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Research paper

Juvenile hormone regulates the differential expression of putative *juvenile hormone esterases* via *methoprene-tolerant* in non-diapause-destined and diapause-destined adult female beetle

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ABSTRACT

Juvenile hormone (JH) plays an essential role in regulating molting, metamorphosis, reproduction, and diapause (dormancy), in many insects and crustaceans. JH esterases (JHEs) can control JH titer by regulating JH degradation. Although the biochemistry and structure of JHEs have been well studied, regulation of their expression remains unclear. We identified three putative *JHEs* (*JHE1*, *JHE2*, *JHE3*) in the cabbage beetle *Colaphellus bowringi*, and investigated the regulation of their expression by JH signaling in non-diapause-destined (NDD, reproductive) and diapause-destined (DD) female adults. Sequence and phylogenetic tree analyses indicate that the three putative JHEs shared conserved motifs with the JHEs of other insects and one crustacean, and were similar to Coleopteran, Dipteran, Orthopteran, Hymenopteran, and Decapodan JHEs. They were, however, less closely related to Hemipteran and Lepidopteran JHEs. *JHEs* were more highly expressed in NDD female adults than in DD female adults. JH analog induction in DD female adults significantly upregulated the expression of *JHE1* and *JHE2*, but had no effect on the expression of *JHE3*. Knockdown of the JH candidate receptor *methoprene-tolerant* (*Met*) in NDD female adults downregulated the expression of all three *JHEs*. These results suggest that *JHE* expression is positively correlated with JH signaling, and that *Met* may be involved in the JH-mediated differential expression of *JHE* in DD and NDD adult female *C. bowringi*.

1. Introduction

In many insects and crustaceans, juvenile hormone (JH) is considered one of the most important hormones regulating molting, metamorphosis, reproduction, diapause, and even behavior (Chang, 1993; Giray et al., 2005; Jindra et al., 2013; Denlinger and Armbruster, 2014). Accurate regulation of JH levels is therefore critical and achieved by biosynthesis and degradation (De Kort and Granger, 1996; Belles et al., 2005). Previous studies in insects have demonstrated that a specific carboxylesterase, JH esterase (JHE), can downregulate JH titer by converting active JH (JH III) to inactive JH acid and JH acid diol (Kamita and Hammock, 2010). Although crustaceans do not produce JH III, they use the JH III precursor, methyl farnesoate, to regulate development. It has been suggested that JHE may also degrade crustacean JH by hydrolyzing methyl farnesoate into farnesoic acid (Homola and Chang, 1997; Lee et al., 2011; Sin et al., 2015). The conserved function of JHE suggests that it has a similarly conserved structure among arthropods (Lee et al., 2011; Sin et al., 2015), but this requires confirmation.

Site-directed mutagenesis, biochemistry analysis, and multiple sequence alignment shows that the JHEs of insects have five conserved functional motifs; RF, DQ, GQSAG, E, and GxxHxxD/E (Kamita and Hammock, 2010). Moreover, because JHE generally plays a role in hemolymph (Vince and Gilbert, 1977; Kamita and Hammock, 2010), the presence of the N-terminal signal peptide is another diagnostic indicator of JHE. The structure of JHE has been well studied, at least in insects. However, how JHE expression is regulated remains unclear. Previous studies of *Drosophila melanogaster* (Kethidi et al., 2005), the cabbage looper *Trichoplusia ni* (Venkataraman et al., 1994; Jones et al., 1998), and the diamondback moth *Plutella xylostella* (Duan et al., 2016) found that either JH, or JH analog (JHA), could induce *JHE* expression, but the regulatory mechanism responsible for this was not determined. Recent research on JH signaling has demonstrated that the bHLH-PAS transcription factor methoprene-tolerant (Met) is the intracellular

Abbreviations: DD, diapause-destined; dsRNA, double-stranded RNA; JH, Juvenile hormone; JHA, JH analog; JHE, JH esterase; LD, long-day; Met, methoprene-tolerant; NDD, non-diapause-destined; RT-qPCR, reverse transcription-quantitative PCR; RNAi, RNA interference; SD, short-day

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Table 1
Primers for gene cloning, RT-qPCR, and RNAi.

Genes	Forward primers (5′–3′)	Reverse primers (5′–3′)	PCR efficiency (%)	Standard curve R^2
Gene cloning				
JHE1	accgccagatagtgactgac	caataattactagtgaggta	n.a.	n.a.
JHE2	cttgtttgaatcatgttgct	tctgcgattagttttatttc	n.a.	n.a.
JHE3	caatgcttccgaagaatcta	tttctatttattctcaaatagg	n.a.	n.a.
qRT-PCR				
RPL19	gtaatgcgatgcggcaagaa	aaacctgtagcggtgcactc	102.2	0.998
JHE1	ggtagtgccagcatgacgaa	gctttgcaatccgtttgtcg	109	0.978
JHE2	cctgccagtgacattttggc	tcatttggcgcaactttcgg	99.9	0.997
JHE3	gtccgcatttgttcccgttt	tgccagatcttctacgtgcc	109.2	0.991
Met	caattgctcaacacccagcc	ccttcgttgagcgacagtct	98.2	0.998
RNAi				
Met	gcgtaatacgactcactataggatgattgaggaagtgtcggg	gcgtaatacgactcactatagggattctcgtggtggaccagt	n.a.	n.a.
GFP	gcgtaatacgactcactataggtggtcccaattctcgtggaac	gcgtaatacgactcactataggcttgaagttgaccttgatgcc	n.a.	n.a.

Note: n.a., not applied.

nuclear receptor of JH in many insects (Charles et al., 2011; Jindra et al., 2015). This new finding prompted us to investigate whether Met is involved in the regulation of JH-mediated JHE expression. High JHE expression is considered vital for the photoperiodic downregulation of JH titer in some insects (Kramer and De Kort, 1976; Vermunt et al., 1999; Ishikawa et al., 2012), but whether this true of insects in general is not clear.

The beetle *Colaphellus bowringi* is a serious pest of cruciferous vegetables in Asia. At 25 °C under short-day (SD) conditions, adult female *C. bowringi* prepare for reproduction and do not enter diapause, which is arrested reproduction. Such females are therefore termed non-diapause-destined (NDD), or reproductive, females. However, at 25 °C under long-day (LD) conditions, adult females enter reproductive diapause and are consequently termed diapause-destined (DD) females (Xue et al., 2002; Wang et al., 2004). JH stimulates NDD females to express vitellogenin and complete ovarian development within 4 days of eclosion, and the absence of JH allows DD females to complete preparation for diapause within the same period (Liu et al., 2016; Tan et al., 2016). Therefore, the JH levels of NDD and DD females would be expected to differ significantly. Using *C. bowringi* as a model insect, we identified three putative *JHEs* in this species and investigated how their expression was regulated by JH-Met signaling in NDD and DD females. The results suggest that *JHE* expression is positively correlated with JH signaling in both NDD and DD females, and that *Met* may be involved in JH-induced *JHE* expression in *C. bowringi*. This suggests that JH-Met signaling regulates the differential expression of *JHEs* in DD and NDD adult female *C. bowringi*.

2. Materials and methods

2.1. Insect rearing

The founders of our laboratory colony of *C. bowringi* were collected as larvae in Xiushui County (29°10N, 114°40E), Jiangxi Province, China and fed on radish *Raphanus sativus* L. var. *longipinnatus* (Brassicales: Brassicaceae) (Tan et al., 2015). Offspring of this population were maintained in our lab and used for this study. NDD female adults were produced by keeping larvae at 25 °C under a 12:12 h light:dark photoperiod (SD), and DD female adults were produced by keeping larvae at 25 °C under a 16:8 h light:dark photoperiod (LD).

2.2. cDNA cloning and sequence analysis

Based on the gene annotation of our *C. bowringi* transcriptome (Tan et al., 2015), we found three unigenes that could be putative *JHEs*. These three genes were amplified via PCR with corresponding primers, and inserted into the pMD™-18 T Vector (TaKaRa, Japan) for sequencing. Predicted amino acid sequences were deduced with the ExPASy Translate tool (http://web.expasy.org/translate/) and the sequences were aligned and compared with JHE sequences from other insect taxa and a shrimp using MEGA 4.1 software (Fig. S1). Conserved JHE motifs were identified by comparing the results with previous studies (Ward et al., 1992; Kamita and Hammock, 2010). The sequence identity and similarity of the three putative *C. bowringi* JHEs with those from different insect taxa and the shrimp were determined by pairwise sequence alignment using EMBOSS Matcher (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/). A rooted phylogenetic tree was constructed using the neighbor-joining method in MEGA 4.1 software with the carboxylesterase 4A-like protein of *Tribolium castaneum* as the outgroup. The cDNA sequences of the three putative JHEs of *C. bowringi* are available from GenBank (*JHE1*, KY229689; *JHE2*, KY229690; *JHE3*, KY229691).

2.3. mRNA expression analysis

Reverse transcription-quantitative PCR (RT-qPCR) was used to determine the mRNA abundance in this study. We performed RT-qPCR following the Minimum Information for publication of Quantitative real time PCR Experiments (MIQE) guidelines (Bustin et al., 2010) and our previous protocol (Liu et al., 2016). Because JHE generally is high expressed in the fat body (similar to vertebrate liver) (Vermunt et al., 1999), we investigated the regulation of its expression by using this tissue in this study. Briefly, total RNA was extracted from the fat body of a pool of 15 female adults using RNAiso Plus (TaKaRa, Dalian, China). One μg of RNA was treated with DNAase to remove residual genomic DNA and then used for first-strand cDNA synthesis using the PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. One μL of a 20-fold diluted cDNA solution was subjected to RT-qPCR reactions with corresponding primers (Table 1) and SYBR® Premix ExTaq™ II (TaKaRa, Japan) using a Bio-Rad Detection iQ2 System. Based on the evaluation of reference genes in *C. bowringi* (Tan et al., 2015), *ribosomal protein L19* (*RPL19*) was used as the reference gene to normalize the *JHEs* expression. Relative expression was analyzed by the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) based on three independent biological replicates and three technical replicates.

2.4. JHA induction in DD female adults

DD females were treated with JHA methoprene as per our previous protocol (Liu et al., 2016). Briefly, 15 μg of methoprene in 200 nL was injected into newly emerged DD female adults. The control group was a similar number of females that were injected with the same amount of acetone. Fat bodies were collected from females for RT-qPCR analyses

24 h after injection.

2.5. RNA interference (RNAi) knockdown of Met in NDD female adults

Our RNAi protocol for *C. bowringi* has been described previously (Liu et al., 2016). Briefly, double-stranded RNA (dsRNA) against *C. bowringi Met* (dsMet) was synthesized with primers (Table 1) and a T7 transcription kit (Fermentas, Lithuania) according to the manufacturer's instructions. One μg of dsRNA in 200 nL was injected into the newly emerged NDD female adults. The control was a group of females injected with the same amount of dsRNA against *green fluorescent protein* (dsGFP). Total RNA from the fat bodies of females was extracted for RT-qPCR analyses 72 h after dsRNA injection.

2.6. Statistical analysis

Statistical analyses of mRNA expression differences were performed by using Microsoft Excel and Graphpad Prism 5 software package. Values are means ± standard deviation (s.d.); the statistical significance of differences between means was assessed with Student's *t*-test ($*P < 0.05$; $**P < 0.01$).

3. Results

3.1. Cloning and identification of putative JHEs in C. bowringi

We identified and cloned three putative *JHE* genes; *JHE1*, *JHE2*, *JHE3*. *JHE1* and *JHE2* have full open reading frames, whereas *JHE3* lacks a termination codon. The predicted molecular weights of these three JHEs are 63.6 kDa, 63.0 kDa, and 60.8 kDa, respectively. Sequence alignment shows that JHE1 has 41.2% identity and 56.1% similarity to the *Gryllus assimilis* (Orthoptera) JHE, whereas JHE3 has 45.9% identity and 62.6% similarity to *T. castaneum* (Coleoptera) JHE (Table 2). Interestingly, *C. bowringi* JHE2 shares 44.2% identity and 61.6% similarity with the JHE of the shrimp *Pandalopsis japonica* (Decapoda). All three putative *C. bowringi* JHEs have the RF and DQ motifs, which are conserved in both the other insect JHEs examined and that of the shrimp *P. japonica* (Table 3 and Fig. S1). *C. bowringi* JHE2 and JHE3 also have the E motif, but this is replaced with D in JHE1. This replacement has also been documented in the mosquitoes *Aedes aegypti* (EAT43357) (Bai et al., 2007) and *Culex quinquefasciatus* (AEW07365) (Kamita et al., 2011). Of the three putative *C. bowringi* JHEs, only JHE2 has the GQSAG motif, whereas JHE1 and JHE3 have variants of GESAG and GHSSG, respectively. The GxxHxxD/E motif is present in both JHE1 and JHE2, but is replaced by the RxxHxxD motif in *C. bowringi* JHE2. In addition, another important indicator of JHE, the N-terminal signal peptide, can be found in all three *C. bowringi* putative JHEs. Therefore, notwithstanding some minor species-specific differences, the three putative *C. bowringi* JHEs share a number of highly conserved motifs with those of other insects and even a species of shrimp.

Table 2
Sequence identities and similarities of the three putative *C. bowringi* JHEs compared to those of other insects and a shrimp.

Order	Species	Sequence identity and similarity (%)		
		JHE1	JHE2	JHE3
Coleoptera	*C. bowringi*			
	T. castaneum	37.6; 55.8	33.1; 52.1	45.9; 62.6
	T. molitor	37.4; 53.6	40.4; 58.1	38.1; 54.7
	P. hilaris	35.1; 52.4	32.0; 51.3	30.9; 49.4
Diptera	*A. aegypti*	35.7; 52.4	40.0; 58.5	32.0; 51.0
	C. quinquefasciatus	36.6; 51.6	39.4; 56.4	38.5; 54.0
	D. melanogaster	35.1; 52.9	40.3; 57.5	30.0; 47.2
Orthoptera	*G. assimilis*	41.2; 56.1	40.7; 55.6	38.3; 53.1
Hymenoptera	*A. mellifera*	35.0; 53.4	34.3; 49.9	32.6; 49.0
Lepidoptera	*B. mori*	30.5; 49.0	28.8; 44.5	27.7; 45.5
	C. fumiferana	28.8; 46.0	30.2; 46.8	29.0; 46.8
	H. armigera	29.5; 47.9	29.2; 48.9	28.0; 46.4
	H. virescens	29.9; 48.4	30.8; 48.1	27.9; 47.4
	M. sexta	31.4; 48.6	39.2; 59.0	29.5; 47.9
Hemiptera	*N. lugens*	31.0; 46.0	33.2; 49.5	30.9; 45.9
Decapoda	*P. japonica* (JHE1)	36.8; 53.3	43.9; 62.2	37.4; 56.1
	P. japonica (JHE2)	34.3; 50.3	44.2; 61.6	41.2; 58.4

The identities and similarities of JHE sequences were determined by pairwise sequence alignment using EMBOSS Matcher (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/). JHEs from different insects include those from the Coleoptera (*Tribolium castaneum*, GenBank No. NP_001180223; *Tenebrio molitor*, AAL41023; *Psacothea hilaris*, BAE94685), the Diptera (*Aedes aegypti*, EAT43357; *Culex quinquefasciatus*, AEW07365; *Drosophila melanogaster*, AAK07833), the Orthoptera (*Gryllus assimilis*, ABQ23214), the Hymenoptera (*Apis mellifera*, AAU81605), the Lepidoptera (*Bombyx mori*, AAL55240; *Choristoneura fumiferana*, AAD34172; *Helicoverpa armigera*, AEB77712; *Heliothis virescens*, AAB96654; *Manduca sexta*, AAG42021), and the Hemiptera (*Nilaparvata lugens*, ACB14344). JHEs from one shrimp are Decapoda *Pandalopsis japonica* ADZ96217 and ADZ96218.

Table 3
Conserved sequence motifs of JHEs from *C. bowringi*, other insects, including those from three different orders, and a shrimp.

Order	Species	SP[a]	Motifs[b]				
			RF	DQ	GQSAG	E	GxxHxxD/E
Coleoptera	*C. bowringi* (JHE1)	19	+	+	GESAG	D	GvcHadD
	C. bowringi (JHE2)	18	+	+	+	+	RvgHaeD
	C. bowringi (JHE3)	21	+	+	GHSSG	+	GvgHveD
	T. castaneum	23	+	+	+	+	GvsHcdD
	T. molitor	22	+	+	+	+	GvsHcdD
	P. hilaris	21	+	+	+	+	GvsHcdD
Diptera	*A. aegypti*	–	+	+	+	D	GvvHcdE
	C. quinquefasciatus	19	+	+	+	D	GvvHcdE
	D. melanogaster	20	+	+	+	+	GvvHcdD
Orthoptera	*G. assimilis*	–	+	+	+	+	GvsHcdD
Hymenoptera	*A. mellifera*	18	+	+	GLSAG	+	GvcHadD
Lepidoptera	*B. mori*	19	+	DM	+	+	GagHadD
	C. fumiferana	21	+	DM	+	+	GtgHseD
	H. armigera	19	+	+	+	+	GagHieD
	H. virescens	17	+	+	+	+	GagHieD
	M. sexta	22	+	DM	+	+	GagHieD
Hemiptera	*N. lugens*	20	+	+	+	+	GpaHadD
Decapoda	*P. japonica* (JHE1)	19	+	+	GESAG	+	WvsHgdE
	P. japonica (JHE2)	19	+	+	GESAG	+	WvfHvdD

JHEs are the same as those shown in Table 2.

[a] Length of the N-terminal signal peptide predicted by SignalP 4.1 Server, "-"suggests the lack of signal peptide.

[b] The conserved sequence motifs of JHEs, the "+" indicates the same sequence is maintained.

To further clarify the phylogenetic relationships of the three *C. bowringi* JHEs, we constructed a rooted phylogenetic tree using 16 different insect JHEs and one shrimp JHE (Fig. 1) with the carboxylesterase 4A-like protein of *T. castaneum* as the outgroup. All JHEs were clearly classified into two main branches, one comprised of JHEs from the Hemiptera (*Nilaparvata lugens*) and Lepidoptera and the other comprised of those from *C. bowringi* and the other insects. Interestingly, *C. bowringi* JHE2 and JHE3 appear most closely related to that of the shrimp *P. japonica*. Identification of these *C. bowringi* JHEs provides further evidence of the conserved structure of JHEs in arthropods.

3.2. Higher expression of JHEs in NDD than in DD females

Low JH levels are considered the primary reason for the occurrence of reproductive diapause in insects (Denlinger and Armbruster, 2014; Smykal et al., 2014). Previous studies suggest that in addition to the inhibition of JH biosynthesis, JHE-mediated JH degradation may also contribute to low JH levels in DD adults (Kramer and De Kort, 1976; Vermunt et al., 1999). Therefore, to verify the role of JHE-mediated JH degradation in the reproductive diapause of *C. bowringi* we examined the expression profiles of the three putative *C. bowringi JHEs* in NDD and DD females during the four day period after eclosion when females

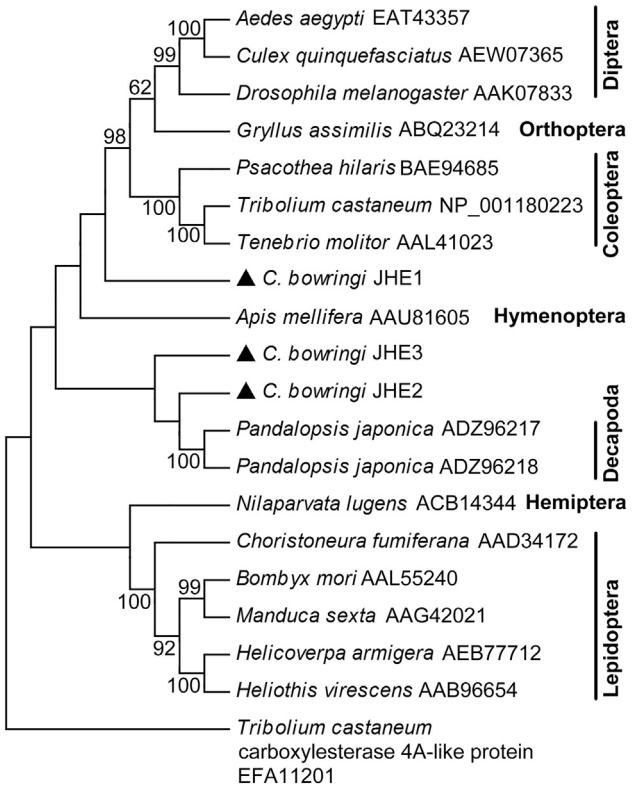

Fig. 1. The rooted phylogenetic tree of JHEs from *Colaphellus bowringi*, 16 other insects and a shrimp. The carboxylesterase 4A-like protein of *Tribolium castaneum* was the outgroup. Bootstrap values (> 50) are marked on the nodes.

either prepare for reproduction or diapause. Generally, NDD adult females had higher JH titers than DD adult females in many insects (Denlinger and Armbruster, 2014; Smykal et al., 2014; Liu et al., 2016; Tan et al., 2017), so it seems to be reasonable that JHE should be more highly expressed in DD females to degrade JH. Contrary to our expectation, all three *JHEs* were more highly expressed in NDD females than in DD females (Fig. 2). This finding suggests that the occurrence of diapause in *C. bowringi* may be not due to the JHE-mediated JH degradation.

3.3. JHA may upregulate JHE expression in the fat body of female adults through Met

The differential expression of *JHEs* in NDD and DD females suggested that *JHE* expression may be positively correlated with JH signaling in *C. bowringi*. Hence, we speculated that JH signaling may regulate *JHE* expression in this beetle. We therefore injected DD females (low JH levels) with JHA methoprene to test if this would upregulate JHE expression. Transcripts of *JHE1* and *JHE2* significantly increased in the fat bodies of injected females (Figs. 3A–B) compared to the control group (acetone) but *JHE3* abundance was not affected (Fig. 3C). This result indicates that JH can upregulate the expression of at least some *JHEs* in *C. bowringi*.

To determine the possible regulatory mechanism by which JH signaling regulates *JHE* expression, we depleted the JH intracellular receptor *Met* in NDD females by injecting them with dsRNA against *C. bowringi Met*. *Met* expression in the fat body subsequently decreased by over 50% compared to the dsGFP control group (Fig. 4A). Furthermore, all three putative *JHEs* were significantly downregulated after *Met* depletion (Figs. 4B–C). These results suggest that JH induces *JHEs* expression via *Met* in *C. bowringi*.

4. Discussion

Because of its vital function in JH degradation, the expression of JHE is usually regulated in many physiological processes, such as development and diapause (Vermunt et al., 1999; Tsubota et al., 2010; Gu et al., 2015). However, the regulatory mechanisms responsible for this remain unclear. We identified three putative *JHEs* in *C. bowringi* and investigated the regulation of their expression by JH signaling. Our results indicate that *JHE* expression is positively correlated with JH levels in NDD and DD females, and that JH may upregulate *JHE* expression via its intracellular receptor *Met*.

Previous studies have suggested that the JHEs of almost all insects have 5 conserved motifs; RF, DQ, GQSAG, E, and GxxHxxD/E (Kamita and Hammock, 2010). R and D in the RF and DQ motifs are required for efficient functioning of JHE in the tobacco budworm *Heliothis virescens* (Ward et al., 1992). The S (in GQSAG), E, and H (in GxxHxxD/E) are the most conserved because they collectively comprise the catalytic triad (Ward et al., 1992). However, the E motif can be replaced by D in the mosquitoes *A. aegypti* (EAT43357) (Bai et al., 2007) and *C. quinquefasciatus* (AEW07365) (Kamita et al., 2011). The GQSAG motif hardly appears in general esterases and is thought to be invariant among JHEs (Kamita and Hammock, 2010). We found that all three putative *C. bowringi* JHEs have the RF and DQ motifs. Both JHE2 and JHE3 have the E motif, and S and H residues, however, as in *A. aegypti* (EAT43357) (Bai et al., 2007) and *C. quinquefasciatus* (AEW07365) (Kamita et al., 2011), the E motif is replaced with D in JHE1. Of the three *C. bowringi* JHEs, only JHE2 has the GQSAG motif, whereas JHE1 and JHE2 have GESAG and GHSSG, respectively. The GQSAG motif may not be as invariable as previously thought. For example, honey bee *Apis mellifera* JHE (AAU81605) has GLSAG instead of GQSAG (Mackert et al., 2008). Interestingly, GESAG also appears in two JHEs in the shrimp *P. japonica* (ADZ96217 and ADZ96218) (Lee et al., 2011). In addition, GxxHxxD/E occurs in *C. bowringi* JHE1 and JHE2, but RxxHxxD appears in JHE2. Although almost all insect JHEs have the conserved GxxHxxD/E motif, in the shrimp *P. japonica* this has been replaced by WxxHxxE (Lee et al., 2011). Therefore, we suggest that the three putative *C. bowringi* JHE genes cloned in this study are true JHE genes because, notwithstanding some species-specific variations, they have the conserved motifs

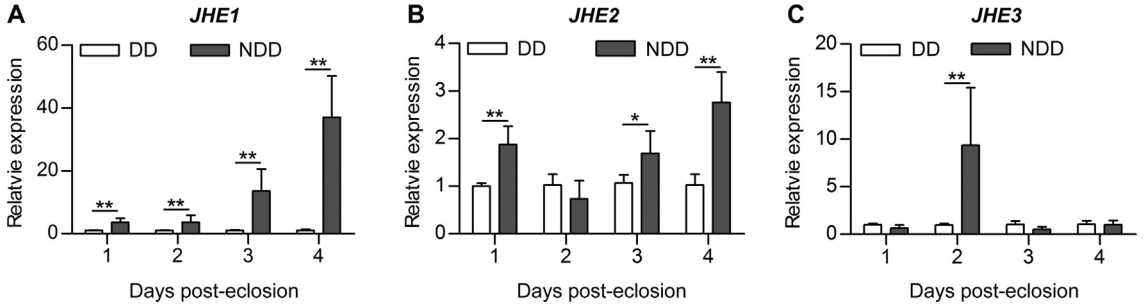

Fig. 2. Differential expression of putative *JHEs* in non-diapause destined (NDD) and diapause-destined (DD) adult female *C. bowringi*. Relative expression of (A) *JHE1*, (B) *JHE2*, (C) *JHE3*, was determined by RT-qPCR in the fat body of NDD and DD females after normalization to the expression of *RPL19* mRNA.

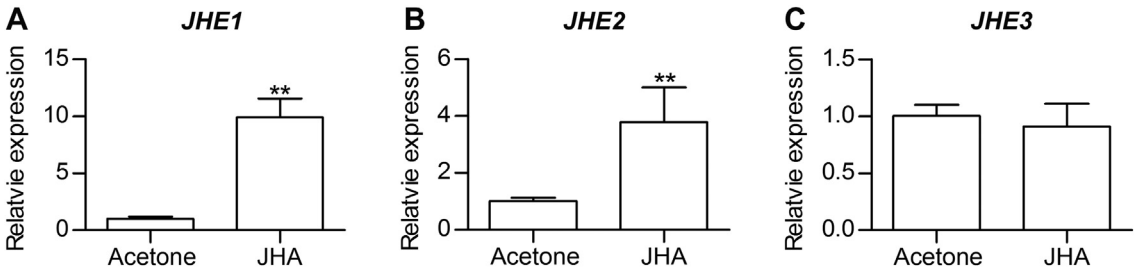

Fig. 3. JHA induced expression of *JHEs* in diapause-destined (DD) adult female *C. bowringi*. Relative expression of (A) *JHE1*, (B) *JHE2*, and (C) *JHE3* was examined in the fat bodies of DD female adults 24 h after injecting them with either JHA methoprene (15 μg) or acetone (control).

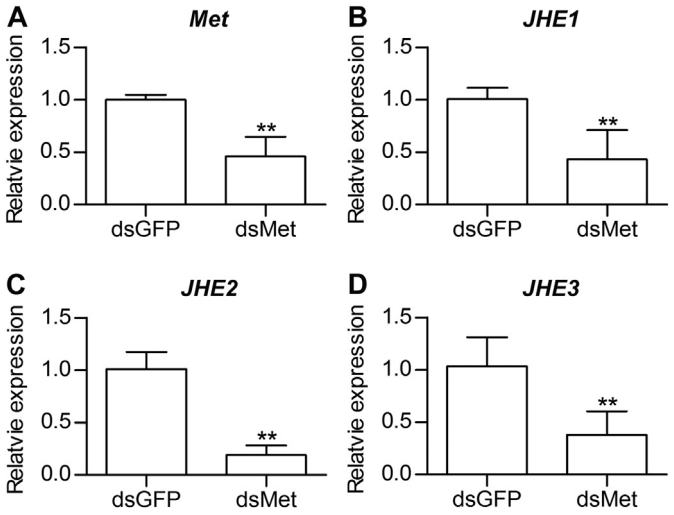

Fig. 4. Suppression of *JHE* expression by RNAi knockdown of *Met* in non-diapause destined (NDD) adult female *C. bowringi*. Relative expression of (A) *Met*, (B) *JHE1*, (C) *JHE2*, and (D) *JHE3* was analyzed in the fat bodies of NDD female adults 72 h after injecting them with either dsMet or dsGFP (control).

common to JHEs from a range of insect groups, and even one decapod species.

Sequence alignment and the phylogenetic tree show that *C. bowringi* JHE2 and JHE3 share high identity and similarity to JHEs of the shrimp *P. japonica*. Moreover, *P. japonica* JHEs cluster with those of a number of insect taxa in one distinct clade whereas Hemipteran (*N. lugens*) and Lepidopteran JHEs are in a separate clade. These results suggest that some Coleopteran, Dipteran, Orthopteran, and Hymenopteran JHEs are more closely related to those of the decapod *P. japonica* than they are to those of the Hemiptera and Lepidoptera. This is new evidence of the highly conserved structure of JHEs in both insects and crustaceans.

Although the biochemistry and structure of insects JHEs have been well studied (Kamita and Hammock, 2010), little was known about the regulation of their expression. It has been reported that hormones, such as ecdysone 20-hydroxyecdysone (20E) and JH may regulate JHE expression. In the silkworm *Bombyx mori*, 20E upregulated *JHE* expression in the anterior silk gland but inhibited *JHE* expression in the fat body. However, JH induced *JHE* expression in both these organs (Kamimura et al., 2007). In the tobacco budworm *H. virescens*, the JH mimic epofenonane significantly promoted *JHE* transcription (Wroblewski et al., 1990). Similarly, the JH mimic pyriproxyfen also upregulated the activity and expression of JHE in the diamondback moth *P. xylostella* (Duan et al., 2016). Although these studies clarified the effect of JH on JHE expression, exactly how JH signaling regulates JHE expression remained unclear. Consistent with previous studies in other insects (Wroblewski et al., 1990; Kamimura et al., 2007; Duan et al., 2016), we found that treatment with JHA methoprene induced the expression of *JHE1* and *JHE2* in *C. bowringi* within 24 h. Although *JHE3* expression was not increased by JHA, we cannot exclude the possibility that insufficient time was allowed to induce this effect. This conjecture is partly supported by the results of our RNAi experiment which showed that downregulation of the JH receptor *Met* significantly suppressed the expression of all three *C. bowringi JHEs*. This suggests that JH induces *JHE* expression in *C. bowringi* via its intracellular receptor *Met*. JH may act via an unknown nuclear receptor to rapidly upregulate *JHE* expression in the cabbage looper *T. ni* (Venkataraman et al., 1994; Jones et al., 1998) and spruce budworm *Choristoneura fumiferana* (Kethidi et al., 2004), raising the possibility of the direct regulation of *JHE* transcription by JH signaling. Our work further verifies that Met may be a transcriptional factor candidate that directly regulates *JHE* expression, but this needs to be confirmed by analysis of its promoter binding and transcriptional activation.

The results of this study suggest that JH signaling is positively correlated with *JHE* expression in *C. bowringi*. *JHE* expression increased in NDD females which had a relatively high JH level, and decreased in DD females which had a relatively low JH level. We infer that the high expression of *JHE* in NDD females is essential for the control of excessive JH level (Kethidi et al., 2005), and then contributes to reproductive development. Hence, the induction of *JHE* expression by JHA seems to be reasonable (Feng et al., 1999; Kethidi et al., 2005; Kamimura et al., 2007; Liu et al., 2008). Our finding with respect to the low expression of *JHE* in DD females suggests that JHE may not, however, be responsible for the low JH titer of DD females. Similarly, JHE is also not crucial for the switch between larval diapause and development in the Mediterranean corn borer, *Sesamia nonagrioides* (Schafellner et al., 2008). In fact, in addition to JH degradation, block of JH biosynthesis has been well proved to be the very important reason for reproductive diapause induction (Kort, 1990; Sim and Denlinger, 2008; Bradshaw and Holzapfel, 2010; Kang et al., 2014). Therefore, we speculate that JH biosynthesis, rather than JHE-mediated JH degradation, may be regulated during diapause induction of *C. bowringi*. However, this conjecture needs to be demonstrated in our future work. Interestingly, our results contrast with those obtained from a study of photoperiodic diapause in the Colorado potato beetle, *L. decemlineata* (Vermunt et al., 1999). In that study, adult *L. decemlineata* expressed high *JHE* to promote JH degradation during diapause induction phase. Moreover, the JH mimic pyriproxyfen suppressed *JHE* expression during this phase. Therefore, the high expression of *JHE* plays an important role in diapause induction in *L. decemlineata*. Meanwhile, JH biosynthesis is blocked during reproductive diapause induction in this beetle (Kort, 1990). Hence, it is possible that the low JH level in DD *L. decemlineata* is due to both the block of JH biosynthesis and the JHE-mediated JH degradation. These different results suggest that the regulation of JH biosynthesis and degradation during reproductive diapause induction is variable in different insects.

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