduced amino acid sequence of ORF is shown in single letter code below
492 the nucleotide sequence. The typical SAM binding motif “VLDIGCGSG” is boxed by
493 solid line, the stop codon is indicated with asterisk (*), and the polyA signal is
494 underlined.

495 **Fig. 2 Multiple alignment of PtJHAMT with representative JHAMT sequences.**
496 Species abbreviations included in the sequence labels are as follows: Nd, *Neocaridina*
497 *denticulate*; Dp, *Daphnia pulex*; Sg, *Schistocerca gregaria*; Dm, *Drosophila*
498 *melanogaster*; Bm, *Bombyx mori*; Am, *Apis mellifera*. Residues highlighted by the red
499 box are the conserved SAM binding motif. The small triangles indicate the residues in
500 contact with SAM and substrates (JHA or FA). Secondary structure elements of
501 PtJHAMT are showed as cylinders (α helices) and arrows (β strands). Numbers after
502 each sequence represent % identity relative to PtJHAMT.

503 **Fig. 3 Phylogenetic analysis of JHAMT and FAMeT homologs from different**
504 **species based on amino acid sequences.** Sequences are labeled with the species
505 Latin names plus GenBank accession numbers. The tree was built by the Neighbor
506 joining method (1000 bootstrap), and displayed topologically.

507 **Fig. 4 Tissues distribution of *PtJHAMT* transcripts.** Tissue abbreviations are as
508 follows: MO, mandibular organ; ES, eyestalk; YO, Y-organ; MS, muscle; TG, thoracic
509 ganglia; HP, hepatopancreas; OV, ovary; HT, heart.

510 **Fig. 5 Relative expression of *PtJHAMT* transcript in MO during the second**
511 **ovarian development of *P. trituberculatus*.** Developmental stages are
512 pre-vitellogenic stage (I), early-vitellogenic stage (II), late-vitellogenic stage (III) and
513 mature stage (IV). A replicate from stage I was used as calibrator. Bars represent mean
514 + SEM (n=5). Asterisks (*) represent values statistically different (P<0.05) from the
515 pre-vitellogenic stage.

516 **Fig. 6 RNAi efficiency of *PtHMGR* (A), *PtFAMeT* (B), and *PtJHAMT* (C) dsRNA.**
517 A replicate from 0 h post injection was used as calibrator within each group. Bars
518 represent mean + SEM (n=5). Asterisks (*) represent values statistically (P<0.05)
519 different from the control group.

520 **Fig. 7 Effect of dsRNA treatment on hemolymph MF titers.** Bars represent mean +
521 SEM (n=5). Asterisks (*) represent values statistically different (P<0.05) from the
522 control group.

523 **Fig. 8 Change of *PtFAMeT* and *PtJHAMT* expression with *PtHMGR* dsRNA**
524 **treatment.** The change in expression of *PtHMGR* was indicated by grey line. Bars
525 represent mean + SEM (n=5). Asterisks (*) represent values statistically different
526 (P<0.05) from the 0 h group.

Fig. 1

[illegible]

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8