Cloning Three *Harmonia axyridis* (Coleoptera: Coccinellidae) Heat Shock Protein 70 Family Genes: Regulatory Function Related to Heat and Starvation Stress[1]

Qida Shen, Lina Zhao, Guoqiang Xie, Ping Wei, Mengmeng Yang, Shigui Wang, Fan Zhang[2], and Bin Tang[3]

Hangzhou Key Laboratory of Animal Adaptation and Evolution, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, Zhejiang 310036, China.

J. Entomol. Sci. 50(3): 168–185 (july 2015)

Abstract Heat shock protein (HSP) is a very conservative group of proteins expressed in response to stress in organisms or cells in culture. There are four major HSP gene families in insects: small HSP, HSP60, HSP70, and HSP83. To gain insight into the various functions of the HSP70 family genes in response to stress in insects, two new HSP70 cDNAs were cloned from the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (hereafter *Harax* when referring to genes, proteins, or cDNAs). *HaraxHSP68* has an open reading frame (ORF) of 638 amino acids and an isoelectric point (pI) of 6.53. *HaraxHSP70B* has an ORF of 661 amino acids and a pI of 6.20. *HaraxHSP70A*, obtained in earlier work, has an ORF of 651 amino acids and a pI of 5.36. HSP70 proteins have the three signatures IDLGTTYSCVGV, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQ; the signature ATP/GTP binding site motif AEAYLG(K/T)T; and an MEEVD motif at the C terminus. The three HSP70 proteins do not have a high degree of similarity, however, and *HaraxHSP70B* can be distinguished clearly from the other HSP70 proteins. *HaraxHSP68* was found to be highly expressed in the early larval stages. *HaraxHSP70A* is highly expressed and *HaraxHSP70A* is moderately expressed in both the pupal and adult stages. In general, relative levels of expression of the three genes increased with increasing temperature, and *HaraxHSP70B* was highly expressed at 0°C. The relative expression levels of the three HSP70 genes reached a maximum after starvation for 8 h. These results revealed the three HSP70 proteins might have significant function(s) during development, heating, and starvation.

Key Words heat shock protein, cloning, characterization, expression pattern, *Harmonia axyridis*

Heat shock protein (HSP) is a highly conserved group of stress proteins rapidly expressed in organisms (and cells in culture) when subjected to deleterious environmental factors (stress) (Sørensen et al. 2003, Colinet et al. 2010). HSP was discovered first in a thermally shocked salivary gland chromosome of the fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Ritossa 1962). Since

[1]Received 12 November 2014; accepted for publication 8 April 2015.

[2]Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100089, China.

[3]Corresponding author (email: tbzm611@yahoo.com).

HSP was discovered, many HSP genes have been cloned and studied in various insect orders, including Diptera (Yocum et al. 1998, Goto and Kimura 2004), Lepidoptera (Sakano et al. 2006, Zhang and Denlinger 2010), Orthoptera (Qin et al. 2003, Warchalowska-Sliwa et al. 2005), Hymenoptera (Elekonich 2009), and Coleoptera (Mahroof et al. 2005, Huang and Kang 2007, Xu et al. 2009). There are four major HSP gene families in insects: small HSP (molecular mass ~20–30 kDa), HSP60 (molecular mass ~60 kDa), HSP70 (molecular mass ~70 kDa), and HSP83 (high molecular mass) (Kim et al. 1998, Sørensen et al. 2003, Li et al. 2009).

HSP70, currently the most intensively studied heat shock protein family, is one of the most conserved gene families (Boorstein et al. 1994). There are two kinds of expression in the HSP70 gene family (Karlin and Brocchieri 1998). One expression involves the HSP70 gene with which the relative expression is usually at a low level, but the relative expression elevates when an organism is subjected to external sources of stress. The other type of expression involves the heat shock cognate protein 70 (HSC70) gene, whose relative expression is usually at a high level. HSC70 accounts for 5–10% of the total protein in a healthy organism, but it does not respond to external stress (Kiang and Tsokos 1998).

The two types of HSP70 have common structural characteristics, although they have different expression behaviors. Both have two major domains with an amino-terminal ATP-binding domain (molecular mass 44 kDa) with very well conserved N-terminal amino acid residues, and a carboxy-terminal substrate-binding domain with a polypeptide (molecular mass 30 kDa) (Kiang and Tsokos 1998). A motif (EEVD) for cellular localization is located at the terminus of the C-terminal domain (Gupta 1995).

The multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (hereafter *Harax* when referring to genes, proteins, or cDNAs), is used worldwide as a focus of agriculture and forestry production pest control strategies (Sebaey and Gantiry 1999). Its temperature adaptability indicates its strong resistance mechanism that responds to environmental stress. It is an excellent biological system for the study of biological resistance (Koch 2003, Zhu et al. 2010). In this study, conserved cDNAs of HSP70 genes from many kinds of insects were regarded as templates. Two sequences of HSP70 gene cDNAs were obtained from *H. axyridis* by rapid amplification of cDNA ends (RACE). In the present study, HSP68, HSP70B, and HSP70A genes (Yang et al. 2009) were cloned from *H. axyridis* (GenBank accessions KJ136115, KJ136116, and ABR92405, respectively). We observed differential expression of these three genes in different insect developmental stages and different temperatures. A functional analysis of gene response during heat shock and starvation stress was also performed.

Materials and Methods

A colony of *H. axyridis* originally collected in the Xiasha Region, Hangzhou, Zhejiang Province, China, had been cultured in the laboratory for three generations and served as the source of all insects used in this study. All *H. axyridis* were kept in rectangular plastic feeding boxes (15 × 12 × 7 cm) at 25 ± 1°C and 80 ± 5% relative humidity, with a 14:10 light:dark photoperiod (light from 0800 to 2200 hours

daily) (Zhang et al. 2013). Insect stages subjected to analyses were first-instar larvae (1 d after eclosion), second-instar larvae (1 and 2 d after molt), third-instar larvae (1 and 2 d after molt), fourth-instar larvae (1, 2, 3, and 4 d after molt), prepupae, pupa (1 2, 3, and 4 d after molt), and adult (1, 2, and 3 d after emergence).

Temperature stress was assessed by two methods. For one method, the insects were initially placed in artificial climate box at 0°C. The temperature was increased from 0 to 20°C at +5°C/h. Individual insects were collected at 0, 5, 10, 15, and 20°C, with three replicates at each temperature, and three individuals for each replicate for each developmental stage. Some individuals were kept at 25°C as controls. Total RNA was extracted from the insects removed at each temperature.

In the second method, individual insects were placed in artificial climate box maintained at a constant −5, 0, 5, 10, or 15°C for 1 h after which they were removed for total RNA extraction (three replicates with three individuals per replicate) at each temperature and developmental stage. Appropriate controls were held at 25°C.

Insects were removed from food for various times to assess the impact of starvation. Individual insects were randomly assigned to one of five treatment groups (starved for periods of 4, 8, 12, 18, and 24 h) and withheld from diet for those times. Controls were insects reared under normal conditions and that were not withheld from diet. Total RNA was extracted (three replicates with three individuals per replicate) for each treatment.

Total RNA was extracted from processed material using TRIzol® reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was conducted with a PrimeScript® RT reagent kit with gDNA Eraser (Takara, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA (1 μl) was used as the template for the polymerase chain reaction (PCR). *HaraxHSP68-5F1*, *HaraxHSP68-3R1*, *HaraxHSP70B-5F1*, and *HaraxHSP70B-3R1* (Table 1) were designed on the basis of the conserved amino acid sequences of several known forms of HSP70.

Rapid Amplification of cDNA Ends (RACE) was required for the 5′- and 3′-RACE of *HaraxHSP68* and 5′- and 3′-RACE of *HaraxHSP70B*. For 5′- and 3′-RACE, cDNA was synthesized with a SMART™ kit (Takara) according to the manufacturer's protocol. Specific primers for 5′-RACE (*HaraxHSP70B-5RA*, *HaraxHSP70B-5RB*, *HaraxHSP70B-5RC*, *HaraxHSP68-5RA*, *HaraxHSP68-5RB*, and *HaraxHSP68-5RC*) and 3′-RACE (*HaraxHSP70B-3FA*, *HaraxHSP70B-3FB*, *HaraxHSP70B-3FC*, *HaraxHSP68-3FA*, *HaraxHSP68-3FB*, and *HaraxHSP68-3FC*) (Table 1) were synthesized on the basis of the cDNA sequence of the PCR fragment and used to amplify the 5′ and 3′ sequences of the HSP70B gene and the 5′ and 3′ sequences of the HSP68 gene, respectively. 5′-RACE was performed with 2.5 μl of 5′-ready-cDNA with Universal Primer Mix (UPM) (long 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′, short 5′-CTAATACGACTCACTATAGGGC-3′; Takara) and *HaraxHSP70B-5RA*/*HaraxHSP68-5RA*. Nested PCR was performed with Nested Universal Primer A (NUP) (5′-AAGCAGTGGTAACAACGCAGAGT-3′; Takara), and *HaraxHSP70B-5RB*/*HaraxHSP68-5RB*. 3′-RACE was performed with 2.5 μl of 3′-ready-cDNA with UPM and *HaraxHSP70B-3FA*/*HaraxHSP68-3FA*, then with NUP and *HaraxHSP70B-3FB*/*HaraxHSP68-3FB*. The PCR conditions were as follows: 10 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 48°C, and 60 s at 72°C, and finally, 10 min at 72°C. The PCR products were cloned into the pMD18-T vector (Takara) and

sequenced. *HaraxHSP70B-5RC*, *HaraxHSP70B-3FC*, *HaraxHSP68-5RC*, and *HaraxHSP68-3FC* were used as primers for bacterial PCR testing in 5′- and 3′-RACE experiments.

HaraxHSP68, *HaraxHSP70A*, and *HaraxHSP70B* cDNA sequences (KJ136115, ABR92405, and KJ136116, respectively) were compared with other HSP sequences deposited in GenBank by using the BLAST tools on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/). Three amino acid sequences of HSP were deduced from the corresponding cDNA sequences by the translation tool at the ExPASy Proteomics website (http://expasy.org/tools/dna.html). The neighbor-joining method was used to construct a phylogenetic tree with Molecular Evolutionary Genetics Analysis, Version 6.0 (MEGA6.0) software on the basis of several amino acid sequences of known HSPs. Other tools for the analysis of protein sequence used in this study, such as molecular mass, isoelectric point (pI), and *N*-glycosylation sites, were obtained from the ExPASy Proteomics website (http://expasy.org/). Multiple sequence alignment of insect HSPs was performed with the multiple sequence alignment website (http://bioinfo.genotoul.fr/multalin/multalin.html).

Real-time, fluorescence-based quantitative PCR (QRT-PCR). Specific primers for QRT-PCR (*HaraxHSP68-5F2*, *HaraxHSP68-3R2*, *HaraxHSP70A-5F2*, *HaraxHSP70A-3R2*, *HaraxHSP70B-5F2*, and *HaraxHSP70B-3R2*) (Table 1) were synthesized on the basis of the full-length sequence of the genes, and these primers were used to amplify sequences of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* genes, respectively. The annealing temperature was 56 $\pm$ 0.5°C, the fragment length was ~100–200 base pairs (bp), and *18S rRNA* was chosen as the reference gene for robust QRT-PCR. The PCR protocol was as follows: 3 min at 95°C followed by 39 cycles of 10 s at 95°C, and 25 s at 56°C. The plate was read and then exposed to temperature increments from 65 to 95°C for 5 s. The plate was then reread. A reaction system was prepared in a volume of 20 μl.

Data were obtained from the PCR instrument, and relative expression was measured by the $2^{-\Delta\Delta C_T}$ method. All data are presented as mean and SE as calculated by Basic Statistic in Statistic 8.0 (Tang and Jia 2008). Statistically significant difference was set at $P \leq 0.05$.

Results

Two *HaraxHSP* cDNAs were obtained by PCR and by 5′- and 3′-RACE and were verified as HSP70 by cloning and sequencing. Both *HaraxHSP68* and *HaraxHSP70B* had the highest level of similarity with the HSP70 of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), with 85 and 74% similarity, respectively.

HaraxHSP68 cDNA (KJ136115) has an open reading frame (ORF) of 1,917 nucleotides (nt) that encodes a protein of 638 amino acids (predicted molecular mass ~70.22 kDa, pI of 6.53), in which the AATAAA polyadenylation signal is 13 bp upstream from poly(A) (Fig. 1A). *HaraxHSP70B* cDNA (KJ136116) has an ORF of 1,986 nt that encodes a protein of 661 amino acids (predicted molecular mass ~71.93 kDa, pI of 6.20), in which the AATAAA polymerization signal is 14 bp upstream from poly(A) (Fig. 1B). *HaraxHSP68* and *HaraxHSP70B* have the

Table 1. Primer sequences used for amplification of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B*.

Primer	Direction*	Type**	Sequence (5′-3′)	Purpose
HaraxHSP68-5F1	F	D	5′-CAAGGCAAGGTGGAGATCAT-3′	RT-PCR
HaraxHSP68-3R1	R	D	5′-TGCTTCATATCCTGCTGCAC-3′	RT-PCR
HaraxHSP70B-5F1	F	D	5′-TCCGAGCAACACTGTTTTTG-3′	RT-PCR
HaraxHSP70B-3R1	R	D	5′-CCAGAAAACTAGCCGCAGTC-3′	RT-PCR
HaraxHSP68-3FA	F	G	5′-CGAAGAGTACGAGCACAAGC-3′	3′-RACE
HaraxHSP68-3FB	F	G	5′-CCTGTGATGACGAAACTGC-3′	3′-RACE
HaraxHSP68-3FC	F	G	5′-GCGGTCCAACCATAGAAGAAGTCG-3′	3′-RACE
HaraxHSP68-5RA	R	G	5′-GTTTGCCATTAACATTGACCAC-3′	5′-RACE
HaraxHSP68-5RB	R	G	5′-ACTTCCTGCCAATGAGCC-3′	5′-RACE
HaraxHSP68-5RC	R	G	5′-GGAGTAAGTGGTGCCCAGGT-3′	5′-RACE
HaraxHSP70B-3FA	F	G	5′-GGAAAGATGTGTAACTGAAGG-3′	3′-RACE
HaraxHSP70B-3FB	F	G	5′-CACGGAATTAAGAAGATCCAG-3′	3′-RACE
HaraxHSP70B-3FC	F	G	5′-GCGACTAACAGCAACTCTC-3′	3′-RACE
HaraxHSP70B-5RA	R	G	5′-CCACGCAAGAATAAGTTGTTCC-3′	5′-RACE
HaraxHSP70B-5RB	R	G	5′-GAGTCGAAGACGATGCGTTTCC-3′	5′-RACE
HaraxHSP70B-5RC	R	G	5′-GAGCTTGAGGCCATCGTGTG-3′	5′-RACE
HaraxHSP68-5F2	F	G	5′-CTGTTATAACCGTGCCAGCCT-3′	QRT-PCR

Table 1. Continued.

Primer	Direction*	Type**	Sequence (5′-3′)	Purpose
HaraxHSP68-3R2	R	G	5′-CGAAGGACGTTTATGCCTGC-3′	QRT-PCR
HaraxHSP70A-5F2	F	G	5′-CGATATGGGTGGTGGAACATTCG-3′	QRT-PCR
HaraxHSP70A-3R2	R	G	5′-CTGCCCACCGAGATGAGTATCACC-3′	QRT-PCR
HaraxHSP70B-5F2	F	G	5′-GTCCAAGCTGATATGAAGCAC-3′	QRT-PCR
HaraxHSP70B-3R2	R	G	5′-GTGATAACAGCATTGGTGACAG-3′	QRT-PCR

* F, forward; R, reverse.
** D, degenerate primer; G, gene-specific primer.
*** RT-PCR, real-time, fluorescence-based polymerase chain reaction; RACE, rapid amplification of cDNA ends; QRT-PCR, real-time, fluorescence-based quantitative polymerase chain reaction.

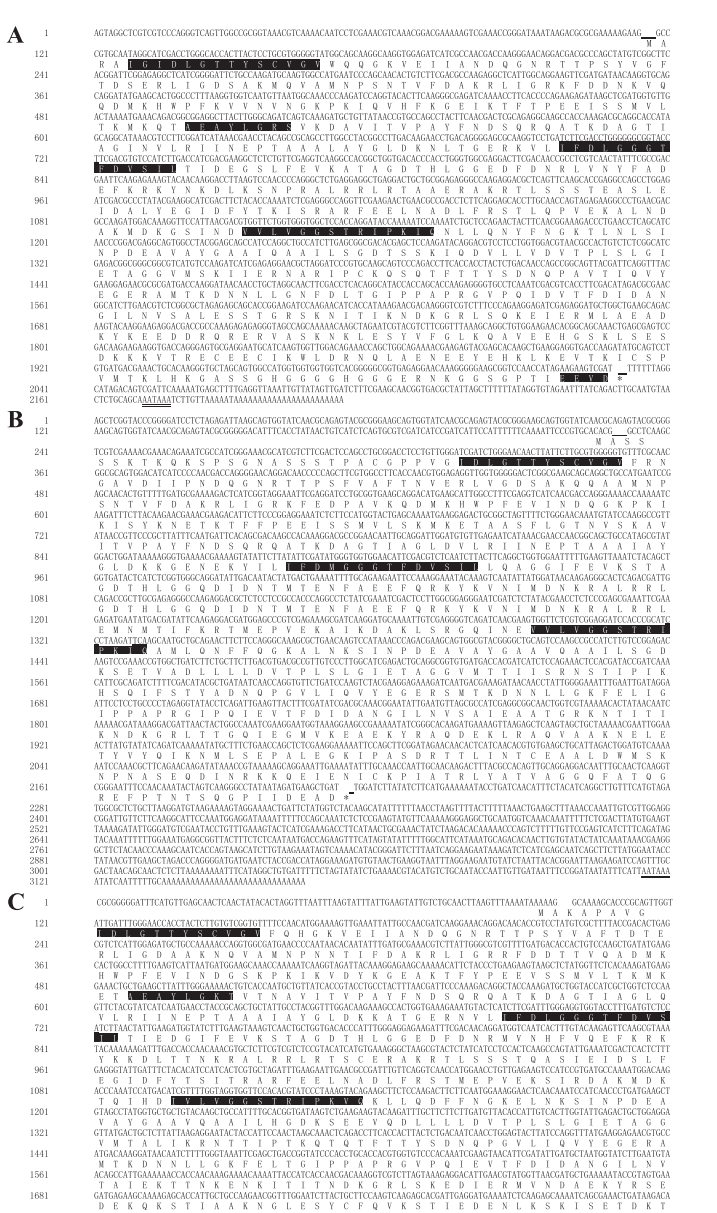

Fig. 1. Nucleotide and deduced amino acid sequences and gene structures of three HSP proteins. The initiation and termination codons are indicated in bold italics. The deduced amino acid sequences are shown with numbering beginning at the initiation methionine residue. AATAAA

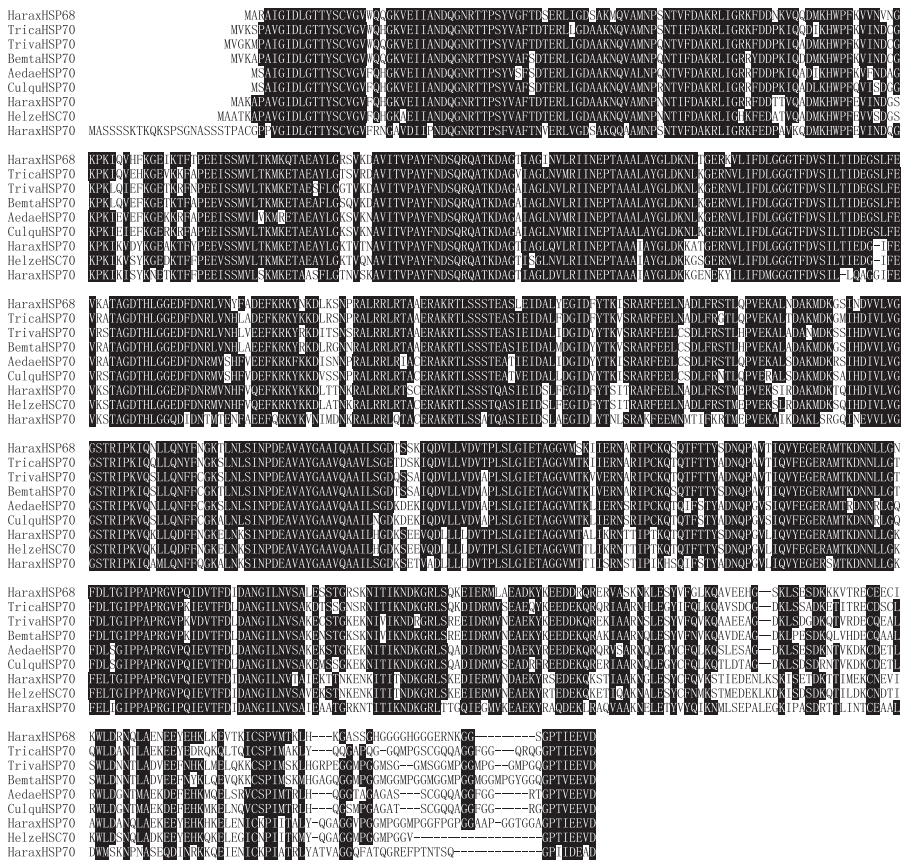

Fig. 2. AedaeHSP70 (FJ177310), CulquHSP70 (XM_001861401), HaraxHSP70A (ABR92405), HelzeHSC70 (GQ389712), HaraxHSP70B (KJ136116), BemtaHSP70 (DQ093385), TrivaHSP70 (EU934244), HaraxHSP68 (KJ136115), and TricaHSP70 (Nm_001170728) proteins were aligned. Highly conserved regions are shaded in black.

conserved ATPase structure of the HSP70 gene in the N terminus as well an AATAAA polymerization signal between the terminator codon and poly(A). These results suggest the full-length coding sequences were obtained. HaraxHSP70A cDNA (ABR92405) (Yang et al. 2009) has an ORF of 1,956 nt that encodes a

←

sequences in the 3′-untranslated regions (3′-UTR) are doubly underlined. The conserved motifs sequences are shaded. The nucleotide sequences reported here have been submitted to GenBank. (A) *HaraxHSP68*, accession KJ136115. (B) *HaraxHSP70B*, accession KJ136116. (C) *HaraxHSP70A*, accession ABR92405.

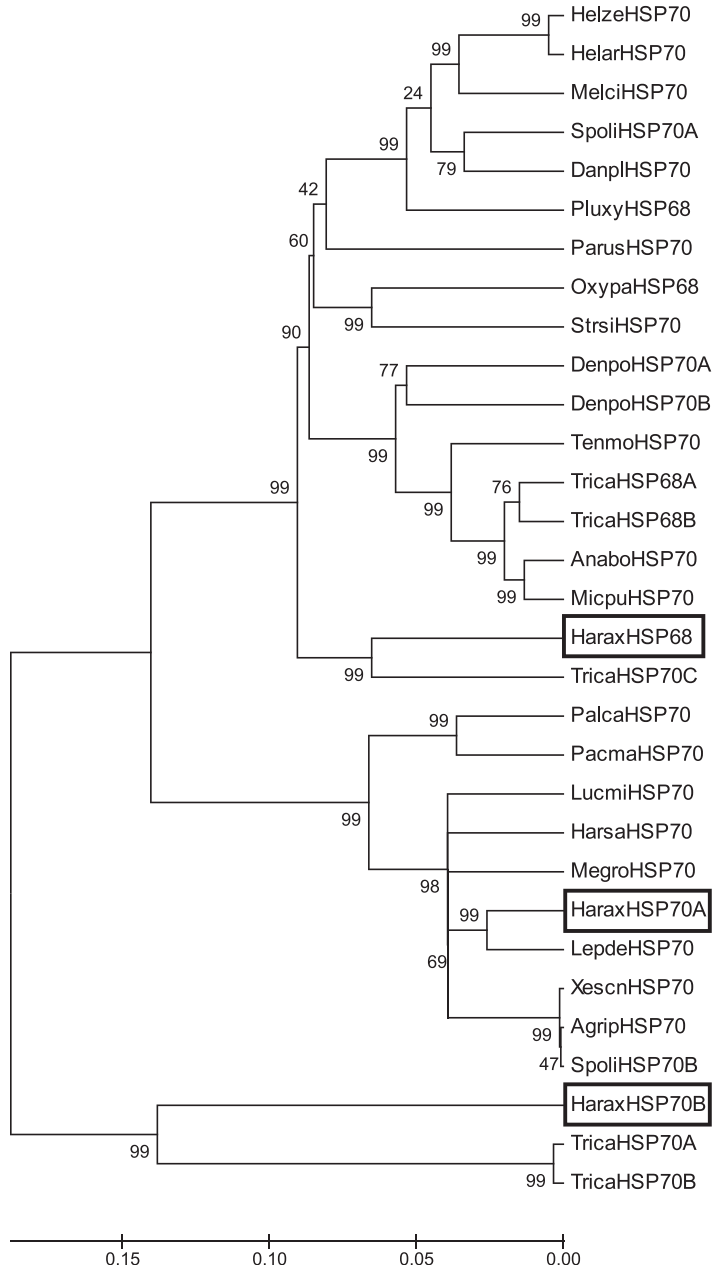

Fig. 3. MEGA6.0 was used for phylogenetic analysis of HSP70 proteins from other species and construction of a phylogenetic tree based on the amino acid sequences of known (Herbst) HSP70 proteins. HSP70 family proteins were from *Tribolium castaneum* (TricaHSP70C: NP_

protein of 651 amino acids (predicted molecular mass ~71.32 kDa, pI of 5.36), in which the AATAAA polymerization signal is 13 bp upstream from poly (A) (Fig. 1C).

Alignment of protein sequences in insects revealed HSP70 proteins have the three signatures IDLGTTYSCVGV, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQ; the putative ATP/GTP binding site motif AEAYLG(K/T)T; and an MEEVD motif at the C terminus (Fig. 2). It also showed that HSP70 was a highly conservational protein family, especially in the middle of the putative catalytic domain (Fig. 2).

BLAST was used to analyze HaraxHSP68 and HaraxHSP70B. Many proteins similar to HaraxHSP68 were found as a result of this analysis. The 17 most similar proteins were chosen for further examination: TricaHSP70C (similarity 85%), MelciHSP70 (82%), OxypaHSP68 (82%), TricaHSP68A (84%), SpoliHSP70A (81%), TricaHSP68B (80%), DenpoHSP70A (80%), TenmoHSP70 (80%), HelzeHSP70 (81%), AnaboHSP70 (83%), ParusHSP70 (80%), DanplHSP70 (81%), PluxyHSP68 (80%), MicpuHSP70 (80%), StrsiHSP70 (81%), HelarHSP70 (81%), and DenpoHSP70B (79%).

Many proteins also were found that were similar to HaraxHSP70B. The 10 most similar proteins were chosen: TricaHSP70A (similarity 74%), TricaHSP70B (74%), LepdeHSP70 (71%), XescnHSP70 (71%), MegroHSP70 (71%), AgripHSP70 (71%), SpoliHSP70B (71%), HaraxHSP70A (71%), LucmiHSP70 (72%), and HarsaHSP70 (69%). HaraxHSP68 is most similar to TricaHSP70C, HaraxHSP70A is most similar to LepdeHSP70, and HaraxHSP70B is most similar to TricaHSP70A and TricaHSP70B (Fig. 3). HaraxHSP70B can be distinguished clearly from HaraxHSP68 and HaraxHSP70A (Fig. 3).

The *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* genes were expressed in all developmental stages of *H. axyridis* (Fig. 4A, B, C). The relative expression of HaraxHSP68 was low during the larval stages but relative expression increased after the fourth-instar (2 d after molting) stage and remained high during the pupal and adult stages (Fig. 4A). The relative expression of *HaraxHSP70A* was high

---

←

001164098, TricaHSP68A: XP_974442, NP_001164199, XP_001811933, EFA00016), *Melitaea cinxia* (L.) (MelciHSP70: AGR84224), *Oxycera pardalina* Meigen (OxypaHSP70: ADJ96611), *Spodoptera litura* (F.) (ADV03160, ADM66138), *Dendroctonus ponderosae* Hopkins (AEE62651, ENN76738), *Tenebrio molitor* L. (Tenmo HSP70: AFE88579), *Helicoverpa zea* (Boddie) (HelzeHSP70: ACV32640), *Anatolica polita borealis* Koszab (AnaboHSP70: ABQ39970), *Paratlanticus ussuriensis* (Uvarov) (AFP54305), *Danaus plexippus* (L.) (EHJ73891), *Plutella xylostella* (L.) (AFQ37587), *Microdera dzhungarica punctipennis* Kasz (AEB52075), *Stratiomys singularior* (Harris) (ADX42270), *Helicoverpa armigera* (Hübner) (ADP37711), *Leptinotarsa decemlineata* (Say) (AHA36968), *Xestia c-nigrum* (L.) (AGQ50302), *Megachile rotundata* (F.) (XP_003705538), *Agrotis ipsilon* (Hufnagel) (AEG78288), *Locusta migratoria* (L.) (AAO21473), *Harpegnathos saltator* (T. C. Jerdon) (EFN75098); and *Palaemon carinicauda* (Holthuis) (prawn) (ADN78256) and *Pachygrapsus marmoratus* (F.) (crab) (CAL68994) were chosen as out-groups.

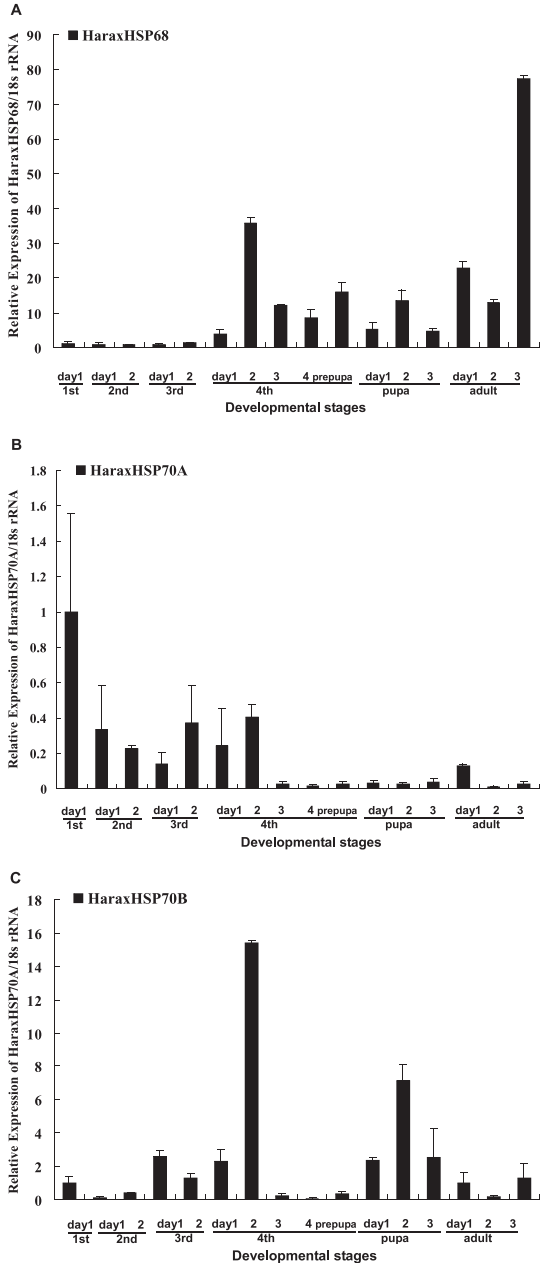

Fig. 4. mRNA relative expression level of (A) *HaraxHSP68*, (B) *HaraxHSP70A*, and (C) *HaraxHSP70B* at different developmental stages. In (A), filled columns indicate relative expression of *HaraxHSP68/18s* rRNA. In (B), filled columns indicate relative expression of *HaraxHSP70A*/*18s* rRNA.

during the larval stages, but relative expression decreased after the fourth-instar (2 d after molting) stage and remained low during the pupal and adult stages (Fig. 4B). The *HaraxHSP70B* gene had high relative expression in the fourth-instar (2 d after molting) stage and in the pupal stages (Fig. 4C).

The relative expression of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* changed markedly with exposure to increasing temperature (Fig. 5). The relative expression of *HaraxHSP68* increased with increasing temperature until 15°C and then remained constant at temperatures >15°C (Fig. 5). The relative expression of *HaraxHSP70A* continued to increase with rising temperature. The relative expression of *HaraxHSP70B* followed the temperature fluctuations and reached a maximum at 15°C (Fig. 5).

The relative expression of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* varied with temperature (Fig. 6). The relative expression of *HaraxHSP68* was very low at 0, 5, and 15°C, but *HaraxHSP68* was expressed only slightly at −5, 10, and 25°C (control) (Fig. 6). The relative expression of *HaraxHSP70A* was very low at −5, 0, 5, and 15°C, but *HaraxHSP70A* was expressed slightly at 10 and 25°C (control) (Fig. 6). *HaraxHSP70B* also was slightly expressed at each temperature, and the relative expression of *HaraxHSP70B* was high at 0°C (Fig. 6). The relative expressions of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* reached maximum at 8 h of starvation and were low before and after this time point (Fig. 7).

Discussion

HSPs usually act as molecular chaperones and are involved in many cellular processes (Hartl and Hayer-Hartl 2002), including protein folding and assembly as well as intracellular transport. They are also part of a protective cellular mechanism that involves accumulating HSPs under normal cellular conditions, fixing denatured proteins, and preventing proteins from misfolding or aggregating (Clark and Worland 2008). The primary structure of HSP proteins includes an amino-terminal ATP-binding domain and a carboxy-terminal substrate-binding domain (Demand et al. 1998, Hartl and Hayar-Hartl 2002). However, homologies of HaraxHSP68, HaraxHSP70A, and HaraxHSP70B are lower than those of HaraxHSP68 and HaraxHSP70A. In addition, HaraxHSP68 and HaraxHSP70A have the conserved EEVD sequence found in most proteins of the HSP70 family (Gupta 1995), but HaraxHSP70B, which has the DEAD sequence, does not (Fig. 1); thus, these proteins are easily distinguished (Fig. 2). This is perhaps the result of species evolution and more kinds of HSP genes evolving under different environmental stresses. The difference among the three HSP70 proteins (Fig. 3) suggests that there is a division of labor in physiological function and that more HSP70s likely will be found in *H. axyridis*, as has been documented in *T. castaneum* (Mahroof et al. 2005).

←

In (C), filled columns indicate relative expression of *HaraxHSP70B*/*18s* rRNA. The *x*-axis indicates different developmental stages, including 1-, 2-, 3-, and 4-d larval stages and pupal and adult stages. All data are presented as mean ± SE ($P > 0.05$).

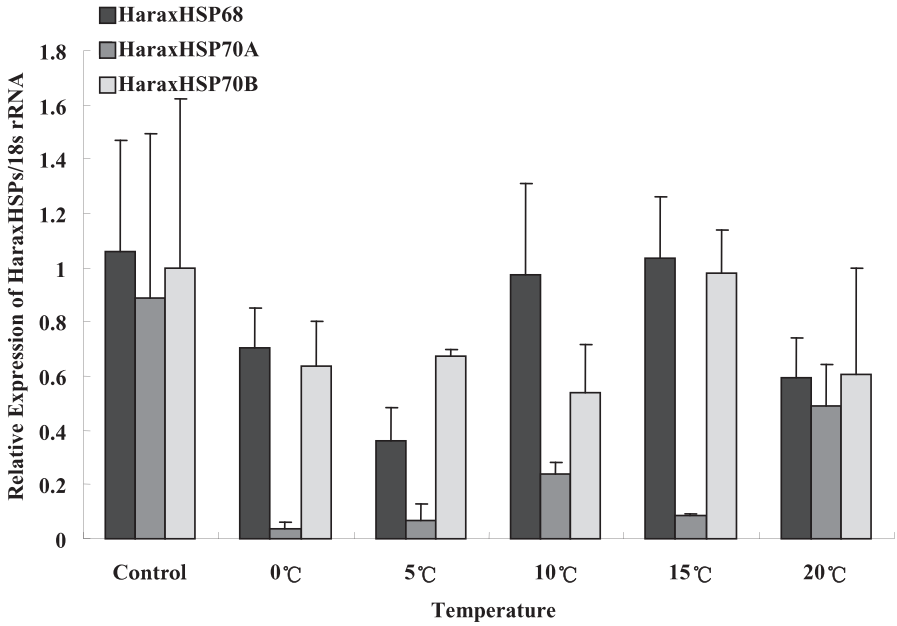

Fig. 5. mRNA relative expression level of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* at different temperatures. Red, relative expression of *HaraxHSP68*/*18s* rRNA; green, relative expression of *HaraxHSP70A*/*18s* rRNA; and yellow, relative expression of *HaraxHSP70B*/*18s* rRNA. Control check of *HaraxHSP70B*/*18s* rRNA was regarded as 1. All data are presented as mean ± SE ($P > 0.05$).

The well-conserved C-terminal motif EEVD argues that these motifs enable HSP70s to bind other cochaperones and indicates HSP70s are cytosolic HSPs (Gupta 1995, Zhang and Denlinger 2010). Comparison of HSP70 and HSC70 reveals no GGXP motif near the 3′ terminus of HSP70 (Fig. 2) (Wang et al. 2008), whereas HSC70 contains two GGXP repeats involved in cochaperone binding activities (Demand et al. 1998). The C-terminal repeats $(GGM)_n$ suggest HaraxHSP70A might be a mitochondrial HSC70. Therefore, HaraxHSP68 and HaraxHSP70B are both HSP70s (Huang and Kang 2007).

The results of this study suggest that HaraxHSP68 usually operates during the pupal and adult stages of *H. axyridis* (Fig. 4A), HaraxHSP70A usually operates during the larval stages (Fig. 4B), and HaraxHSP70B operates throughout development (Fig. 4C). Interestingly, HaraxHSP68, HaraxHSP70A, and HaraxHSP70B were highly expressed in the fourth-instar (2 d after molt) stage (Fig. 4A, B, C), a key developmental stage in *H. axyridis*. The reason why three proteins of HSP70 were highly expressed at this stage might be correlated with the quantity of diet consumed by *H. axyridis* immediately before pupation. In the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), however, HSP70 expression decreased with the development of larvae and pupae in the

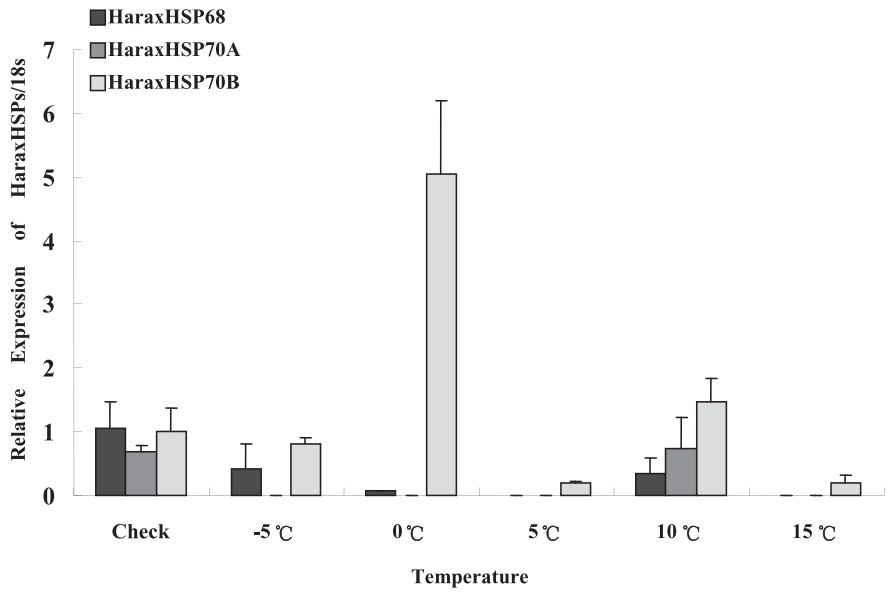

Fig. 6. mRNA relative expression level of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* at different temperatures. Red, relative expression of *HaraxHSP68*/*18s* rRNA; green, relative expression of *HaraxHSP70A*/*18s* rRNA; and yellow, relative expression of *HaraxHSP70B*/*18s* rRNA. Control check of *HaraxHSP70B*/*18s* rRNA was regarded as 1. All data are presented as mean ± SE ($P > 0.05$).

absence of thermal stress, and the expression decreased dramatically with larval age (Xu et al. 2011). The lowest level of expression was observed in the fourth-instar larvae (Jiang et al. 2012), an expression that is also observed in the western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (Li and Du 2013).

Generally, the relative expression of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* showed a tendency to increase with temperature rising (Fig. 5), thereby conforming to the behavioral characteristic of HSPs that are expressed quickly when organisms experience environmental stresses (Sørensen et al. 2003). We found that *HaraxHSP70B* was highly expressed at 0°C (Fig. 6). We postulate that insects likely increase food consumption before entering diapause to overwinter.

Starvation induces expression of HSP genes (Yengkokpam et al. 2008). Substantial changes were recorded in the expression of three HSP genes in *H. axyridis* after starvation for 24 h (Fig. 7). Starvation is a complex stressor that includes reduced energy resources and substantial challenges to ion and water homeostasis (Wang et al. 2012). We found that the relative expression of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* reached maximal levels of activity after starvation of the insects for 8 h (Fig. 7). At this time, insects might

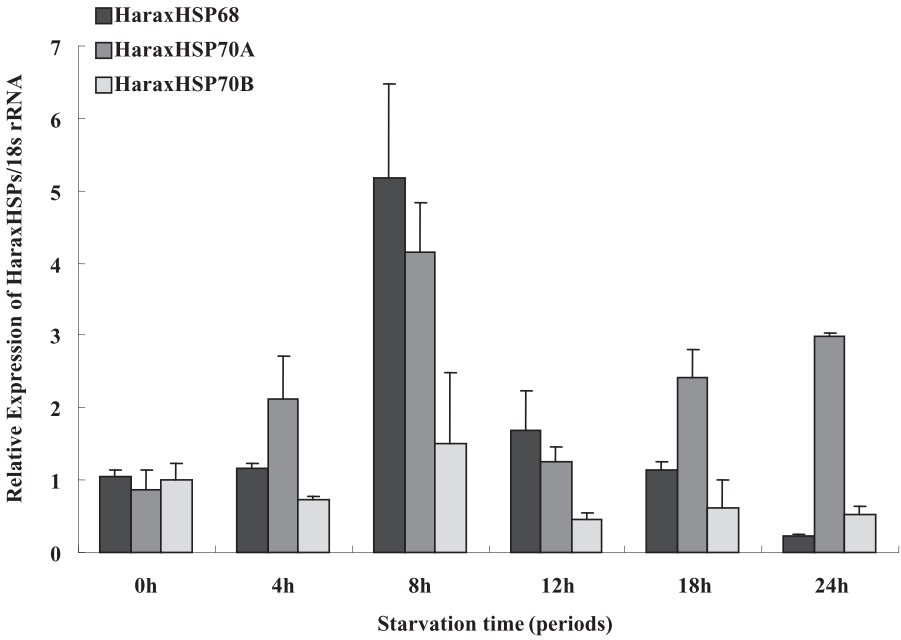

Fig. 7. mRNA relative expression level of *HaraxHSP68*, *HaraxHSP70A*, and *HaraxHSP70B* under the stress of starvation. Red, relative expression of *HaraxHSP68*/*18s* rRNA; green, relative expression of *HaraxHSP70A*/*18s* rRNA; and yellow, relative expression of *HaraxHSP70B*/*18s* rRNA. Control check of *HaraxHSP70B*/*18s* rRNA was regarded as 1. All data are presented as mean ± SE ($P > 0.05$).

actively search for food. This phenomenon occurred also in a study of trehalose-6-phosphate synthase in *H. axyridis*, in which insects moved the furthest after starving for 8 h (Qin et al. 2012, Tang et al. 2012).

HSPs act in cellular responses to environmental stressors, including sublethal heat and cold shocks, infections, environmental contaminants, and starvation (Feder et al. 1997). HSPs are expressed in insects when they are subjected to thermal pressure and synthesis of normal protein is restrained (Colinet et al. 2010). For HSPs involved in the reaction process of thermal tolerance, to study the relationship between HSPs and temperature tolerance in insects is in favor of understanding the regulation of insect development depending on temperature. Specific HSP actions include assisting in folding of nascent proteins, preventing protein aggregations, and chaperoning existing proteins (Fink 1999). In insects, apart from extreme temperature, HSPs are induced by exposure to a variety of factors, including pollution by heavy metals (Warchalowska-Sliwa et al. 2005) and parasitic infection (Rinehart et al. 2002). Therefore, study of the production of HSPs and variation of HSPs is in favor of understanding the regulation of insect development depending on all kinds of environmental factors, especially the study

of the mechanism(s) of thermal tolerance for natural enemies that might be used in classical or augmentative biological control programs.

Acknowledgments

This work was supported by grants from the National Basic Research Program of China (2013CB127605), National Natural Science Foundation of China (31071731 and 31371996), and the Program for Excellent Young Teachers in Hangzhou Normal University (JTAS 2011-01-031).

References Cited

Boorstein, W.R., T. Ziegelhoffer and E.A. Craig. 1994. Molecular evolution of the HSP70 multigene family. J. Mol. Evol. 38: 1–17.

Clark, M.S. and M.R. Worland. 2008. How insects survive the cold: Molecular mechanisms—A review. J. Comp. Physiol. B 178: 917–933.

Colinet, H., S.F. Lee and A. Hoffmann. 2010. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. Fed. Eur. Biochem. Soc. J. 277: 174–185.

Demand, J., J. Luders and J. Hohfeld. 1998. The carboxy-terminal domain of Hsc70 provides binding sites for a distinct set of chaperone cofactors. Mol. Cell. Biol. 18: 2023–2028.

Elekonich, M.M. 2009. Extreme thermotolerance and behavioral induction of 70-kDa heat shock proteins and their encoding genes in honey bees. Cell Stress Chaperones 14: 219–226.

Feder, M.E., N. Blair and H. Figueras. 1997. Natural thermal stress and heat-shock protein expression in *Drosophila* larvae and pupae. Funct. Ecol. 11: 90–100.

Fink, A.L. 1999. Chaperone-mediated protein folding. Physiol. Rev. 79: 425–449.

Goto, S.G. and M.T. Kimura. 2004. Heat-shock-responsive genes are not involved in the adult diapause of *Drosophila triauraria*. Gene 326: 117–122.

Gupta, R.S. 1995. Phylogenetic analysis of the 90 Kd heat-shock family of protein sequences and an examination of the relationship among animals, plants, and fungi species. Mol. Biol. Evol. 12: 1063–1073.

Hartl, F.U. and M. Hayar-Hartl. 2002. Molecular chaperones in the cytosol: From nascent chain to folded protein. Science 295: 1852–1858.

Huang, L.H. and L. Kang. 2007. Cloning and interspecific altered expression of heat shock protein genes in tow leaf miner species in response to thermal stress. Insect Mol. Biol. 16: 491–500.

Jiang, X.F., H.F. Zhai, L. Wang, L.Z. Luo, W.S. Thomas and L. Zhang. 2012. Cloning of the heat shock protein 90 and 70 genes from the beet armyworm, *Spodoptera exigua*, and expression characteristics in relation to thermal stress and development. Cell Stress Chaperones 17: 67–80.

Karlin, S. and L. Brocchieri. 1998. Heat shock protein 70 family: Multiple sequence comparisons, function, and evolution. J. Mol. Evol. 47: 565–577.

Kiang, J. and G. Tsokos. 1998. Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology. Pharmacol. Ther. 80: 183–201.

Kim, K.K., R. Kim and S. Kim. 1998. Crystal structure of small heat-shock protein. Nature 394: 595–599.

Koch, R.L. 2003. The multicolored Asian lady beetle, *Harmonia axyridis*: A review of its biology, uses in biological control, and non-target impacts. Insect Sci. 3: 1–16.

Li, H.B. and Y.Z. Du. 2013. Molecular cloning and characterization of an Hsp90/70 organizing protein gene from *Frankliniella occidentalis* (Insecta: Thysanoptera, Thripidae). Gene 520: 148–155.

Li, Z.W., X. Li, Q.Y. Yu, Z.H. Xiang, H. Kishino and Z. Zhang. 2009. The small heat shock protein (sHSP) genes in the silkworm, *Bombyx mori*, and comparative analysis with other insect sHSP genes. Biomed. Central Evol. Biol. 9: 215.

Mahroof, R., Z.K. Yan, L. Neven, B. Subramanyam and J. Bai. 2005. Expression patterns of three heat shock protein 70 genes among developmental stages of the red our beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). Comp. Biochem. Physiol. A 141: 247–256.

Qin, W., M.G. Tyshenko, B.S. Wu, V.K. Walker and R.M. Robertson. 2003. Cloning and characterization of a member of the hsp 70 gene family from *Locusta migratoria*, a highly thermotolerant insect. Cell Stress Chaperones 8: 144–152.

Qin, Z., S. Wang, P. Wei, X.D. Xu, B. Tang and F. Zhang. 2012. Molecular cloning and cold-induced expression of trehalose-6-phosphate synthase gene in *Harmomia axyridis* (Coleoptera: Coccinellidae). Acta Entomol. Sin. 55: 651–658.

Rinehart, J.P., D.L. Denlinger and D.B. Rivers. 2002. Upregulation of transcripts encoding select heat shock proteins in the flesh fly *Sarcophaga crassipalpis* in response to venom from the ectoparasitoid wasp *Nasonia vitripennis*. J. Invertebr. Pathol. 79: 62–63.

Ritossa, F. 1962. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. Cell. Mol. Life Sci. 18: 571–573.

Sakano, D., B. Li, Q. Xia and K. Yamamoto. 2006. Genes encoding small heat shock proteins of the silkworm, *Bombyx mori*. Biosci. Biotechnol. Biochem. 70: 2443–2450.

Sebaey, I.I.A. and A.M. Gantiry. 1999. Biological aspects and description of different stages of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). Bull. Fac. Agric. Cairo Univ. 50: 87–97.

Sørensen, J.G., G.T.N. Kristensen and V. Loeschcke. 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6: 1025–1037.

Tang, B., Q. Xu, Q. Zou, Q. Fang, S. Wang and G. Ye. 2012. Sequencing and characterization of glycogen synthase and glycogen phosphorylase genes from *Spodoptera exigua* and analysis of their function in starvation and excessive sugar intake. Arch. Insect Biochem. Physiol. 80: 42–62.

Tang, Y.K. and Y.Y Jia. 2008. Method of processing real time PCR data. Biotechnology 3: 89–91.

Wang, H., S.Z. Dong, K. Li, G. Hu and G.Y. Ye. 2008. A heat shock cognate 70 gene in the endoparasitoid, *Pteromalus puparum*, and its expression in relation to thermal stress. Biochem. Mol. Biol. Rep. 41: 388–393.

Wang, H., K. Li, J.Y. Zhu, Q. Fang and G.Y. Ye. 2012. Cloning and expression pattern of heat shock protein genes from the endoparasitoid wasp, *Pteromalus puparum* in response to environmental stresses. Arch. Insect Biochem. Physiol. 79: 247–263.

Warchalowska-Sliwa, E., M. Niklinska, A. Gorlich, P. Michailova and E. Pyza. 2005. Heavy metal accumulation, heat shock protein expression and cytogenetic changes in *Tetrix tenuicornis* (L.) (Tetrigidae, Orthoptera) from polluted areas. Environ. Pollut. 133: 373–381.

Xu, J., J. Shu, X. Qiu, Z. Wang, F. Zhao, Z. Zhang and Q. Zhang. 2009. Effects of heat shock on ovary development and hsp83 expression in *Tribolium castaneum* (Coleoptera: Tenebrionidae). Arch. Insect Biochem. Physiol. 70: 204–216.

Xu, Q., Q. Zou, H.Z. Zheng, F. Zhang, B. Tang and S.G. Wang. 2011. Three heat shock proteins from *Spodoptera exigua*: Gene cloning, characterization and comparative stress response during heat and cold shocks. Comp. Biochem. Physiol. B 159: 92–102.

Yang, C.H., H. Pang and F. Zhang. 2009. Cloning and sequencing of Hsp70 gene of *Harmonia axyridis*. Environ. Entomol. 31: 124–131.

Yengkokpam, S., A.K. Pal, N.P. Sahu, K.K. Jain, R. Dalvi, S. Misra and D. Debnath. 2008. Metabolic modulation in *Labeo rohita* fingerlings during starvation: Hsp70 expression and oxygen consumption. Aquaculture 285: 234–237.

Yocum, G.D., K.H, Joplin and D.L. Denlinger. 1998. Upregulation of a 23 kDa small heat shock proteins transcript during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. Insect Biochem. Mol. Biol. 28: 671–676.

Zhang, Q. and D.L. Denlinger. 2010. Molecular characterization of heat shock protein 90, 70 and 70 cognate cDNAs and their expression patterns during thermal stress and pupal diapause in the corn earworm. J. Insect Physiol. 56: 138–150.

Zhang, W., Y.Z. He, D. Li, L. Liu and Q.J. Qin. 2013. Influences of photoperiod and diet on growth and development of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). J. Agric. Univ. Hebei 36: 80–84.

Zhu, J.Y., Q. Fang, L. Wang, C. Hu and G.Y. Ye. 2010. Proteomic analysis of the venom from the endoparasitoid wasp *Pteromalus puparum* (Hymenoptera: Pteromalidae). Arch. Insect Biochem. Physiol. 75: 28–44.