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ORIGINAL ARTICLE

Transcript analysis and expression profiling of three heat shock protein 70 genes in the ectoparasitoid *Habrobracon hebetor* (Hymenoptera: Braconidae)

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Abstract Heat shock proteins (HSPs) are known as chaperones that help with folding of other proteins when cells are under environmental stresses. The upregulation of HSPs is essential for cold survival during insect diapause. The ectoparasitoid *Habrobracon hebetor*, a potential biological control agent, can enter reproductive diapause when reared at low temperature and short photoperiod. However, the expression of HSPs during diapause of *H. hebetor* has not been studied. In this study, we sequenced and characterized the full-length complementary DNAs of three *Hsp70* genes (*HhHsp70I*, *HhHsp70II* and *HhHsp70III*) from *H. hebetor*. Their deduced amino acid sequences showed more than 80% identities to their counterparts from other insect species. However, the multiple sequence alignment among the three deduced amino acid sequences of HhHsp70s showed only 46% identities. A phylogenetic analysis of the three HhHsp70s and all other known Hsp70 sequences from Hymenoptera clustered all the Hsp70s into four groups, and the three HhHsp70s were distributed into three different groups. Real-time quantitative polymerase chain reaction analysis showed that the expression of the three *HhHsp70* genes in *H. hebetor* reared at different conditions was quite different. *HhHsp70I* showed higher relative expression when *H. hebetor* were reared at 27.5°C than at two lower temperatures (17.5°C and 20°C) regardless of the photoperiod, whereas *HhHsp70II* showed higher expression when *H. hebetor* were reared at 20°C and 10 : 14 L : D than when reared at 17.5°C and either 16 : 8 L : D or 10 : 14 L : D. In contrast, *HhHSP70III* was expressed at similar levels regardless of the rearing conditions. These results may suggest functional differences among the three *HhHsp70* genes in *H. hebetor*.

Key words ectoparasitoid, gene expression, *Habrobracon hebetor*, heat shock protein 70, phylogenetic analysis, quantitative RT-PCR

Introduction

The ectoparasitoid *Habrobracon hebetor* (Say) (= *Bracon hebetor*) (Hymenoptera: Braconidae) is considered as a potential biological control agent of several pyralid

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moth pests infesting warehouses due to its rapid population growth (Amir-Maafi & Chi, 2006; Balevski, 1984; Brower & Press, 1990; Huang, 1986; Keever *et al*., 1986; Press *et al*., 1982). It has been used to suppress moth populations in stored products (Balevski, 1984; Brower & Press, 1990; Cline & Press, 1990; Garba & Gaoh, 2008; Huang, 1986; Press *et al*., 1982) and in field crops (Cheng, 1991; Gerling, 1971; Imam *et al*., 2007; Uwais *et al*., 2006). Long-term storage of *H. hebetor* would facilitate its use in biological control programs, and we have recently shown that adult females appear to enter reproductive diapause when reared at 17.5°C or 20°C at 10 : 14 L : D (Chen *et al*., 2012) and that these females can be stored for up to 2 months without loss of vitality (Chen *et al*., 2013).

Diapause has been defined as a delay in development in response to regularly and recurring periods of adverse environmental conditions (Chapman, 1998; Tauber *et al*., 1986) and diapause can occur at different life stages in different insect species. Usually, tolerance to stresses, including heat, cold and desiccation, is greater in diapausing than in normal individuals (Tauber *et al*., 1986; Danks, 1987; Denlinger, 1991; Pullin, 1996; Goto *et al*., 1998; Foerster & Doetzer, 2006; Denlinger, 2008). Much work has been done on the mechanisms of acquired stress tolerance, such as accumulation of polyols or sugars (Storey & Storey, 1991), production of proteins to protect against freezing and desiccation (Kroeker & Walker, 1991; Lee, 1991), and regulation of the unsaturation of fatty acyl chains in phospholipids (Wada *et al*., 1990).

Expression of heat shock proteins (HSPs) is another important adaptive strategy in stress tolerance (Yocum *et al*., 1998; Feder & Hoffman, 1999; Rinehart *et al*., 2007; Zhang & Denlinger, 2010; Aruda *et al*., 2011), and the upregulation of HSPs is essential for cold survival during insect diapause (Rinehart *et al*., 2007). For example, two heat shock protein 70 (*Hsp70*) genes in the flesh fly, *Sarcophaga crassipalpis*, and one *Hsp70* in the cotton bollworm, *Helicoverpa armigera*, were upregulated during pupal diapause (Rinehart *et al.*, 2000; Rinehart *et al*., 2007; Bao & Xu, 2011), and one *Hsp70* was upregulated in adult diapause of the Colorado potato beetle, *Leptinotarsa decemlineata* (Yocum, 2001). In addition, *Hsp*s were the only genes identified as essential to recovery from low-temperature damage (Michaud, 2007). Knowing which *Hsp* genes are upregulated or downregulated during diapause may allow enhancement of diapause and prolonging the duration of low-temperature storage of insects by finding ways to artificially control the expression of these genes.

In an effort to better understand the diapause process in *H. hebetor* with the intent of lengthening the period that the females can be stored, in this study we investigated transcriptional response of three *Hsp70* genes in females reared at conditions that appear to induce reproductive diapause. We first obtained partial complementary DNA (cDNA) sequences of *Hsp70* by using degenerate primers, and then used 3′ and 5′ RACE (rapid amplification of cDNA ends). After we obtained the full-length cDNAs putatively encoding Hsp70s, we conducted reverse transcription quantitative PCR (RT-qPCR) to examine transcriptional responses of the three *Hsp70* genes in adults of *H. hebetor* reared at different conditions.

Materials and methods

Insects

A field strain of *H. hebetor* was collected in Parlier, CA, USA, in October 2009, and the colony was maintained in the laboratory on last instar larvae of the Indian meal moth, *Plodia interpunctella* (Hübner), at 27.5 ± 0.5°C, 65% ± 5% relative humidity (RH) and 16 : 8 L : D photoperiod. We placed adult parasitoids into 55.5-mL plastic vials (3.2 cm diameter by 8.3 cm high) containing 30 *P. interpunctella* last instar larvae so that they would be experienced in host searching and egg laying before they were used in experiments. The vials were capped with lids that had a 12-mm diameter hole covered by a fine screen. Fresh eggs of *H. hebetor* for use in experiments then were obtained by placing one pair of experienced *H. hebetor* adults with 30 *P. interpunctella* last instar larvae, and then removing the adults from the vial after 24 h. The *P. interpunctella* larvae with *H. hebetor* eggs were reared at 17.5°C, 20.0°C and 27.5°C, under each of two photoperiods (10 : 14 L : D and 16 : 8 L : D). Adults were collected every 24 h at 27.5°C and every 3.5 days at 17.5°C and 20.0°C, and then the adults were stored at –80°C until needed for analyses described below.

Total RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from 100 mg of *H. hebetor* adults using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA) according to the procedure described by Zhu (2009). Total RNA was treated with deoxyribonuclease (Dnase I: Fermentas Inc., Burlington, ON, Canada) to remove possible residual genomic DNA. The concentration of RNA was determined by Nanodrop (Thermo Fisher Scientific Inc, Waltham, MA, USA). One microgram of total RNA was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (Fermentas) according to the manufacturer's protocol.

Generation of Hsp70 *and* Rps3 *partial cDNAs*

Degenerate primers of *Hsp70*s were designed according to the highly conserved amino acid sequences of known insect *Hsp70*s from the honey bee, *Apis mellifera* (GenBank ID: NM_001160072) (Williams *et al*., 2008), apple maggot fly, *Rhagoletis pomonella* (GenBank ID: EF103584) (Rinehart *et al*., 2007) and the endoparasitoid, *Pteromalus puparum* (GenBank ID: FJ798091) (Wang *et al*., 2008), by using a strategy similar to that described by Mahroof *et al*. (2005). The amino acid sequences of the red flour beetle, *Tribolium castaneum* (GenBank ID: NP_001165863.1) (Lord *et al*., 2010), the fruit fly, *Drosophila melanogaster* (GenBank ID: AAF56129.1) (Adams *et al*., 2000), and the European corn borer, *Ostrinia nubilalis* (GenBank ID: AAR98922.1) (Li *et al*., 2005) were used to design degenerate primers for *H. hebetor* ribosomal protein S3 (*Rps3*) gene to be used as an internal reference (Table 1). Specific *Hsp70* and *Rps3* fragments were amplified from cDNA by using PCR Smart Mix (Invitrogen), and PCR amplifications were hot-started at 94°C for 3 min, followed by three cycles at 94°C for 1 min, annealing at 40°C for 1 min and extension at 72°C for 2 min. These then were followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. PCR was performed using a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA).

Amplified DNA fragments of predicted sizes were purified by using QIAEX II Agarose Gel Extraction kit (Qiagen, Valencia, CA, USA) and subcloned into pCR® 2.1 vector (Invitrogen) according to the manufacturer's protocol. Plasmid DNA was then purified by using Zyppy™ Plasmid Miniprep kit (Zymo, Orange, CA, USA). *Eco*RI was used to digest the plasmid DNA, which was then subjected to gel electrophoresis to verify whether or not the target was inserted. Once the insert targets were confirmed, the nucleotide sequences of plasmid DNA that included the target fragment DNA were determined by using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were further examined for their identities by BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RACE

A 3′ and 5′-full RACE kit (Takara Biotechnology Inc, Dalian, China) was used to obtain the 3′ and 5′ ends of the *Hsp70* cDNAs. The basic strategy for 3′ RACE was to synthesize first strand cDNA by reverse transcription of total RNA with 3′ RACE adaptor primer, and then use the *Hsp*70 gene-specific outer primer and 3′ RACE outer primer to perform the first round PCR. The *Hsp70* gene-specific inner primer, 3′ RACE inner primer, and first-round PCR product were then used to run the second-round PCR. For 5′ RACE, the 5′ RACE adaptor was ligated to the messenger RNA (mRNA) using T4 RNA ligase, and the mRNA was then reverse transcribed to cDNA by using reverse transcriptase M-MLV (Moloney murine leukemia virus) and random 9 mers. When the cDNA was prepared, the first- and second-round PCRs were performed using outer primers and inner primers. The primers for 3′ RACE (Hsp70I-3O, Hsp70I-3I, Hsp70II-3O, Hsp70II-3I, Hsp70III-3O and Hsp70III-3I) and 5′ RACE (Hsp70I-5O, Hsp70I-5I, Hsp70II-5O, Hsp70II-5I, Hsp70III-5O and Hsp70III-5I) (Table 1) were designed and synthesized based on the partial *Hsp70* cDNA sequences. The 3′ and 5′ RACE were performed based on the manufacturer's procedure of 3′ and 5′-full RACE kit. The PCR products were subjected to electrophoresis, and the expected bands were cut from the gel and purified using Zomen Agarose Gel Extraction kit (Zomen Biotechnology, Beijing, China). After the extracted DNA was subcloned into *pEASY*-T1 plasmid vector (Transgen Biotechnology, Beijing, China) according to the manufacturer's protocol, the plasmid vector was used to transform *Escherichia coli*. The inserted cDNAs were sequenced by using a 3730 DNA Analyzer (Applied Biosystems) and M13 forward and reverse primers. The sequences were then analyzed by a blast search (NCBI: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Analysis of deduced amino acid sequences of Hsp70 *cDNAs*

After the amino acid sequences were deduced from *Hsp70* cDNA sequences, multiple sequence alignment was performed using the ExPASy Proteomics website (http://expasy.org). The neighbor-joining method in MEGA 5.05 (http://www.megasoftware.net/mega.php) was used to construct phylogenetic trees based on the Hsp70 amino acid sequences from this study and from other species of Hymenoptera. A bootstrap analysis was conducted, and each cluster was verified using 5 000 replicates (Felsenstein, 1985). Other protein sequence analysis tools used in this study (molecular mass, isoelectric point and motif sequences site search) were obtained from the ExPASy Proteomics website (http://expasy.org/).

RT-qPCR

RT-qPCR was performed by MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules,

Table 1 The degenerate and gene-specific primers used for cloning the partial and full-length *Hsp70* cDNA sequences and for quantitative PCR analysis.

Application of primers	Gene name	Primer name	Type	Nucleotide sequence (5′–3′)	Temp. °C	PCR product size (bp)
Amplification of cDNA fragments	*Hsp70*	Hsp70F	D	GGNGGNGARGAYTTYGA		684
		Hsp70R	D	RTTRTTRTCYTTNGTCAT		
Internal reference gene	Ribosomal protein S3	RPS3F	D	GARATHATHATHATGGC		327
		RPS3R	D	GGYAGNTGYTTRAAGTA		
3′ RACE	*Hsp70I*	Hsp70I-3′O	S	ATTGAAACTGCTGGTGGC	54.0	971
	Hsp70II	Hsp70II-3′O	S	CGTTGCTTATGGTGCTGC	56.0	713
	Hsp70III	Hsp70III-3′O	S	ACTTCTTCAACGGCAAGG	54.0	1281
		3′RACE Outer Primer		TACCGTCGTTCCACTAGTGATTT	58.6	
	Hsp70I	Hsp70I-3′I	S	CGATAATCAGCCTGGAGT	54.0	882
	Hsp70II	Hsp70II-3′I	S	CATCCAGGTGTACGAGGGC	56.0	506
	Hsp70III	Hsp70III-3′I	S	TGTCTTGATCCAGGTCTACG	60.0	1025
		3′RACE Inner Primer		CGCGGATCCTCCACTAGTGATTTCACTATAGG	66.1	
5′ RACE	*Hsp70I*	Hsp70I-5′O	S	CGATCTCGATGGTAGCCTCTGT	51.0	956
	Hsp70II	Hsp70II-5′O	S	ATTTCAATCCTGACCTGGTGGC	51.0	1105
	Hsp70III	Hsp70III-5′O	S	TCGCACGCTCACAAGCTGTCCT	51.1	958
		5′RACE Outer Primer		CATGGCTACATGCTGACAGCCTA	61.6	
	Hsp70I	Hsp70I-5′I	S	CGCAGTCTCCTCAAAGCCCTAG	51.1	894
	Hsp70II	Hsp70II-5′I	S	TCCTTTCTGATGTCCTTGCCCT	51.0	1021
	Hsp70III	Hsp70III-5′I	S	CCTTCTTGTACTTGCGCTT	56.0	899
		5′RACE Inner Primer		CGCGGATCCACAGCCTACTGATGATCAGTCGATG	70.7	
RT-qPCR analysis	*Hsp70I*	Hsp70IF	S	GAGAAAGTATAGAAAGGATATGA	57.9	101
		Hsp70IR	S	CCTCTGTACTAGATGACAA	58.1	
	Hsp70II	Hsp70IIF	S	GCTCTACAAGAAGAAGAAG	57.8	197
		Hsp70IIR	S	AATCAATGTTCAACTCCTC	58.0	
	Hsp70III	Hsp70IIIF	S	CTCAACAAGTCCATCAAC	58.5	190
		Hsp70IIIR	S	TTGGAATGGTTGTGTTAC	57.9	
	Ribosomal protein S3	RPS3F	S	GACGAATTTCATGCTCTT	58.2	151
		RPS3R	S	GTCACTCAGATACAAACTC	57.9	

O, outer primer; I, inner primer; F, forward; R, reverse; D, degenerate primer; S, gene-specific primer; RACE, rapid amplification of cDNA ends; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

CA, USA), and the expression of *Hsp70* genes in each sample was tested through comparative CT method, $2^{\Delta\Delta C_T}$ (Livak & Schmittgen, 2001). *Rps3* was chosen as the reference gene. The concentration of cDNA was adjusted by *Rps3* before RT-qPCR was performed. Each treatment was replicated with four biological samples, and each biological sample included two technical replicates. The sequences of the *Hsp70* and *Rps3* primers used for RT-qPCR analyses are shown in Table 1.

Statistical analysis

Relative levels of *Hsp70* transcripts in *H. hebetor* reared at different conditions were compared with that from *H. hebetor* reared at 27.5°C and 16 : 8 L : D, which was considered as a control. For data obtained from RT-qPCR, the changes in expression of the target genes were normalized to *Rps3* first, and then the relative expression levels in percentage for each gene were calculated by dividing the relative expression value (REV) of each gene in each rearing condition by the REV of the same gene in all conditions. The REV values were transformed using the arcsine square root transformation, and the transformed data were compared using one-way analysis of variance (ANOVA) and Tukey's b test ($P = 0.05$) across the different rearing conditions using SPSS software (SPSS, Inc., 2007).

Results

Sequencing and characterization of Hsp70 *cDNAs*

By using the degenerated primers in our PCR amplification of *Hsp70* cDNAs from *H. hebetor*, we obtained three PCR fragments with lengths of 649, 637 and 645 bp. The deduced amino acid sequences of these PCR fragments showed 94%–99% identities to the same regions of Hsp70s from *Microplitis mediator* (GenBank ID: ABN54440), *P. puparum* (GenBank ID: ACO57618), *T. castaneum* (GenBank ID: EEZ98819), *Loxostege sticticalis* (GenBank ID: ACD63049), *Megachile rotundata* (GenBank ID: AAS57865) and *Camponotus floridanus* (GenBank ID: EFN65945).

Based on the cDNA sequence of three PCR products, we performed RACE PCR and successfully obtained the full-length cDNAs of the three putative *Hsp70* genes from *H. hebetor*. These genes were named as *HhHsp70I(B4)*, *HhHsp70II(B6)* and *HhHsp70III(A6)* with GenBank accession numbers of JQ839279, JQ839280 and JQ839281, respectively. The cDNA lengths of *HhHsp70I(B4)*, *HhHsp70II(B6)* and *HhHsp70III(A6)* were 2220, 2004 and 2417 bp with open reading frames encoding 639, 616 and 649 amino acid residues, respectively (Table 2). A BLAST search in the NCBI showed that the deduced amino acid sequences of the three putative *HhHsp70* genes (Table 2) were highly similar to the known *hsp70* genes from other insect species.

Amino acid sequence comparisons and phylogenetic analysis

Three Hsp70 family signatures, signature 1 (consensus sequences: [IV]-D-L-G-T-[ST]-x-[SC]), signature 2 (consensus sequences: [LIVMF]-[LIVMFY]-[DN]-[LIVMFS]-G-[GSH]-[GS]-[AST]-x(3)-[ST]-[LIVM]-[LIVMFC]) and signature 3 (consensus sequences: [LIVMY]-x-[LIVMF]-x-G-G-x-[ST]-{LS}-[LIVM]-P-x-[LIVM]-x-[DEQKRSTA]) were found in our deduced HhHsp70 amino acid sequences by PROSITE analysis

Table 2 Nucleotide and putative amino acid sequences of *HhHsp70* genes after 3′ and 5′ RACE.

	HhHsp70I	*HhHsp70II*	*HhHsp70III*
Full-length cDNA (bp)	2220	2004	2417
5′ UTR (bp)	75	142	111
3′UTR (bp)	228	14	359
Canonical polyadenylation signal sequence (AATAAA)	No	No	Yes
Poly (A) tail	Yes	Yes	Yes
Open reading frame (ORF) (bp)	1917	1848	1947
Polypeptide comprised	639	616	649
Predicted molecular mass (kDa)	73.57	68.05	73.72
Theoretical isoelectric point (pI)	5.09	5.24	4.84
Locations of three Hsp70 signatures site[†]	6–13, 194–207, 332–345	35–42, 223–236, 360–373	9–16, 197–210, 335–348
Locations of ATP/GTP-binding motif site	128–135	158–165	131–138
Locations of non-organellar consensus motif site	297–303	325–331	300–306

[†]The locations represent the regions of the deduced amino acid sequences as presented in Fig. 1. RACE, rapid amplification of cDNA ends; UTR, untranslated region.

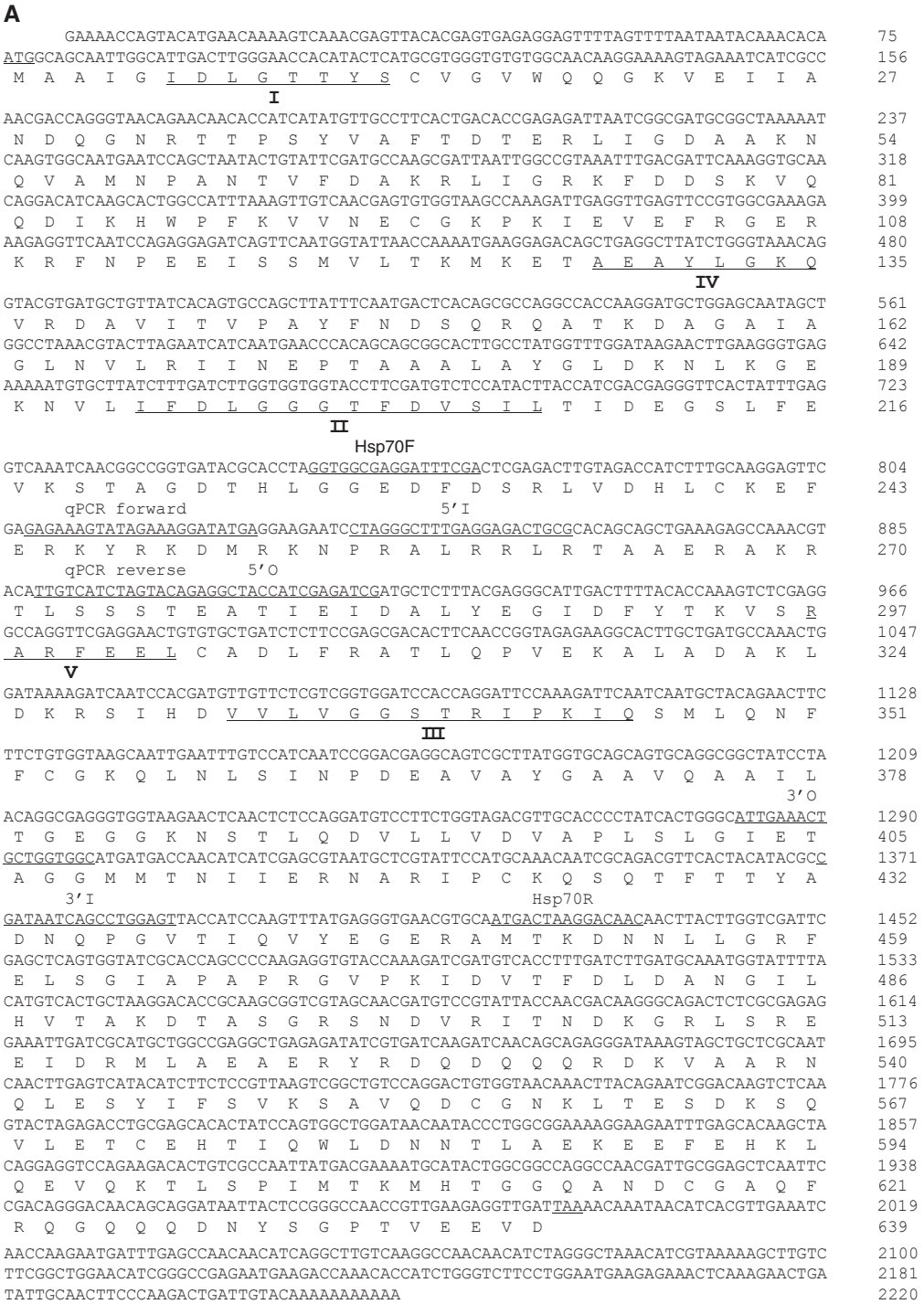

Fig. 1 Full-length cDNA sequences and deduced amino acid sequences of (A) *HhHsp70I*, (B) *HhHsp70II*, and (C) *HhHsp70III* from *H. hebetor*. Start codons (ATG), stop codons (TAA) and the putative polyadenylation signals (AATAAA) in *HhHsp70III* are underlined. Three signature *Hsp70* sequences are underlined and labeled I, II, and III, and ATP-GTP binding site motif and non-organellar consensus motif are underlined and labeled as IV and V, respectively. The forward and reverse degenerate primers in *Hsp70s*, primers for RACE and primers for RT-qPCR are underlined and labeled.

B

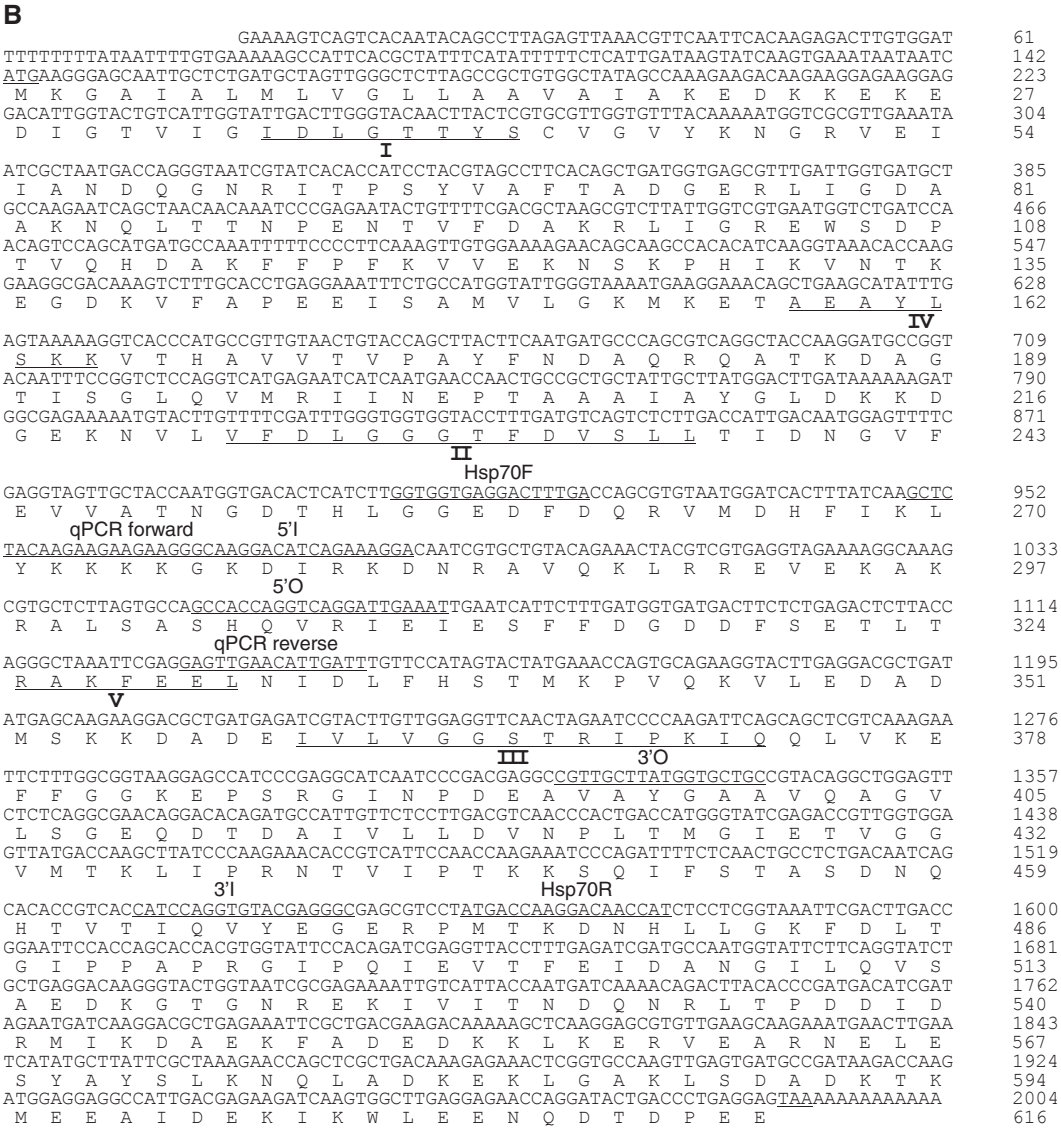

Fig. 1 Continued.

(www.expasy.org/prosite) (Fig. 1, Table 2). A notable feature in the protein is the presence of three consecutive repeats of the tetrapeptide motif GGMP in its C-terminal region for HhHsp70III.

ATP/GTP-binding site motif (Sonoda *et al*., 2006) and non-organellar consensus motif (Tungjitwitayakul *et al*., 2008) were also observed (Fig. 1, Table 2). Multiple sequence alignments showed very high homologies among the three deduced amino acid sequences of HhHsp70s and Hsp70s in other insect species. HhHsp70I was 85% identical to *Macrocentrus cingulum* Hsp70 (GenBank ID: ACD84944) and *P. puparum* Hsp70 (GenBank ID: ACO57618), and 83% identical to *Nasonia vitripennis* Hsp70 (GenBank ID: XP_001604951); HhHsp70II was 84% identical to *N. vitripennis* Hsp70 (GenBank ID: XP_001606463) and *C. floridanus* Hsp70 (GenBank ID: EFN61604) and 83% identical to *Acromyrmex echinatior* Hsp70 (GenBank ID: EGI70210); and HhHsp70III was 88% identical to *P. puparum* Hsp70 (GenBank ID: ACA53150) and *N. vitripennis* Hsp70 (GenBank ID: NP_001166228) and 87% identical to *C. floridanus* Hsp70 (GenBank ID: EFN65945). However, the identity among the HhHsp70s was relatively low. Multiple sequence alignments of the HhHsp70s reported in this article showed the identity was as low as 46%: HhHsp70II was 53% identical both to HhHsp70I and HhHsp70III,

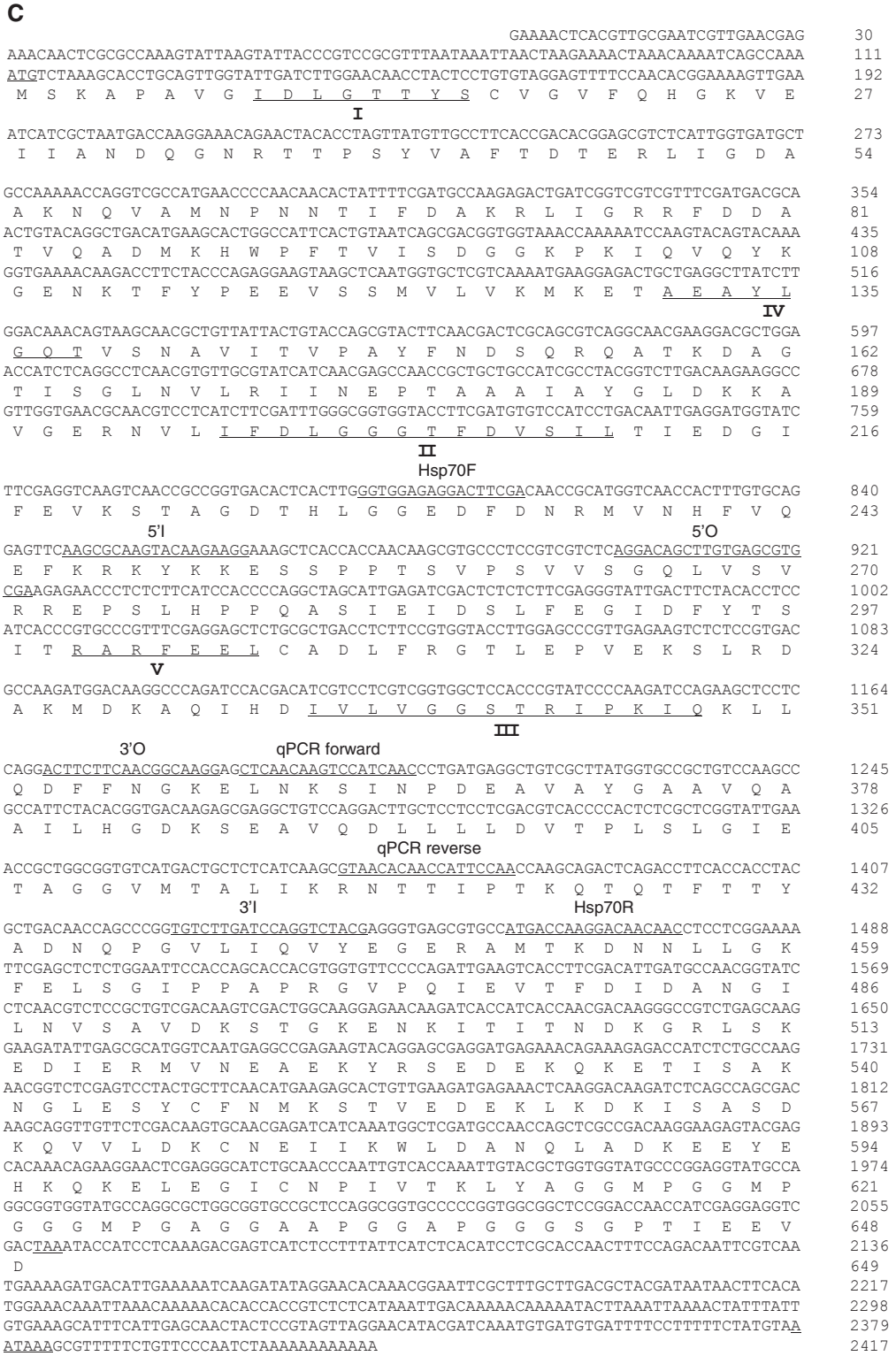

Fig. 1 Continued.

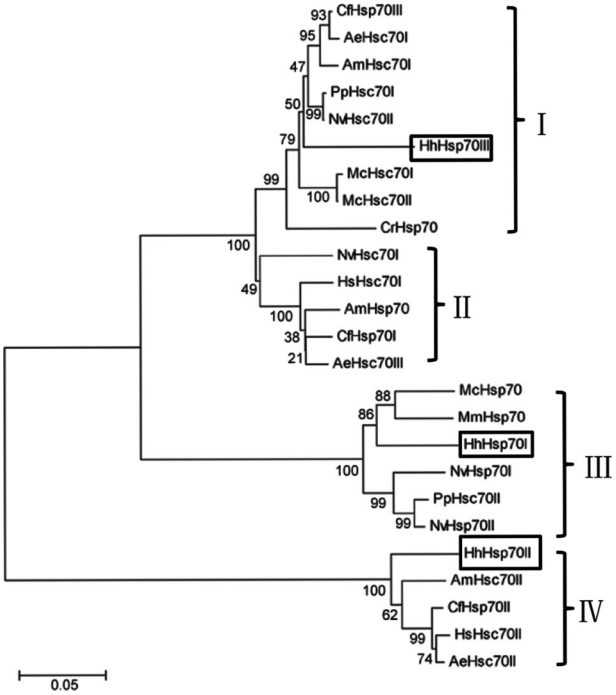

Fig. 2 A phylogenetic tree of three Hsp70s from *Habrobracon hebetor* and heat shock protein 70 and heat shock cognate 70 family members from other hymenopteran species constructed with the neighbor-joining method. Numbers at each branch indicate the percentage of times a node was supported in 5 000 bootstrap pseudoreplications by neighbor joining.

and HhHsp70I was 66% identical to HhHsp70III. Based on the three deduced amino acids sequences of HhHsp70 and all other known Hsp70 sequences in Hymenoptera, a phylogenetic tree was constructed using the program MEGA 5. Results show that the Hsp70s in Hymenoptera clustered into four groups, and the three HhHsp70s were distributed into three different groups (i.e., I, III and IV, Fig. 2).

Transcriptional responses of Hsp70 *genes in* H. hebetor *reared under different conditions*

HhHsp70I showed a higher transcript level when *H. hebetor* were reared at 27.5°C than at low temperatures (17.5°C and 20°C) regardless of the photoperiod (Fig. 3). The highest transcript level for *HhHsp70I* was found when *H. hebetor* were reared at 27.5°C and 10 : 14 L : D. For *HhHsp70II*, the transcript level was higher when *H. hebetor* were reared at 20°C and 10 : 14 L : D than when reared at17.5°C and either 16 : 8 L : D or 10 : 14 L : D. However, there were no significant differences among all the conditions at the two higher temperatures (27.5°C and 20°C). In contrast, the transcript level of *HhHsp70III* was stable across all rearing temperatures and photoperiods examined in this study.

Discussion

We sequenced and characterized the full-length cDNAs of three putative *Hsp70* genes (*HhHsp70I*, *HhHsp70II* and *HhHsp70III*) from *H. hebetor*. The lengths of these cDNAs, the open reading frames and the predicted molecular masses of their deduced amino acid sequences were similar to those of other known *Hsp70*s. All the important characteristics, including three Hsp70 family signatures and an ATP-GTP binding site in each of their deduced amino acid sequences, strongly suggest that these three genes belong to the Hsp70 family. This notion is further supported by the presence of another conserved motif (EEVD) in HhHsp70I and HhHsp70III as the members of the Hsp70 family.

Generally, members of the Hsp70 family are highly conserved, and our results underscore this high homology. However, it should be noted that their C-terminal regions are often highly divergent. The variation in sequences of the C-terminus may determine the functional specificity of individual HSPs (Demand *et al*., 1998; Fuertes *et al*., 2004; Park *et al*., 2007). For example, a conserved EEVD motif at the C-terminus of HhHsp70I and HhHsp70III might allow them to bind other co-chaperones (Daugarrd *et al*., 2007). Furthermore, some Hsp70s have GGMP repeats at the C-terminal, whereas other Hsp70 family members lack such structural elements (Boorstein *et al*., 1994; Boutet *et al*., 2003a, 2003b; Kourtidis *et al*., 2006). In our study, only HhHsp70III was found to possess three repeats of the tetrapeptide GGMP.

All eukaryotes investigated have more than one *Hsp70* gene, with eight *Hsp70* homologues documented for yeast and at least eight unique gene products for humans (Werner-Washburne & Craig, 1989; Daugarrd *et al*., 2007). Phylogenetic analysis using the Hsp70 deduced amino acid sequences has been applied to many insect species (Sim *et al*., 2007; Wang *et al*., 2008; Garbuz *et al*., 2011). We used the neighbor-joining method to conduct a phylogenetic analysis of 21 Hsp70s from Hymenoptera for which full-length amino acid sequences have been identified. The three Hsp70s from *H. hebetor* were grouped with other Hsp70s and clustered into three different groups (Fig. 2). Such clustering patterns are also consistent with those of *A. mellifera* and *C. floridanus* (Fig. 2). Thus, these *Hsp70* genes appear to be paralogous with each other in that they may have independently evolved from their ancestors.

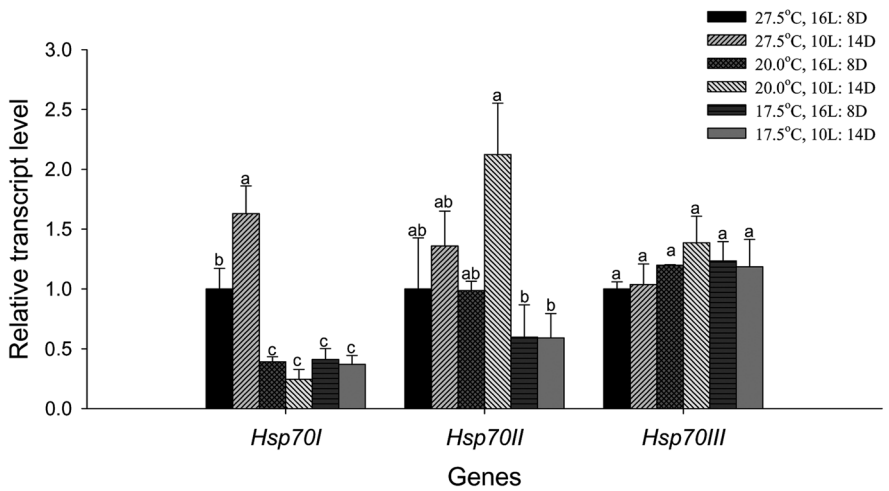

Fig. 3 Relative expression of *Hsp70*-like genes to *Rps3* in *Habrobracon hebetor* reared at different conditions. Bars denoted by the same letter indicate that there are no significant differences in gene expression among the different rearing conditions (Tukey's b $P > 0.05$).

The expression of *Hsp70* genes is commonly upregulated when an insect enters diapause (Rinehart *et al*., 2007). For example, expression of *Hsp70*s is upregulated throughout the adult diapausing stage of the Colorado potato beetle, *L. decemlineata* (Yocum, 2001), and in the pupal diapausing stage of the solitary bee, *M. rotundata* (Yocum *et al*., 2005), the cotton bollworm, *H. armigera* (Bao & Xu, 2011), the flesh fly, *S. crassipalpis* (Rinehart *et al*., 2000) and the onion maggot, *Delia antiqua* (Chen *et al*., 2006). In contrast, *Hsp70* is not upregulated in the adult diapausing stage of *Drosophila triauraria* (Goto *et al*., 1998) and the house mosquito, *Culex pipiens* (Rinehart *et al*., 2006), or in the larval dipausing stage of the green bottle fly, *Lucilia sericata* (Tachibana *et al*., 2005) and the Asiatic rice borer, *Chilo suppressalis* (Sonoda *et al*., 2006). Such varied expression responses of *Hsp70* genes in different developmental stages of diapausing insects suggest insect-specific traits related to *Hsp70*s.

The expression of *Hsp70*s may also be influenced by different environmental conditions. In our study, expression of three *Hsp70* genes in *H. hebetor* reared at different conditions was quite different. Chen *et al*. (2012) showed that reproductive diapause can be induced at a short photoperiod at 20°C, but not at a long photoperiod. However, in this study the expression of *HhHsp70I* was found not to be different when the insect was reared at different photoperiods at 20°C. However, the expression of *HhHsp70I* in *H. hebetor* reared at 27.5°C was higher than that when *H. hebetor* was reared at 17.5°C and 20°C, regardless of the photoperiod. This implies that temperature has a greater influence on expression of *HhHsp70I* than the insect's diapause status. For *HhHsp70II*, the transcript level was higher when *H. hebetor* were reared at 20°C and 10 : 14 L : D than when reared at 17.5°C and either 16 : 18 L : D or 10 : 14 L : D. We also found (Chen *et al*., 2013) that *H. hebetor* perform best after long-term cold storage when the wasp was reared at 20°C and a 10-h photophase. Further study should be conducted on the relationship between this gene and cold tolerance in *H. hebetor*. In contrast, the expression of *HhHsp70III* was influenced neither by the temperatures nor by the photoperiods examined in this study.

The expression of HSP genes involved in diapause may change during the course of diapause. For example, the expressions of *Hsp23* and *Hsp70* in *S. crassipalpis* are turned on at the onset of pupal diapause, and continue until the pupae receive a signal to resume development (Rinehart *et al*., 2007). On the other hand, the expression of *Hsp90* in *Helicoverpa zea* is not consistent during pupal diapause; it is downregulated after the first 3 days, stays at a low expression level for about 1 month, and then increases when the pupae enter deep diapause (Zhang & Denlinger, 2010). Also, *Hsp70*s may not be consistently involved in diapause. For example, one *Hsp70* is upregulated during adult diapause of *L. decemlineata*, but another *Hsp70* is unchanged during diapause (Yocum, 2001). In addition, the expression of *Hsp* genes may be upregulated in the pre-adult stage in species with adult (reproductive) diapause (Rinehart *et al*., 2007). Thus, unlike the upregulation of *Hsp* genes in some of other diapausing insects,

the *Hsp70*s in *H. hebetor* are not simply upregulated during diapause. Our results suggest that the upregulation of *Hsp70*s is not universal among diapausing insects and that there may be functional differences among the three *Hh Hsp70* genes in *H. hebetor*.

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Disclosure

This manuscript and its authors are not involved in any potential conflicts of interest, including financial interests and relationships and affiliations.

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