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

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Abstract

Ambient temperature is one of the most crucial factors influencing 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 *Hsps* by quantitative real-time polymerase chain reaction. In the third instar larvae, *MaHsp60, MaHsp70*, and *MaHsp90* expression was upregulated in response to cold and heat, but the three *Hsps* 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 *Hsps*.

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 euksaryotes and prokaryotes. Hsps can facilitate proper protein folding, 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 (Colin et al. 2010; Sorensen and Loschcke 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) *Hsps* increases during the process of development and after cold hardening (Huang et al. 2010; Ma et al. 2006). In southern China, *M. alternatus* must face extreme cold and heat. The freezing temperatures during the winter 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 face extreme cold and heat. The freezing temperatures during the winter can reach −15°C (Ma et al. 2006). It also survives winter by avoiding freezing or increasing their cold tolerance (Ma et al. 2006).
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). Moreover, Hsps play key roles in thermal tolerance and diapause in insects such as *Antheraea pernyi* Guérin-Méneville (Lepidoptera: Saturniidae) (Liu et al. 2013), *Paratlassius issaei* (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 *Pinus massoniana* Lam in the Guangzhou, China (23°32′N, 113°33′E) and was reared in an artificial climate incubator under conditions of 25°C, 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 tubes 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°C. 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°C (Ohsawa and Mitsuteru 2014). Therefore, temperatures of −15 and 40°C 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°C for 1 h, respectively, and then were allowed to recover at 25°C for 1 h. Groups of five late adults were exposed to 30°C for a long time, exposed to 35 or 25°C for 1 h, and then exposed to 30°C.

To investigate Hsp gene patterns during developmental stages under cold stress, we used 5°C because it represents the minimum temperature experienced in Southern Chinese year in year out. There were two reasons why we used 35°C and not 40°C to study the effect of Hsps gene’s heat shock. Firstly, 35°C often represents the stable temperature in the forest of Southern Chinese during the summer. Secondly, exposure to 35°C does not impact on the physiological activity of *M. alternatus*. Therefore, second, third, fourth, fifth larva, 1-d pupae (Early pupae), 9-d pupae (Late pupae), 1-d (EA), and 12-d adults (Late adult) were kept at 5°C, 35°C, and 25°C (as control) for 1 h, and then allowed to recover at 25°C 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°C.

#### 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

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**Table 1. Primers used for RACE and RT-qPCR**

| Abbreviation | Primer sequence (5′–3′) | Technique used |
|--------------|-------------------------|----------------|
| MaHsp60-F1   | CTTTGATGAGTCTGCAAGC    | 3′RACE         |
| MaHsp60-F2   | GGTGTCGTCGCTGGTTTCG    | 3′RACE         |
| MaHsp60-F    | GCTGCTATGTAGCCCTTGACT  | RT-qPCR        |
| MaHsp60-R    | ACTGCTTGGTGGGATGTT     | RT-qPCR        |
| MaHsp70-F1   | TGGCGGCAAACCGAAGAT     | 3′RACE         |
| MaHsp70-F2   | TCCGATGAAAGAGAGACG     | 3′RACE         |
| MaHsp70-F    | TCCGATGAAAGAGAGACG     | 3′RACE         |
| MaHsp70-R    | TGGCGGCAAACCGAAGAT     | RT-qPCR        |
| MaHsp70-R    | CGTGGCGACCTGAAATG      | RT-qPCR        |
| MaHsp90-F1   | AGCACATCTACCTCCACAGGGG | 3′RACE         |
| MaHsp90-F2   | GTTATCGATGAAACTCCAG    | 3′RACE         |
| MaHsp90-F    | GAGGAAGGTATGAGGCGAG    | RT-qPCR        |
| MaHsp90-R    | AGGCGTTCGTCAAGAGGAG    | RT-qPCR        |
| actin-F      | CTCTGCTATGTAGGCTTACATT | RT-qPCR        |
| actin-R      | GCAGTTGAGGTGGTTGCTG    | 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°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 20 s; and cooling at 40°C for 30 s. Reactions with diethyl 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.

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**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^ΔΔCt 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 pl 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...
(Coleoptera: Coccinellidae) (87%), *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) (81%), *Drosophila fuscipha* (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 fuscipha* (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), *Lissorhopterus oryzophilus* (AHE77367.1), and *Colaphellus bowringi* (AMK38874.1). (C) Phylogenetic analysis of Hsp90s. Amino acid sequence alignment of Hsp90 was performed in *Tenebrio molitor* (AFN02498.1), *Lissorhopterus oryzophilus* (AHE77367.1), and *Colaphellus bowringi* (AMK38874.1). (D) Phylogenetic analysis of Hsp90s. Amino acid sequence alignment of Hsp90 was performed in *Tenebrio molitor* (AFN02498.1), *Lissorhopterus oryzophilus* (AHE77367.1), *Leptinotarsa decemlineata* (AHB18587.1), *Harmonia axyridis* (ACL50550.1), and *Propylea japonica* (AHW79725.1).
Expression of Hsp Genes in Larvae
The expression levels of the three Hsp genes were different among various tissues (body wall, fat body, Malpighian tube) from third instar larvae. Hsp genes mRNA expression was significantly different among the tissues of M. alternatus (F_{2,48}=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 abdominal tissue compared to other adult tissues.

Expression of Hsp Genes at Different Temperatures
In the present study, Hsps gene expression was significantly different among various tissues (body wall, fat body, Malpighian tube) 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℃ were maintained as control. To justify it. In Fig. 6, Hsps gene were significantly different among various temperature exposure(F_{6,56}=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.

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_{2,24}=777.754, 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 larval (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_{2,24}=353.635, P<0.001) (Fig. 9B).

The expression of MaHsp90 did not differ among developmental stages (F_{2,24}=748.242, P<0.001) (Fig. 9C). The highest expression
of MaHsp90 at 5°C and 35°C was measured in EA stages, and the highest expression of MaHsp90 at 25°C occurred in LP stages.

Discussion

Hsps are highly conserved in insects (Feder and Hofmann 1999), and our analysis of three Hsps emphasize the importance of these proteins. In this study, we isolated three full-length genes encoding Hsps from M. alternatus, including MaHsp60, MaHsp70, and MaHsp90. Sequence alignments revealed that the three M. alternatus Hsps were highly similar to Hsps 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 Hsps 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, Liriomyza sativae, during cold hardening (Huang et al. 2009). Hsp90 decreases during cold hardening but accumulates in heated and chilled nondiapause and diapause 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 MaHsps 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 Hsps 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 littoralis, the highest expression of Hsp60 and Hsp90 is observed in female adults.
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\(^{\circ}\)C/25\(^{\circ}\)C, also in the late pupa after 35\(^{\circ}\)C treatment. The expression of MaHsp70 and MaHsp90 reached the highest level in EAs after 35\(^{\circ}\)C treatment. These results indicate that Hsp expression in the late pupal and EA stages of M. alternatus 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\(^{\circ}\)C) and lower altitudes (higher temperatures) but cannot reproduce at altitudes above 1150 m (lower than 8.2\(^{\circ}\)C). 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°C. 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.

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