Expression Patterns of Heat Shock Protein Genes and Antioxidase Genes in *Apis cerana cerana* (Hymenoptera: Apidae) under Heat Stress[1]

Xinyu Li[2], Weihua Ma[3], and Yusuo Jiang[4,5]

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Abstract Heat stress elicits the expression of various antistress proteins (e.g., heat shock proteins [HSPs] and antioxidase enzymes) in honeybees (Hymenoptera: Apidae), which are important in protecting cells from heat-induced stresses. In this study, we used real-time quantitative reverse transcription–polymerase chain reaction to analyze the expression patterns of the heat shock protein 90 (Hsp90), heat shock protein 70Ab (Hsp70Ab), peroxidase (Pod), and cytochrome P450 (Cyp450) in response of *Apis cerana cerana* F. to different temperatures and different heat exposure times. We observed that, with the increase of temperature and exposure time, the expression of the four genes also increased, thus confirming that heat stress can activate heat-resistant mechanisms of *A. cerana cerana* and that temperature and exposure time are key factors affecting the accumulation of HSPs. Our results provide information on the expression patterns of four genes during heat stress to serve as a basis for determining the mechanisms by which *A. cerana cerana* adapts to thermal stress.

Key Words *Apis cerana cerana*, heat shock protein, antioxidase, thermal stress

Honeybees (Hymenoptera: Apidae) are important pollinators of numerous plants and crops. They are vulnerable to a variety of environmental stressors, such as temperature, humidity, pollutants, pesticides, parasites, and food quality, which have varying degrees of impact on their physiological metabolism (Alqarni et al. 2019, Annoscia et al. 2020, Ayton et al. 2016, Chaimanee et al. 2012, Di Pasquale et al. 2013, Monchanin et al. 2021). Of those environmental factors that affect honeybee physiology, temperature is one of the most important factors, especially heat stress (Abou-Shaara et al. 2012, Medina et al. 2020). With global warming and the widespread use of honeybees to pollinate greenhouse crops, the probability of honeybees suffering from heat stress has greatly increased (Blayt-Erekien et al. 2010). Greenhouses are characterized by high internal temperature and humidity, which can significantly impact the life of pollinating bees. Honeybees can keep the temperature in the nest within suitable range, but for foraging bees, the variable environment is a challenge.

Heat shock proteins (HSPs) are highly conserved proteins that exist in all biological cells and are newly synthesized or increased in content when cells or

[1]Received 31 May 2022; accepted for publication 14 July 2022.
[2]Shandong Vocational College of Light Industry, Zibo, Shandong Province, China.
[3]College of Horticulture, Shanxi Agricultural University, Taiyuan, Shanxi Province, China.
[4]College of Animal Sciences, Shanxi Agricultural University, Taigu, Shanxi Province, China.
[5]Corresponding author (email: jiangys-001@163.com).

organisms are subjected to heat or other stresses (Samali and Cotter 1996). Likewise, heat stress can induce the synthesis of HSPs in honeybees, which may protect the honeybee from thermal damage. Temperature level and exposure time are the key factors affecting the accumulation of HSPs (Ma et al. 2019). Previous studies have reported that *Apis mellifera* L. increased expression of HSPs in vivo when exposed to thermal stress (Koo et al. 2015). The widespread expression of HSPs in honeybees proves that they play an important role in the response of honeybees to heat stress (Zhao and Jones 2012).

Classified according to molecular weight, HSPs are divided into Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp20, and Hsp10 (Feder and Hofmann 1999). The Hsp90 and Hsp70 families are the most common HSPs in insects. Hsp90 acts as a molecular chaperone to bind to denatured proteins and maintain their normal folded state (Schopf et al. 2017). The main functions of Hsp70 are to help protein fold correctly, hydrolyze unstable proteins, guide protein transmembrane transport, and assist protein localization (Fernández-Fernández and Valpuesta 2018).

Some environmental stimuli can lead to oxidative stress in insects and produce reactive oxygen species (ROS) and metabolites. Heat stress is also an important inducement to cause oxidative stress in insects (Yan et al. 2013). Antioxidant enzymes have the function of removing excess ROS in the body (He et al. 2017). Detoxification enzymes can regulate the metabolism and degradation of endogenous or exogenous compounds (Liska 1998). These enzymes can protect the body from environmental stress. Under adverse environments, such as high temperature, the activity of antioxidant enzymes in insects increases significantly, and multiple enzymes work together to regulate metabolism and respond to the stress. Studies with honeybees show that high temperature increases the activities of various antioxidant enzymes and detoxification enzymes, which improves the tolerance of honeybees (Li et al. 2019).

Heat stress is an important abiotic factor that affects the physiological metabolism of honeybees and has an extremely important impact on the entire life history of honeybees. To resist thermal stress, different species of honeybees have gradually developed their own unique defense mechanisms during the long evolutionary process. *Apis cerana cerana* F. is a unique honeybee genetic resource in China. Compared with *A. mellifera*, *A. cerana cerana* has a keen sense of chemoreception, exhibits resistance to parasitic mites, is an effective forager in collecting scattered nectar resources, and resists adverse conditions such as cold resistance and heat resistance. However, with the widespread rearing of *A. mellifera* in China, relatively little attention has been paid to the native honeybee species, *A. cerana cerana*. Therefore, this study investigated the expression patterns of two HSP genes and two antioxidase genes in *A. cerana cerana* under different temperatures and different heat exposure times. The results of this study will provide greater understanding of the physiological mechanisms of heat tolerance in *A. cerana cerana* and information on the potential roles of these genes in heat stress resistance in the honeybee.

Materials and Methods

We selected one to two sealed brood combs as colony-level replicates from three healthy *A. cerana cerana* colonies in the experimental apiary of Shanxi

Table 1. Primers used for real-time quantitative reverse transcription–polymerase chain reaction.

Primer Name	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
*Acer*Hsp90	TGGCAAACAGTTGGTCTCTG	TTGGCTTTGTCTTCTTCACG
*Acer*Hsp70Ab	GATTCGCAAAGGCAAGCTAC	ATCCAAGCCATAAGCAATCG
*Acer*Pod	TTCTACCCGTTGTTCTCGGC	TGGACGAGAAATTGGCCGAA
*Acer*Cyp450	ATGGAAGCCGTTGAGAACAC	GGTTCGAGCACTCGAGAATC
β-actin	ACTACGGCCGAACGTGAAAT	GGAAAAGAGCCTCGGGACAA

Agricultural University (Taigu, China). Brood combs were placed in an incubator maintained at 34 ± 0.5°C and approximately 75–80% relative humidity (RH). After emergence from the cells, individual bees were marked on the back with a nontoxic, odorless paint and then returned to the original colony. At age of 20 d, the marked bees were collected with insect traps at the entrance of the hive.

Temperature stress. Six hundred adults ($n = 200$/hive) were collected, and each individual bee was placed into 15-ml centrifuge tubes with small pores. Bees were then divided into five groups ($n = 20$/group/hive) and exposed for 2 h at either 25°C, 30°C, 35°C, 40°C, or 45°C. RH was fixed at 30%. Similarly, five groups of bees ($n = 20$/group/hive) were exposed at 45°C, 30% RH for either 0, 0.5, 1, 1.5, or 2 h. We randomly selected five bees as a biological replicate from each group and each hive, three biological replicates for each treatment. These samples were frozen in liquid nitrogen and stored at −80°C for RNA extraction.

RNA extraction, cDNA synthesis, and qRT-PCR. Total RNA was extracted from the entire tissue using TRIzol Reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA degradation and contamination of each sample was monitored by separating the samples on a 1.5% agarose gel. The RNA concentration was determined using a QubitI RNA Assay Kit and QubitI 2.0 fluorometer (Life Technologies, Rockville, MD, USA).

First-strand cDNA was synthesized from 1 μg of total RNA using the ReverTra Ace-a First Strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan). Primers for the real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) were designed using Primer 3 software. Primers designed for each gene are provided in Table 1. The qRT-PCR thermal cycle procedure was as follows: 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. All samples were tested in triplicate. The relative quantification of PCR results was conducted using the $2^{-\Delta\Delta CT}$ method. The *β-actin* gene was used as an internal standard. The control treatment was at 25°C.

Statistical analyses. The data were statistically analyzed by analysis of variance (ANOVA), and the means were compared by the least significant difference test ($P < 0.05$). ANOVA was used to analyze the data and perform normality tests and isovariance tests. For data that failed these tests, we used the Kruskal–Wallis H test ($P < 0.05$).

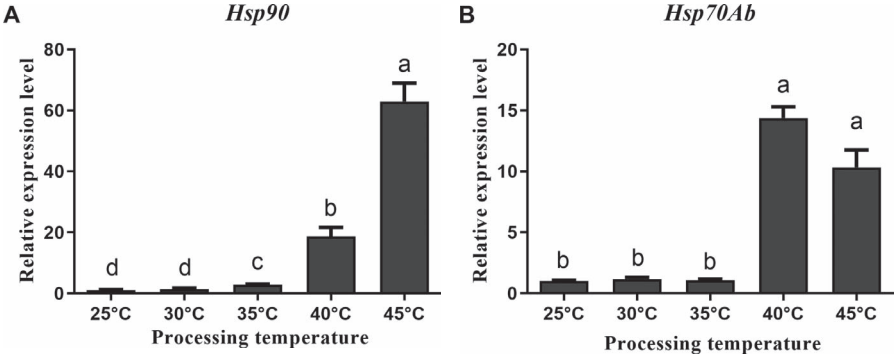

Fig. 1. Expression pattern of Hsp90 (A) and Hsp70Ab (B) at different temperatures as determined by real-time quantitative reverse transcription–polymerase chain reaction. The different letters indicate significant differences at different treatment temperatures ($P < 0.05$). Data are mean ± SEM.

Results

Temperature and exposure time effects on HSP genes expression. There were significant differences in the expression levels of Hsp90 and Hsp70Ab in 20-d-old adult *A. cerana cerana* exposed to temperatures of either 25°C, 30°C, 35°C, 40°C, or 45°C for 2 h (Fig. 1). Expression of the HSPs generally increased with increasing temperature, and it was apparent that heat stress could up-regulate the expression of the two genes, especially at 40°C and 45°C. The expression level of Hsp90 was highest at 45°C and was significantly higher ($F = 101.694$; df = 4; $P < 0.001$) than that for the other temperatures (Fig. 1A). The results of Hsp70Ab showed that expression level was highest at 40°C and were significantly higher ($F = 148.972$; df = 4; $P_{40} < 0.001$ and $P_{45} < 0.001$) at 40°C and 45°C than at the 25°C (Fig. 1B).

There were significant differences in the expression levels of the HSPs among the exposure times at 45°C (Fig. 2). The expression levels of Hsp90 and Hsp70Ab were comparatively low between 0 to 2 h of exposure, but increased exposure time up-regulated the expression of the two genes, especially following 1.5 or 2 h of exposure to 45°C. The results with Hsp90 showed an expression level that was highest at 2 h, and that expression levels were significantly higher ($F = 42.815$; df = 4; $P_{1.5} < 0.001$ and $P_2 < 0.001$) at 1.5 and 2 h than that for 0 h (Fig. 2A). The expression pattern of Hsp70Ab was similar to that for Hsp90, with the highest expression level at 2 h, and 1.5-h and 2-h expression levels being significantly higher ($F = 15.950$; df = 4; $P_{1.5} < 0.001$ and $P_2 < 0.001$) than those for 0 h (Fig. 2B).

Temperature and exposure time effects on antioxidase genes expression. Heat stress up-regulated the expression of Pod and Cyp450 compared with the expression at 25°C. The expression patterns of Pod and Cyp450 from 25°C to 45°C were similar to Hsp90 and Hsp70Ab, and there was a general trend of increasing expression level with increasing temperature. Pod showed the highest expression

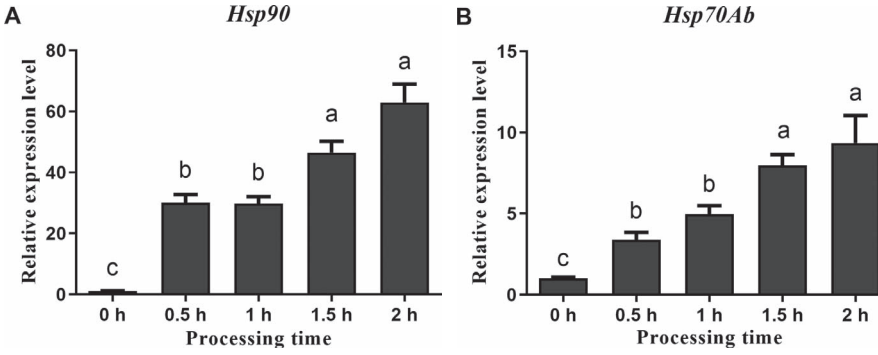

Fig. 2. Expression pattern of Hsp90 (A) and Hsp70Ab (B) at different exposure times as determined by real-time quantitative reverse transcription–polymerase chain reaction. The different letters indicate significant differences at different exposure times ($P < 0.05$). Data are mean ± SEM.

at 45°C, and there were no significant differences between the treatment groups (Fig. 3A). Cyp450 had highest expression at 40°C, and the expression levels at 40°C and 45°C were higher than other treatment groups (Fig. 3B).

For different heat exposure times, longer-term heat stress caused Pod and Cyp450 expression to increase with significant difference in the expression level among the different exposure times. Pod expression levels were highest at 2 h, with levels at 1.5 and 2 h of exposure significantly higher ($F = 4.928$; df = 4; $P_{1.5} = 0.009$ and $P_2 = 0.004$) than that at 0 h (Fig. 4A). The expression level of Cyp450 was similar to those for Pod, with the highest expression level at 2 h which was significantly higher ($F = 4.781$; df = 4; $P_{0.5} < 0.001$, $P_1 = 0.039$ and $P_{1.5} < 0.001$) than those at 0.5, 1, and 1.5 h (Fig. 4B).

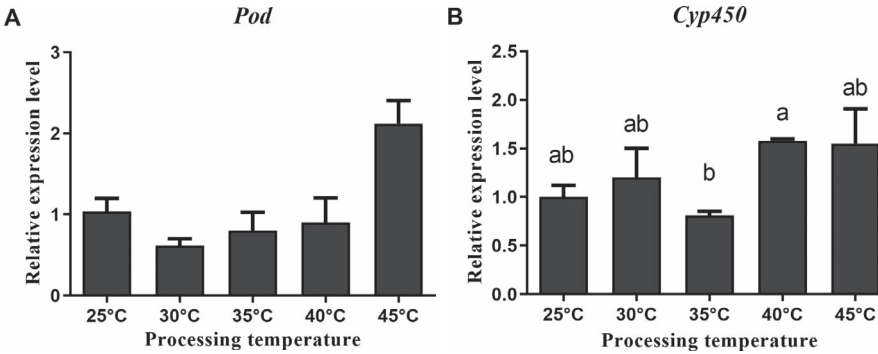

Fig. 3. Expression pattern of Pod (A) and Cyp450 (B) at different temperatures as determined by real-time quantitative reverse transcription–polymerase chain reaction. The different letters indicate significant differences at different treatment temperatures ($P < 0.05$). Data are mean ± SEM.

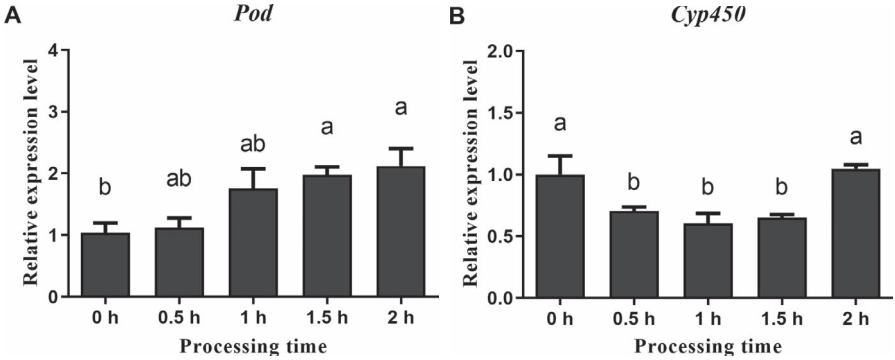

Fig. 4. Expression pattern of Pod (A) and Cyp450 (B) at different treatment times as determined by real-time quantitative reverse transcription–polymerase chain reaction. The different letters indicate significant differences at different exposure times ($P < 0.05$). Data are mean ± SEM.

Discussion

There are increasing reports from around the world of honeybees suffering from various stressors, including extreme temperatures, disease, parasites, pesticides, and malnutrition (Elekonich 2009, Koo et al. 2015). Honeybees have developed effective coping methods to adapt, and physiological and metabolic changes within honeybees provide them means to quickly respond to stressors (Even et al. 2012). Previous research reported some HSPs and antioxidase expression in *A. cerana cerana* in response to stress factors such as heat, pesticides, and infection (Li et al. 2022, Liu et al. 2014, Zhang et al. 2019). Our results in this current study demonstrated that thermal stresses induce the expression of HSP genes and antioxidase genes in *A. cerana cerana* foragers, with temperature level and exposure time to a constant temperature being two key factors affecting their expression.

Treatment temperature was the primary factor affecting the gene expression of HSPs and antioxidase in honeybee. There was little difference in the expression of Hsp90 and Hsp70Ab among the honeybees exposed to either 25°C, 30°C, or 35°C for 2 h. However, the expression of the HSP genes was significantly increased after exposure to temperatures of 40°C and 45°C for 2 h. Honeybees within hives use such behaviors as wing fanning to help maintain hive temperature when experiencing hot weather conditions. The normal temperature range within a colony is 33°C to 36°C (Kleinhenz et al. 2003, Petz et al. 2004), but when temperature rises above 36°C, larval development may be affected (Severson et al. 1990). Our results reflect these facts as temperatures of 25°C to 35°C had little impact on HSP expression in *A. cerana cerana*; yet, temperatures above 35°C triggered adaptive mechanisms to resist heat stress, prevent cellular damage, and to maintain cellular homeostasis. Both the Hsp90

family and the Hsp70 family have important effects on honeybee heat tolerance. Comparing the expression results of the two HSP genes, the relative expression level of Hsp90 in the high-temperature treatment levels (40°C and 45°C) was higher than that of Hsp70Ab. This result may indicate that Hsp90 is more sensitive to heat stress in *A. cerana cerana* and may play a greater role in *A. cerana cerana* tolerance to heat stress.

The elevated temperature also increased the expression level of antioxidase genes, with expression levels following exposure to the highest temperatures (40°C and 45°C) being higher (although not significantly) than that of other temperatures tested. These results indicate that heat stress can induce the response of *A. cerana cerana* antioxidant system, but from the expression level observed, heat stress may not be the main factor causing the antioxidant response.

Time of exposure to a constant high temperature also significantly affected the expression of HSP genes and antioxidase genes. As exposure time increased, expression of Hsp90 and Hsp70Ab also increased, especially with 1.5 h and 2 h or exposure where the expression of the two genes was significantly higher than other exposure times. Although the expression levels of the two genes after 0.5 h and 1 h of exposure were lower than those exposed for 1.5 h and 2 h, they also were significantly higher than those in the controls (0 h). Such results indicate that when *A. cerana cerana* are subjected to heat stress, large amounts of HSPs can be expressed in short periods of time and with the increase of exposure time, HSPs gradually accumulate to help *A. cerana cerana* cope with heat stress. Comparing the expression results of the two HSP genes, the relative expression level of Hsp90 was higher than that of Hsp70, similar to the expression pattern in different temperatures. This further confirms our hypothesis that heat stress is more likely to trigger the Hsp90 response, which may have an extremely important role in the heat tolerance of *A. cerana cerana*.

The expression level of Pod increased with exposure time, but the effect on Cyp450 was not obvious. This suggests that sustained heat stress leads to the accumulation of antioxidase in *A. cerana cerana*, but the effect is limited. Pod appears to be more responsive to heat stress than Cyp450, which may be due to the increase of ROS caused by heat stress.

Heat shock can trigger HSPs and antioxidase, inducing the expression of related genes in *A. cerana cerana*. In our results, the expression pattern of Hsp90 was the most prominent, which is also consistent with previous studies of *A. mellifera* (Ma et al. 2019). This indicates that Hsp90 plays an important role in the response of the honeybee to heat stress. The increased expression of antioxidase genes may be in response to the accumulation of ROS caused by high temperature. Extensive HSP activity in honeybees may have important implications for their ability to adapt to heat. The roles of different HSPs and antioxidase in honeybee heat stress resistance requires further study to determine their expression patterns and molecular characteristics.

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