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Authors: Yang, Wen-Jia, Xu, Kang-Kang, Shang, Feng, Dou, Wei, and Wang, Jin-Jun

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Identification and characterization of three juvenile hormone genes from *Bactrocera dorsalis* (Diptera: Tephritidae)

Wen-Jia Yang[1,2], Kang-Kang Xu[1,2], Feng Shang[1], Wei Dou[1], and Jin-Jun Wang[1,]*

Abstract

Juvenile hormone (JH) plays an important role in regulating growth, development, and reproduction of insects. Three key enzymes, namely, JH esterase (JHE), JH epoxide hydrolase (JHEH), and JH diol kinase (JHDK), are involved in JH degradation. In this study, we identified the full-length cDNAs of the 3 genes *BdJHEH2*, *BdJHEH3*, and *BdJHDK* encoding JHEH and JHDK from the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). We used quantitative real-time polymerase chain reaction to investigate mRNA expression profiles of these 3 genes in various development stages and tissues, and in response to both 20-hydroxyecdysone (20E) and starvation. Both *BdJHEH2* and *BdJHDK* were highly expressed during the larval–pupal transition, whereas *BdJHEH3* was mainly expressed in the early 3rd instars. All 3 genes were highly expressed in 7-d-old and 10-d-old adults, but exhibited sex-specific expression patterns. *BdJHEH2* was highly expressed in fat body, whereas *BdJHEH3* and *BdJHDK* were most abundant in Malpighian tubules. In response to 20E exposure, the 3 genes were significantly up-regulated at various time points compared with the control. However, the transcript levels of *BdJHDK* decreased significantly during the initial exposure to 20E. After starvation treatment, expression of *BdJHEH2* and *BdJHEH3* significantly decreased, whereas *BdJHDK* was up-regulated. No significant change was observed after feeding resumption. These 3 genes have distinct roles in regulating growth and development of *B. dorsalis*.

Key Words: juvenile hormone; JH epoxide hydrolase; JH diol kinase; expression pattern; 20-hydroxyecdysone; starvation

Resumen

La hormona juvenil (JH) juega un papel importante en la regulación de crecimiento, el desarrollo y la reproducción de los insectos. Tres enzimas clave, JH esterasa (JHE), JH epóxido hidrolasa (JHEH), y JH diol quinasa (JHDK), están implicadas en la degradación de JH. En este estudio, hemos identificado los ADNc de longitud completa de los 3 genes *BdJHEH2, BdJHEH3* y BdJHDK que codifican JHEH y JHDK de la mosca oriental de la fruta, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). Se utilizó PCR cuantitativa en tiempo real para investigar los perfiles de expresión de mRNA de estos 3 genes en diferentes estadios de desarrollo y tejidos, y en respuesta a tanto 20-hidroxiecdisona (20E) y la inanicion. Tanto *BdJHEH2* y *BdJHDK* fueron altamente expresado durante la transición larval-pupal, mientras que *BdJHEH3* se expresó principalmente en el 3er estadio temprano. Todos los 3 genes fueron altamente expresado en los adultos de 7 días de edad y de 10 días de edad, pero exhiben patrones de expresión específicos al sexo. *BdJHEH2* fue altamente expresado en la grasa corporal, mientras que *BdJHEH3* y *BdJHDK* fueron más abundantes en los túbulos de Malpighi. En respuesta a la exposición 20E, los 3 genes fueron significativamente hasta reguladas en varios puntos de tiempo en comparación con el control. Sin embargo, los niveles de transcripción de *BdJHDK* disminuyeron significativamente durante la exposición inicial a 20E. Después del tratamiento de inanicion, la expresión de *BdJHEH2* y *BdJHEH3* disminuyó significativamente, mientras que BdJHDK su regulacion aumento. No se observó ningún cambio significativo después de la reanudación de la alimentación. Estos 3 genes tienen funciones distintas en la regulación del crecimiento y el desarrollo de *B. dorsalis*.

Palabras Clave: hormona juvenil; JH epóxido hidrolasa; JH diol quinasa; patrón de expresión; 20-hidroxiecdisona; inanición

Juvenile hormone (JH) is a sesquiterpenoid hormone that plays important roles in the control of insect physiological processes, such as molting, metamorphosis, diapause, and reproduction (Wyatt & Davey 1996; Riddiford et al. 2003). The regulation of the JH titer is important during the entire insect life cycle. JH titers in the hemolymph are precisely adjusted through biosynthesis and degradation (Gilbert et al. 2000; Li et al. 2003). At least 3 enzymes are metabolically responsible for JH degradation. These are JH esterase (JHE), JH epoxide hydrolase (JHEH), and JH diol kinase (JHDK). JHE (EC 3.1.1.1) belongs to the carboxylesterase family and converts JH to JH acid (JHa) in a reversible reaction, and catalyzes the conversion of JH diol (JHd) to yield JH acid diol (JHad) (Share & Roe 1988). JHEH (EC 3.3.2.3) is a member of the microsomal epoxide hydrolase family that catalyzes the conversion of JH to form JHd, and converts JHa to JHad (Hammock 1985). JHDK (EC 2.1.7.3) belongs to the calcium-binding protein family. It catalyzes the conversion of JHd to JH diol phosphate (JHdp), an inactive JH metabo-

[1]Key Laboratory of Entomology and Pest Control Engineering, College of Plant Protection, Southwest University, Chongqing 400716, P. R. China; E-mail: yangwenjia10@126.com (W.-J. Y.), kkxu1988@163.com (K.-K. X.), fengshang1994@yahoo.com (F. S.), douwei80@swu.edu.cn (W. D.), jjwang7008@yahoo.com (J.-J. W.)

[2]Key & Special Laboratory of Guizhou Education Department for Pest Control and Resource Utilization, College of Biology and Environmental Engineering, Guiyang University, Guiyang, Guizhou 550005, P. R. China

*Corresponding author; E-mail: jjwang7008@yahoo.com or wangjinjun@swu.edu.cn. (J.-J. W.)

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lite (Maxwell et al. 2002a,b). Most studies of JH metabolizing enzymes have focused on JHE, but little is known about the roles of JHEH and JHDK in the regulation of JH titers.

JHEHs are crucial enzymes responsible for JH degradation, and are potential targets for selective insecticidal compounds. The cDNAs of *JHEH* genes from several insect species have been cloned, including *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) (Harris et al. 1999), *Ctenocephalides felis* (Bouche) (Siphonaptera: Pulicidae) (Keiser et al. 2002), *Bombyx mori* L. (Lepidoptera: Bombycidae) (Zhang et al. 2005), *Apis mellifera* L. (Hymenoptera: Apidae) (Mackert et al. 2010), *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Tsubota et al. 2010b), *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) (Kamita et al. 2013), *Manduca sexta* L. (Lepidopetra: Sphingidae) (Wojtasek & Prestwich 1996), and *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Lü et al. 2015). The ability of recombinant JHEHs to degrade JH in vitro has been confirmed in several insect species (Keiser et al. 2002; Zhang et al. 2005; Tsubota et al. 2010b; Kamita et al. 2013). Multiple *JHEH*-like genes are found in some insect species. For example, there are 5 genes in *B. mori* (Cheng et al. 2014), 3 in *Drosophila melanogaster* Macquart (Diptera: Drosophilidae) (Taniai et al. 2003), 5 in *T. castaneum* (Tsubota et al. 2010b), and 2 in *L. decemlineata* (Lü et al. 2015). Although these multiple *JHEHs* are similar in domain architecture, not all act as JH-metabolizing enzymes. In *D. melanogaster*, 1 *JHEH*-like gene is involved in detoxification of xenobiotics (Taniai et al. 2003). In *T. castaneum*, only *TcJHEH-r3* and *TcJHEH-r4* exhibited JH-degrading activity, whereas the other 3 *TcJHEH-r* genes probably act as detoxification enzymes (Tsubota et al. 2010b). RNA interference (RNAi) experiments in *L. decemlineata* demonstrated that silencing *LdJHEH1*, *LdJHEH2*, or both genes affected JH degradation and impaired adult emergence (Lü et al. 2015).

The first JHDK was purified from the Malpighian tubules of *M. sexta* larvae, and subsequent sequencing showed that *M. sexta JHDK* was a homology of the sarcoplasmic calcium-binding protein-2 (dSCP2) of *D. melanogaster*, which was similar to GTP-binding proteins. There were 3 conserved sequence elements responsible for purine nucleotide binding and 3 elongation factor (EF)-hand motifs in both dSCP2 and GTP-binding proteins (Maxwell et al. 2002a,b). Several *JHDK* cDNAs have been identified from insect species, such as *Danaus plexippus* L. (Lepidoptera: Nymphalidae), *Plutella xylostella* L. (Lepidoptera: Plutellidae), and *B. mori* (Li et al. 2005), *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae) (Feng & Zhai 2011), *Spodoptera litura* F. (Lepidoptera: Noctuidae) (Zeng et al. 2015), and *L. decemlineata* (Fu et al. 2015). The recombinant BmJHDK of *B. mori* in Sf9 cells using a baculovirus system converted 10S-JH diol to form JHdp, suggesting its enzymatic function in JH regulation (Li et al. 2005). In *L. decemlineata*, knockdown of *LdJHDK* significantly increased the hemolymph JH titer and negatively affected adult emergence (Fu et al. 2015).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is a destructive agricultural pest in many regions of world (Clarke et al. 2005; Li et al. 2007). Frequent application of various types of insecticides has led to resistance development and reduced control (Hsu et al. 2012; Wang et al. 2015). Previous studies sought to develop new insect pest management strategies by targeting vital genes that function mainly during development (Baum et al. 2007; Yang et al. 2013a). The activities of JH-metabolizing enzymes are likely to be critical for successful functional regulation of JH in insect growth and development (Yang et al. 2011; Fu et al. 2015; Lü et al. 2015). *Bactrocera dorsalis* is a typical holometabolous insect that undergoes dramatic morphological changes during development from egg to adult. However, little is known about the roles of JH-metabolizing enzymes during development in this species. Previous studies demonstrated that both 20-hydroxyecdysone (20E) and starvation induced precocious metamorphosis in *B. dorsalis* larvae (Yang et al. 2013b). However, the roles of JH-metabolizing genes in JH regulation are unknown. This study identifies and characterizes the JH metabolism–related genes of *B. dorsalis* and provides basic information potentially useful for the selection of new insecticidal targets.

Materials and Methods

INSECT AND SAMPLE COLLECTION

The oriental fruit fly, *B. dorsalis*, was reared in the laboratory at 27 ± 1 °C and 70 ± 5% relative humidity with a 14:10 h L:D photoperiod, using previously described artificial diet (Cong et al. 2012). Individuals of various development stages including 3rd instars, pupae, and adults (both males and females) were collected separately and stored at −80 °C. The 10-d-old adults were dissected on ice, and various body parts or tissues, including head, thorax, midgut, Malpighian tubules, and fat body, were isolated, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

The 2-d-old 3rd instar larvae were used for 20E (Sigma-Aldrich, St. Louis, Missouri) and starvation treatment based on previous experiments (Yang et al. 2013b). A stock solution (10 µg/µL) of 20E dissolved in 95% ethanol was diluted to 1 µg/µL with distilled water and used for injection treatments. The micro-injection technique was used for 20E injection as previously described (Yang et al. 2013a). A 0.5 µL droplet of 20E solution was applied topically to each larva, and the controls were injected with 0.5 µL of 0.1% ethanol. Five larvae were randomly selected from each group at 1, 4, 8, and 12 h after treatment, and the samples were frozen in liquid nitrogen and stored at −80 °C.

For the starvation treatment, larvae were divided into 3 groups. The 1st group was maintained on artificial diet and served as the control. The 2nd group was starved for 24 or 48 h before sample collection. The 3rd group was starved for 24 h and then re-fed for the following 24 h. Each sample included more than 30 individual insects, and the experiment was repeated 3 times.

RNA ISOLATION AND REVERSE TRANSCRIPTION

Total RNA was isolated separately from each sample using the TRIzol kit (Invitrogen, Carlsbad, California) according to the manufacturer's protocol and treated with DNase I (Promega, Madison, Wisconsin) to remove contaminating genomic DNA. First-strand cDNA was synthesized with 1 µg RNA from each sample using random hexamer primers and oligo-(dT) and the PrimeScript® RT Reagent Kit (TaKaRa, Dalian, China).

IDENTIFICATION OF *BDJHEH2*, *BDJHEH3*, AND *BDJHDK* cDNA

Based on the conserved domains of *JHEH* and *JHDK* genes in insects, 2 *B. dorsalis* transcriptome databases (Shen et al. 2011; Yang et al. 2014) were analyzed using bioinformatics methods. Two distinct cDNA fragments of putative *JHEH* genes and one *JHDK* homologous sequence were identified. Alignment with other insect *JHEH* or *JHDK* sequences suggested a full-length cDNA of *BdJHEH2*, and partial cDNAs of *BdJHEH3* and *BdJHDK* in the databases. To obtain the complete coding sequences of these 2 genes, we isolated the cDNA clones using the rapid amplification of cDNA ends (RACE) method with the SMARTer RACE cDNA Amplification Kit (Clontech, Palo Alto, California). The primers for full-length cDNA amplification and RACE polymerase chain reaction (PCR) are shown in Table 1. The following cycling parameters were

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Table 1. Primers used in this study.

Gene Identity		Primer Name	Primer Sequence (5′ to 3′)
BdJHEH2	Full-length confirmation	JHEH2-F1 JHEH2-R1	TCGAAAGTGAAATGGGCACCTT CGAACTGCTATTGCTTTGGTTGT
	qPCR	q-JHEH2-F q-JHEH2-R	GCGTTTGGGTCATGAGAAAT AAAGAAGCTCGGGAACCAAT
BdJHEH3	5′ RACE	JHEH3-51 JHEH3-52	CCCAATCGCCACCCTGT CGTAACATTAGATTCCGCAT
	3′ RACE	JHEH3-31 JHEH3-32	GCTCTTGGTGGAGGTGTC TGGGTTCTCGGACGCTG
	Full-length confirmation	JHEH3-F1 JHEH3-R1	CGTTAATTATTTCTGTTGCGTTAG CTATATTGTGTATTTTCCAGCTTTA
	qPCR	q-JHEH3-F q-JHEH3-R	GGGTTCTGTGCGTGAATTTT GCCATCTCTAGCGAATCGAA
BdJHDK	3′ RACE	JHDK-31 JHDK-32	GAACACACCCAAGAACAAGGAG CGGAGGAGTTCACATTGGTCT
	Full-length confirmation	JHDK-F1 JHDK-R1	ATCATGTCGATCTCCGACTTCC GAGCGTTAAAATGCATTTTTGC
	qPCR	q-JHDK-F q-JHDK-R	AGCGTTATGGACTGGCAAAC CCTCTGCTGCGAAGTATTCC
Internal standard for qPCR		α-Tubulin-F α-Tubulin-R	CGCATTCATGGTTGATAACG GGGCACCAAGTTAGTCTGGA

used: 3 min at 94 °C, followed by 34 cycles of 30 s at 94 °C, 30 s at 55 to 60 °C (based on the primer annealing temperatures) and 1 to 2 min (based on the size of expected fragment) at 72 °C, then 10 min at 72 °C. The PCR products were purified on 1.5% agarose gels, sub-cloned into the pGEM-T Easy vector (Promega), and then sequenced in both directions at BGI Company (Beijing, China).

SEQUENCE ANALYSIS

DNAMAN 6.0 (LynnonBiosoft, Quebec, Canada) was used to edit sequences and perform multiple sequence alignment. Sequence similarity and conserved domains were analyzed using the Blastp tool at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The open reading frame (ORF) of each gene was identified using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight and isoelectric point (*pI*) were predicted by using ExPASy tools (http://www.expasy.ch/). Signal peptide was predicted with the SignalP4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Potential *N*-glycosylation sites were predicted with the NetNGlyc1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). The phylogenetic tree was constructed with MEGA 5 (Tamura et al. 2011) using the neighbor-joining method with 1,000 bootstrap replicates.

QUANTITATIVE REAL-TIME PCR

A quantitative real-time PCR (qPCR) assay was performed to investigate mRNA expression profiles of the 3 genes in different development stages and tissues, as well as in response to both 20E and starvation treatment. The PCR amplification was performed in a final reaction mixture of 20 µL including 10 µL GoTaq qPCR Master Mix (Promega), 0.2 mM each of gene-specific primers (Table 1), and the cDNA templates. Thermal cycling was completed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, California) using the program of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. After the cycling protocol, a melting curve analysis from 60 °C to 95 °C was applied to all reactions to verify a single PCR product. Each qPCR experiment consisted of 3 independent biological replicates, each with 2 technical replicates. The α-*tubulin* (GenBank accession no. GU269902) was used as a reference gene to normalize the target gene expression profiles among the samples based on previous evaluations (Shen et al. 2010). Relative expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

DATA ANALYSIS

The expression data were analyzed with SPSS 16.0 software (SPSS Inc., Chicago, Illinois) and given as mean ± standard error (SE). Significant differences between samples were analyzed either by a *t*-test (for comparison of 2 means) or, for multiple comparisons, by 1-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test.

Results

Supplementary figures for this article are available online at http://purl.fcla.edu/fcla/entomologist/browse. The figures in the supplementary document are mentioned in the text below as Suppl. Figs. 1 to 3.

IDENTIFICATION AND SEQUENCE ANALYSIS OF *BDJHEH2*, *BDJHEH3*, AND *BDJHDK*

Based on the *B. dorsalis* transcriptome, 3 full-length cDNAs of JH metabolism–related genes, including *BdJHEH2*, *BdJHEH3*, and *BdJHDK*, were obtained and verified by RACE-PCR. The cDNA sequences of *BdJHEH2* (GenBank accession no. AGB51283) and *BdJHEH3* (AEN03029) have ORFs of 1,398 and 1,377 nucleotides encoding proteins of 465 and 459 amino acid residues, respectively. The predicted molecular weight and *pI* were 52.9 kDa and 8.64 for BdJHEH2, and 52.6 kDa and 6.33 for BdJHEH3, respectively. SignalP4.1 analysis predicted that BdJHEH2 has a signal peptide at the N-terminus (Suppl. Fig. 1). Two potential

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N-glycosylation sites (N-X-S/T) at positions 35 and 73 were observed in BdJHEH3, but not in BdJHEH2 (Suppl. Fig. 2). As found in other insect JHEHs, the 2 BdJHEHs had 3 active site residues (Asp232, Glu409, and His436), which may form the catalytic triad in EHs. Two tyrosine residues (Tyr 304 and Tyr380) and a conserved HGXP motif (where X indicates any amino acid residue) that compose the oxyanion hole of EHs were also identified in both BdJHEHs (Fig. 1A). The deduced amino acid sequences of *BdJHEH2* and *BdJHEH3* had 45.9% identity, and 43 to 88% identity to JHEHs of *Bactrocera minax* (Enderlein) (Diptera: Tephritidae), *D. melanogaster*, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), and *Aedes aegypti* (L.) (Diptera: Culicidae). A phylogenetic tree was generated using the JHEH-like proteins from 11 insect species. As expected, the JHEHs from the same insect order grouped together, and the 2 *B. dorsalis* JHEHs were most closely related to the *D. melanogaster* JHEHs (Fig. 1B).

The complete cDNA of *BdJHDK* consisted of 1,065 nucleotides, including an ORF of 552 nucleotides encoding 184 amino acid residues, and 260 and 250 nucleotide non-coding regions at 5′ and 3′ ends, respectively (Suppl. Fig. 3). The putative protein of *BdJHDK* had a predicted molecular weight of 21.1 kDa with a theoretical *pI* of 4.45. One potential *N*-glycosylation site at position 22 was predicted. Three predicted GTP-binding motifs and 3 conserved calcium-binding motifs were found in BdJHDK (Fig. 2A). Multiple protein alignments showed that BdJHDK had homology to the known and predicted JHDK proteins in other insect species (Fig. 2). For instance, BdJHDK shared 85, 53, 52, and 51% identity with the *SCP2* sequence of *D. melanogaster* (NM_079657), and *JHDK* sequences of *O. furnacalis* (ADK94879), *B. mori* (AAQ63486), and *P. xylostella* (AEP25399), respectively. Phylogenetic analysis also revealed that insect JHDKs from the same insect order grouped together, and BdJHDK was most closely related to the *D. melanogaster* SCP2 protein (Fig. 2B).

DEVELOPMENTAL EXPRESSION ANALYSIS

The expression patterns of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* were examined at various ages of 3rd instars, pupae, and adults by using qPCR. Both *BdJHEH2* and *BdJHDK* were highly expressed during the larval–pupal transition, whereas *BdJHEH3* was mainly expressed in early 3rd instars. Low levels of *BdJHEH3* and *BdJHDK* mRNA were detected during the pupal stages (Fig. 3A). For the adult stage, the expression was examined individually in males and females. Specifically, high levels of *BdJHEH2* were detected in 10-d-old adults of both sexes, whereas the levels of *BdJHEH3* were relatively high in 4-d-old and 7-d-old females. Interestingly, the levels of *BdJHEH2* and *BdJHDK* in 7-d-old males were greater than those in females (Fig. 3B).

TISSUE-SPECIFIC EXPRESSION ANALYSIS

Tissue-specific expression patterns of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* were also analyzed by qPCR. These mRNAs were detected in the head, thorax, midgut, Malpighian tubules, and fat body of 10-d-old adults. *BdJHEH2* was expressed significantly higher in fat body than in the other tissues. Both *BdJHEH3* and *BdJHDK* were expressed at the highest level in Malpighian tubules. *BdJHEH3* was also highly expressed in fat body and midgut, whereas *BdJHDK* was highly expressed in the head, with slight or no detectable expression in other tissues (Fig. 4).

RESPONSE OF *BDJHEH2*, *BDJHEH3*, AND *BDJHDK* TO 20E AND STARVATION

At 3 initial time points after injection of 20E, the expression levels of *BdJHEH2* and *BdJHEH3* were significantly increased compared with the control. The expression patterns of *BdJHEH2* and *BdJHEH3* were similar, but the response of *BdJHEH3* was significantly greater than that of *BdJHEH2*. In contrast, the transcript level of *BdJHDK* decreased significantly during initial exposure to 20E but at 12 h post-injection, the expression level of *BdJHDK* had significantly increased (Fig. 5). In addition, responses of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* to starvation were dissimilar. *BdJHEH2* and *BdJHEH3* were significantly decreased after starvation, but the expression of *BdJHDK* increased significantly after 48 h starvation compared with the fed larvae. No significant change was observed in the 3 genes when the insects were re-fed for 24 h following 24 h of starvation (Fig. 6).

Discussion

The JH metabolism pathway plays an essential role in JH regulation during insect growth and development (Munyiri & Ishikawa 2007; Cheng et al. 2014; Fu et al. 2015; Lü et al. 2015). Three JH-metabolizing enzymes, including JHE, JHEH, and JHDK are functionally involved in JH degradation (Li et al. 2004). It is assumed that these JH-metabolizing enzymes act synergistically in JH degradation (Li et al. 2004; Yang et al. 2011). Due to their specific and critical roles during insect growth, JH metabolism genes are potential targets for pest control. We identified and characterized 3 JH metabolism genes from *B. dorsalis*, a commercially important pest species worldwide. The temporal and spatial expression patterns of these genes were investigated. Their responses to treatment of both 20E stimulus and starvation stress were also analyzed.

We observed expression of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* during several stages of insect growth, such as the transition from larvae to pupae, and during adult development. Both *BdJHEH2* and *BdJHDK* were expressed primarily during the larval–pupal transition, suggesting that these genes facilitate JH degradation during metamorphosis. This degradation is necessary for *B. dorsalis* larvae to pupate because decreased JH is necessary for pupation in holometabolous insects. We expect that maintaining high hemolymph levels of BdJHEH2 and BdJHDK might be critical for successful larval–pupal metamorphosis. Similar situations have been found in *L. decemlineata* (Fu et al. 2015; Lü et al. 2015) and *B. mori* (Cheng et al. 2014). Specifically, high peaks of *LdJHEH1* and *LdJHEH2* transcripts occurred 32 h after ecdysis of the final instars (Lü et al. 2015), whereas abundant amounts of *LdJHDK* transcripts were observed immediately before and after molting (Fu et al. 2015). In *B. mori*, 4 *JHEH* and 3 *JHDK* genes were highly expressed before pre-pupation wandering, and *JHEH-l1* and *JHEH-l3* displayed high expression in females during the pupa-to-adult transition (Cheng et al. 2014). Additionally, high mRNA levels of *BdJHEH3* were found in early 3rd instars because there is a high demand for JH degradation for successful completion of the larval–larval molt. Consistent with our results, a previous study on *S. litura* showed that the expression levels of *SlJHDK* were higher in the early stages than in the later stages of each larval stadium (Zeng et al. 2015).

JH-metabolizing enzymes are necessary for vitellogenin (Vg) production and endocrine regulation (Wyatt & Davey 1996). In *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), the expression level of *JHE* was higher in adults than in nymphs, reached a peak 11 d after eclosion, and had an expression pattern similar to that of the *Vg* gene (Long et al. 2013). Similar phenomena have been found in other insects, including *A. mellifera*, where *AmJHE-like* expression levels closely paralleled the changes observed in *Vg* gene expression in adult worker bees (Piulachs et al. 2003; Mackert et al. 2008). This finding suggests that JH-metabolizing enzymes are associated

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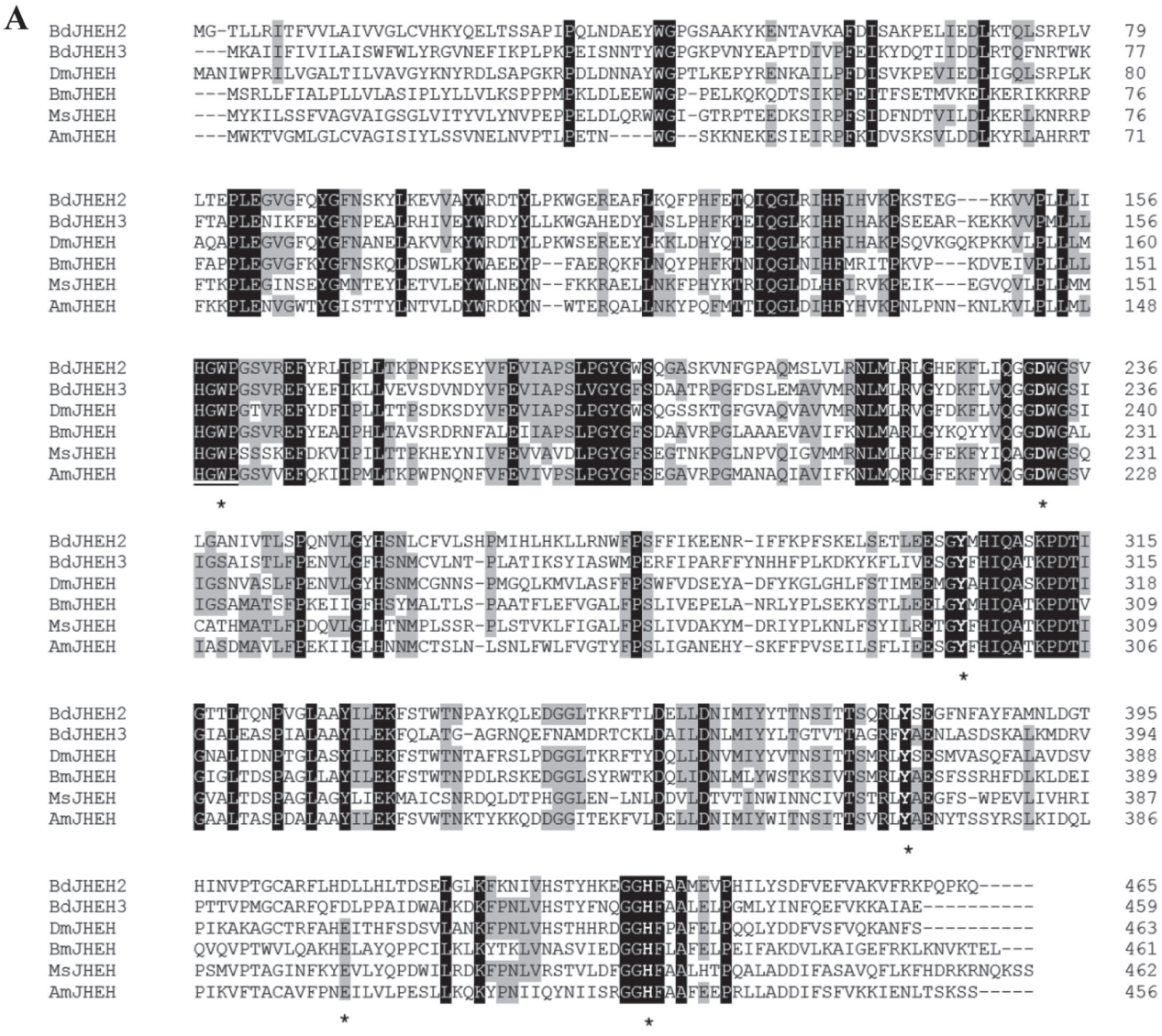


Fig. 1. Multiple sequence alignment and phylogenetic analysis of JHEHs from *Bactrocera dorsalis* and other insects. (A) Sequence alignment. The sequences of *B. dorsalis* JHEHs are compared with JHEH from *Drosophila melanogaster* (Dm), *Bombyx mori* (Bm), *Manduca sexta* (Ms), and *Apis mellifera* (Am). The catalytic triad (Asp232, Glu409, and His436), 2 tyrosine residues (Tyr 304 and Tyr380), and HGXP motif are labeled with asterisks. The HGXP motif is underlined. (B) Phylogenetic analysis of JHEH homologs. The tree was generated with MEGA 5 using the neighbor-joining method. Nodes with >50% bootstrap values (1,000 replicates) are indicated on branches. GenBank accession numbers of all sequences are listed in the tree.

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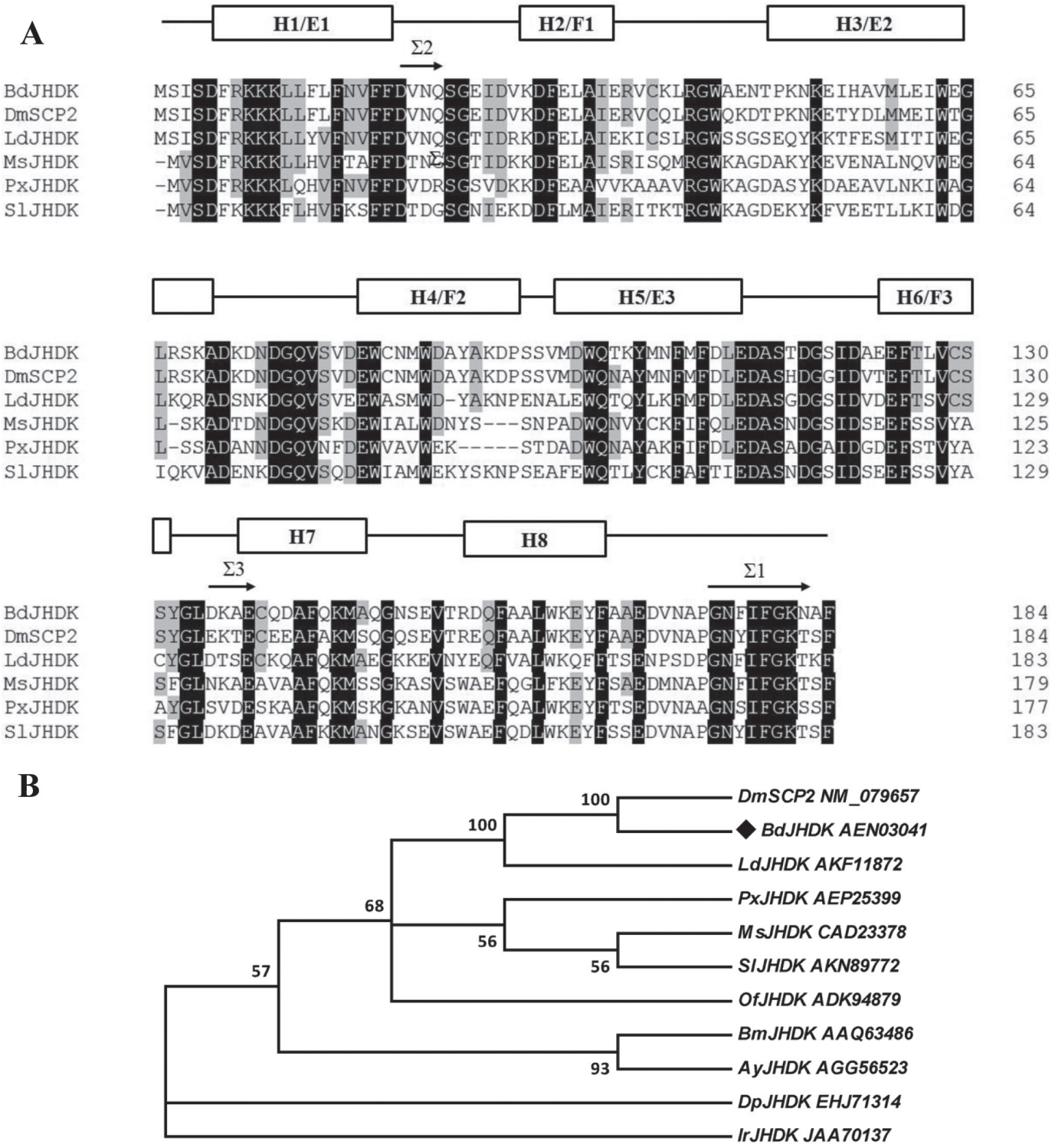


Fig. 2. Multiple sequence alignment and phylogenetic analysis of JHDK from *Bactrocera dorsalis* and other insects. (A) Sequence alignment. The sequence of *B. dorsalis* JHDK is compared to SCP2 from *Drosophila melanogaster* (Dm), and JHDK from *Leptinotarsa decemlineata* (Ld), *Manduca sexta* (Ms), *Plutella xylostella* (Px), and *Spodoptera litura* (Sl). The **α**-helices (H1-8) and EF hands are indicated above the alignment according to results from *B. mori* (Li et al. 2005).Three predicted GTP-binding motifs (Σ1–3) are labeled with an arrow. (B) Phylogenetic analysis of JHDK homologs. The tree was generated with MEGA 5 using the neighbor-joining method. Nodes with >50% bootstrap values (1,000 replicates) are indicated on branches. GenBank accession numbers of all sequences are listed in the tree.

with the development of the insect reproductive systems. In *D. melanogaster*, *JHE* expression was detected in both male and female adults (Kethidi et al. 2005). In *T. castaneum*, *TcJHE* was expressed more strongly in adult females than in males, suggesting that it may be attributable to the higher JH titer in females (Tsubota et al. 2010a). In our study, 3 JH metabolism–related genes were highly expressed in 7-d-old and 10-d-old adults, which is a key period of sexual development. It is possible that these genes may be associated with adult sexual maturation, and this possibility deserves further investigation.

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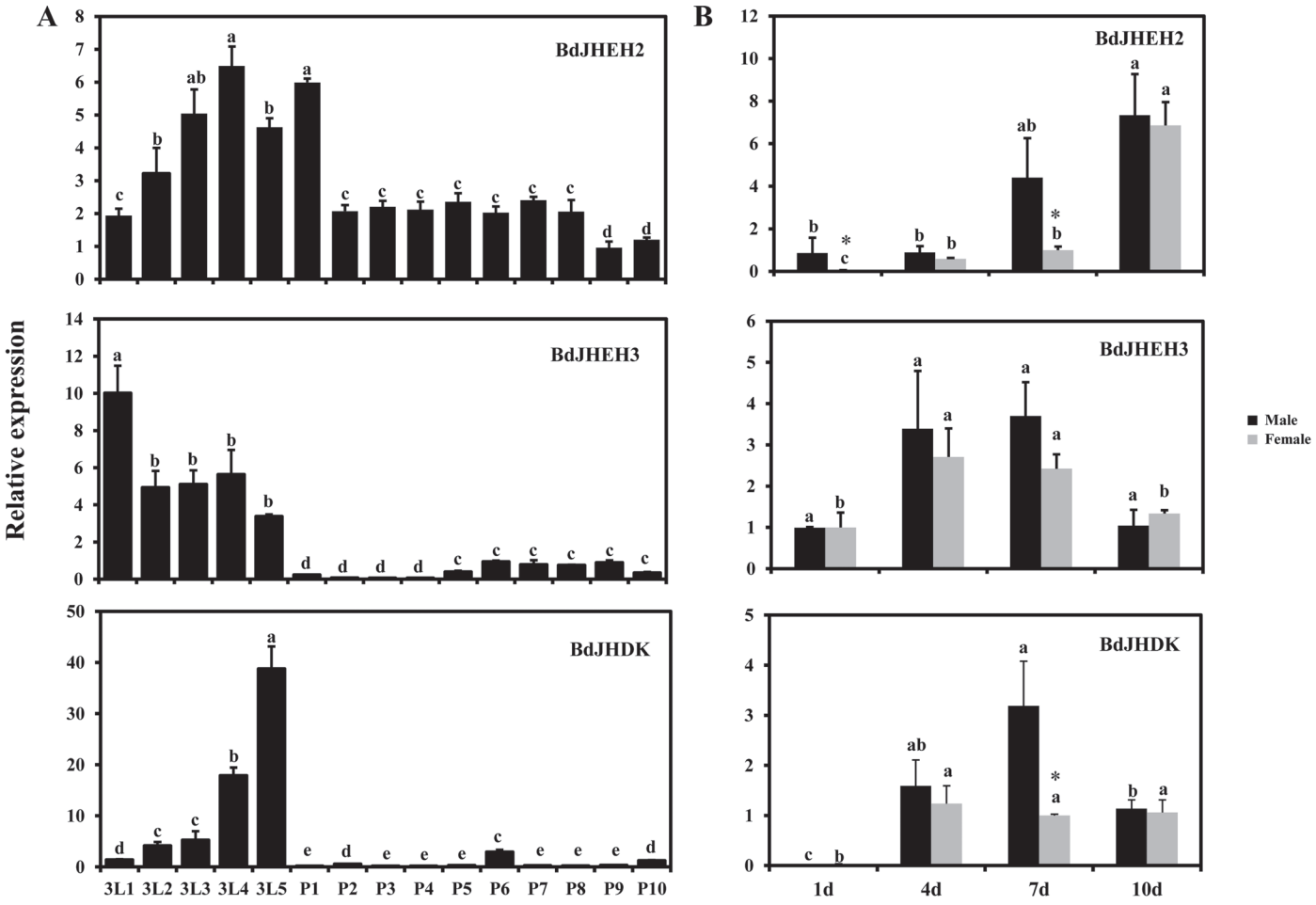


Fig. 3. Relative expression levels of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* in developmental stages of *Bactrocera dorsalis*. Expression levels at 19 time points in **(A)** 3rd instar larvae and pupae and **(B)** adults were detected by qPCR. 3L1: 1-d-old 3rd instar larvae; P1: 1-d-old pupae. Females or males were collected for qPCR analysis at 1, 4, 7, and 10 d after eclosion. Different letters indicate significant differences among females or males based on 1-way ANOVA followed by an LSD test ($P < 0.05$). Significant differences between the females and males determined with a *t*-test are indicated by * ($P < 0.05$).

The expression of insect JH-metabolizing enzymes has clear tissue specificity. In *B. mori*, *JHEH* mRNA was detectable, at comparable levels, in all tissues examined except for the silk gland and hemocytes, in which no expression was observed (Yang et al. 2011). In *L. decemlineata*, *LdJHEH1* was highly expressed in the thoracic muscles, foregut, midgut, and hindgut, whereas *LdJHEH2* was highly expressed in the brain-corpora cardiaca-corpora allata complex, hindgut, Malpighian tubules, and female ovaries (Lü et al. 2015). In the current study, *BdJHEH2* was mainly expressed in the fat body whereas *BdJHEH3* was predominately expressed in the Malpighian tubules. Taken together, these data demonstrate that JHEH might degrade JH in various tissues depending on the species. Our data revealed that *BdJHDK* was highly expressed in the Malpighian tubules. This result is consistent with previous findings in *D. melanogaster* (Maxwell et al. 2002a), *M. sexta* (Maxwell et al. 2002b), *B. mori* (Cheng et al. 2014), and *L. decemlineata* (Fu et al. 2015). The Malpighian tubules are important excretory organs, where JH metabolites hydrolyzed by JHDK could be excreted from the insect body (Yang et al. 2011). Future studies are necessary to determine the roles of JH metabolism–related genes in *B. dorsalis*.

Previous studies have shown that the expression of JH-metabolizing genes can be regulated by 20E in several insects. In *D. melanogaster*, *DmJHE* was induced by JH III, but the induction was suppressed by the simultaneous application of 20E (Kethidi et al. 2005). Similarly, functional analysis in *A. mellifera* showed that *AmJHE-like* expression was induced by JH III and suppressed by 20E (Mackert et al. 2008). In *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae), the *JHE* gene was induced by JH I and the JH I induction was suppressed by 20E (Feng et al. 1999). In 5th instars of *B. mori*, application of 20E resulted in a suppression of JHE activity in the hemolymph, and final instar larvae underwent a supernumerary molt (Hirai et al. 2002). Interestingly, the mRNA level of *JHDK* was not regulated by JH in *B. mori*, and the JH response element was not found in the promoter region of the gene (Li et al. 2005). We found that 20E up-regulated mRNA levels in *BdJHEHs* and *BdJHDK* at different time points. This observation is not consistent with previous studies, in which JH metabolic gene expression was suppressed by 20E (Feng et al. 1999; Kethidi et al. 2005; Mackert et al. 2008). *Bactrocera dorsalis* larvae can grow faster following 20E application and this can cause precocious metamorphosis (Bai et al. 2014). Hormonal adaptation of insects appears to be precisely controlled by regulation of the expression of different JH metabolic genes.

In insects, the nutritional state is a key factor in determining larval development. In *B. dorsalis* and *Psacothea hilaris* (Pascoe) (Coleoptera:

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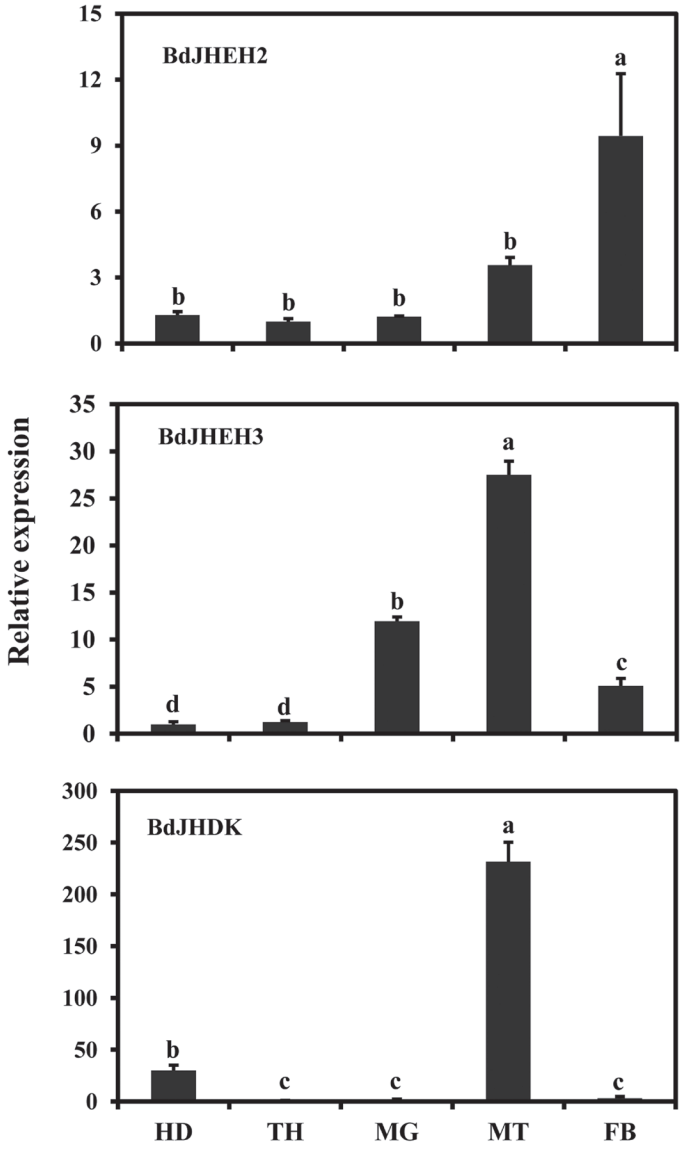


Fig. 4. Relative expression levels of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* in tissues of *Bactrocera dorsalis*. Expression levels in the head (HD), thorax (TH), midgut (MG), Malpighian tubules (MT), and fat body (FB) were detected by qPCR. Different letters indicate significant differences among tissues based on 1-way ANOVA followed by an LSD test ($P < 0.05$).

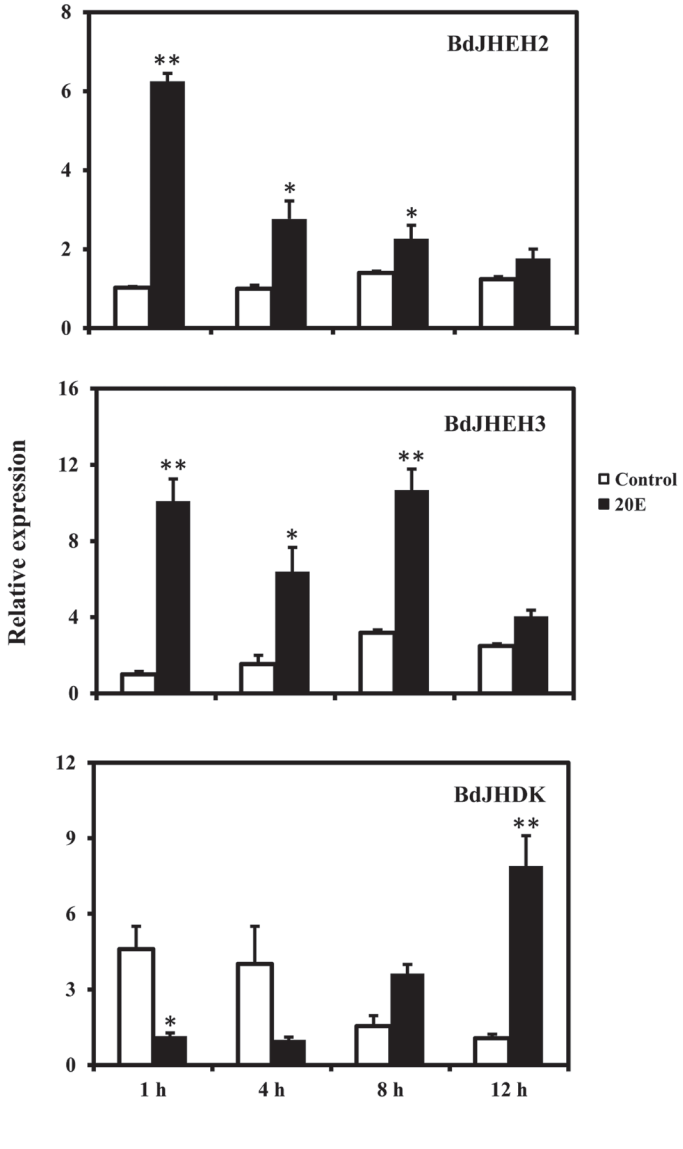


Fig. 5. Influence of 20E on the expression of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* in *Bactrocera dorsalis*. The 2-d-old 3rd instars were collected for qPCR analysis at 1, 4, 8, and 12 h post-injection with 20E. Control: control insects; 20E: insects injected with 20E. Significant differences between treatment and control was determined using a *t*-test and indicated by * ($P < 0.05$) or ** ($P < 0.01$).

Cerambycidae), larval starvation can lead to precocious metamorphosis and formation of unusually small but morphological normal adults (Munyiri et al. 2003; Yang et al. 2013b). A rapid decrease of hemolymph JH and a pulse of 20E during the final instar initiate larval–pupal transition (Goodman & Cusson 2012). Therefore, there may be a functional connection between starvation and JH regulation. Previous studies have emphasized the role of JH-metabolizing enzymes in modulating the starvation tolerance of insects. For example, prolonged starvation of *P. hilaris* larvae increased the expression of *PhJHE* mRNA whereas refeeding with glucose decreased expression (Munyiri & Ishikawa 2007). In *M. sexta* and *Galleria mellonella* L. (Lepidoptera: Pyralidae), starvation of the final instar larvae from 1 d following ecdysis prevented the increase of the JHE activity (Reddy et al. 1979; Cymborowski et al. 1982). In the present study, expression of the 2 *BdJHEHs* decreased during starvation and increased when feeding resumed. However, the expression of *BdJHDK* increased significantly during starvation. This result suggests that the 3 JH metabolism–related genes are readily influenced by the nutritional state. The up-regulation of *BdJHDK* may result in a rapid decrease of JH titers in the hemolymph, and this is consistent with the occurrence of precocious metamorphosis caused by starvation.

In conclusion, we identified 3 JH metabolism–related genes (*BdJHEH2*, *BdJHEH3*, and *BdJHDK*) in *B. dorsalis* and characterized their expression profiles in various development stages and tissues. Transcript levels of these genes were significantly increased by 20E. The expression level of *BdJHDK* was closely related to the nutritional state. These findings suggest that the JH metabolism–related genes of *B. dorsalis* have diverse and important roles during insect development. More in-depth investigations are needed to clarify the physiological mechanism of these genes.

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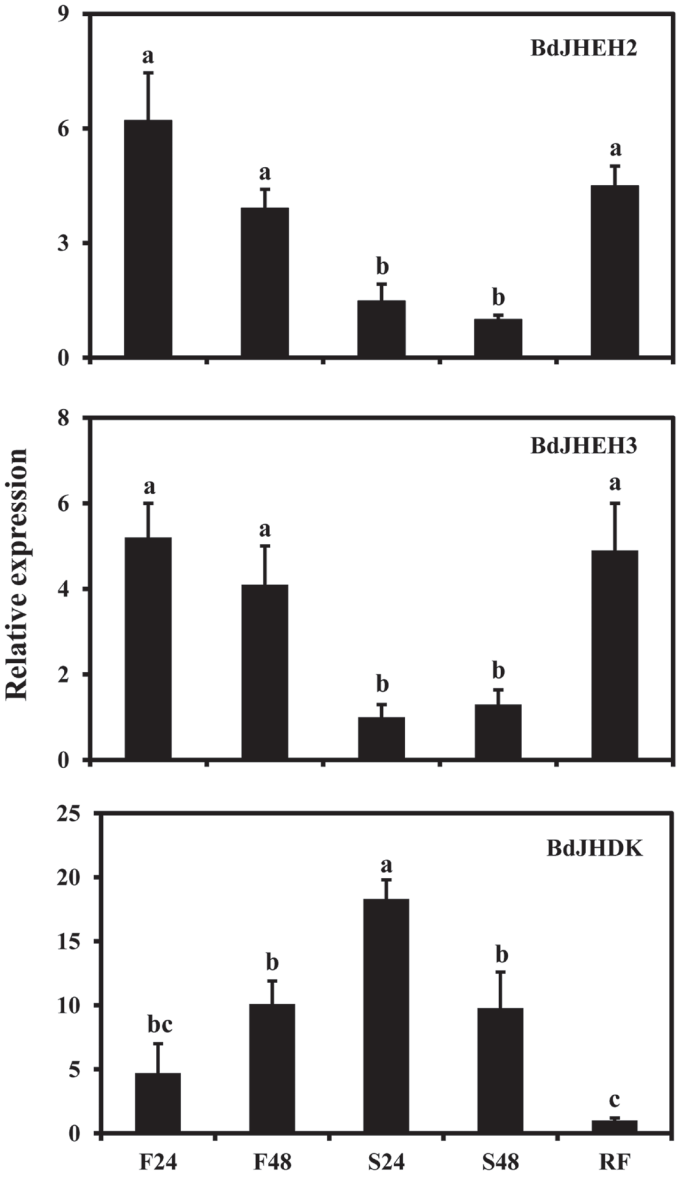


Fig. 6. Effects of starvation on the expression of *BdJHEH2*, *BdJHEH3*, and *BdJHDK* in *Bactrocera dorsalis*. The 2-d-old 3rd instars were fed or starved for 24 and 48 h before collection. The re-fed larvae were initially starved for 24 h, then re-fed for an additional 24 h prior to collection. F24: feeding 24 h; F48: feeding 48 h; S24: starvation 24 h; S48: starvation 48 h; RF: re-fed. Different letters indicate significant differences based on 1-way ANOVA followed by an LSD test ($P < 0.05$).

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