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Gene Expressions of Heat Shock Proteins in *Bombyx mori* Egg Parasitized by a Parasitoid Wasp, *Telenomus theophilae*

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Authors' Contributions
LW and CL conceived and designed the experiments. LW and YZ performed the experiments. CQ and BZ analyzed the data. GW contributed reagents/materials/ analysis tools. LW and YZ wrote the paper.

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Egg, parasitoid, heat shock protein, *Bombyx mori*, parasitoid wasp.

ABSTRACT

Parasitoid wasps inject their eggs into host along with virulent factors to manipulate hosts physiology and immune system. Heat shock proteins (HSPs) are as molecular chaperones and stress proteins induced by heat, cold, anoxia and parasitism. There was no report about the roles of HSP in insect eggs against egg parasitoids. Here, we studied the expression profiles of several HSPs in silkworm eggs after parasitization by *Telenomus theophilae*. The results showed that expressions of Hsp19.9, Hsp20.1, Hsp20.8 or Hsp23.7 were significant up-regulated at 24 h post-parasitization, but the expressions of Hsp20.4, Hsp21.4, Hsp70, heat shock cognate protein and Hsp90 after 24 h parasitization had no significant difference compared with the control. After 3 h post-prarsitization, the expressions of nine HSPs detected in eggs had no significant changes. Our results indicated HSPs were involved in the host-parasitoid interaction. This helps us to understand the functions of HSP in host against egg parasitoids.

INTRODUCTION

Egg parasitoid wasps are important natural enemies of pests, and have been used in many countries for biological control. Parasitoids inject their eggs into host eggs, along with a variety of substances including venoms, polydnaviruses, ovarian fluids, and other maternal factors (Asgari and Rivers, 2011; Pennacchio and Strand, 2006). At the same time, insect eggs protect themselves from natural enemies, such as viruses, bacterial, fungi and parasitoids. Different with larvae stage, eggs have hard physical barriers egg shell, maternal or paternal endowment with natural chemical products (Abdel-Latief and Hilker, 2008; Jamil *et al*., 2015; Stanley and Miller, 2006). In contrast to the immune response of larval or adult stage response to parasitoid, little is known about how attack of insect eggs by parasitoids or molecular mechanism of egg defending parasitism (Abdel-Latief and Hilker, 2008; Hamed and Nadeem, 2012).

Heat shock proteins (HSPs) are conserved proteins, which are found in almost all organisms (Zhang *et al*., 2015). HSPs can be divided into five families, including small heat shock protein (12-42 kDa, sHSP), Hsp60, Hsp70, Hsp90 and Hsp100, based on sequence homology and typical molecular weight (Li *et al*., 2009). HSPs are known as stress proteins and molecular chaperones, and are induced by several insults, including heat, cold, desiccation, starvation, and anoxia (King and MacRae, 2015). HSPs in host or parasitoid have been reported to be involved in host-parasitoid interactions (Kraaijeveld and Godfray, 2009; Zhu *et al*., 2013).

Endoparasitoid *Telenomus theophilae* Wu et Chen (Hymenoptera: Scelionidae), is the predominant parasitoid of *Bombyx mandarina* eggs, and it also could parasite successful in domestic silkworm *Bombyx mori* (Sun *et al*., 2007). In previous studies, nine HSPs (Hsp19.9, Hsp20.1, Hsp20.4, Hsp20.8, Hsp21.4, Hsp23.7, Hsp70, Hsp90 and heat shock cognate protein) were reported expressed highly in *B. mori* eggs (Fan *et al*., 2013; Hong *et al*., 2006). In this study, we examined these nine HSPs gene expression patterns in response to parasitization by *Telenomus theophilae*. Our aim was to understand the function of HSP in host-parasitoid interactions.

MATERIALS AND METHODS

Experimental insects

Egg parasitoids *Telenomus theophilae* Wu et Chen, were reared from wild silkworm *Bombyx mandarina* eggs collected at Tongxiang city, Zhejiang province in China. Once parasitoid eclosion, *T. theophilae* adults were held together for mating and fed with 50% (v/v) honey solution absorbed on cotton at 25±1°C. Silkworm

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Bombyx mori (Dazao) larvae were reared with fresh mulberry leaves. Pupae were kept at 25°C. After silkworm moths emerged, they were mated. Eggs were collected within 12 h for *T. theophilae* parasitism.

Parasitism

The silkworm eggs were collected within 1 h and divided into 50 eggs each sample. Each egg sample was exposed to 5 mated female wasps of *T. theophilae* for parasitism. After 3 and 24 h parasitization, the eggs were collected for RNA extraction. The eggs without parasitism were taken as control. Each treatment was repeated 3 times.

RNA extraction and cDNA synthesis

The eggs were homogenized in mortar with liquid nitrogen. Total RNA was isolated from silkworm eggs with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and quantity of the extracted RNA were quantified by the ratio of OD_{260}/OD_{280} by a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Wilmington, DE). The RNA samples were treated with RQI RNase-free DNase (Promega) to remove any contaminating DNA following the manufacturer's instructions. Purified RNA (1 µg) was reverse-transcribed in a 20 µl reaction mixture with random hexamers primer using using M-MLV reverse transcriptase (TAKARA, Japan) according to the manufacture's instruction.

Real time quantitative PCR

Primers for HSPs and Actin A3 (cytoplasmic actin A3, Genbank accession number: U49854) of *B.mori* were designed using the online Primer3 internet based interface (http://frodo.wi.mit.edu) (Table I). The real time quantitative PCR (RT-qPCR) was performed in 20 µl reactions containing 10 µl 2 × SYBR Premix Ex TaqII (Tli RNase Plus) (Takara), 1 µl of each primer, 1 µl of 1:10 diluted cDNA templates and 7 µl RNase-free H_2O. RT-qPCR was performed using a CFX96TM real-time detection system (Bio-Rad, California, USA), using the following procedure: initial denaturation at 95°C for 30 s, followed by 40 cycles of amplification (95°C for 5 s, and 60°C for 30 s) and a final extension at 72°C for 25 s. A melting curve analysis (65–95°C) for each reaction was determined to confirm the unique and specific PCR product. The relative expression level was determined according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All cDNA samples were normalized using *B. mori* Actin A3 as an internal control. Each biological treatment was repeated three times. The data were presented as the means ± standard error (S.E).

Statistical analysis

Data were analyzed using one-way ANOVA analysis with Tukey's test by DPS software (version 9.50) (Tang and Zhang, 2013). All data were represented as means ± standard deviation (S.D.). Differences were considered significant at $P < 0.05$.

Table I.- Primers used for real-time PCR in this study.

Primer name	Sequence (5'---3')
HSP19.9-S	CCGGAAGATTTTCTCAGTGC
HSP19.9-A	TTGCCTTCAACCACGATGTA
HSP20.1-S	GCCAACGATGTCCAGAGATT
HSP20.1-A	CTGCCTCTCCTCGTGCTTAC
HSP20.4-S	AAGAAAGACGAGCACGGGTA
HSP20.4-A	TCTTCGCTCTGGTCCTTGAT
HSP20.8-S	GACCTCGGTTCCAGCATAAA
HSP20.8-A	GAACCCCGTCTGATGACAGT
HSP21.4-S	CCGAAATGAGGAAGATGGAA
HSP21.4-A	GAATGAGCGGCGAGTTTAAG
HSP23.7-S	GGACGAGCACGGATACATTT
HSP23.7-A	CCGGGCCAGTTTTAGTGATA
HSP70-S	TTCAGCAGGACATGAAGCAC
HSP70-A	ATGCCGGAACTGTGACTACC
HSP90-S	CAAGTCCATGCTTCCCGTAT
HSP90-A	ACACCGATGCACAAAAACAA
Hsc70-4-S	AAGTCTGAGGAGGTGCAGGA
Hsc70-4-A	GCTCGAATTTACCGAGCAAG
Actin A3-S	GCGGCTACTCGTTCACTACC
Actin A3-A	TGGCTTCCATACCCAAGAAC

RESULTS AND DISCUSSION

Expression of small heat shock proteins after parasitization

Small heat shock protein (sHSP) as molecular chaperones, protect proteins from being denatured during extreme conditions (Li *et al.*, 2009). Among 16 silkworm sHSPs, six were highly expressed in eggs. Compared with the control, the gene expressions of Hsp19.9, Hsp20.1, Hsp20.8 or Hsp23.7 were significantly up-regulated at 24 h post-parasitization (Fig. 1A,B,D,F). The expressions of Hsp20.4 and Hsp21.4 after 24 h parasitization were also up-regulated but had no significant difference (Fig. 1C,E). At 3 h post-parasitization, mRNA expressions of six sHSP were up- or down-regulated, but all had no significant difference compared with non-parasitization control (Fig. 1). sHSP genes (Hsp19.9, Hsp20.1, Hsp20.4, Hsp20.8, Hsp23.7) from *B. mori* were induced in the larval fat body, testis and ovary by heat stress, where Hsp21.4 was down-regulated (Li *et al.*, 2012; Sakano *et al.*, 2006). In flesh fly *Sarcophaga crassipalpis* response to envenomation by

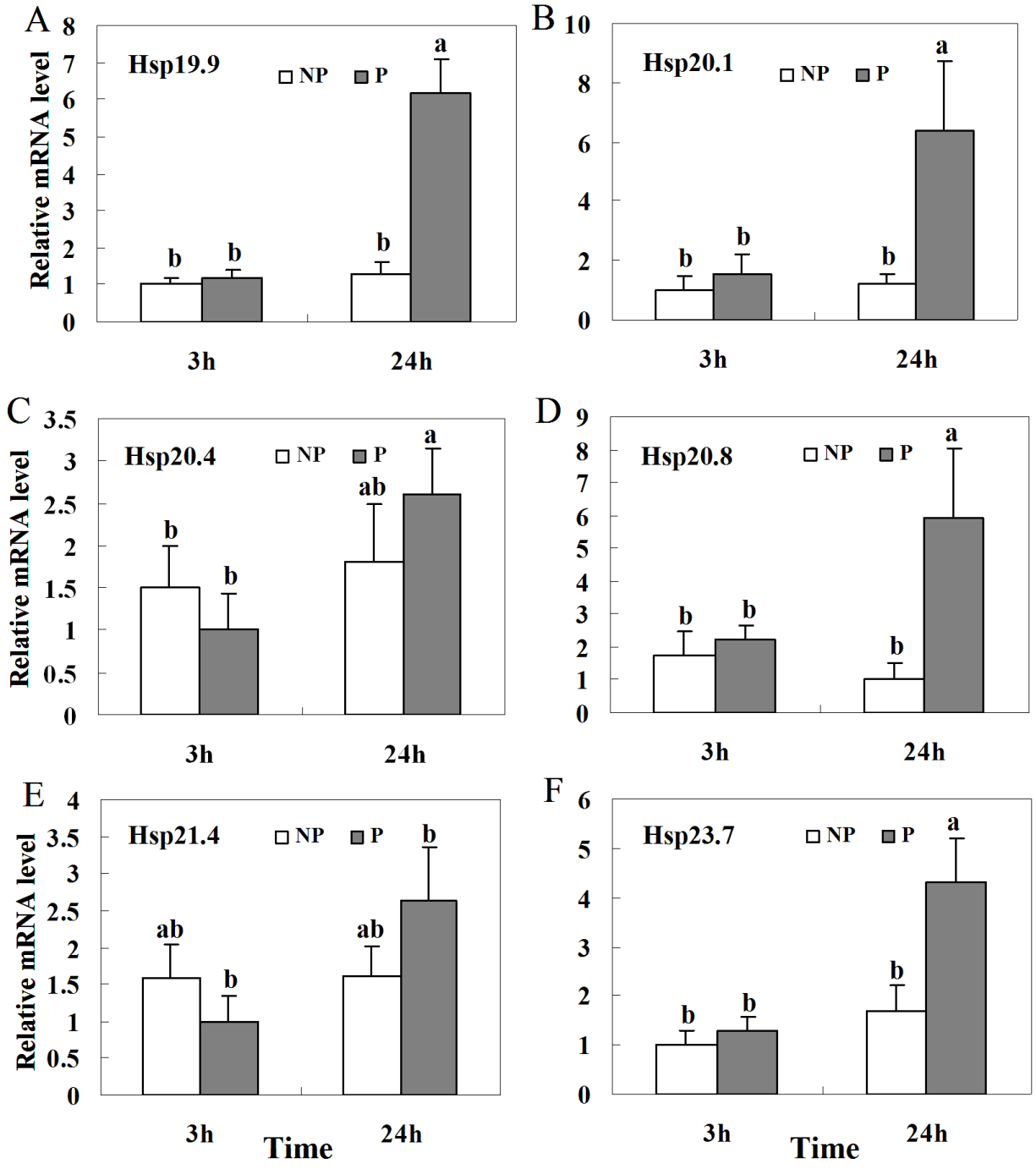

Fig. 1. Transcript expression levels of small heat shock proteins in the eggs of *Bombyx mori* following parasitization by *Telenomus theophilae*. The mRNA expression levels for all samples were assessed by real time quantitative polymerase chain reaction. Each treatment was repeated 3 times. All transcript levels were normalized to the control gene *Actin A3*. All data were represented as means ± standard deviation (S.D.). Difference letters indicated significant differences at P <0.05. NP, non-parasitization; P, parasitization. A, Hsp19.9; B, Hsp20.1; C, Hsp20.4; D, Hsp20.8; E, Hsp21.4; F, Hsp23.7.

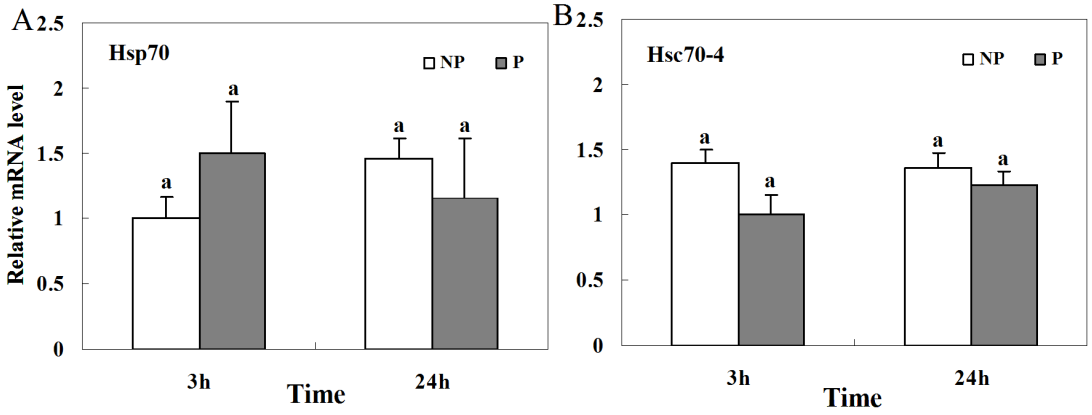

Fig. 2 .Transcript expression levels of heat shock protein 70s in the eggs of *Bombyx mori* following parasitization by *Telenomus theophilae*. Details are as the same as Fig. 1. A, Hsp70; B, Hsc70-4.

the ectoparasitic wasp *Nasonia vitripennis*, Hsp23 expression was highly upregulated 13 h after envenomation (Rinehart *et al.*, 2002). The expression of sHSP gene (GenBank Accession No. U94328) in *Plodia interpunctella* larvae was increased, and did not decrease until 4 days after by envenomation of ectoparasitoid *Bracon hebetor* (Shim *et al.*, 2008). Hsp20 mRNA in *Pieris rapae* pupae was clearly up-regulated between 12-48 h post-parasitization by *Pteromalus puparum* (Zhu *et al*., 2013).

Expression of Hsp70s after parasitization

The level of Hsp70 in *B. mori* eggs was up-regulated at 3 h post-parasitization, down-regulated at 24 h, but both had no significant difference compared with control (Fig. 2A). The expressions of Hsc70-4 gene were down-regulated at 3 h and 24 h, and had no significant difference with non-parasitization (Fig. 2B). Hsp70 in the fat body, testis and ovary of *B. mori* was down-regulated in heat-treated larvae (Li *et al*., 2012). BmHsc70-4 was expressed at steady-state levels throughout the BmNPV infection (Iwanaga *et al.*, 2014). Hsp70 in *S. crassipalpis* was highly upregulated 13 h, but Hsc70 in *S. crassipalpis* was downregulated slightly after envenomation by *N. vitripennis* (Rinehart *et al.*, 2002). The level of Hsp70 in *P. interpunctella* larvae was gradually increased and with a high level until 4 days after envenomation by *B. hebetor* (Shim *et al*., 2008). The transcription of Hsp75 in *P. rapae* was down-regulated by *P. puparum* parasitization (Zhu *et al*., 2013).

Expression of Hsp90 after parasitization

The expression of Hsp90 in *B. mori* eggs after parasitization had no signification difference compared with control (Fig. 3). Hsp90 mRNA in nondiapausing larvae of the apple maggot, *Rbagoletis pomonella*, was strong up-regulated in response to heat (Lopez-Martinez and Denlinger, 2008). The amount Hsp90 in *Spodoptera frugiperda* was unchanged during infection, but with supportive role in virus replication (Lyupina *et al*., 2011). Hsp90 in *S. crassipalpis* were downregulated slightly when compared to unenvenomated controls (Rinehart *et al*., 2002). The level of Hsp90 gene in *P. interpunctella* larvae was not influenced by *B. hebetor* (Shim *et al*., 2008). The expression of Hsp75 gene in *P. rapae* was down-regulated by *P. puparum* parasitization (Zhu *et al*., 2013).

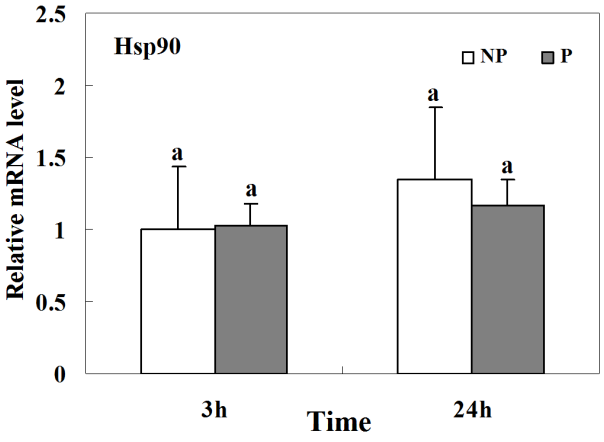

Fig. 3 Transcript expression levels of heat shock protein 90 in the eggs of *Bombyx mori* following parasitization by *Telenomus theophilae*. For other details, see Figure 1.

In conclusion, this study reported the gene expression of the HSPs in *B. mori* eggs by *T. theophilae* parasitization. Each HSP gene was differentially influenced by parasitization. Previous studies showed different HSP participated in different physiological process (King and MaCrae, 2015). Upregulation of HSP genes may play important roles in silkworm eggs against parasitoids. The alternated transcript of HSP may be a component of the host syndrome after parasitization, or a physiological change for growth-arrested host (Shim *et al*., 2008). The information would be helpful to understand the roles of HSPs in host-parasitoid relationship.

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REFERENCES

Abdel-Latief, M. and Hilker, M., 2008. Innate immunity: Eggs of *Manduca sexta* are able to respond to parasitism by *Trichogramma evanescens*. *Insect Biochem. Mol. Biol.*, **38**: 136-145.

Asgari, S. and Rivers, D.B., 2011. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. *Annu. Rev. Ent.*, **56**: 313-335.

Fan, L.F., Lin, J.R., Zhong, Y.S. and Liu, J.Y., 2013. Shotgun proteomic analysis on the diapause and non-diapause eggs of domesticated silkworm *Bombyx mori*. *PLoS One*, **8**: e60386.

Hamed, M., Nadeem, S. 2012. Effect of cereals on the development of *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) and subsequent quality of the egg parasitoid, *Trichogramma chilonis* (Ishii) (Hymenoptera: Trichogrammatidae). *Pakistan J. Zool.*, **44**: 923-929.

Hong, S.M., Nho, S.K., Kim, N.S., Lee, J.S. and Kang, S.W., 2006. Gene expression profiling in the silkworm, *Bombyx mori*, during early embryonic development. *Zool. Sci.*, **23**: 517-528.

Iwanaga, M., Shibano, Y., Ohsawa, T., Fujita, T., Katsuma, S. and Kawasaki, H., 2014. Involvement of HSC70-4 and other inducible HSPs in *Bombyx mori* nucleopolyhedrovirus infection. *Virus Res.*, **179**: 113-118.

Jamil, A., Ul Abdin, Z., Arshad, M., Falabella, P., Abbas, S.K., Tahir, M., Jamil, A., Manzoor, A. and Shaina, H., 2015. Multiple effects of host density on egg density and the sex ratio of progeny of *Bracon hebetor* (Say.) (Hymenoptera: Braconidae). *Pakistan J. Zool.*, **47**: 455-460.

King, A.M. and MaCrae, T.H., 2015. Insect heat shock proteins during stress and diapause. *Annu. Rev. Ent.*, **60**: 59-75.

Kraaijeveld, A.R. and Godfray, H.C.J., 2009. Evolution of host resistance and parasitoid counter-resistance. *Adv. Parasit.*, **70**: 257-280.

Li, J., Moghaddam, S.H.H., Du, X., Zhong, B.X. and Chen, Y.Y., 2012. Comparative analysis on the expression of inducible HSPs in the silkworm, *Bombyx mori*. *Mol. Biol. Rep.*, 39: 3915-3923.

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

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, **25**: 402-408.

Lopez-Martinez, G. and Denlinger, D.L., 2008. Regulation of heat shock proteins in the apple maggot *Rhagoletis pomonella* during hot summer days and overwintering diapause. *Physiol. Ent.*, **33**: 346-352.

Lyupina, Y.V., Zatsepina, O.G., Timokhova, A.V., Orlova, O.V., Kostyuchenko, M.V., Beljelarskaya, S.N., Evgen'ev, M.B. and Mikhailov, V.S., 2011. New insights into the induction of the heat shock proteins in baculovirus infected insect cells. *Virology*, **421**: 34-41.

Pennacchio, F. and Strand, M.R., 2006. Evolution of developmental strategies in parasitic hymenoptera. *Annu. Rev. Ent.*, **51**: 233-258.

Rinehart, J.P., Denlinger, D.L. and Rivers, D.B., 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. Inverteb. Pathol.*, **79**: 62-63.

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

Shim, J.K., Ha, D.M., Nho, S.K., Song, K.S. and Lee, K.Y., 2008. Upregulation of heat shock protein genes by envenomation of ectoparasitoid *Bracon hebetor* in larval host of Indian meal moth *Plodia interpunctella*. *J. Inverteb. Pathol.*, **97**: 306-309.

Stanley, D.W. and Miller, J.S., 2006. Eicosanoid actions in insect cellular immune functions. *Ent. exp. Appl.*, **119:** 1-13.

Sun, S.H., Lou, B.G., Yang, T.T. and Gao, Q.K., 2007. Separation and purification of host recognition kairomone of *Telenomus theophilae* by reversed phase high performance liquid chromatography. *Chinese J. Anal. Chem.*, **35**: 1004-1007.

Tang, Q.Y. and Zhang, C.X., 2013. Data processing system (DPS) software with experimental design, statistical analysis and data mining developed for use in

entomological research. *Insect Sci.*, **20**: 254-260.

Zhang, C.F., Dai, L.S., Wang, L., Qian, C., Wei, G.Q., Li, J., Zhu, B.J. and Liu, C.L., 2015. Inhibitors of eicosanoid biosynthesis influencing the transcripts level of sHSP21.4 gene induced by pathogen infections, in *Antheraea pernyi*. *PLoS One*, **10**: e0121296.

Zhu, J.Y., Wu, G.X., Ye, G.Y. and Hu, C., 2013. Heat shock protein genes (hsp20, hsp75 and hsp90) from *Pieris rapae*: Molecular cloning and transcription in response to parasitization by *Pteromalus puparum*. *Insect Sci.*, **20**: 183-193.