Journal of Insect Science, (2023) 23(1): 4; 1–8
https://doi.org/10.1093/jisesa/iead002
Research

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Honeybees (Hymenoptera: Apidae) Adapt to the Shock of High Temperature and High Humidity Through Changes in Sugars and Polyols and Free Amino Acids

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Subject Editor: Lieceng Zhu

Received 19 August 2022; Editorial decision 19 December 2022.

Abstract

Temperature and humidity are important factors affecting the honeybees physiological metabolism. When honeybees are stressed by high temperature and high humidity, various physiological stress mechanisms evolved by bees are activated in response to injury. The accumulation of some sugars, polyols, and free amino acids can effectively protect cell structure stability and resist temperature stress. In this study, the changes of glucose, trehalose, cholesterol, sorbitol, sorbitol dehydrogenase, mannitol, and free amino acids content of worker honeybees [*Apis cerana cerana* Fabricius and *Apis mellifera* Ligustica (Hymenoptera: Apidae)] under different temperature and humidity conditions were measured. Our research results show that high temperature has an important impact on the metabolism of honeybees. Heat stress can cause the accumulation of various antistress substances in worker. The contents of sugars, polyols, and some free amino acids accumulated in high temperature were significantly higher than those in the control, while the influence of high humidity was less. Although high humidity was improved compared with the control, the difference was not obvious. It provides a theoretical basis for exploring the physiological mechanism of individual heat resistance of honeybees.

Key words: high temperature, high humidity, physiological metabolism, *Apis cerana*, *Apis mellifera*

Honeybees are a type of economic insects that are very important to human. Humans have obtained abundant bees products through beekeeping, and use bees to pollinate crops to improve the yield and quality of crops (Catarino et al. 2019). Throughout the life cycle of honeybees, they encounter numerous environmental stressors of physical, chemical, or biological origin, but they survive through physiological adaptations (Huang et al. 2012, Koo et al. 2015, Milone et al. 2020).

Temperature and humidity are important abiotic factors that affect honeybees physiology (Abou-Shaara et al. 2017). In natural, changes in temperature and humidity have an important impact on the physiological metabolism of bees (Dalmon et al. 2019, Giannoni-Guzmán et al. 2021). In particular, the high temperature and high humidity environment will have adverse effects on the survival, reproduction, and metabolism of bees (Li et al. 2019, Medina et al. 2020). Therefore, the heat tolerance of bees is very important for them to cope with extreme temperatures (Abou-Shaara et al. 2012).

With global warming and more extreme weather and the widespread use of greenhouse pollination, these are increasing the risk of heat stress for bees (Blazyte-Cereskiene et al. 2010). In order to reduce the damage of extreme temperature to themselves, in the process of evolution, honeybees have a series of adaptive regulation mechanisms. These mechanisms improve the heat tolerance of honeybees, allowing bees to adapt to extreme environmental changes (Elekonich 2009, Ma et al. 2019).

Under heat stress, the metabolism of insects will change, such as the increase of consumption rate of carbohydrates, the increase of lipid oxidation, and concentration of cholesterol, etc., which will also cause the increase of the synthesis of some polyols (sorbitol, mannitol) (Salvucci et al. 1999, Salvucci et al. 2000). The accumulation of polyols in organisms is very important to improve temperature tolerance. It can stabilize the natural conformation of proteins and offset the adverse effects of extreme temperatures (Salvucci et al. 2000). Sorbitol and mannitol are both six-carbon sugar alcohols, which are important substances for organisms to resist heat and cold. Studies on whiteflies and aphids (Hendrix and Salvucci 1998) have shown that the content of sorbitol and mannitol in their bodies is constantly changing throughout the day. When the temperature is higher at noon, the content is higher, and in the early morning and evening, the content is lower. This shows that polyols can be used as

heat inhibitors to protect these insects from the harmful effects of high temperatures. Sorbitol dehydrogenase (SDH) can reversibly catalyze the oxidation of D-sorbitol to D-fructose. It is the rate-limiting enzyme in the polyol metabolism pathway and plays a key role in the glycolysis bypass (Wang et al. 2013).

Glucose and trehalose are the main energy substance of bees. They not only provide energy for bees, but also a synthetic raw material for polyols (Tosi et al. 2017). The level of carbohydrate physiological indicators can directly reflect the individual energy metabolism of bees (Sinclair 2014). Secondly, trehalose is important product of stress metabolism and can form a special protective film on the cell surface under severe conditions such as high temperature, which can effectively protect the structure of biomolecules from being destroyed (Mizunoe et al. 2017). Under heat stress, lipid metabolism in insects will also change. Studies have reported that *Schitocerca gregaria* under heat stress significantly increases the cholesterol concentration in muscle cells and intensifies the peroxidation reaction (Downer and Kallapur 1981). On the other hand, studies have shown that free amino acid changes are related to the heat tolerance of insects. For example, the increase of free amino acids is closely related to the heat tolerance of *Drosophila melanogaster* (Schou et al. 2017).

High temperature and high humidity will significantly affect the physiological metabolism of insects, and insects can resist stress by synthesizing a series of antistress small molecules. Among them, polyols and free amino acids are important resistance substances for organisms to cope with adversity. In general, the relevant research mostly focuses on solitary insects, and little is known about social insects. *Apis cerana* and *Apis mellifera*, as typical social insects, are also the two most commonly raised bees by humans, and are important pollinators in greenhouses. Therefore, we used worker honeybee (*A. cerana* and *A. mellifera*) as experimental materials to measured the changes in the contents of glucose, trehalose, cholesterol, sorbitol, sorbitol dehydrogenase, mannitol, and free amino acids in honeybee under different temperature and humidity conditions, and explored the metabolic physiology changes of bee in high temperature and high humidity, and then understand the physiological mechanism of resisting high temperature and humidity.

Materials and Methods

Collection of Bees

We separately selected three healthy colonies of *A. cerana* and *A. mellifera* and 1~2 sealed brood combs from each colony as colony-level replication in this study, which were placed it in an incubator at 34 ± 0.5°C and 75%~80% relative humidity (RH). After emergence from the cell, the bees were marked on the back with nontoxic, odorless paint. Put the marked bees returned to the original colony, and then at the age of 20 d, the marked bees were collected at the entrance of the hive.

Experimental Design

The colony is regarded as a warm-blooded animal, because the temperature in the colony is relatively stable, and the bees in the colony are not easily subjected to temperature stress, but the changeable environment is a huge challenge for the outgoing foraging bees. In particular, pollinator bees in greenhouses are more susceptible to heat stress. Therefore, we choose the normal outdoor temperature of 25°C as the control temperature. When the temperature exceeds 40°C, bees stop going out to forage, so we set the extreme temperature of 45°C as the highest temperature. On the other hand, for the temperature and humidity data in the greenhouse, we choose 30%RH as the low humidity and 80%RH as the high humidity.

Three hundred adults (n = 100/hive) were collected and each individual bee was placed into 15 ml centrifuge tubes with small pores. Bees were then divided into four groups (n = 25/group) and treated for 2 h at 25°C, 30%RH (control: CK), 25°C, 80%RH (high humidity: HRH), 45°C, 30%RH (high temperature: HT), 45°C, 80%RH (high temperature add high humidity: HT + HRH). We randomly picked 10 bees as a biological replicate from each group, three biological replicates for each treatment. These samples were fully ground in liquid nitrogen and stored at −80°C.

Determination of Glucose Content

The 100 mg sample was taken and made up to 1.0 ml with phosphate buffer (0.1 M, pH 7.4) was mixed by shaking and kept 10 min. It was centrifuged at a speed of 3,000 rpm for 10 min. To the 50 µL supernatant, an equal volume of reagent (4-Aminoantipyrine 0.5 mmol/L, Glucose oxidase > 10 KU/L, Peroxidase > 1 KU/L, Phenol 5 mmol/L) were mixed and kept at 37°C for 15 min. The sample absorbance was then measured at 505 nm (Synergy H1 Microplate Reader, BioTek).

Determination of Trehalose Content

To the 100 mg sample, an 1 ml 10% Trichloro acetic acid was added and mixed and kept at room temperature for 45 min. It was centrifuged at a speed of 8,000 rpm for 10 min. To the 50 µL supernatant and 200 µL reagent (0.1 g Anthrone dissolved in 100 ml concentrated sulfuric acid) were mixed and kept at 95°C for 10 min. Measured the absorbance of each sample at 620 nm (Synergy H1 Microplate Reader, BioTek).

Determination of Total Cholesterol Content

The 100 mg sample was taken and made up to 1.0 ml with phosphate buffer (0.1 M, pH 7.4) was mixed by shaking and kept 10 min. It was centrifuged at a speed of 3,000 rpm for 10 min. To the 50 µL supernatant and 200 µL reagent (4-Aminoantipyrine 0.3 mmol/L, Esterase cholesterol > 50 KU/L, Cholesterol oxidase > 25 KU/L, Peroxidase > 1.3 KU/L, Phenol 5 mmol/L) were mixed and kept at 37°C for 10 min. Measured the absorbance of each sample at 510 nm (Synergy H1 Microplate Reader, BioTek).

Determination of Sorbitol Content

To the 100 mg sample, an 1 ml distilled water was added and mixed and in a 95°C water bath for 10 min. After cooling, it was centrifuged at a speed of 8,000 rpm for 10 min. The supernatant was taken as sample and kept at 25°C for 10 min. 200 µL supernatant and 60 µL reagent (Copper Sulfate 0.05 g/ml, Sodium hydroxide 0.1 g/ml) were mixed and kept at 25°C for 10 min. It was centrifuged at a speed of 10,000 rpm for 10 min. Measured the absorbance of each sample at 655 nm (Synergy H1 Microplate Reader, BioTek).

The standard (10 mg/ml) dissolve in 1 ml distilled water and diluted to 2, 1, 0.5, 0.25, 0.125, and 0 mg/ml respectively. Established standard curve based on the concentration and absorbance of the standard solution to calculated the content of sorbitol in the sample.

Determination of SDH Activity

The 100 mg sample was taken and made up to 1.0 ml with distilled water was mixed by shaking. It was centrifuged at a speed of 8,000 rpm at 4°C for 10 min. About 50 µL supernatant and 1 ml reagent (Sorbitol 2 g/L, Nicotinamide adenine dinucleotide 0.5 mmol/L)

were mixed and kept at 25°C for 5 min. Measured the absorbance of each sample at 340 nm (ND-1000 UV Spectrophotometer, NanoDrop) at the 20th s (A_1) and at 140s (A_2).

Determination of Mannitol Content

To the 100 mg sample, 70% methanol solution (in the ratio of weight (g): volume (ml) = 1:2) was added and mixed and filtered with filter paper. To 25 µL of supernatant, 25 µL sample diluent was added and mixed by shaking and kept 30 min at 37°C. The liquid was discarded, and the microplate was washed by washing solution. To 50 µL of chromogenic solution A and 50 µL chromogenic solution B was added and mixed and kept 10 min at 37°C. About 50 µL of stop solution was added and mixed the solution well. The sample absorbance was then measured at 450 nm (Synergy H1 Microplate Reader, BioTek). Established standard curve based on the concentration and absorbance of the standard solution to calculated the content of mannitol in the sample.

Determination of Free Amino Acids Content

To the 1 g sample, 8 ml hydrochloric acid (0.02 mol/L) was added and mixed. The sample kept in the dark for 2 h. It was centrifuged at a speed of 4,000 rpm for 10 min. To 1 ml of supernatant, 1 ml sulfosalicylic acid (6–8%), and 250 µL triethylamine acetonitrile solution (1 mol/L) was added and mixed. About 250 µL of phenyl isothiocyanate acetonitrile solution (0.1 mol/L) was added and mixed the solution well and kept at room temperature for 1 h. The supernatant and 2 ml n-hexan were added and mixed and kept at room temperature for 10 min. The lower layer solution was taken and detected (HPLC, Agilent 1260).

Statistical Analyses

The study was designed using the principle of complete randomization. The data were statistically analyzed by analysis of variance and the means were compared by the least significant difference test ($P < 0.05$).

Results

Glucose Content

Exposure of *A. cerana* and *A. mellifera* to high temperature and high humidity resulted in significant changed of glucose content compared to control. For *A. cerana*, high temperature add high humidity and high temperature resulted in significant elevation of glucose content compared to control and high humidity ($F = 53.393$; df = 3, df = 8; $P < 0.001$). For *A. mellifera*, high temperature was significantly higher than that of high humidity of glucose content ($F = 3.174$; df = 3, df = 8; $P = 0.016$).

Increase in glucose content was observed in high temperature add high humidity stressed *A. cerana* compared to *A. mellifera* ($F = 12.366$; df = 7, df = 15; $P = 0.009$), while in high humidity, the resulted was the opposite ($F = 12.366$; df = 7, df = 15; $P = 0.015$) (Fig. 1).

Trehalose Content

Keeping the *A. cerana* and *A. mellifera* at different temperature and humidity, did create change in the content of trehalose. For *A. cerana*, high temperature resulted in significant elevation of trehalose content compared to other treatment ($F = 4.936$; df = 3, df = 8; $P_{CK} = 0.005$, $P_{HRH} = 0.049$, $P_{HT*HRH} = 0.046$). For *A. mellifera*, there was no significant difference in trehalose content in each treatment.

The results showed that in control, the trehalose content of *A. mellifera* was higher than that of *A. cerana*, while in other treatment, the trehalose content of *A. cerana* was slightly higher than that of *A. mellifera* (Fig. 2).

Total Cholesterol Content

Keeping the *A. cerana* and *A. mellifera* at different temperature and humidity, did not create significant change in the content of total cholesterol. For *A. cerana*, high temperature add high humidity and high temperature resulted in elevation of total cholesterol content compared to control and high humidity. For *A. mellifera*, high temperature add high humidity was higher than that of other of total cholesterol content.

The results showed that the total cholesterol content of *A. mellifera* in high humidity was significantly higher than that of *A. cerana* ($F = 14.232$; df = 7, df = 15; $P = 0.008$), the total cholesterol content of *A. mellifera* was higher than that of *A. cerana* under high temperature add high humidity, while in high temperature and control, the total cholesterol content of *A. cerana* was significantly higher than that of *A. mellifera* ($F = 14.232$; df = 7, df = 15; $P_{CK} = 0.02$, $P_{HT} = 0.047$) (Fig. 3).

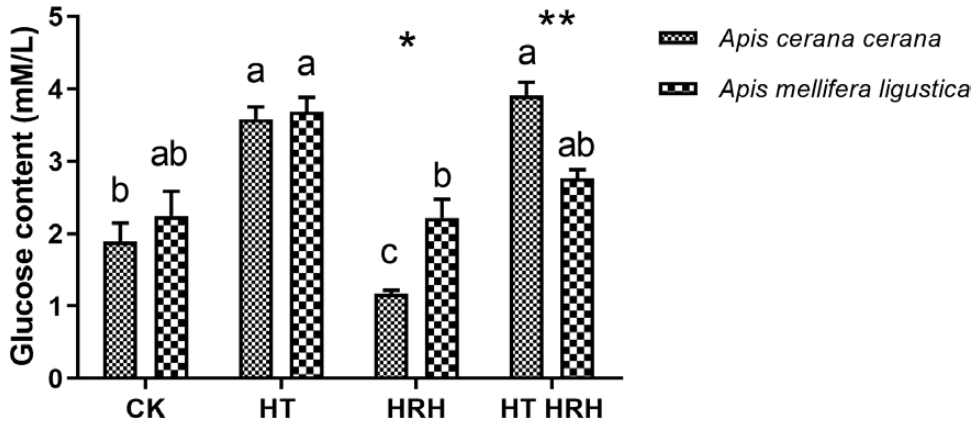

Fig. 1. Glucose content in *Apis cerana* and *Apis mellifera* after exposure to different temperature and humidity. Lowercase letters indicate significant differences between different temperatures and humidity ($P < 0.05$). **Indicates that the difference between different varieties under the same temperature and humidity treatment is extremely significant ($P < 0.01$). *Indicates that different varieties are significantly different under the same temperature and humidity treatment ($P < 0.05$). Data are mean ± SEM.

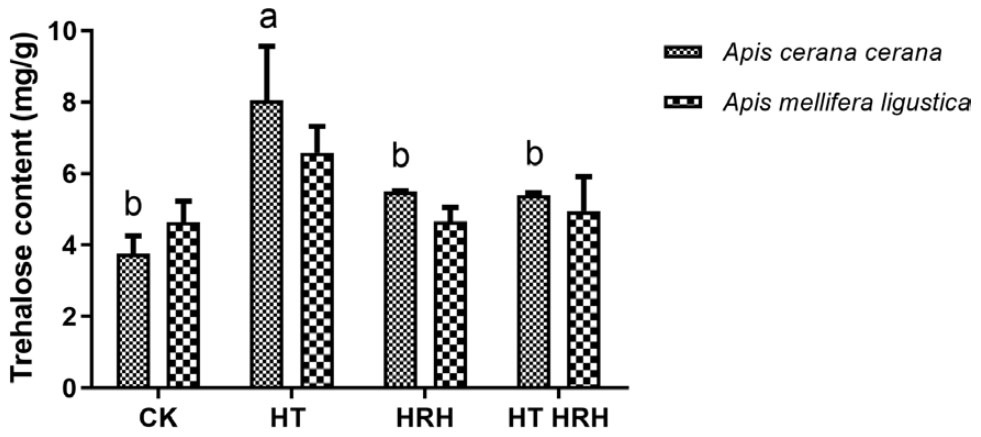

Fig. 2. Trehalose content in *Apis cerana* and *Apis mellifera* after exposure to different temperature and humidity. Lowercase letters indicate significant differences between different temperatures and humidity ($P < 0.05$). Data are mean ± SEM.

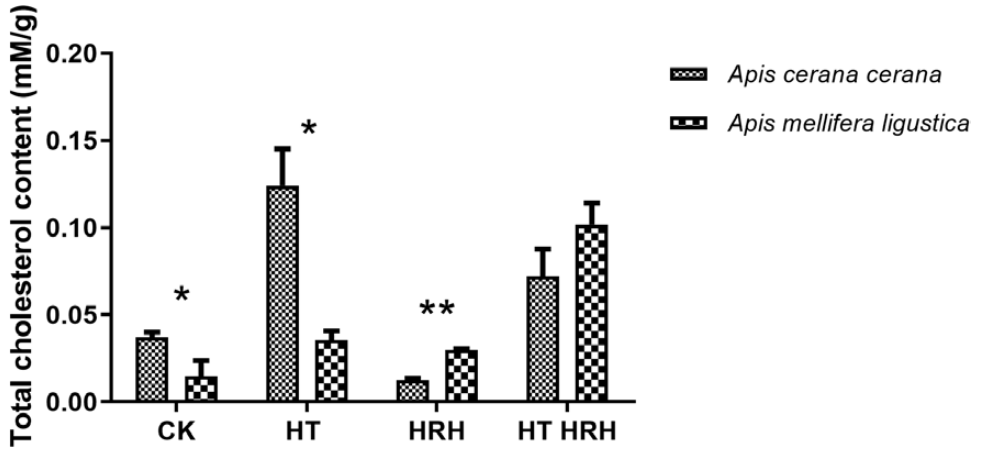

Fig. 3. Total cholesterol content in *Apis cerana cerana* and *Apis mellifera ligustica* after exposure to different temperature and humidity. **Indicates that the difference between different varieties under the same temperature and humidity treatment is extremely significant ($P < 0.01$). *Indicates that different varieties are significantly different under the same temperature and humidity treatment ($P < 0.05$). Data are mean ± SEM.

Sorbitol Content

Change in sorbitol content was resulted in different temperature and humidity stressed *A. cerana* and *A. mellifera*. For *A. cerana*, high temperature and high temperature add high humidity were significantly higher than that of control of sorbitol content ($F = 4.471$; df = 3, df = 8; $P_{HT} = 0.011$, $P_{HT+HRH} = 0.036$). For *A. mellifera*, high temperature resulted in significant elevation of sorbitol content compared to control ($F = 3.378$; df = 3, df = 8; $P = 0.02$).

In each treatment, the sorbitol content of *A. mellifera* was higher than that of *A. cerana*. Among them, in high temperature, there was a significant difference between the two ($F = 4.591$; df = 7, df = 15; $P = 0.028$) (Fig. 4).

Activity of Sorbitol Dehydrogenase

Response of sorbitol dehydrogenase to different temperature and humidity stress was different. For *A. cerana*, high temperature add high humidity resulted in significant elevation of SDH activity compared to high humidity ($F = 13.014$; df = 3, df = 8; $P = 0.001$). For *A. mellifera*, high temperature was significantly higher than that of other treatment of SDH activity ($F = 11.797$; df = 3, df = 8; $P_{CK} = 0.043$, $P_{HRH} = 0.044$, $P_{HT+HRH} = 0.02$).

Increase in SDH activity was observed in high temperature add high humidity stressed *A. cerana* compared to *A. mellifera* ($F = 141.758$; df = 7, df = 15; $P = 0.003$), while in high temperature, the resulted was the opposite ($F = 141.758$; df = 7, df = 15; $P = 0.031$) (Fig. 5).

Mannitol Content

Exposure of *A. cerana* and *A. mellifera* to high temperature and high humidity resulted in significant changed of mannitol content compared to control. For *A. cerana*, high temperature add high humidity and high temperature resulted in significant elevation of mannitol content compared to control and high humidity ($F = 36.310$; df = 3, df = 8; $P < 0.001$). For *A. mellifera*, high temperature add high humidity was significantly higher than that of control and high humidity of mannitol content ($F = 4.782$; df = 3, df = 8; $P_{CK} = 0.014$, $P_{HRH} = 0.015$).

In each treatment, the mannitol content of *A. cerana* was higher than that of *A. mellifera*. Among them, in high temperature, there was a significant difference between the two ($F = 36.310$; df = 7, df = 15; $P = 0.021$) (Fig. 6).

Free Amino Acids Content

Change in free amino acids content was resulted in different temperature and humidity stressed *A. cerana* and *A. mellifera*. For *A. cerana*, the content of each amino acid was significantly different

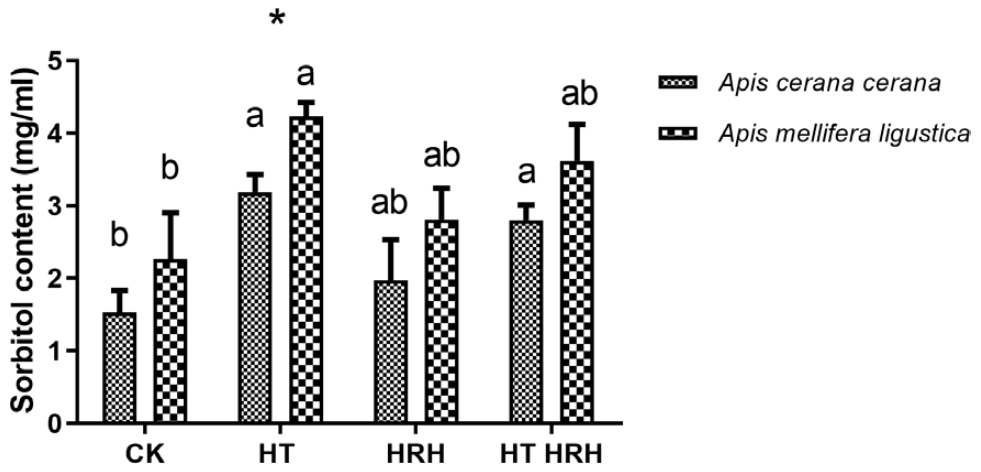

Fig. 4. Sorbitol content in *Apis cerana cerana* and *Apis mellifera ligustica* after exposure to different temperature and humidity. Lowercase letters indicate significant differences between different temperatures and humidity ($P < 0.05$). *Indicates that different varieties are significantly different under the same temperature and humidity treatment ($P < 0.05$). Data are mean ± SEM.

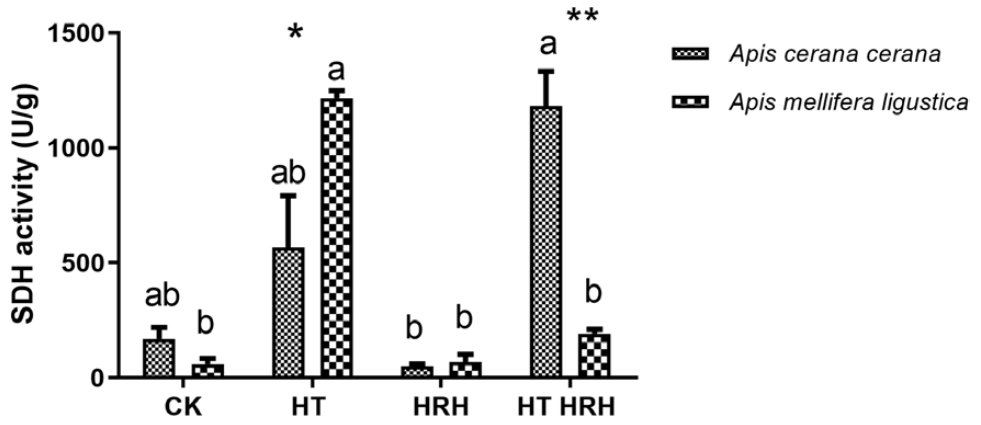

Fig. 5. Sorbitol dehydrogenase activity in *Apis cerana cerana* and *Apis mellifera ligustica* after exposure to different temperature and humidity. Lowercase letters indicate significant differences between different temperatures and humidity ($P < 0.05$). **Indicates that the difference between different varieties under the same temperature and humidity treatment is extremely significant ($P < 0.01$). *Indicates that different varieties are significantly different under the same temperature and humidity treatment ($P < 0.05$). Data are mean ± SEM.

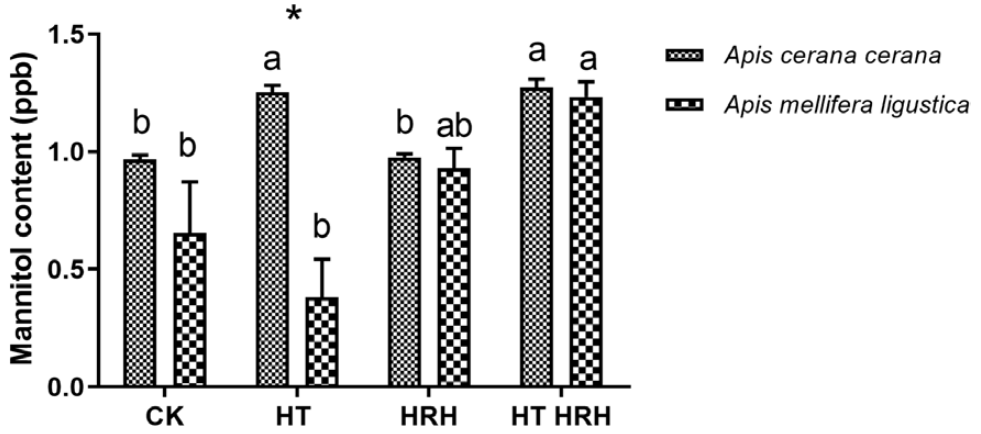

Fig. 6. Mannitol content in *Apis cerana cerana* and *Apis mellifera ligustica* after exposure to different temperature and humidity. Lowercase letters indicate significant differences between different temperatures and humidity ($P < 0.05$). *Indicates that different varieties are significantly different under the same temperature and humidity treatment ($P < 0.05$). Data are mean ± SEM.

between each treatment. Among them, the content of glycine and histidine was the highest in high temperature, and the content of glutamic acid, cystine, arginine, alanine, and lysine was the highest in the high humidity, and all other amino acids showed the highest content in the control; except for tyrosine, the content was the lowest in the high temperature, and the content of

other amino acids was the lowest in the high temperature add high humidity. For *A. mellifera*, except cystine, the contents of other amino acids were significantly different in each treatment. Among them, the contents of aspartic acid, tyrosine, and phenylalanine were the highest in the control, and all other amino acids showed the highest content in the high temperature; aspartic acid, lysine, and proline have the lowest content in the high temperature and high humidity, and other amino acids are the lowest in the high humidity.

By comparing the content of each amino acid in the two bee species, it can be found that, except for cystine, there are significant differences in the content of the other amino acids in the two bee species. In general, the results of the control and the high humidity were similar, while the results of high temperature and high temperature and high humidity were similar (Table 1).

Discussion

High temperature and high humidity will directly affect the development and reproduction of bees (Jones et al. 2005), as well as the pollination and collection behavior of bees (Joshi and Joshi 2010), greatly restricting the development of the colony. In order to resist heat stress, bee can synthesize some stress-resistant substances such as trehalose, polyols, and free amino acids, which play an important role in resistance to heat stress. We investigated how *A. cerana* and *A. mellifera* adapt to high temperature and high humidity shock through changes in glucose, trehalose, cholesterol, sorbitol, mannitol, and free amino acids catabolism.

Sugar is the main energy substance in bees. There are three main types of 'blood sugar' in bees: trehalose, glucose, and fructose. Among them, trehalose is the most, followed by glucose (Avonce et al. 2006). Glucose the changes in its physiological indicators reflect the individual energy metabolism of bees. Trehalose not only supplies energy for cells, but also synthesizes resistance substances such as sorbitol and it can protect cell membranes (Shukla et al. 2015). In this study, we measured the change of glucose and trehalose content in honeybees under high temperature and high humidity stress. High temperature did cause increase in glucose content with respect to that observed in control. This indicates that heat stress affects honeybee energy metabolism, which may be an important manifestation of high temperature accelerating honeybee metabolism, further indicating that the change of honeybee glucose content is an important response to high temperature stress. On the other hand, considering the protective effect of trehalose, high temperature leads to an increase in trehalose metabolism, indicating that trehalose accumulation may an important stress response for bees to cope with high temperature stress. Previous results of *Zaprionus indianus* exposure to heat stress also support our conclusions (Kalra et al. 2017).

Under heat stress, the oxidation reaction in insects is enhanced, and active oxygen increases. Some lipids are very sensitive to the peroxidation reaction caused by high temperature, and their corresponding metabolism will also change. Researchers (Wang et al. 2006) have observed increase of fat peroxidation in *Musca domestica* due to high temperature stress. This result is also consistent with this experiment, where we observed an increase in total cholesterol content in temperature-stressed honeybees (high temperature and high temperature add high humidity). This may prove from one side that heat stress leads to intensified peroxidation of bees.

Previous studies have proved that polyols can help insects resist temperature stress, and the representative substances are sorbitol and mannitol. Polyols have the function of stabilizing protein conformation and can prevent protein denaturation caused by high temperature (Minhyun et al. 2017). The accumulation of polyols is an important physiological mechanism for insects to respond to temperature stress (Doucet et al. 2009). We tried to reveal the changes in polyol metabolism of honeybees under high temperature stress. In the present study, high temperature and high temperature add high

Table 1. Each amino acid content in *Apis cerana* and *Apis mellifera* after exposure to different temperature and humidity

	Apis cerana				Apis mellifera				
	Control	High temperature	High humidity	High temperature add high humidity	Control	High temperature	High humidity	High temperature add high humidity	Varietal difference
Asp	1225.7 ± 289.9^{a}	825.9 ± 166.1^{ab}	1152.9 ± 145.5^{a}	456.9 ± 136^{b}	758 ± 49.9^{a}	732.1 ± 93.3^{a}	606.3 ± 57.9^{ab}	448.5 ± 18.3^{b}	*
Glu	5930.5 ± 791.8^{a}	6344.5 ± 146.5^{a}	6356.2 ± 265^{a}	4569.7 ± 386.5^{a}	4674.9 ± 482.8^{b}	6951.6 ± 579.3^{a}	3187 ± 237.9^{c}	4056.2 ± 338.7^{bc}	**
Cys	115.4 ± 15.1^{ab}	77.2 ± 5.6^{ab}	122.9 ± 16.6^{a}	67 ± 20.7^{b}	103.4 ± 21.2^{a}	115.5 ± 14.5^{a}	89.9 ± 5.2^{a}	92.3 ± 11.2^{a}	*ns*
Ser	446.8 ± 23.5^{a}	284.1 ± 24.3^{b}	441.3 ± 8.4^{a}	232.7 ± 32.8^{b}	301.8 ± 28.3^{ab}	383.3 ± 36.8^{a}	227.5 ± 22.7^{b}	301.8 ± 23.3^{ab}	**
Gly	964.8 ± 69.3^{a}	993.4 ± 15.1^{a}	928.7 ± 30.5^{a}	855.8 ± 44.5^{a}	868.5 ± 65.8^{bc}	1259.5 ± 93.9^{a}	693.1 ± 48.3^{c}	980.9 ± 86^{b}	*
Thr	934.4 ± 28.7^{a}	1124.6 ± 94.2^{a}	914.3 ± 42.3^{a}	910.7 ± 68.6^{a}	805.2 ± 43.4^{b}	1031 ± 74.3^{a}	700.2 ± 54.2^{b}	884.8 ± 82.2^{ab}	*
Ala	3246.1 ± 46.1^{a}	2774.4 ± 100.1^{b}	3158.8 ± 85.5^{a}	2444.1 ± 111.7^{c}	2369.9 ± 144.1^{bc}	3117.6 ± 190.8^{a}	1894.1 ± 110^{c}	2715.4 ± 272^{ab}	**
Pro	417.1 ± 15.5^{a}	258.8 ± 12.5^{b}	399 ± 12.4^{a}	236.1 ± 32.9^{b}	308.9 ± 33.8^{ab}	374.2 ± 26.9^{a}	248.5 ± 18.5^{b}	308.1 ± 28.6^{ab}	**
Tyr	630.9 ± 11.3^{a}	629.7 ± 28.5^{a}	637.7 ± 25.6^{a}	524.3 ± 84.4^{a}	427.5 ± 48.4^{b}	597.5 ± 19.3^{a}	318 ± 36.3^{b}	451.3 ± 46.9^{b}	**
Val	1878.1 ± 111.5^{a}	1519 ± 57.3^{bc}	1739.7 ± 95.2^{ab}	1245.2 ± 86.4^{c}	1929 ± 113.5^{ab}	2010.6 ± 73.6^{a}	1616.6 ± 93.6^{bc}	1510.1 ± 105.5^{c}	**
Met	584.9 ± 64.3^{a}	356.6 ± 60.1^{bc}	517.3 ± 43.1^{ab}	255.3 ± 67.8^{c}	299 ± 41.6^{a}	269.2 ± 29.1^{a}	208.3 ± 26.8^{a}	230.6 ± 43.01^{a}	**
Ile	471.2 ± 21.7^{a}	336.6 ± 9.4^{ab}	454.3 ± 13.4^{ab}	293.8 ± 41.7^{b}	329.5 ± 41.9^{ab}	407.7 ± 32.8^{a}	244.3 ± 14.2^{b}	307.4 ± 22.7^{ab}	**
Leu	324.7 ± 25.1^{a}	180.6 ± 13.6^{b}	302.5 ± 14.2^{a}	138.5 ± 16.5^{b}	192.8 ± 23.5^{a}	207.3 ± 22.1^{a}	140.2 ± 16.9^{a}	169.3 ± 18.34^{a}	**
Phe	461.9 ± 33.1^{a}	311.2 ± 6.9^{b}	441 ± 13.6^{a}	259.7 ± 24.6^{b}	300.9 ± 34.8^{ab}	379.6 ± 28.3^{a}	216.2 ± 20^{b}	288.7 ± 23.2^{ab}	**
Lys	901.7 ± 73.4^{a}	533.5 ± 35.4^{b}	855.6 ± 26.8^{a}	393.6 ± 39.3^{b}	523.2 ± 70.1^{ab}	576.6 ± 60.2^{a}	374.2 ± 44.6^{b}	412.7 ± 35.5^{ab}	**
His	688.6 ± 11.9^{a}	317.2 ± 8.5^{c}	605 ± 26.9^{b}	272.8 ± 26.4^{c}	333.8 ± 43.2^{a}	295.2 ± 34.2^{a}	248.6 ± 28.4^{a}	264.1 ± 28.1^{a}	**
Arg	892.4 ± 32.3^{a}	724.2 ± 193.8^{a}	910 ± 51.6^{a}	434.1 ± 56.4^{b}	595.5 ± 56.7^{a}	670.9 ± 77^{a}	597 ± 105.67^{a}	464.3 ± 39.5^{a}	**

Lowercase letters indicate significant differences in bees under different temperatures and humidity ($P < 0.05$). **Indicates that the difference between different varieties under the same temperature and humidity treatment is extremely significant ($P < 0.01$). *Indicates that different varieties are significantly different under the same temperature and humidity treatment ($P < 0.05$).

humidity did cause increase in sorbitol, mannitol content, and SDH activity with respect to that observed in control and high humidity. This result shows that high temperature stress can stimulate the accumulation of sorbitol and mannitol in the bees. This result is consistent with the changes in the contents of sorbitol and trehalose in the body of aphids and whiteflies after high temperature stimulation (Hendrix and Salvucci 1998). The accumulation of polyols may help honeybees to improve heat tolerance, an important physiological response to heat stress that is consistent with solitary insects.

The hemolymph of insects is rich in free amino acids. Under stress, these free amino acids may be important raw materials for the synthesis of antistress proteins, such as heat shock proteins (Liu et al. 2013, Sun et al. 2014, Garbuz 2017) and antimicrobial peptides (Chaimanee et al. 2012). This study found that the stress of high temperature and high humidity can greatly change the content of free amino acids in *A. cerana* and *A. mellifera*. For *A. cerana*, in addition to the four amino acids alanine, histidine, glycine, and glutamic acid, the other 13 amino acids all show a lower content in the high temperature or high temperature add high humidity, indicating that they have undergone depletion and conversion under high temperature stress. They may be involved in the synthesis of related stress-resistant proteins. For *A. mellifera*, the content of aspartic acid and proline in the high temperature and high humidity is lower, indicating that they may involved in the synthesis and metabolism of the anti-stress related substances of *A. mellifera*; glycine, glutamic acid, histidine, arginine, and alanine are higher in high temperature. We believe that the accumulation of these 5 amino acids may help protect cell membranes. In addition, other amino acids did not change significantly between different treatments, indicating that these amino acids did not have a high degree of participation in *A. mellifera* heat resistance.

According to previous studies, there is a certain difference in heat tolerance between *A. cerana* and *A. mellifera*, which may originate from complex and different physiological metabolic. For example, our previous research found that *A. cerana* were more tolerant than *A. mellifera* under extremely high temperature conditions, there were differences in the oxidative stress response between the two species (Li et al. 2019). Comparing the results between the two species, in general, they showed similar results after exposure to heat stress, albeit with some differences. This is consistent with the fact that these two bee species are closely related. The results of this experiment showed that the types of stress resistance accumulated by honeybees, as social insects, were basically the same as those of solitary insects under heat stress, indicating that the accumulation of these stress resistance substances is an important physiological regulation mechanism for insects to cope with stress. For different treatment, the results of high temperature and high temperature add high humidity are similar. Although the high humidity has improved compared with the control, the difference is not obvious. This result shows that the impact of high temperature on the metabolism of bees is much higher than that of high humidity, and the impact of high humidity on bees may still inhibition the loss of water by the bees, which intensifies heat stress.

Elevation of resistant substance related to heat stress with increase of sugar, polyol, and free amino acids are indicative of the physiological mechanisms that the bees employ in response to a stressor. However, under heat stress, which specific signaling pathways are activated? What is the relationship between oxidative stress and Hsp expression and these physiological responses in this study? We need further in-depth research to reveal how honeybees respond to thermal stimuli in their physiological metabolism.

Acknowledgments

This work was supported by the earmarked fund for China Agriculture Research System (CARS-44-KXJ22). The authors declare no conflict of interest.

Author Contributions

Xinyu Li designed and conducted experiments; Xinyu Li wrote first draft of manuscript and Weihua Ma and Yusuo Jiang contributed to revisions of manuscript.

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