Journal of Insect Science, (2017) 17(6): 113; 1–11
doi: 10.1093/jisesa/iex082
Research Article

Overexpression of Three Heat Shock Proteins Protects *Monochamus alternatus* (Coleoptera: Cerambycidae) From Thermal Stress

Ziling Cai, Jingxiang Chen, Jie Cheng, and Tong Lin[1]

South China Agricultural University, College of Forestry and Landscape Architecture, Guangzhou, 510642, China, [1]Corresponding author, e-mail: lintong@scau.edu.cn

Subject Editor: Jurgen Ziesmann

Received 22 March 2017; Editorial decision 29 August 2017

Abstract

Ambient temperature is an important factor limiting the abundance and distribution of insects, and heat shock protein (*Hsp*) gene expression is sensitive to extremes of cold and heat. In order to explore the role of Hsps during thermal stress and development in *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), we cloned and characterized full-length *Hsp* genes, including *MaHsp60*, *MaHsp70,* and *MaHsp90*. *M. alternatus* were exposed to different temperatures (–15, –5, 5, 15, 25, 35, and 40°C) for 1 h and was allowed to recover at 25°C for 1 h. Following the treatments, we investigated the expression of the *Hsp*s by quantitative real-time polymerase chain reaction. In third instar larvae, *MaHsp60*, *MaHsp70,* and *MaHsp90* expression was upregulated in response to cold and heat, but the three *Hsp*s were especially sensitive to heat, specifically at 35°C and 40°C. After heating *M. alternatus* to 35°C, the expression of *MaHsp60*, *MaHsp70,* and *MaHsp90* was higher than at 5°C and 25°C in nearly all developmental stages. *MaHsp60*, *MaHsp70,* and *MaHsp90* expression was highest in later pupal, early adult, and early adult stages, respectively. These results suggest that compared with normal ambient temperatures, thermal stress could induce high expression of the three *Hsp*s.

Key words: Heat Shock Protein, *Monochamus alternatus*, Expression, Thermal Stress

Pine sawyers, specifically *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), widely distributed throughout the world, are the dispersing vectors of *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Aphelenchida: Parasitaphelenchidae), a nematode that causes serious losses in coniferous forests (Togashi et al. 2016). The beetles drop their supercooling point in order to live at low temperatures during the winter, which can reach –15°C (Ma et al. 2006). It also survives winter by avoiding freezing or increasing their cold tolerance (Ma et al. 2006). In southern China, *M. alternatus* must stand high temperatures, which can reach higher than 40°C. *M. alternatus* survives these extreme temperatures through certain survival mechanisms, such as the cellular stress response or heat shock response (Sanders and Martin 1993).

Ambient temperature is one of the most crucial factors influencing the development, survival, generation, and predation rates of insects. *M. alternatus* is not an exception (Ohsawa and Mitsuteru 2014). Heat shock proteins (Hsps) are ubiquitous proteins that are highly conserved in eukaryotes and prokaryotes. Hsps can facilitate proper protein folding and localization while preventing protein aggregation (Feder and Hofmann 1999; Hartl and Hayer-Hartl 2002). In insects, the expression of *Hsp* mRNA or protein is induced by environmental stressors such as sublethal heat and cold shock (Colinet et al. 2010; Sorensen and Loeschcke 2007), heavy metal pollution (Warchalowska-Sliwa et al. 2005), and parasitic infection (Rinehart et al. 2002). Based on sequence similarity, typical molecular weight and function, Hsps are subdivided into four families, i.e., small Hsps (sHsps), Hsp60, Hsp70, and Hsp90, (Feder and Hofmann, 1999). The adenosine triphosphate (ATP)-independent sHsps, which prevent the denaturation of substrate proteins when cells encounter stress, are the first line of cell defense (Basha et al. 2012). The large ATP-dependent proteins, Hsp60, Hsp70, and Hsp90, interact with proteins and promote proper protein folding, degradation, disaggregation, and localization, thereby influencing essential biological processes, including protein synthesis, transcription, cell signaling, and metabolism (Clare and Saibil 2013; Roehl et al. 2013).

In insects and other animals, exposure to stressors such as anoxia, crowding, heat, and cold, leads to a decline in the synthesis rate of most proteins, but *Hsp* expression increases. For example, taxa-specific Hsps are overexpressed in *Chortoicetes terminifera* upon crowding (Chapuis et al. 2011). The *Tribolium castaneum* (Coleoptera: Tenebrionidae) Hsp is upregulated in response to harmful UV-A radiation (Sang et al. 2012). The expression of *Liromyza sativa* and *Bombyx mori* (Lepidoptera: Bombycidae) *Hsp*s increases during the process of development and after cold hardening (Huang

et al. 2009; Moribe et al. 2010). Hsps are abundantly upregulated in *Sarcophaga crassipalpis* (Li et al. 2009) during diapause. Unlike several other species, *Hsp70* is not upregulated during diapause in *Helicoverpa zea* (Lepidoptera: Noctuidae), but the expression pattern of Hsp90 is consistent with several other species (Zhang and Denlinger 2010). Morover, Hsps play key roles in thermal tolerance and diapause in insects such as *Antheraea pernyi* Guérin-Méneville (Lepidoptera: Saturniidae) (Liu et al. 2013), *Paratlanticus ussuriensis* (Uvarov) (Orthoptera: Tettigoniidae) (Shim et al. 2012), *Apis cerana cerana* Fabricius (Hymenoptera: Apidae) (Zhang et al. 2014), and *Calanus finmarchicus* (Gunnerus) (Calanoida: Calanidae) (Aruda et al. 2011).

In light of the important function of *Hsp* proteins and the influence of temperature on the distribution of *M. alternatus*, we hypothesized that there might be a link between Hsp proteins and thermal resistance in *M. alternatus*. Therefore, in this study, we cloned and characterized the full-length *M. alternatus Hsp60*, *Hsp70,* and *Hsp90* cDNA sequences. The expression patterns of their transcripts were examined in response to thermal stressors using real-time quantitative polymerase chain reaction (RT-qPCR). These results represent the first characterization of *Hsp* expression associated with thermal stress in *M. alternatus* and help to characterize the response of this pest to temperature stress.

Methods

Insect Preparation

M. alternatus was obtained from dead infested *Pinas massoniana* Lamb in the Guangzhou, China (23°32′N, 113°33′E) and was reared in an artificial climate incubator under conditions of 25℃, 70% relative humidity, and 12:12 h (L:D) photoperiod (Togashi 2014).

According to the entomotomy, the third instar larvae can dissect into body wall, fat body, and Malpighian tubules roughly. Adults also can divide into head, thorax, abdomen, tarsi, antennae, ovary, and elytra. Those sample were then frozen using liquid nitrogen and stored at −80℃. Eggs, second instar larvae, third instar larvae, fourth instar larvae, fifth instar larvae, 1-d pupae (Early pupae), 9-d pupae (Late pupae), 1-d (Early adult [EA]), and 12-d adults (Late adult) were collected separately for temperature stress treatments.

Temperature Exposure

M. alternatus is widely distributed and can survive in temperatures ranging from −15 to 40℃ (Ohsawa and Mitsuteru 2014). Therefore, temperatures of −15 and 40℃ were selected as thermal stress end points. Groups of five third instar larvae, keeping relative stabilization in physiology condition within five larval instars, were exposed to −15, −5, 5, 15, 25, 35, or 40℃ for 1 h, respectively, and then were allowed to recover at 25℃ for 1 h. Groups of five late adults were exposed to 30℃ for a long time, exposed to 35 or 25℃ for 1 h, and then exposed to 30℃.

To investigate Hsp gene patterns during developmental stages under cold stress, we used 5℃ because it represents the minimum temperature experienced in Southern Chinese year in year out. There were two reasons why we used 35℃ and not 40℃ to study the effect of Hsps gene's heat shock. Firstly, 35℃ often represents the stable temperature in the forest of Sonthern Chinese during the summer. Secondly, exposure to 35℃ does not impact on the physiological activity of *M. alternatus*. Therefore, second, third, fourth, fifth larvae, 1-d pupae (Early pupae), 9-d pupae (Late pupae), 1-d (EA), and 12-d adults (Late adult) were kept at 5℃, 35℃, and 25℃ (as control) for 1 h, and then allowed to recover at 25℃ for 1 h. The treatment groups included five beetles per one treatment. The treatment groups were enclosed in a petri dish set to the desired temperature using an artificial climate incubator. Each treatment was repeated three times. After the thermal treatments, samples were immediately frozen in liquid nitrogen and stored at −80℃.

Cloning of *Hsp* Genes

Total RNA was extracted using the E.Z.N.A. TM Total RNA Kit II (Omega, USA) following the supplier's instructions. After isolating the RNA, the quality of RNA for each individual sample was visualized on a denaturing agarose gel, and RNA concentrations were determined using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA). First-strand cDNA was synthesized and RACE-PCRs were conducted using the 3′-Full RACE Core Set with PrimeScript RTase (Clontech, USA) according to the manufacturer's instructions. All RACE primers are shown in Table 1. After amplification, PCR products were cloned into a pMD18-T vector (Takara Bio, Otsu, Japan) and transformed into *Escherichia coli* DH5α Competent Cells (Takara Bio, Otsu, Japan) for sequencing (Invitrogen Biotechnology, Shanghai, China).

Bioinformatics Analysis Tools

The open reading frames (ORFs) of full-length cDNA sequences were found using the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), and the conserved domains

Table 1. Primers used for RACE and RT-qPCR

Abbreviation	Primer sequence (5′–3′)	Technique used
MaHsp60-F1	AAATGCTGGAGTGGATGG	3′RACE
MaHsp60-F2	CATCCCTCTTGACGACCG	3′RACE
MaHsp60-F	GCTGTATGTCCGCCGTGTA	RT-qPCR
MaHsp60-R	GGGAGTCTTCGTGAATGCC	RT-qPCR
MaHsp70-F1	GACGAGAAGCAAAGACAGAG	3′RACE
MaHsp70-F2	TCCGATGACAAGAGCACG	3′RACE
MaHsp70-F	TGGCGGCAAACCGAAGAT	RT-qPCR
MaHsp70-R	CGCTGGCACCGTAATGAC	RT-qPCR
MaHsp90-F1	AGCACATCTACTTCATCACGGG	3′RACE
MaHsp90-F2	GGTTACCGATGAAACTCCAG	3′RACE
MaHsp90-F	GAGGAAGGTATTGTAGCAGG	RT-qPCR
MaHsp90-R	AGCGGTCGTCAAGAGGGATG	RT-qPCR
actin-F	CTCTGCTATGTAGCCCTTGACTT	RT-qPCR
actin-R	GCAGTTGTAGGTGGTTTCGTG	RT-qPCR

were analyzed using SMART (http://smart.embl-heidelberg.de/). Homologous sequences from other species were obtained using NCBI Protein BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The retrieved sequences were aligned using the ClustalX multiple alignment tool. Molecular phylogenetic trees were constructed using the neighbor-joining method in MEGA version 4.0 (Kumar et al. 2008). A Poisson model and 1,000 bootstrap repetitions were applied for the phylogeny analysis.

Quantitative Real-Time PCR

Total RNA was extracted as performed in the cloning of *Hsp* genes, and 2 µg of RNA was used to synthesize first-strand cDNA using the PrimeScript RT reagent Kit With gDNA Eraser (Takara Bio, Otsu, Japan). The actin of *M. alternatus* was chosen as endogenous control for normalizing RNA expression between samples (Niu et al. 2008). All primers for *Hsp* genes and actin, designed by Primer Premier 5.0, are listed in Table 1. RT-qPCR was performed using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) in a 20-µl volume using an LightCycler480. The RT-qPCR amplification conditions were as follows: initial denaturation at 95℃ for 5 min; 40 cycles of 95℃ for 10 s, 60℃ for 20 s; and cooling at 40℃ for 30 s. Reactions with diethy pyrocarbonate water rather than cDNA templates served as the negative controls. Each individual sample was analyzed three times, and three individual samples were prepared for each treatment.

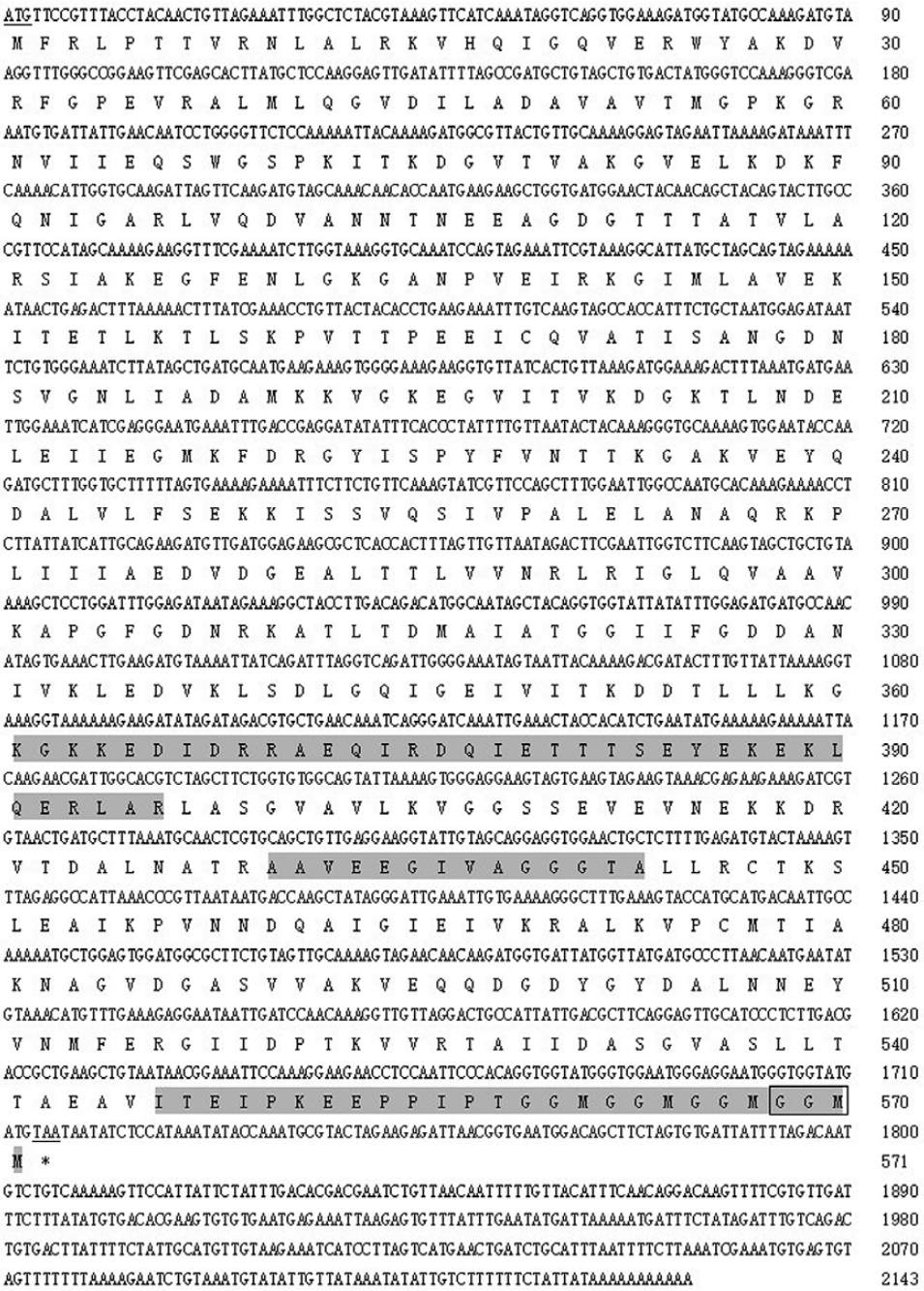

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *MaHsp60*. The character shading (ATG) and translational termination codon (TAA) are underlined for emphasis. Four highly conserved regions in MaHsp60 are shown in gray and the conserved motif (GGM) at the C-terminus is boxed.

The relative expression level of the cDNA templates was determined using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). Statistical analyses were performed using the Tukey honestly significant difference test within the SPSS software package (version 11).

Results

Cloning and Characterization of Hsp60, Hsp70, and Hsp90

RT-PCR and RACE were used to obtain the full-length cDNA of *Hsp60*, *Hsp70,* and *Hsp90* from *M. alternatus*, named *MaHsp60* (GenBank Accession KU323593), *MaHsp70* (GenBank Accession KU159184), and *MaHsp90* (GenBank Accession KU159185), respectively. The full-length *MaHsp60* cDNA is 2143 bp, including an ORF of 1716 bp and a 3′terminal UTR of 427 bp with a poly(A) tail. *MaHsp60 has* four highly conserved regions and conserved motif (GGM) (Fig. 1). The ORF of *MaHsp60* encodes 571 amino acids, with a predicted molecular weight of 61.079 KDa and a theoretical isoelectric point (pI) of 5.39. The ORF of *MaHsp70* is 1947 bp in length and encodes 648 amino acids with a calculated molecular mass of 70.797 KDa and theoretical pI of 5.49. *MaHsp60* has four highly conserved regions and conserved motif (GGM) (Fig. 2). The full length of the *MaHsp90* cDNA is 2385 bp,

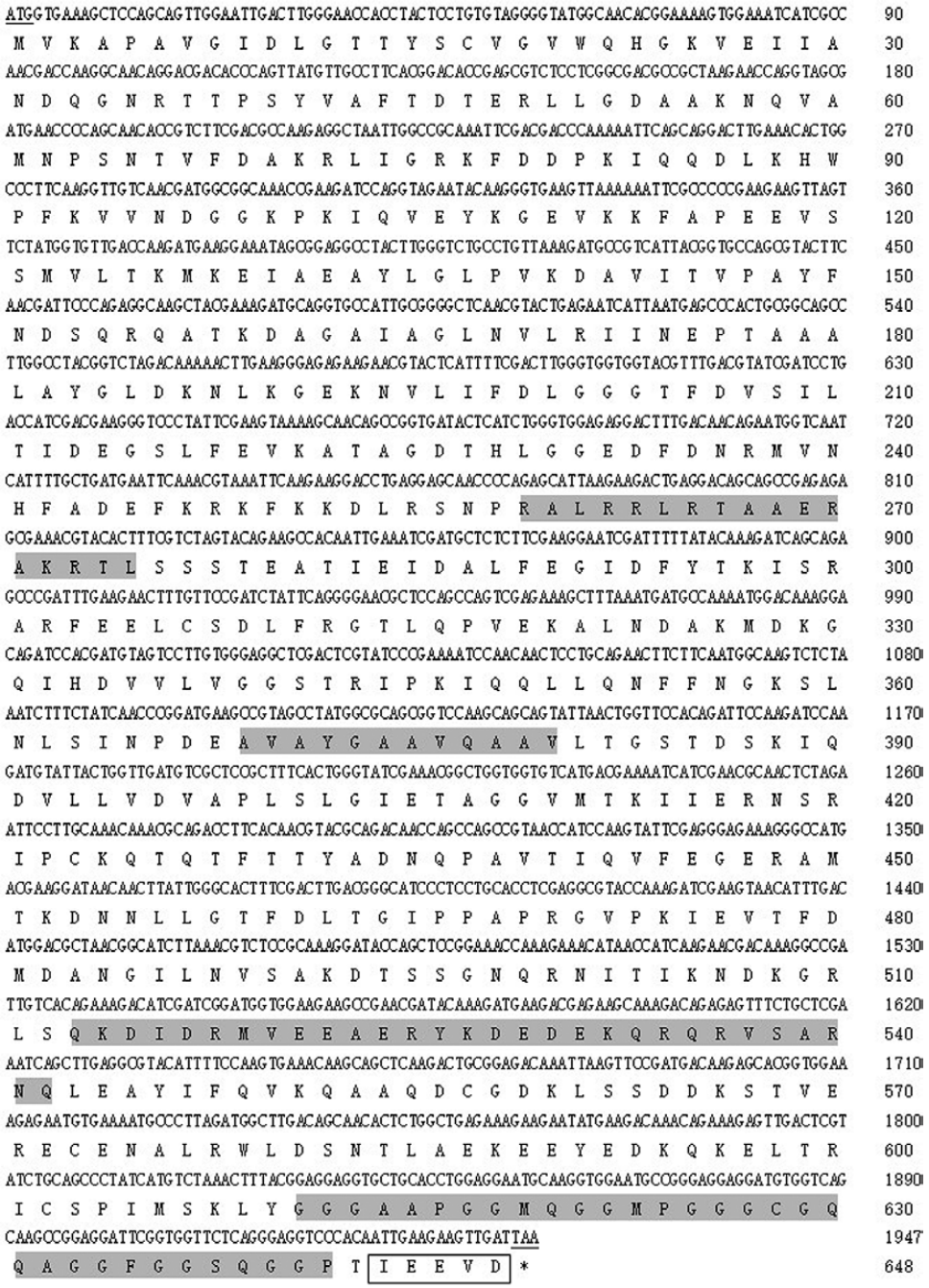

Fig. 2. Nucleotide sequence and deduced amino acid sequence of *MaHsp70*. The character shading (ATG) and translational termination codon (TAA) are underlined for emphasis. Four highly conserved regions in MaHsp70 are shown in gray and the conserved motif (IEEVD) at the C-terminus is boxed.

which includes a 2154 bp ORF and a 231 bp 3′terminal UTR. *MaHsp60* has two highly conserved regions in MaHsp90 and conserved motif (MEEVD) (Fig. 3). The ORF of *MaHsp90* encodes 717 amino acids with a molecular weight of 82.309 KDa and a theoretical pI of 4.97.

As shown in Fig. 4A, alignment of MaHsp60 with Hsp60 reference sequences from other insect species revealed that the predicted *Hsp60* protein in *M. alternatus* is highly homologous to the *Hsp60* proteins from *T. castaneum* (91%), *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) (93%), *Cryptolaemus montrouzieri*

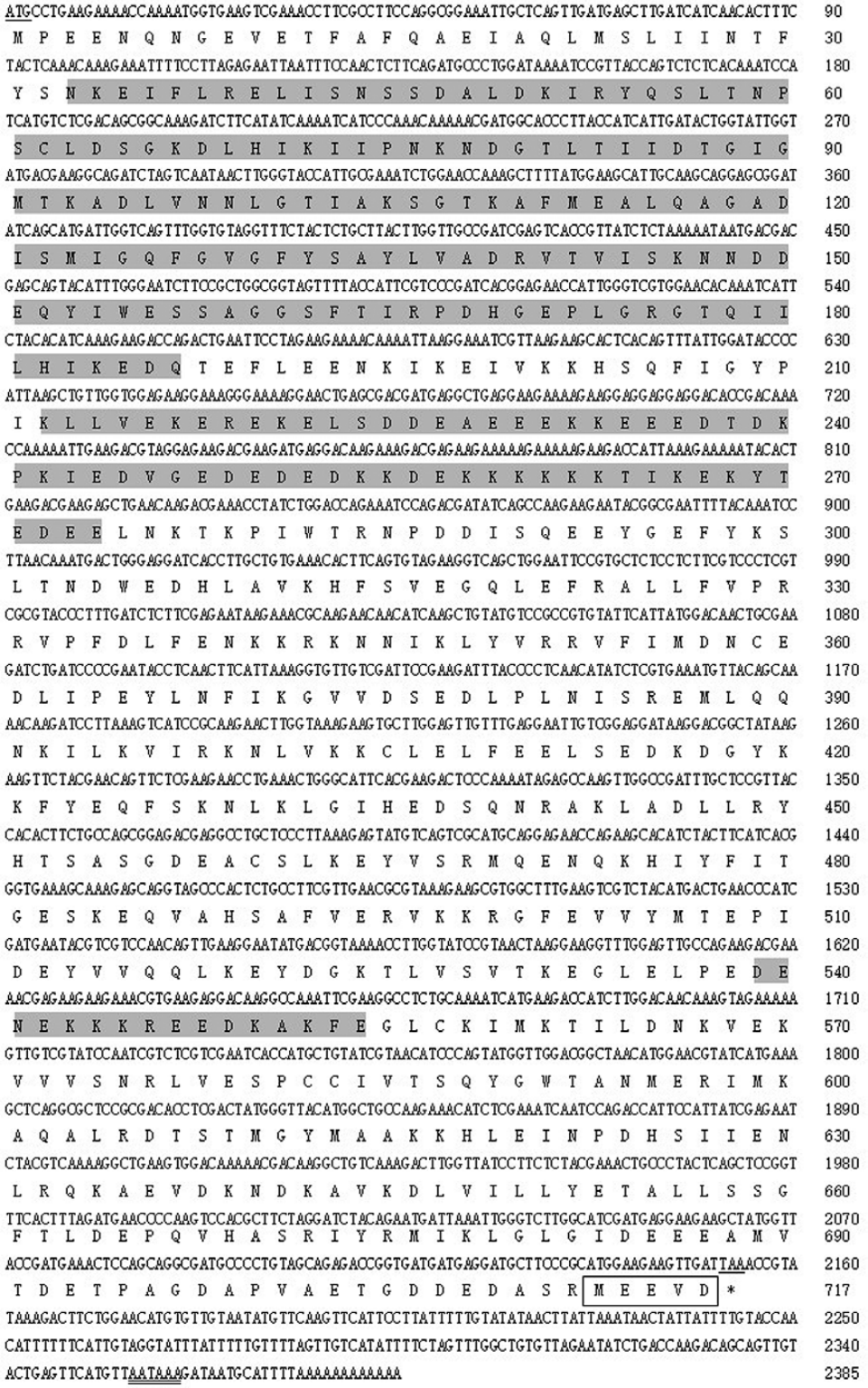

Fig. 3. Nucleotide sequence and deduced amino acid sequence of *MaHsp90*. The character shading (ATG) and translational termination codon (TAA) are underlined for emphasis. The HATPase c domain in position 33 to 187 is shown in gray, two highly conserved regions in MaHsp90 are shown in gray, and the conserved motif (MEEVD) at the C-terminus is boxed.

(Coleoptera: Coccinellidae) (87%), *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) (81%), *Drosophila ficusphila* (82), and *Bactrocera dorsalis* (Diptera: Tephritidae) (80%). The similarity of *M. alternatus* Hsp70 sequence was very high (>87%) compared to other species, such as *Anatolica polita borealis* (91%), *Microdera punctipennis* (88%), and *T. castaneum* (89%) (Fig. 4B). The deduced MaHsp90 sequence is 89–94% identical to Hsp90 in other insects (Fig. 4C). These sequence alignments suggest that Hsp60, Hsp70, and Hsp90 are highly conserved across insect species.

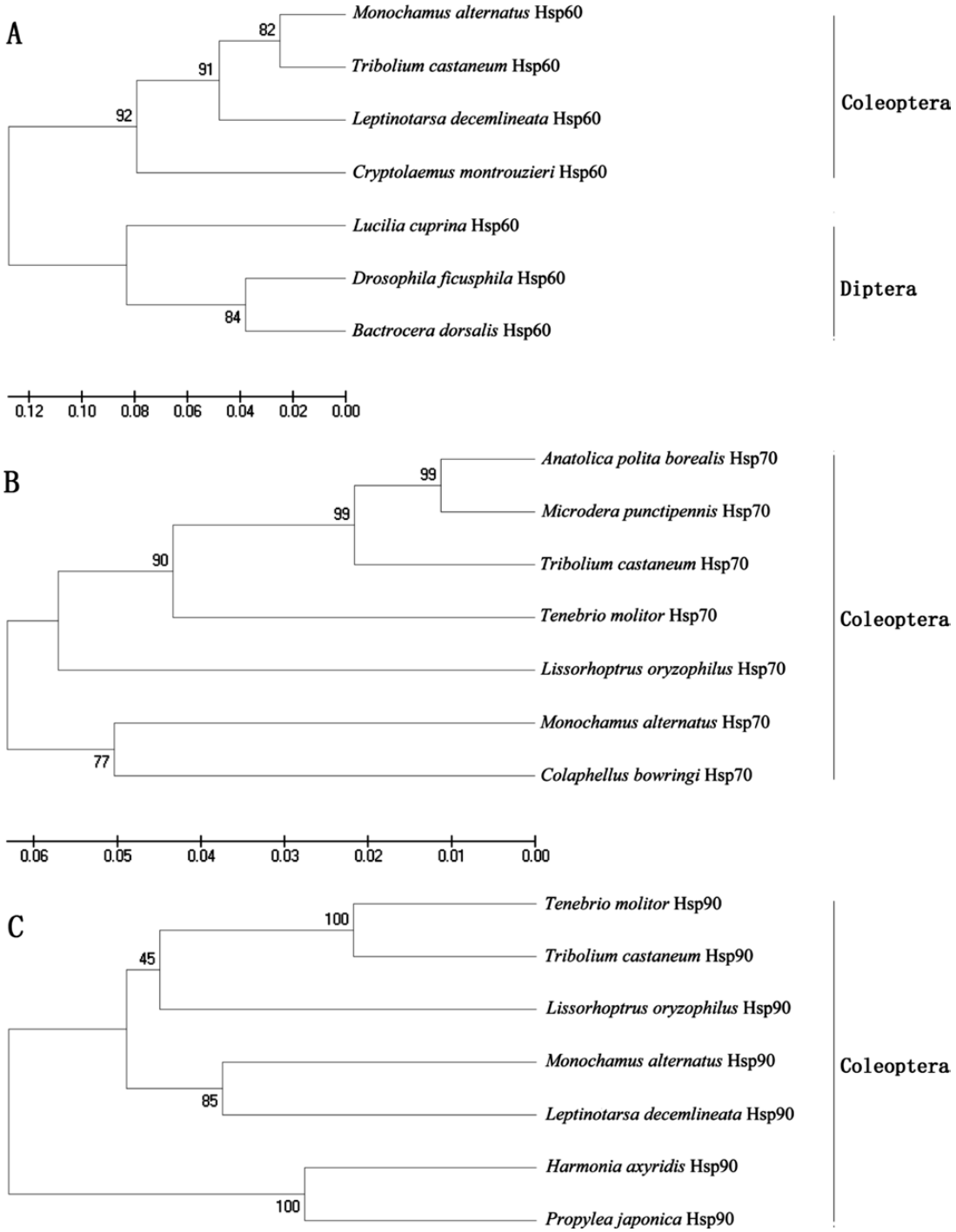

Fig. 4. Phylogenetic analysis of Hsp amino acid sequences from Diptera and Coleoptera. This tree, used for predicting the relationships between different Hsp, was generated via neighbor-joining analysis. The bootstrap values of 1000 replicates are displayed for each branch. Distance bar indicates branch length scale of the tree, or the number of substitutions per amino acid site. (A) Phylogenetic analysis of Hsp60s. Amino acid sequence alignment of Hsp60 was performed in *M. alternatus*, *Tribolium castaneum* (XP_971630.1), *Leptinotarsa decemlineata* (AHB18586.1), *Cryptolaemus montrouzieri* (ALW95353.1), *Lucilia cuprina* (ABO09590.1), *Drosophila ficusphila* (XP_017045694.1), and *Bactrocera dorsalis* (NP_001304341.1). (B) Phylogenetic analysis of Hsp70s. Amino acid sequence alignment of Hsp70 was performed in *Anatolica polita borealis*(ABQ39970.1), *Microdera punctipennis* (AEB52075.1), *Tribolium castaneum* (NP_001164199.1), *Tenebrio molitor* (AFE88579.1), *Lissorhoptrus oryzophilus*(AHE77387.1), and *Colaphellus bowringi* (AMK38874.1). (C) Phylogenetic analysis of Hsp90s. Amino acid sequence alignment of Hsp90 was performed in *Tenebrio molitor* (AFN02498.1), *Lissorhoptrus oryzophilus* (AHE77376.1), *Leptinotarsa decemlineata* (AHB18587.1), *Harmonia axyridis* (ACL50550.1), and *Propylea japonica* (AHW57925.1).

Expression of *Hsp* Genes in Larvae

The expression levels of the three *Hsp* genes were different among various tissues (body wall, fat body, Malpighian tuble) from third instar larvae. *Hsp* genes mRNA expression was significantly different among the tissues of *M. alternatus* ($F_{2,24}$>1211.198, P<0.001) (Fig. 5). For example, the relative mRNA level of *MaHsp60* in the fat body was 3.869-fold (compared with the control), which was much higher than *MaHsp70* and *MaHsp90* mRNA levels in the body wall. *MaHsp70* was highly expressed in the body wall (2.835-fold), while *MaHsp60* and *MaHsp90* were maintained at low levels in the body wall (0.9953-fold).

Expression of *Hsp* Genes in Adults

As shown in Fig. 6, *MaHsp70*($F_{6,56}$=136.401, P<0.001) exhibited the most diverse and highest expression in all adult tissues, while *MaHsp60* ($F_{6,56}$=58.189, P<0.001) and *MaHsp90* ($F_{6,56}$=1158.338, P<0.001) persisted at constant expression levels. *MaHsp70* expression was increased in the abdomen significantly but remained constant in head, thorax, tarsi, antennae, ovary, and elytra tissue. Additionally, *MaHsp60* was more highly expressed in abdomen tissue compared to other adult tissues.

Expression of *Hsp* Genes at Different Temperatures

In the present study, *Hsps* gene expression was significantly different between different temperatures (*MaHsp60* [$F_{6,56}$=161.077, P<0.001], *MaHsp70* [$F_{6,56}$=6699.774, P<0.001], and *MaHsp90* [$F_{6,56}$=1.68, P=0.198]) (Fig. 7). The responses of *MaHsp60*, *MaHsp70*, and *MaHsp90* to thermal stress were quite different, but all three *Hsp* genes were upregulated in the thermal stress-treated groups compared to the 25℃ control treatment. After exposure to −15℃ for 1 h and recovery at 25℃ for 1 h, *MaHsp60*, *MaHsp70*, and *MaHsp90* were upregulated in third instar larvae approximately 1.851-, 5.204-, and 1.990-fold, respectively. *MaHsp60*, *MaHsp70*, and *MaHsp90* were upregulated approximately 1.801-, 4.352-, and 1.827-fold, respectively, after exposure to −5℃. They were also upregulated 3.518-, 2.981-, and 3.115-fold when treated at 5℃; 3.085-, 9.583-, and 3.071-fold when treated at 15℃; 11.87-, 64.04-, and 14.27-fold when treated at 35℃; and 4.664-, 92.02-, and 10.73-fold when treated at 40℃. Responding to heat intensively, the three *Hsp* genes were all induced by heat significantly. *MaHsp70* had the highest expression level after exposure to 40℃, and *MaHsp60* and *MaHsp90* had the highest expression level after exposure to 35℃.

However, the difference of Hsp activity showed in Fig. 7 may be caused by the interaction between temperature-dependent expression of Hsp gene and cold temperature inhibition of enzymes. To assure the link of thermal stress and Hsp proteins, we made an experiment to justify it. In Fig. 8, Hsps gene were significantly different among various temperature exposure(F>348.169, P<0.001). Hsps gene (*MaHsp60*, *MaHsp70*, *MaHsp90*) activity was much higher in 30 to 35℃ (Group2) than in 30 to 25℃ (Group1) treatment.

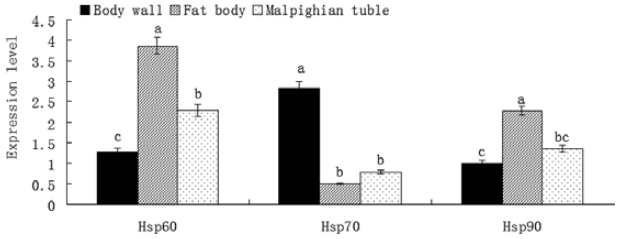

Fig. 5. Expression of *MaHsp60*, *MaHsp70*, and *MaHsp90* in different tissues (body wall, fat body, Malpighian tuble) from third instar larvae of *M. alternatus* at 25℃. To normalize the quantity of Hsp mRNA, the actin was used as reference gene and the third instar larvae at 25℃ served as control. Different letters on the bars indicate significant difference for expression levels of *MaHsps* gene from *M. alternatus*(one-way analysis of variance, Tukey honestly significant difference, P<0.05).The error bars indicate 1 SE.

Expression of *Hsp* Genes in Various Developmental Stages

Expression of *MaHsp60* at 25℃ was lower than at 5℃ and 35℃ in all stages of development. Moreover, the expression of MaHsp60 was at maximal levels in the EA stage. *MaHsp60* showed a greater induction by heat (35℃) than cold (5℃) ($F_{23,192}$=353.635, P<0.001) (Fig. 9A). In addition, the expression of *MaHsp60* at 35℃ was the highest in later pupal (LP) stages and was higher than at 5℃ in all stages except third instar larvae (L3) and later adult (LA) stages.

The expression of *MaHsp70* differed among developmental stages significantly (P<0.05). The expression of *MaHsp70* at 35℃ and 5℃, which was higher than the expression of *MaHsp70* at 25℃ in all stages, was similar to *MaHsp60*. The expression of *MaHsp70* at 25℃, 5℃, and 35℃ was the lowest in fifth instar larvae and the highest in the EAs, fourth instar (L4), and third instar larvae (L3) ($F_{23,192}$=777.754, P<0.001) (Fig. 9B).

The expression of *MaHsp90* did not differ among developmental stages ($F_{23,192}$=748.242, P<0.001) (Fig. 9C). The highest expression

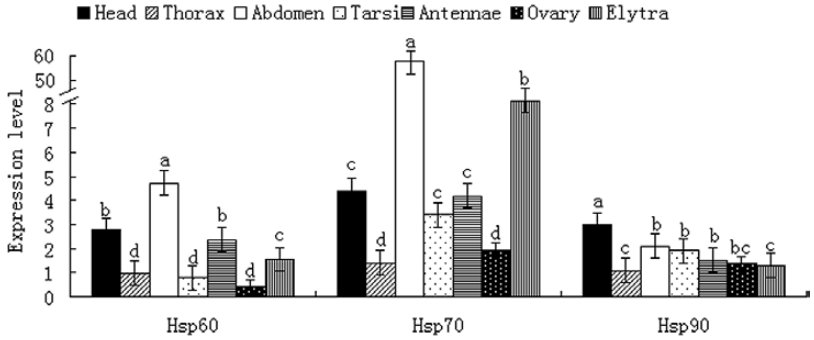

Fig. 6. Expression of *MaHsp60*, *MaHsp70*, and *MaHsp90* in parts of adults at 25℃. The seven parts examined include the head, thorax, abdomen, tarsi, antennae, ovary, and elytra. To normalize the quantity of Hsp mRNA, the actin was used as reference gene and the adults at 25℃ served as control. Different letters on the bars indicate significant difference for expression levels of *MaHsps* gene from *M. alternatus*(one-way analysis of variance, Tukey honestly significant difference, P<0.05). The error bars indicate 1 SE.

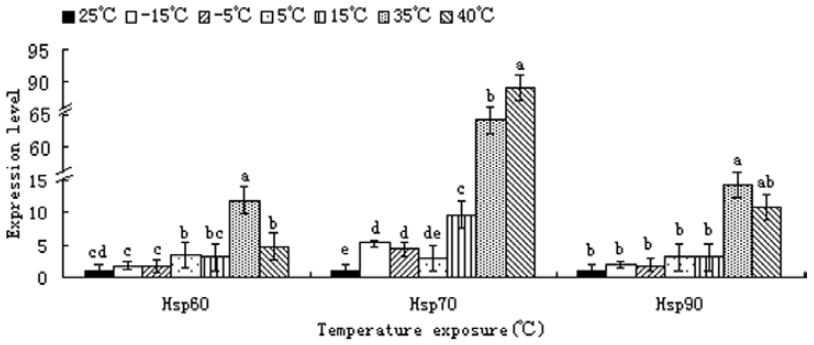

Fig. 7. The expression levels of *MaHsp60, MaHsp70,* and *MaHsp90* from third instar larvae after exposure to different temperatures including 25℃, −15℃, −5℃, 5℃, 15℃, 35℃, 40℃. To normalize the quantity of Hsp mRNA, the actin was used as reference gene and the third instar larvae at 25℃ served as control. Different letters on the bars indicate significant difference for expression levels of *MaHsps* gene from *M. alternatus* (one-way analysis of variance, Tukey honestly significant difference, $P<0.05$). The error bars indicate 1 SE.

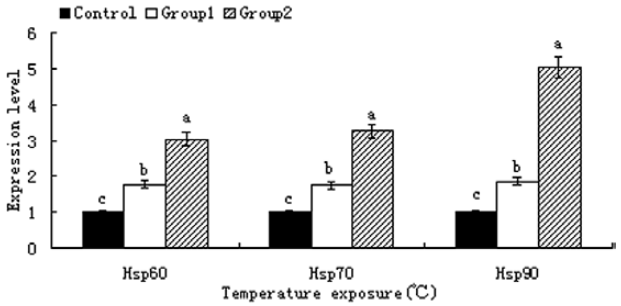

Fig. 8. The expression levels of *MaHsp60, MaHsp70,* and *MaHsp90* from late adult of *M. alternatus* after treated with different temperatures. To normalize the quantity of Hsp mRNA, the actin was used as reference gene and the adults at 25℃ served as control. Group 1 was exposed to 30℃ for a long time, exposed to 35℃ for 1 h while group 2 exposed to 25℃, and then exposed to 30℃. Different letters on the bars indicate significant difference for expression levels of MaHsps gene from *M. alternatus*(one-way analysis of variance, Tukey honestly significant difference, $P<0.05$). The error bars indicate 1 SE.

of *MaHsp90* at 5℃ and 35℃ was measured in EA stages, and the highest expression of *MaHsp90* at 25℃ occurred in LP stages.

Discussion

Hsps are highly conserved in insects (Feder and Hofmann 1999), and our analysis of three *Hsp*s emphasize the importance of these proteins. In this study, we isolated three full-length genes encoding *Hsp*s from *M. alternatus*, including *MaHsp60*, *MaHsp70,* and *Mahsp90*. Sequence alignments revealed that the three *M. alternatus Hsp*s were highly similar to *Hsp*s in other species. The identity ranges were 80–93% for MaHsp60, 87–91% for MaHsp70, and 89–94% for MaHsp90. The MaHsp60 sequence, with conserved mitochondrial Hsp60 signatures and GGM motifs at its C-terminus, belongs to the mitochondrial Hsp60 family. Typical signature structures of Hsp70, including a conserved IEEVD motif at the C-terminus, were also found in the deduced amino acid sequence of *MaHsp70*. The conserved IEEVD motif could allow MaHsp70 to bind with other co-chaperones (Daugarrd et al. 2007). Contained in the C-terminus of MaHsp90 was an MEEVD motif, which can bind to many co-chaperones with tetratricopeptide domains (Scheufler et al. 2000; Li and Du 2013) and indicates that MaHsp90 belongs to the cytosolic Hsp90 family (Pearl and Prodromou 2006). These results suggest that Hsps have been highly conserved throughout evolution at the gene sequence, protein structure, protein function, and gene expression levels (Togashi et al. 2016).

Many studies have revealed that insect Hsps have obvious tissue-specific expression in normal temperatures. In *Sitophilus zeamais* (Coleoptera: Curculionidae), *hsp70* is selectively expressed in the fat bodies, subesophageal ganglion, and Malpighian tubules. *Hsp70* is selectively expressed in the gut, and *hsp90* is selectively expressed in the testes, gut, and subesophageal ganglion (Tungjitwitayakul et al., 2015). In the present study, *MaHsp60*, *MaHsp70*, and *MaHsp90* were most highly expressed in the fat body, body wall, and fat body, respectively (Fig. 5). It has been previously shown that *Hsp*s are rapidly synthesized within cells after exposure to environmental stressors (Schlesinger 1990; Goto and Kimura 1998). However, little is known about Hsp functions when cells are subjected to specific stresses. Thus, *MaHsp60*, *MaHsp70,* and *MaHsp90* were investigated in the whole bodies of larvae (Fig. 5) and adults (Fig. 6). In the various tissues, we have identified Hsp gene has the highest expression level in the fat body. We found that Hsp gene was more highly expressed in the abdomen of adults. Hsps gene was highly expressed in fat body and abdomen, directly in touch with environment, which may be related to its sensitive to the ambient stress. The results of this study demonstrate that three *Hsp* genes are expressed in every tissue but at varying levels, suggesting that the three *Hsp* genes play individual roles in *M. alternatus*.

Hsps have been reported to play an important role in the ability of insects to tolerate cold and heat hardening. The induction of *Hsp* genes varies with the intensity of thermal stress and the physiological state of the insect (King and Macrae 2015). As determined by RT-qPCR, *Hsp60* expression increase in the leafminer, *Leriomyza sativae*, during cold hardening (Huang et al. 2009). *Hsp90* decreases during cold hardening but accumulates in heated and chilled nondiapausing and diapausing *Sarcophaga crassipalpis* (Li and Denlinger 2008; Rinehart and Denlinger 2000). In this work, we found that *MaHsp60*, *MaHsp70,* and *MaHsp90* were heat shock responsive and that the induction of expression was rapid after cold and heat exposure. However, the three *MaHsp*s did not significantly respond to cold temperatures. In contrast, they could be induced by heat quickly and notably.

Hsp60, *Hsp70,* and *Hsp90* play critical roles in insect development (Sharma et al. 2007). Many *Hsp*s regularly express during development stages. For example, in *Neoseiulus cucumeris*, *Hsp90* increases significantly in eggs and adult stages, and *Hsp70* expression is the highest in eggs (Chen et al. 2015). In *Spodoptera litura*, the highest expression of *Hsp60* and *Hsp90* is observed in female adults

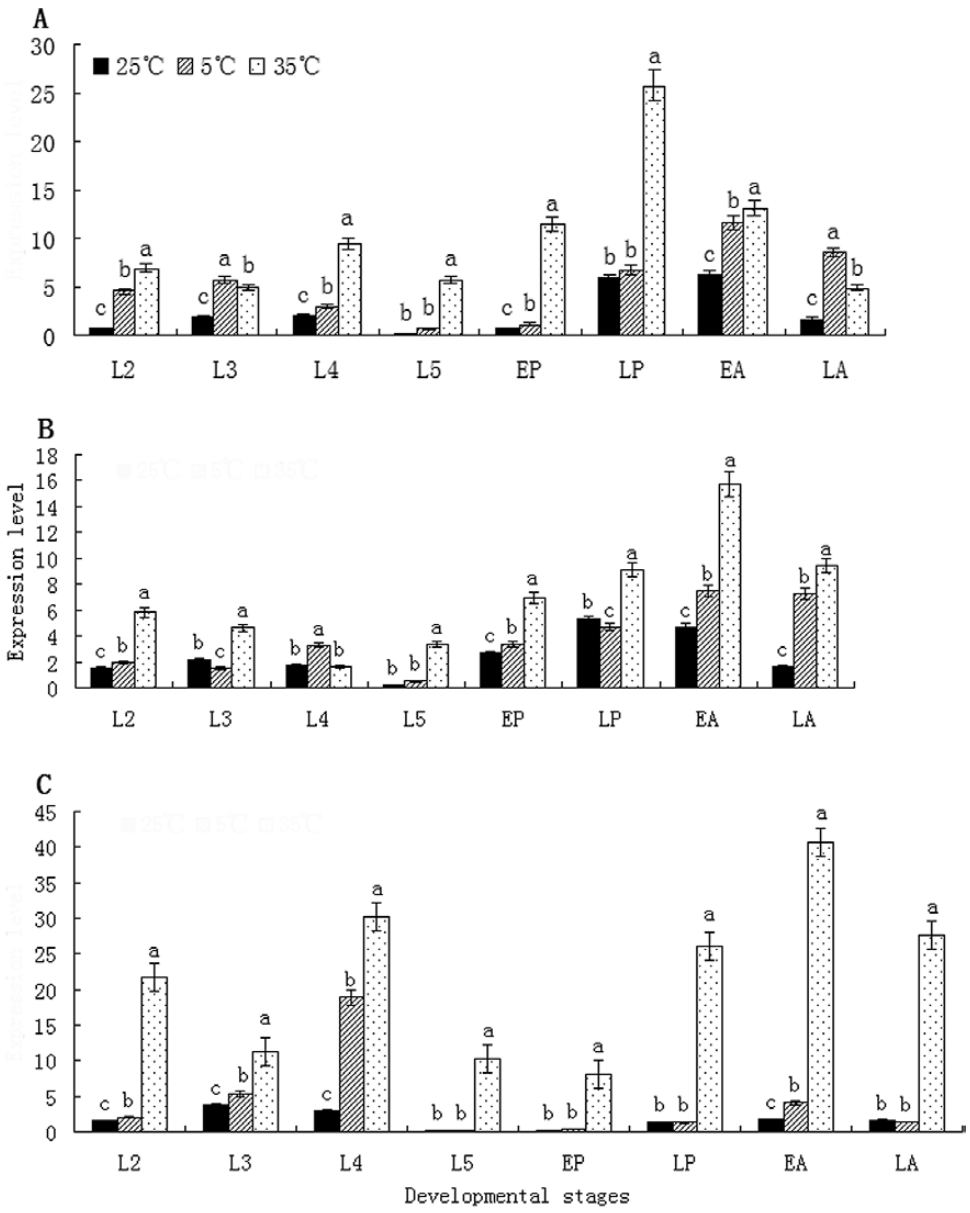

Fig. 9. The expression of *MaHsps* gene ([A] *MaHsp60,* [B] *MaHsp70,* and [C] *MaHsp90*) in various developmental stages of *M. alternatus* treated with different temperatures (25℃, 5℃, 35℃). The eight developmental stages examined include second instar larvae (L2), third instar larvae (L3), fourth instar larvae (L4), fifth instar larvae (L5), early pupae (EP), late pupae (LP), early adult (EA), and late adult (LA). To normalize the quantity of Hsp mRNA, the actin was used as reference gene and the beetles at 25℃ served as control. Different letters on bars indicate significant differences in the relative expression levels of a given *MaHsps* gene (one-way analysis of variance, Tukey honestly significant difference, $P<0.05$). The error bars indicate 1 SE.

(Shu et al. 2011). In *Spodoptera exigua* (Lepidoptera: Noctuidae) and *Chilo suppressalis* (Lepidoptera: Crambidae), *Hsp70* is highly expressed in first instar larvae and second instar larvae (Xu et al. 2011; Lu et al. 2014). We found that *MaHsp60*, *MaHsp70*, and *MaHsp90* expression is consistent with what has been observed in other insects. The highest expression of *MaHsp60* was observed in the EA after exposure to 5℃ /25℃, also in the late pupa after 35℃ treatment. The expression of *MaHsp70* and *MaHsp90* reached the highest level in EAs after 35℃ treatment. These results indicate that Hsp expression in the late pupal and EA stages of *M. altertanus* may be related to maturity.

Temperature is a critical factor in the dynamics of host-pathogen interactions (Calatán et al. 2012; Browne at al. 2014). *Bursaphelenchus xylophilus* causes pine wilt disease and is transmitted by *M. alternatus* adults in Japan (Togashi et al. 2016). Ohsawa and Mitsuteru (2014) found that the beetle population seems to be stable at 850 m (10.2℃) and lower altitudes (higher temperatures) but cannot reproduce at altitudes above 1150 m (lower than 8.2℃). The beetle can endure temperatures lower than those previously reported. Pine wilt disease also occurs at lower temperatures and higher altitudes than expected (Ohsawa and Mitsuteru 2014). In this study, the beetles also displayed remarkable endurance to cold and heat. Moreover, *M. alternatus* produced less Hsps to protect itself against cold in comparison to heat. With these results, we can summarize principal strategies for controlling pine wilt disease at low temperatures suddenly.

In conclusion, *MaHsp60*, *MaHsp70*, and *MaHsp90* can be induced by cold and heat stress. The induction temperature for

three Hsps varies, as does the extent of the response, with *MaHsp70* amplified most intensely. But *MaHsp60*, *MaHsp70,* and *MaHsp90* were all upregulated after a heat shock, especially at 35℃. Such differences suggest functional deviation among the Hsps. *MaHsp60* and *MaHsp70* might play important roles during heat stress, but *MaHsp90* might play an important role during cold stress. Our data establish a highly plausible link between *Hsp* gene expression and thermal adaptation in Coleoptera. However, the precise physiological functions of *Hsp60*, *Hsp70,* and *Hsp90* during thermal stress require further genetic dissection in the future.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant No. 31470653) and the Natural Science Foundation of Guangdong Province (Grant No. 2015A030313416).

References Cited

Aruda, A. M., M. F. Baumgartner, A. M. Reitzel, and A. M. Tarrant. 2011. Heat shock protein expression during stress and diapause in the marine copepod *Calanus finmarchicus*. J. Insect Physiol. 57: 665–675.

Basha, E., H. O'Neill, and E. Vierling. 2012. Small heat shock proteins andα-crystallins: dynamic proteins with flexible functions. Trends Biochem. Sci. 37: 106–117.

Calatán, T. P., A. Wozniak, H. M. Niemeyer, A. M. Kalergis, and F. Bozinovic. 2012. Interplay between thermal and immune ecology: Eeffect of environment temperature on insect immune response and energetic costs after an immune challenge. J. Insect Physiol. 58: 310–317.

Chapuis, M. P., S. J. Simpson, L. Blondin, and G. A. Sword. 2011. Taxa-specific heat shock proteins are over-expressed with crowding in the Australian plague locust. J. Insect Physiol. 57: 1562–1567.

Chen, W., D. S. Li, and M. Zhang. 2015. Cloning and differential expression of five heat shock protein genes associated with thermal stress and development in the polyphagous predatory mite *Neoseiulus cucumeris* (Acari: Phytoseiidae). Exp. Appl. Acarol. 67: 65–85.

Clare, D. K., and H. R. Saibil. 2013. ATP-driven molecular chaperone machines. Biopolymers. 99: 846–859.

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*. FEBS J. 277: 174–185.

Daugarrd, M., M. Rohde, and M. Jäättelä. 2007. The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. FEBS Lett. 581: 3702–3710.

Feder, M. E., and G. E. Hofmann. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61: 243–282.

Goto, S. G., and M. T. Kimura. 1998. Heat- and cold-shock responses and temperature adaptations in subtropical and temperate species of *Drosophila*. J. Insect Physiol.44: 1233–1239.

Hartl, F. U., and M. Hayer-Hartl. 2002. Protein folding-molecular chaperones in the cytosol: from nascent chain to folded protein. Science. 295: 1852–1858.

Huang, L. H., C. Z. Wang, and L. Kang. 2009. Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer, *Liriomyza sativa*. J. Insect Physiol. 55: 279–285.

King, A. M., and T. H. MacRae. 2015. Insect heat shock proteins during stress and diapause. Annu. Rev. Entomol. 60: 59–75.

Kumar, S., M. Nei, J. Dudley, and K. Tamura. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9: 299–306.

Li, A., and D. L. Denlinger. 2008. Rapid cold hardening elicits changes in brain protein profiles of the flesh fly, *Sarcopbaga crassipalpis*. Insect Mol. Biol. 17: 565–572.

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, A., M.R. Michaud, and D.L. Denlinger. 2009. Rapid elevation of Inos and decreases in abundance of other proteins at pupal diapause termination in the flesh fly *Sarcophaga crassipalpis*. BBA-Proteins Proteom. 1794: 663–668.

Liu, Q. N., B. J. Zhu, L. S. Dai, W. W. Fu, K. Z. Lin, and C. L. Liu. 2013. Overexpression of small heat shock protein 21 protects the Chinese oak silkworm *Antheraea pernyi* against thermal stress. J. Insect Physiol. 59: 848–854.

Lu, M. X., Z. X. Liu, Y. D. Cui, and Y. Z. Du. 2014. Expression patterns of three heat shock proteins in *Chilo suppressalis* (Lepidoptera: Pyralidae). Ann. Entomol. Soc. Am. 107: 667–673.

Ma, R. Y., S. G. Hao, and J. Tian. 2006. Seasonal variation in cold-hardiness of the Japanese pine sawyer *Monochamus alternatus* (Coleoptera: Cerambycidae). Environ. Entomol. 35: 881–886.

Moribe, Y., K. Oka, T.Niimi, O. Yamashita, and T. Yaginuma. 2010. Expression of heat shock protein 70a mRNA in *Bombyx mori* diapause eggs. J. Insect Physiol. 56: 1246–1252.

Niu, B. L., W. F. Shen, H. B. Weng, L. H. He, J. J. Mu, Z. L. Wu, P. Jiang, Y. Z., Tao, and Z. Q. Meng. 2008. Cloning and RNAi-mediated functional characterization of MaLac2 of the pine sawyer, *Monochamus alternatus*. Insect Mol. Biol. 17: 303–312.

Ohsawa, M., and A. Mitsuteru. 2014. Possible altitude and temperature limits on pine wilt disease: the reproduction of vector sawyer beetles (*Monochamus alternatus*), survival of causal nematode (*Bursaphelenchus xylophilus*), and occurrence of damage caused by the disease. Eur. J. Forest Res. 133: 225–233.

Pearl, L. H., and C. Prodromou. 2006. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu. Rev. Biochem. 75: 271–294.

Pfaffl, M. W. 2001. A new math ematical model for relative quantification in realtime RT-PCR. Nucleic Acids Res. 29: 2002–2007.

Rinehart, J. P., and D. L. Denlinger. 2000. Heat-shock protin 90 is down-regulated during pupal diapause in the flesh fly, *Sarcopbaga crassipalpis*, but remains responsive to thermal stress. Insect Mol. Biol. 9: 641–45.

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.

Roehl, A., J. Rohrberg, and J. Buchner. 2013. The chaperone Hsp90: changing partners for demanding clients. Trends Biochem. Sci. 38: 253–262.

Sanders, B. M., and L. S. Martin. 1993. Stress proteins as biomarkers of contaminant exposure in archived environmental samples. Sci. Total Environ. 139–140:459–470.

Sang, W., W. H. Ma, L. Qiu, Z. H. Zhu, and C. L. Lei. 2012. The involvement of heat shock protein and cytochrome P450 genes in response to UV-A exposure in the beetle *Tribolium castaneum*. J. Insect Physiol. 58: 830–836.

Schlesinger, M. J. 1990. Heat shock proteins: a mini review. J. Biol. Chem. 265: 12111–12114.

Scheufler, C., A. Brinker, G. Bourenkov, S. Pegoraro, L. Moroder, H. Bartunik, F. U. Hartl, and I. Moarefi. 2000. Structure of TPR domain–peptide complexes. Cell. 101: 199–210.

Sharma, S., M. S. Rohilla, and P. K. Tiwari. 2007. Developmental and hyporthermia-induced expression of the heat shock proteins HSP60 and HSP70 in tissues of the housefly *Musca domestic*a: an in vitro study. Genet. Mol. Biol. 30: 159–168.

Shim, J. K., H. B. Bang, and K. Y. Lee. 2012. High temperature interrupts initial egg diapause in *Paratlanticus ussuriensis* and induces expression of a heat shock protein 70 gene. J. Asia-Pac. Entomol. 15: 5–11.

Shu, Y. H., Y. Du, and J. W. Wang. 2011. Molecular characterization and expression patterns of *Spodoptera litura* heat shock protein 70/90, and their response to zinc stress. Comp. Biochem. Physiol. 158: 102–110.

Sorensen, J. G., and V. Loeschcke. 2007. Studying stress responses in the post-genomic era: its ecological and evolutionary role. J. Biosci. 32: 447–456.

Togashi, K. 2014. Effects of larval food shortage on diapause induction and adult traits in Taiwanese *Monochamus alternatus*. Entomol. Exp. Appl. 151: 34–42.

Togashi, K., O. Miyauchi, and D. Kusumoto. 2016. Commensal relation between *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) and *Monochamus alternatus* (Coleoptera: Cerambycidae) within pine trees. Appl. Entomol. Zool. 51: 53–62.

Tungjitwitayakul, J., N. Tatun, B. Vajarasathira, and S. Sakurai. 2015. Expression of heat shock protein genes in different developmental stages and after temperature stress in the *Maize Weevil* (Coleoptera: Curculionidae). J. Econ. Entomol. 108: 1313–1323.

Warchalowska-Sliwa, E., M. Nikliñska, A. Görlich, 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, Q., Q. Zou, H. Z. Zheng, F. Zhang, B. Tang, and S. Wang. 2011. Three heat shock proteins from *Spodoptera exigua*: gene cloning, characterization and comparative stress response during heat and cold shocks. J. Comp. Physiol. B. 159: 92–102.

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, Y., Y. Liu, X. Guo, Y. Li, H. Gao, and X. Guo. 2014. *sHsp22.6*, an intronless small heat shock protein gene, is involved in stress defence and development in *Apis cerana cerana*. Insect Biochem. Mol. Biol. 53: 1–12.