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Expression of heat shock proteins in adult honey bee (*Apis mellifera* L.) workers under hot-arid subtropical ecosystems

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ABSTRACT

Heat stress elicits the expression of heat shock proteins (HSPs) in honey bee subspecies. These highly conserved proteins have significant role in protecting cells from thermal-induced stresses. Honey bees in subtropical regions face extremely dry and hot environment. The expression of HSPs in the nurses and foragers of indigenous (*Apis mellifera jemenitica*) and imported European (*Apis mellifera ligustica* and *Apis mellifera carnica*) honey bee subspecies after heat shock treatment were compared using SDS-PAGE. Hsp70 and Hsp82 were equally expressed in the nurses of all tested bee subspecies when exposed to 40 °C and 45 °C for 4 h. The forager bees exhibited differential expression of HSPs after heat stress. No HSPs was expressed in the foragers of *A. m. jemenitica*, and Hsp70 was expressed only in the foragers of *A. m. ligustica* and *A. m. carnica* at 40 °C. A prominent diversity in HSPs expression was also exhibited in the foragers at 45 °C with one HSP (Hsp70) in *A. m. jemenitica*, two HSPs (Hsp40 and Hsp70) in *A. m. carnica*, and three HSPs (Hsp40, Hsp60 and Hsp70) in *A. m. ligustica*. No HSPs was expressed in the control nurse and forager bees at any of the tested temperatures. These findings illustrated the differences in HSP expression among nurse and forager bees. It is obvious that the native foragers are more heat tolerant with least HSPs expression than exotic bee races. Further investigations will help to understand the potential role of HSPs in the adaptability, survival, and performance of bee subspecies in harsh climate of the subtropical regions.

1. Introduction

Honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) are dynamic pollinators of numerous crops. These honey bees are vulnerable to many environmental threats that may affect their physiology, performance, biology, and behavior. A range of environmental stresses, such as weather, pollutants, diseases, pollen quality and diversity, nutrition deficiency, and indiscriminate use of pesticides, are linked to sudden colony collapse disorder (CCD) of honey bees (Iqbal and Mueller, 2007; Iqbal, 2009; Klein et al., 2017; Iqbal et al., 2019b). This sudden population decline has aroused concerns of an emerging pollination crisis (Holden, 2006).

The Arabian Peninsula is a subtropical zone characterized by an extremely hot and arid environment (Abou-Shaara et al., 2017; Ali et al., 2017) that might have a significant impact on honey bee life (Abou-Shaara, 2014; Awad et al., 2017; Iqbal et al., 2019a; Joshi and Joshi, 2010). How honeybees survive in such a harsh climate is a big question to explore. The expression of heat shock proteins (HSPs) may be one of the potential targets to be linked with the survival and performance of honey bees in harsh climatic conditions.

HSPs are highly conserved and present in all living organisms from bacteria to human beings, but they vary in the pattern of their expression (Candido, 2001). These proteins are categorized according to their molecular weight (Schlesinger, 1990) and produced when cells are exposed to a temperature above their regular growth temperature. HSPs may also be produced in response to other stressful conditions, such as environmental stress, infection, cold, UV light, pesticides, wound healing and starvation (Zhang et al., 1998; Kanagasabai et al., 2011). Specific genes are activated

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in response to stress stimuli to encode HSPs. The principal HSPs are grouped into conserved classes on the basis of sequence homology and the molecular weight of encoding genes. These include Hsp110, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and some smaller molecular weight HSPs (Feder and Hofmann, 1999; Candido, 2001).

HSPs are synthesized constitutively in several insects, such as the silk worm, red flour beetle, mosquito species, *Drosophila,* Reduviid bug and honey bees (Garcia et al., 2003; Elekonich, 2009; Xu et al., 2010; Zhao et al., 2010). The temperature and its exposure time are very crucial for the synthesis of HSPs, which may protect the insects from thermal damage. The physiological state of the insect also affects HSP synthesis (King and MacRae, 2015). The widespread expression of HSPs in insects indicates their significant role in insect adaptability to a fluctuating environment (Zhao and Jones, 2012). Previous studies have reported the expression of HSPs in *Apis mellifera* L. in response to infection, pesticides and thermal stress (Severson et al., 1990; Gregorc and Bowen, 1999; Elekonich, 2009; Koo et al., 2015).

Apis mellifera jemenitica Ruttner is a widely domesticated indigenous honey bee subspecies in the Arabian Peninsula (Ruttner, 1976). It is also found throughout eastern Africa in Chad, Somalia, and Sudan (Engel, 1999). This is a well-adapted bee subspecies for beekeeping in local severe environment of central Saudi Arabia than the imported European subspecies (*A. m. ligustica* and *A. m. carnica*) (Alqarni, 2006; Alqarni et al., 2014). The Italian subspecies *A. m. ligustica* is distributed along the Italian Peninsula and currently found throughout the world. Similarly, the Carniolan subspecies *A. m. carnica* has been distributed throughout the world. It was originally occurred in south of the Alps, northern Italy, "previously" Yogoslavia and Romania (Engel, 1999). *A. m. jemenitica* is tolerant to heat and water loss in the hot and dry summers. In central Saudi Arabia, the summer temperature frequently reaches above 45 °C with extremely low humidity (Alqarni et al., 2011; Ali et al., 2017; Ali et al., 2019). However, the imported European honey bee subspecies are reported to have problems in terms of their survival under these harsh conditions (Abou-Shaara et al., 2012; Alattal and AlGhamdi, 2015). It was hypothesized that HSPs might have different patterns of expression among different honey bee subspecies to elicit a difference in heat tolerance among them. Therefore, the present study investigated the expression of HSPs in indigenous and imported European honey bee subspecies under controlled laboratory conditions. This study will help to understand the cellular and physiological mechanisms of heat tolerance in *A. m. jemenitica* and for further exploring the behavioral changes associated with expression of HSPs in response to various ecological challenges.

2. Materials and methods

The expression of HSPs was investigated in nurse and forager bees of three honey bee subspecies (*Apis mellifera jemenitica*, *Apis mellifera carnica* and *Apis mellifera ligustica*) during Nov-Dec 2015. The apiaries were kept at experimental farm (24.73°N, 46.61°E and 658 m altitude) of King Saud University (KSU), Riyadh, Saudi Arabia.

2.1. Exposure of honey bees to heat stress

Adult nurse honey bees were collected from the sealed brood area, and foragers were captured from the entrance of the beehives. The temperature range at the time of bee collection was 27–36 °C with relative humidity 30–40%. The bees were kept in falcon tubes at 35 °C for 20 min in a water bath for acclimatization before heat stress. Afterwards, the bees were exposed to heat shock (40 °C or 45 °C) for 4 h in an incubator in two separate groups. A stress treatment for 4 h was adopted due to the probability of maximum HSP response in *Apis mellifera* (Severson et al., 1990). A constant volume (1 μL) of the hemolymph was extracted from each bee and stored at −20 °C in a final volume of 10 μL of buffer solution (Tris-HCl, 10% SDS, 50% glycerol, distilled water and 2-mercaptoethanol) for the subsequent analysis. From the control groups, 1 μL of hemolymph was extracted from each bee immediately after collection and stored in the buffer solution at −20 °C similar to the heat-stressed hemolymph samples.

2.2. Expression of proteins in SDS-Page analysis

The proteins from samples of bee's hemolymph were expressed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Atto corporation, AE-6530 M, Japan) according to the standard protocol (Severson et al., 1990). Ten microliters of the hemolymph samples were loaded on 8% acrylamide gel, and electrophoresis was performed with 20 mA current for 5 h. The standard protein ladders (BenchMark: Novex, life technologies and Pink plus: GeneDireX, Inc.) were also loaded on gels to assess the molecular size (kDa) of the expressed proteins (Wilson and Walker, 2010). The gel was stained with standard Coomassie blue stain (0.1% Coomassie R250, 10% acetic acid and 40% methanol) overnight, rinsed with distilled water and de-stained with de-staining solution (20% methanol, 10% acetic acid and ddH_2O) until there was clear visibility of bands on the gel. The pictures of the gel were taken using a gel documentation system (Biometra GmbH, Germany).

3. Results

Fig. 1 reveals the expression of two HSPs (Hsp70 and Hsp82) in the nurse bees of three subspecies (*A. m. carnica*, *A. m. ligustica* and *A. m. jemenitica)* after exposure to 40 °C and 45 °C. No band of any protein was expressed at both of these temperatures in the control bees of all tested subspecies. Nurse bees have uniform levels of HSP expression in response to 40 °C and 45 °C (Table 1).

Forager bees of the three subspecies (*A. m. carnica*, *A. m. ligustica* and *A. m. jemenitica)* exhibited diverse expression of HSPs when exposed to 40 °C and 45 °C (Fig. 2). The foragers of the indigenous subspecies (*A. m. jemenitica)* did not express any HSPs at 40 °C, whereas the foragers of the exotic European subspecies *(A. m. carnica* and *A. m. ligustica)* expressed one protein (Hsp70) at 40 °C. When forager bees were exposed to 45 °C, the variation in expression was prominent among the tested bee subspecies. In the European bee subspecies, two proteins (Hsp40 and Hsp70) were expressed in *A. m. carnica* and three proteins (Hsp40, Hsp60, and Hsp70) in *A. m. ligustica.* The indigenous bee subspecies (*A. m. jemenitica*) expressed only one protein (Hsp70) at 45 °C (Fig. 2). The control bees (without heat stress) of all three subspecies did not express any HSP protein at both temperatures. Table 1 presents a summary of all HSPs expressed during the current study after exposing the nurse and forager bees to heat stress.

4. Discussion

Heat shock treatment induces the expression of HSPs in nurse and foragers of indigenous (*A. m. jemenitica*) and exotic European (*A. m. carnica* and *A. m. ligustica*) bee subspecies. The previous literature reported some HSP expression (Hsp90, Hsp70, Hsp82, Hsp60, Hsp40, and Hsp20 families) in *Apis mellifera* L. due to a numerous stress factors such as infection, pesticides, and thermal stresses (Severson et al., 1990; Gregorc and Bowen, 1999; Chacon-Almeida et al., 2000; Elekonich, 2009; Koo et al., 2015). Other insects (*Tribolium castaneum*, *Bombyx mori*, *Aedes aegypti*,

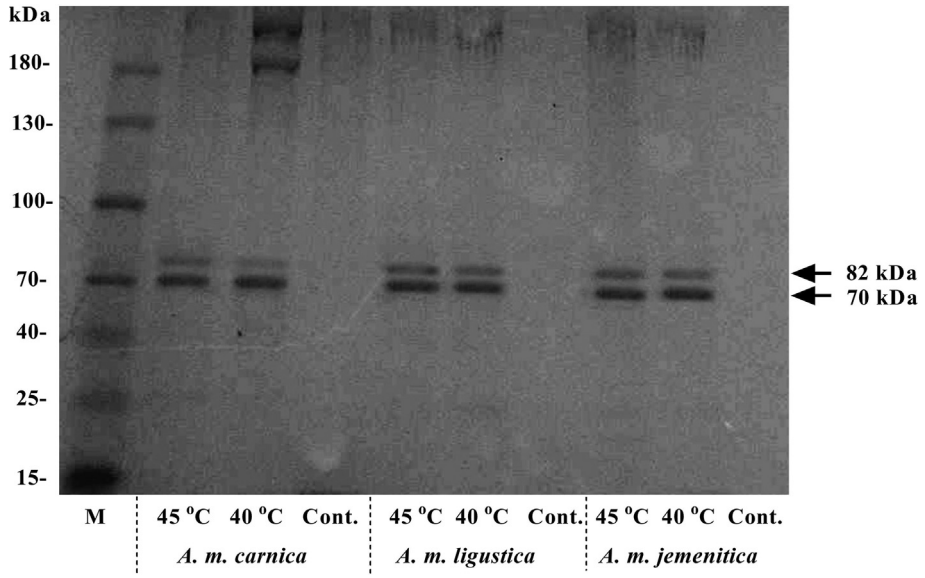

Fig. 1. SDS-PAGE analysis of heat shock-induced heat shock proteins (HSPs) from the hemolymph of nurse bees. The heat shock of 40 °C and 45 °C induces similar HSP expression (Hsp70 and Hsp82) in the nurse bees of three *Apis mellifera* races. The gel was stained with Coomassie blue. M is the molecular weight (kDa) marker (Pink plus protein ladder, GeneDireX, Inc.); Cont. stands for control. Arrows on the right side indicate identified HSPs.

Table 1
Expression of HSPs in nurse and forager bees after exposure to 40 °C and 45 °C.

Heat shock (°C)	Social status	Honey bee race	Expression of HSPs*	Protein size (kDa)
40 °C	Nurse	*A. m. jemenitica* *A. m. carnica* *A. m. ligustica*	Hsp70 Hsp82	70 82
45 °C	Nurse	*A. m. jemenitica* *A. m. carnica* *A. m. ligustica*	Hsp70 Hsp82	70 82
40 °C	Forager	*A. m. jemenitica*	**No Hsp**	-
		A. m. carnica *A. m. ligustica*	Hsp70	70
45 °C	Forager	*A. m. jemenitica*	**Hsp70**	70
		A. m. carnica	Hsp40 Hsp70	40 70
		A. m. ligustica	Hsp40 Hsp60 Hsp70	40 60 70

* HSPs = Heat shock proteins.

Culex spp., *Drosophila* spp., etc.) also express HSPs (Zhao et al., 2009; Sosalegowda et al., 2010; Xu et al., 2010; Zhao and Jones, 2012).

Interestingly, the nurse bees of all tested subspecies expressed similar molecular weight HSPs. However, the differential expression of HSPs was prominent among forager bees of tested bee subspecies after heat exposure at 40 °C and 45 °C. The nurse bees remain inside the colony under a socially regulated temperature (Southwick, 1992). A slight increase in temperature within a normal colony may trigger adaptive mechanism in bees to resist colony overheating and to maintain cellular homeostasis (Severson et al., 1990; Stabentheiner et al., 2010; Zhao and Jones, 2012). This mechanism may justify the expression of similar weight HSPs by nurses of all tested subspecies.

The expressed proteins in nurse bees were high in molecular weight (70 kDa and 82 kDa). Similar proteins (70 kDa and 82 kDa) were inducible in young workers of *A. mellifera* after heat shock of 42 °C for 4 h. (Severson et al., 1990). However, small weight proteins (10–27 kDa) were not detected in this study. The induction of Hsp70 and Hsp82 has also been reported in the fat bodies of 5th instar larval phase honey bees in response to heat shocks of 42 °C or 47 °C for 2 h (Chacon-Almeida et al., 2000)

Our study revealed the differential expression of HSPs among the foragers of the tested bee subspecies after exposure to two heat shock temperatures. The indigenous bee subspecies (*A. m. jemenitica*) did not express any HSPs, in contrast to the exotic European bee subspecies (*A. m. carnica* and *A. m. ligustica*), which expressed Hsp70 when exposed to 40 °C. This result reflects that foragers of *A. m. jemenitica* are more tolerant to an increase in temperature (up to 40 °C) and that their bodies may not need any HSP expression. When the heat shock was raised to 45 °C, the foragers of *A. m. jemenitica* expressed only one HSP (Hsp70), whereas *A. m. carnica* and *A. m. ligustica* expressed more than one HSP (Hsp40 and Hsp70 and Hsp40, Hsp60, and Hsp70, respectively). Thus, the expression of HSPs varied with the degree of temperature exposure. In addition, the metabolism of the organism may also have an important role, as it has been previously related to the expression of HSPs (Erban et al., 2016).

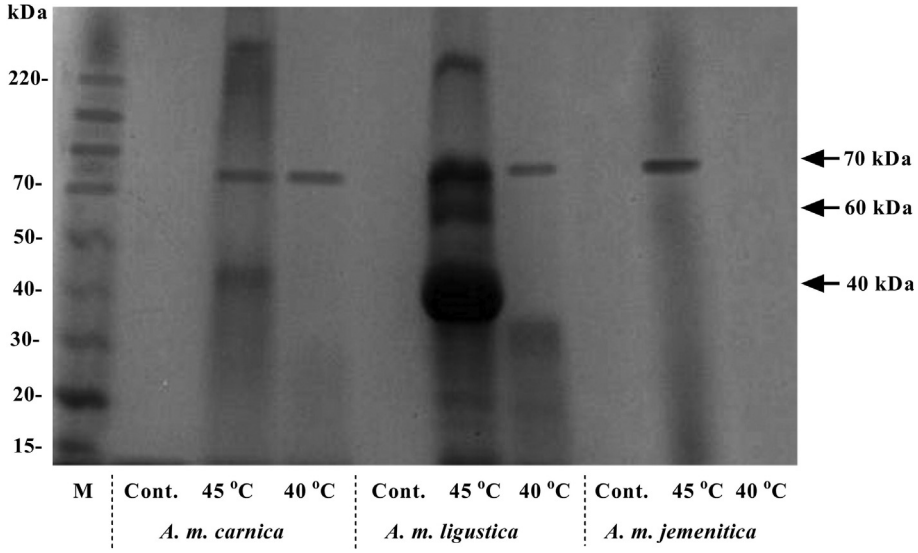

Fig. 2. SDS-PAGE analysis of heat shock-induced heat shock proteins (HSPs) from the hemolymph of forager bees. The heat shock of 40 °C and 45 °C induces differential expression of HSPs (Hsp40, Hsp60 and Hsp70) in the forager bees of three *Apis mellifera* races. The gel was stained with Coomassie blue. M is the molecular weight (kDa) marker (BenchMark™ protein ladder, Novex, life technologies); Cont. stands for control. Arrows on the right side indicate identified HSPs.

It is evident that the indigenous bee subspecies can better cope with increasing temperature. Our results support previous findings regarding the better tolerance and foraging performance of *A. m. jemenitica* in the hot and dry summers of central Saudi Arabia (Alqarni et al., 2011; Ali et al., 2017). The imported European honey bee subspecies faced greater difficulties in their survival under these harsh conditions than did the native race, which is well adapted to the local environment (Abou-Shaara et al., 2012; Alqarni et al., 2014; Alattal and AlGhamdi, 2015). In addition, differential colony losses were observed among bee subspecies, such as 92% in Carniolan bees (*A. m. carnica*), 84% in Italian bees (*A. m. ligustica*) and 46% in indigenous bees (*A. m. jemenitica*), under the temperature conditions of Saudi Arabia (Alattal and AlGhamdi, 2015). Possibly, the differential expression of HSPs among bee subspecies as depicted in our findings may be one of the factors for their differences in colony losses.

The nurse and forager bees have different HSP expression in the present study, which is reinforced by the fact that HSPs change with development in honeybees (Chan and Foster, 2008). The forager bees are more exposed to the potentially harsh outside environment and may need different expression of HSPs than nurse bees to address heat shocks. Newly emerged bees had a lower abundance of HSPs than the later redeye pupal stage during a hemolymph proteomics analysis (Erban et al., 2016). This could also explain the difference in expression of HSPs in the two separate life stages (nurse and forager bees) in the current study.

The expression pattern of Hsp70 is most prominent and is generally used as a sensitive biomarker to assess the stress response in organisms (Candido, 2001; Gibney et al., 2001; Hranitz et al., 2010). In our results, Hsp70 was wide expressed in the hemolymph of forager bees. This result confirms the findings of Elekonich (2009), in which Hsp70 was expressed in all body parts (brain, thorax and head) of *A. mellifera* in response to temperatures ranging between 33 °C and 50 °C. In addition, early emerged nurse bees have ten times lower Hsp70 mRNA than later flight-capable forager bees. This also suggests a quantity-based differential expression of HSPs between nurse and forager bees.

Since no HSPs were expressed in untreated nurse and forager bees of all three tested subspecies, the low surrounding temperature (<35 °C with humidity 30–40%) at the time of bee collection had no effect on the HSPs. This result also confirms that the expression of HSPs in treated bees was due to heat stress. Heat shock can trigger heat shock genes that lead to the expression of HSPs in insects (Zhao et al., 2009).

The widespread HSP activity in different races of honeybees may have a significant role in their adaptability to the harsh, hot weather of the Arabian Peninsula. Further investigation is necessary to determine the expression pattern of HSPs in response to other temperature ranges in different seasons of the year and their molecular characterization.

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Declaration of Competing Interest

The authors declare no potential conflict of interest for this paper.

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