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Differential responses of *Apis mellifera* heat shock protein genes to heat shock, flower-thinning formulations, and imidacloprid

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Short title: stress response and *hsp* levels and of *Apis mellifera*

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ABSTRACT

The honey bee, *Apis mellifera*, is a cosmopolitan pollination insect. Recently, global populations of honey bees have rapidly declined owing to colony collapse disorder (CCD), the mechanism of which is still unknown. Here, we used mRNA levels of heat shock protein (HSP) genes as molecular markers of response to three types of external stress: thermal shock, flower-thinning agents, and pesticides. When worker bees were exposed to temperatures of 4, 27, 40, 45 and 50°C for 1 h, decreased survival occurred only at 50°C. Further, increased levels of *hsp70*, *grp78*, and *hsp90*, but not *hsp40*, were detected, and reached a maximum at 45°C, particularly in the hypopharyngeal glands and fat bodies. Artificial ingestion of two flower-thinning agents containing either 0.1% boron and zinc, or 1% sulfur increased *hsp70* and *grp78* levels at different rates without affecting *hsp40* and *hsp90* levels, and had no effect on workers' mortality. However, ingestion of imidacloprid solution (0.5–50 ppm) increased mortality in workers and decreased the levels of *hsp70*, *grp78*, and *hsp90* in a dose-dependent manner. Our results showed that the responses of honey bees to each *hsp* are differential and highly specific to different stresses. This study suggests that the unique expression profiles of *hsps* can be used as valuable tools for monitoring the susceptibility of honey bees to various environmental impacts.

Key words: Heat shock proteins, Pesticides, Pollinators, Stress responses, Thermotolerance

INTRODUCTION

The honey bee, *Apis mellifera*, is a cosmopolitan pollination insect that contributes to the pollination of wild and cultivated plants globally. In the last two decades, the population of honey bees has been declining as a result of colony collapse disorder (CCD), which is a phenomenon of the abrupt disappearance of European honey bee colonies (vanEngelsdorp et al., 2009). CCD has caused a significant economic impact owing to the decline of pollination of agricultural crops as well as wild plants (Klein et al., 2007; Schacker, 2008). The mechanisms of CCD remains unknown, although many possible causes have been proposed, such as pesticides (primarily neonicotinoids), infectious pathogens (e.g., *Varroa* mites and viruses), malnutrition, genetic factors, immunodeficiency, loss of habitat, changed beekeeping practices, or a combination of these factors (Becher et al., 2013; Smith et al., 2013). However, the effects of these impacts have been evaluated mostly at the whole organism level by determining mortality or infertility in bee colonies. It is equally important to study responses to stress at the cellular and physiological levels to understand why honey bees fail to adapt adequately to various environmental impacts capable of triggering CCD (Johnson et al., 2009).

Heat shock proteins (HSPs) are a group of evolutionarily highly conserved molecules (Lindquist and Craig, 1988; Boorstein et al., 1994; Richter et al., 2010). HSPs are divided into several sub-groups according to their size, structure, and function, namely small HSPs (sHSPs),

HSP60, HSP70, HSP90, and HSP100 (Lindquist and Craig, 1988; Boorstein et al., 1994). Most HSPs are constitutively expressed and perform essential roles in normal cells. The HSP70 family is the largest group and contains many different isoforms that are specifically distributed in cellular organelles. For example, GRP78 is the endoplasmic reticulum (ER) HSP70 and is also called binding immunoglobulin protein (Bip) in mammals or heat shock 70-kDa protein 5 (HSPA5) in humans (Lee, 2001). GRP78 acts as an ER chaperone responsive to ER stress in mammals. Further, its transcription and protein levels are influenced by nutritional as well as thermal stresses (Lee, 2001). Our previous study showed that the level of *grp78* in the Indian mealmoth *Plodia interpunctella* was highly associated with nutrition uptake during the fifth instar larval stage (Shim et al., 2014). The *grp78* level increased in feeding larvae but decreased in wandering larvae, which do not feed, as well as in nutritionally deprived larvae. Expression levels of *grp78* were also upregulated in *P. interpunctella* larvae envenomated by the ectoparasitoid *Bracon hebetor* (Shim et al., 2008).

The role of HSPs in the stress responses of many organisms has been well-documented (Feder and Hofmann, 1999; Denlinger et al., 2001). At the cellular level, HSPs act as chaperones to protect proteins from damage during synthesis, folding, assembly, and localization of proteins in the cell (Feder and Hofmann, 1999; Hartl and Hayar-Hartl, 2002). At the ecological level, HSPs act to protect organisms from various environmental stressors such as heat, cold,

desiccation, toxins, pathogens, and others (reviewed by Feder and Hofmann, 1999; Kregel, 2002). In this regard, levels of transcripts and translated proteins encoded by HSP genes can be used as biomarkers to monitor cellular and physiological responses to various environmental stimuli (Gibney et al., 2001; Nazir et al., 2003).

Honey bees are highly thermotolerant and the roles played by some HSPs against thermal stress have been studied previously (Severson et al., 1990; Gregorc and Bowen, 1999; Elekonich, 2009; Xu et al., 2010). The honey bee genome has been deciphered and it is known to contain at least 36 *hsps* (Honey Bee Genome Sequencing Consortium, 2006; Elsik et al., 2014). However, changes in the expression of different *hsps* in response to various environmental stresses have not been studied in detail in honey bees. The patterns of transcripts and proteins of various *hsps* may provide important information for the understanding of the cellular and physiological mechanisms of susceptibility of honey bees to different environmental challenges. Here, using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) we determined cellular stress response profiles by measuring the expression levels of various *hsps* (*hsp40, hsp70, grp78, hsp90*) in worker honey bees in response to several stress conditions, namely temperature changes, flower-thinning agents, and imidacloprid pesticide.

MATERIALS AND METHODS

Exposure of honey bees to heat and cold shocks, flower-thinning agents, and imidacloprid

Adult workers (10–20 days old) were collected from beehives in Yeongcheon, Korea. The workers were kept in a plastic cage (19 × 12 × 11 cm^3) containing a piece of cotton moistened with a 20% sucrose solution at 26–27°C and 50–60% relative humidity for 1 day before the experiments commenced.

The worker bees were exposed to cold (4°C) and heat (40, 45, and 50°C) shocks for 1 h and maintained at 27°C for 1 h before RNA extraction. The commercial flower-thinning agent Koduri containing 0.1% water-soluble boron and zinc (Apple Co. Ltd., Daegu, Korea) was diluted with a 20% sugar solution to generate 0.1 and 1% dilutions. The resulting solutions contained boron and zinc at final concentrations of 1 and 10 ppm, respectively. The commercial formulation Eco-sulfur containing 1% lime sulfur (Saeil Co. Ltd.; Chilgok, Korea) was also diluted with a 20% sugar solution to prepare 0.1, 1, and 10% dilutions. The final concentrations of lime sulfur in the resulting solutions comprised 10, 100, and 1000 ppm, respectively. The neonicotinoid pesticide Konido containing 10% imidacloprid (Dongbu Farm; Hannong, Seoul, Korea) was diluted with a 20% sugar solution to prepare 0.0005, 0.005, and 0.05% dilutions that contained imidacloprid at final concentrations of 0.5, 5, and 50 ppm, respectively. Cotton balls were soaked in each diluted solution. Worker honey bees (n = 20) were placed in the plastic box and allowed to

ingest the sugar solutions for 4 days at 27°C.

Quantitative real-time RT-PCR

Total RNA was extracted from the abdomen (n = 1) or dissected tissues (n = 5) of workers using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Concentrations of purified RNA samples were determined using a Nanophotometer (Implen, Schatzbogen, Germany). Reverse transcription (RT) reactions were performed with 2 µg of total RNA as a template for cDNA synthesis using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR primers for each gene were designed using sequences identified from the National Center for Biotechnology Information (NCBI) database (Table 1). PCR amplifications were performed in a 25 µL reaction volume containing 0.2 µL cDNAs as a template, 0.2 µL of gene-specific primers at a concentration of 10 pM, 11.9 µL of distilled water, and 12.5 µL of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The amplified signals were monitored continuously with the 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The amplification protocol was as follows: 1 cycle (95°C for 10 min), 40 cycles (95°C for 15 s, 55°C for 20 s, 72°C for 35 s), and 1 cycle for dissociation (95°C for 15 s, 60°C for 30 s, 95°C for 15 s). Threshold cycle (Ct) values were used to calculate the relative quantities of HSPs and actin. Data were analyzed using the formula: $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ \text{treatment}-\Delta Ct\ \text{reference}]}$ (Livak and Schmittgen, 2001). The

levels of HSPs mRNA were normalized by those of actin mRNA in the same samples.

Statistical analysis

Statistical analysis of the data was conducted using the SPSS v. 12.0 software package for Windows (SPSS Inc.; Chicago, IL, USA). The data were analyzed by one-way analysis of variance or the Student's t-test. Data that were not normally distributed were analyzed with Duncan's multiple range test and the same letters are not significantly different ($P < 0.05$).

RESULTS

When worker bees (10–20-day-old adults) were exposed to cold or heat shocks at 4, 27, 40, 45, and 50°C for 1 h, decreased survival rate occurred only at 50°C heat shock into 77.0±10.4% (data not shown). Workers upregulated *hsps* 1 h after treatment but levels of expression were dependent on temperature. Both *hsp70* and *hsp90* levels were highest after the 45°C shock, with their expression being potentiated by 3- to 10-fold more than at any other temperature. However, the *hsp40* level was not changed by cold or heat shocks, but the *grp78* level was significantly increased by both cold and heat shocks at any temperature in comparison with the control temperature (27°C) conditions (Fig. 1).

In a comparison of different tissues, such as brain, hypopharyngeal glands, fat bodies,

midgut, and flight muscle, *hsp* levels were tissue-specific at both normal and stress temperatures. For example, at 27°C *hsp40* was highly expressed in hypopharyngeal glands, but *hsp70* was expressed in flight muscles (Fig. 2). After heat shock at 45°C, *hsp70* was highly upregulated in fat bodies but both *grp78* and *hsp90* were found in the hypopharyngeal glands (Fig. 2). A moderate increase of both *grp78* and *hsp90* was also detected in the brain, fat bodies, and midgut, but not in the flight muscles.

Artificial feeding with two kinds of flower-thinning formulations, Koduri (0.1 and 1% dilutions) and Eco-sulfur (0.1, 1, and 10% dilutions), did not increase mortality when Koduri and Eco-sulfur were used at approximately the commercially recommended concentrations of 0.1 and 1%, respectively (data not shown). In addition, we observed an increase in the expression levels of *hsp70* and *grp78* as a result of these treatments, whereas the expression levels of both *hsp40* and *hsp90* were not altered. Furthermore, both *hsp70* and *grp78* levels were augmented to a similar extent, regardless of the different concentrations of Koduri, but reduced by the higher (1%) dose of Eco-sulfur (Fig. 3).

Exposure to 0.5–50 ppm imidacloprid solutions significantly increased the mortality of workers in a dose-dependent manner (Fig. 4). Complete mortality was detected 2 days after ingestion of 5 ppm and higher doses. We observed a dose-dependent downregulation in the levels of all tested *hsps* (Fig. 5). However, the rates of decease in *hsp70*, *grp78*, and *hsp90* were greater

than in *hsp40*.

DISCUSSION

Honey bees are highly tolerant of thermal stress. The present study showed that all workers survived at 4–45°C, but there was some mortality at 50°C. Further, various *hsps* were upregulated by heat shock. Thermal stress differentially induced *grp78*, *hsp90*, and *hsp70* in the abdomens of worker bees, and maximum levels were reached at 45°C. Thus, these results suggest that the thermal tolerance of honey bees arises from a combination of multiple *hsps*. Elekonich (2009) suggests that *hsp70* is differentially induced according to behavioral changes associated with the age of the worker. For example, exposure to 43°C for 4 h induced a higher increase in the expression of *hsp70* in the brains of old foraging workers than in those of young nursing workers. The age of the workers used in this study was 10–20 days, which is not old for workers, but they might have been active outside the hive in tasks such as ventilating, guarding, and foraging (Elekonich and Roberts, 2005).

A tissue-specific, differential expression pattern of HSPs has been observed in various species (Krebs and Feder, 1997; Michaud et al., 1997). Our results showed that at the normal temperature of 27°C, *hsp40* was highly expressed in hypopharyngeal glands, but *hsp70* was expressed in flight muscles. However, the *hsps* responded to heat shock in a tissue-specific

manner: *hsp70* was highly upregulated in fat bodies but both *grp78* and *hsp90* were upregulated in hypopharyngeal glands. Otherwise, the expression of *hsp40* in hypopharyngeal glands and *hsp70* in flight muscles was not significantly changed by heat shock. This result indicates that the mechanism of thermal stress response is not identical in different tissues at the cellular level. Although the precise meaning of tissue-specific profile is uncertain, *hsps* might be essential for the protection of cellular activity and maintenance of those tissues.

Worker bees are highly active during their flight behavior when foraging for nectar and pollen. Elekonich and Roberts (2005) reported that foraging workers express higher levels of *hsp70* in their thoraces relative to nurse workers. Our results showed that *hsp70* in flight muscle was highly expressed in normal condition and also maintained a high level of expression when subjected to heat shock; other *hsps* (*hsp40*, *grp78*, and *hsp90*) were expressed at a low level and did not respond to heat shock. This suggests that *shp70* might be the most important *hsp* for thermal tolerance in the thoracic muscles of workers.

Foraging workers are frequently exposed to various harmful chemical compounds such as pesticides, flower-thinning agents, or environmental pollutants. Our results showed that the ingestion of two kinds of commercial flower-thinning agents containing boron, zinc, and sulfur was not overly toxic to worker honey bees. Sulfur is known to be non-toxic to bees (Farm Chemicals Handbook, 1994). For example, when honey bees come into contact with sulfur

formulations such as 98% dust and 92% powder and ingest them, these exposures have a low toxic effect (USEPA, 1991). Both boron and zinc are essential minerals required for normal physiological functions and survival, yet higher concentrations have been used for the control of some wood-boring and urban insect pests such as termites, cockroaches, and fleas (Woods, 1994; Rainey et al., 1999; Gentz and Grace, 2006). However, boron toxicity has not yet been determined in honey bees. In this study, the expression of some *hsp* genes was significantly altered by the ingestion of two flower-thinning formulations used in the recommended concentration range. Levels of both *hsp70* and *grp78* were upregulated, but levels of *hsp40* and *hsp90* were not, by exposure to those substances. Therefore, it is likely that they are stressful at a cellular level, although flower-thinning formulations did not disturb the vitality of the honey bees.

One of the putative causes of CCD is the chronic exposure to imidacloprid pesticides (Decourtye et al., 2004, Lu et al., 2014). Numerous studies have demonstrated that imidacloprid has sublethal effects at concentrations of 5–100 ppb and its LD_{50} (the dose required to kill half the members of a tested population after a specified test duration) is ~200 ppb (Blacquiere et al., 2012). Treatment by pesticides such as imidacloprid and coumaphos reduces the size of the hypopharyngeal gland that secretes royal jelly, and also induces programmed cell death in the brain of worker honey bees (Skerl and Gregorc, 2010). Our results show that imidacloprid is highly toxic to worker bees, even at very low concentrations. Complete mortality of worker bees

was observed within 48 h of ingestion of 5–50 ppm imidacloprid solutions, which is the recommended concentration of commercial preparations. Furthermore, a gradual increase in mortality was detected during the treatment with 0.5 ppm imidacloprid. This concentration is extremely low and does not cause lethality in other pest insects. For example, LD_{50} values in German cockroaches and houseflies were 6–8 ppm and 140 ppm, respectively (Wen and Scott, 1997). Furthermore, sweet potato whitefly, *Bemisia tabaci*, has high imidacloprid resistance (Rauch and Nauen, 2003). For example, the LC_{50} value of a B-biotype collected in the USA in 1994 was 1,500 ppm, and that of a Q-biotype collected in Spain in 1999 was 27,000 ppm. Therefore, honey bees are more sensitive to imidacloprid than any other insects.

Our results demonstrated that the levels of three *hsps* studied (*hsp70*, *grp78*, and *hsp90*) gradually decreased when the imidacloprid concentrations were increased. This suggests that the chaperone action of *hsps* cannot overcome the toxicity of imidacloprid. General cellular functions may all be affected by the toxic action of imidacloprid (Ayyanath et al., 2014). The levels of both transcripts and proteins have been utilized as biomarkers for the ecotoxicity of pesticides and other environmental pollutants (Lewis et al., 1999). For example, induction of *hsp70* expression in the midge *Chironomus yoshimatsui* was a characteristic marker for treatment with organophosphate and pyrethroid pesticides (Yoshimi et al. 2002). Various *hsp*s (*hsp19.7*, *hsp20.7*, *hsp70*, and *hsp90*) were also induced in a concentration-dependent manner by chlorfenapyr, an

insecticide causing mitochondrial uncoupling, but not by other insecticides in cultured cells of the cabbage armyworm *Mamestra brassicae* (Sonoda and Tsumuki, 2007). Thus, the determination of *hsp* expression profiles may be used to illustrate the effects of specific compounds on cellular stress responses and reflect the physiological consequences of toxic impacts in target insects. Further studies are required to comprehensively understand the biochemical consequences of stress induced by various insecticides applied at sublethal doses.

In conclusion, our results showed that the expression of *hsps* in worker honey bees differentially changes in response to various stressors such as heat shock, flower-thinning agents, and pesticides. Our study suggests that unique expression profiles of *hsps* may be valuable tools for monitoring the susceptibility of honey bees to various environmental stressors.

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FIGURES LEGENDS

Fig. 1. Effects of cold and heat shocks on the levels of *hsp40*, *hsp70*, *grp78*, and *hsp90* in worker honey bees. Workers were exposed to one of the following temperatures (4, 27, 40, 45, or 50°C) for 1 h and then kept at 27°C for 1 h. Total RNA was extracted from individual abdomens. Relative levels of each *hsp* were measured by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) with the *actin* gene serving as a reference. The different letters indicate statistically significant differences between treatments. Values are presented as mean ± SE (n = 3).

Fig. 2. Tissue-specific changes in *hsp40*, *hsp70*, *grp78*, and *hsp90* levels following heat shock. Workers were exposed to 27°C and 45°C for 1 h and then maintained at 27°C for 1 h. Total RNA was extracted from the brain (B), hypopharyngeal glands (HG), fat bodies (FB), the midgut (MG), and the flight muscles (FM) (n = 5). Relative levels of each *hsp* were measured by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) with the actin gene serving as a reference. Values are presented as mean ± SE (n = 3).

Fig. 3. Effects of flower-thinning formulations on *hsp40*, *hsp70*, *grp78*, and *hsp90* levels in workers. Commercial flower-thinning agents, Koduri and Eco-sulfur, were diluted with a 20% sugar solution to generate 0.1% and 1% dilutions, respectively. Cotton balls were soaked in these diluted solutions. Workers (n = 20) were placed in the plastic box and allowed to ingest sugar solutions for 1 day at 27°C. Total RNA was extracted from individual abdomens. Relative levels of each *hsp* were measured by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) with the actin gene serving as a reference. Values are presented as mean ± SE (*n* = 3).

Fig. 4. Effects of the imidacloprid ingestion on the mortality of workers. A neonicotinoid pesticide, Konido, containing 10% imidacloprid was diluted with a 20% sugar solution to prepare 0.0005, 0.005, and 0.05% dilutions containing imidacloprid at final concentrations of 0.5, 5, and 50 ppm, respectively. Cotton balls were soaked in each diluted solution. Workers (*n* = 20) were placed in the plastic box and mortality was determined over the course of 4 days at 27°C. Values are presented as mean ± SE (*n* = 20).

Fig. 5. Effects of imidacloprid ingestion on *hsp40*, *hsp70*, *grp78*, and *hsp90* levels in workers. A neonicotinoid pesticide, Konido, containing 10% imidacloprid was applied as described in the

legend for Fig. 4. Total RNA was extracted from individual abdomens at 6 h after treatment. Relative levels of each *hsp* were measured by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) with the actin gene serving as a reference. Values are presented as mean ± SE (n = 3).

Table 1. Primers used for qRT-PCR in *Apis mellifera*

Target gene	Synonym	Primer sequence (5′→3′)	Size (bp)	Accession no.	Reference
hsp40	*dnaJC22-like*	F- tcg agg cga cgt gga a R- ccc acg ttg ccc aga a	51	XM_006568544.1	NCBI
hsp70	*hsp70ab-like*	F- cgc ctt cac gga cac aga R- ttc att gcg acc tga ttt ttg	60	NM_001160072.1	Williams et al., 2008
grp78	*hsc70-3*	F- ctt ttc tac tgc atc aga caa cca R- ttt gtc att gga cgt tca cct t	72	NM_001160052.1	Williams et al., 2008
hsp90	*hsp90*	F- agg acg tca cca tgg cta at R- tgt gca att tca gct tgg aa	64	NM_001160064.1	Xu et al., 2010
actin	*actin*	F- tgc caa cac tgt cct ttc tg R- aga att gac cca cca atc ca	156	AB023025	Lourenco et al., 2008

Fig. 1

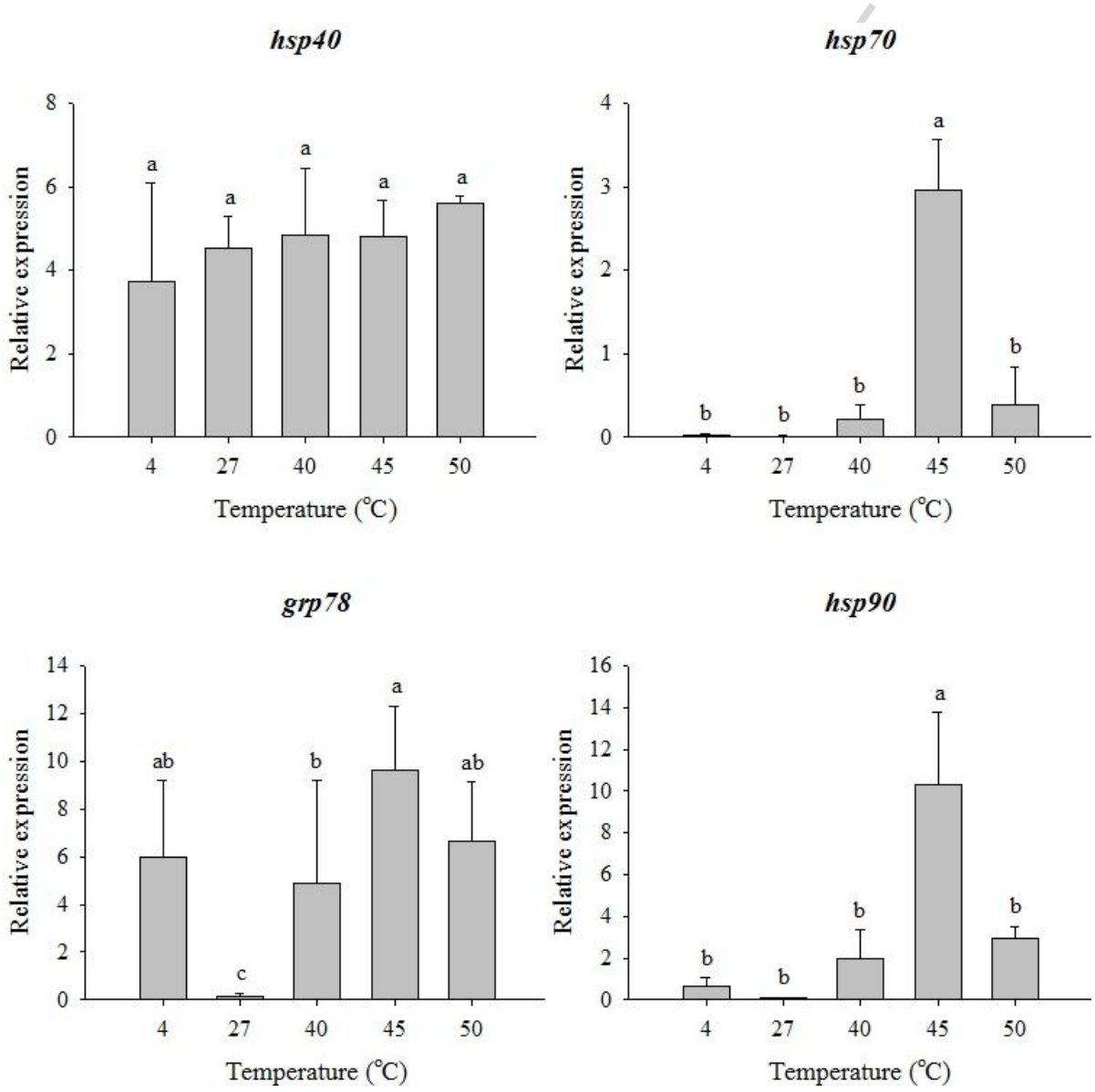


Fig. 2.

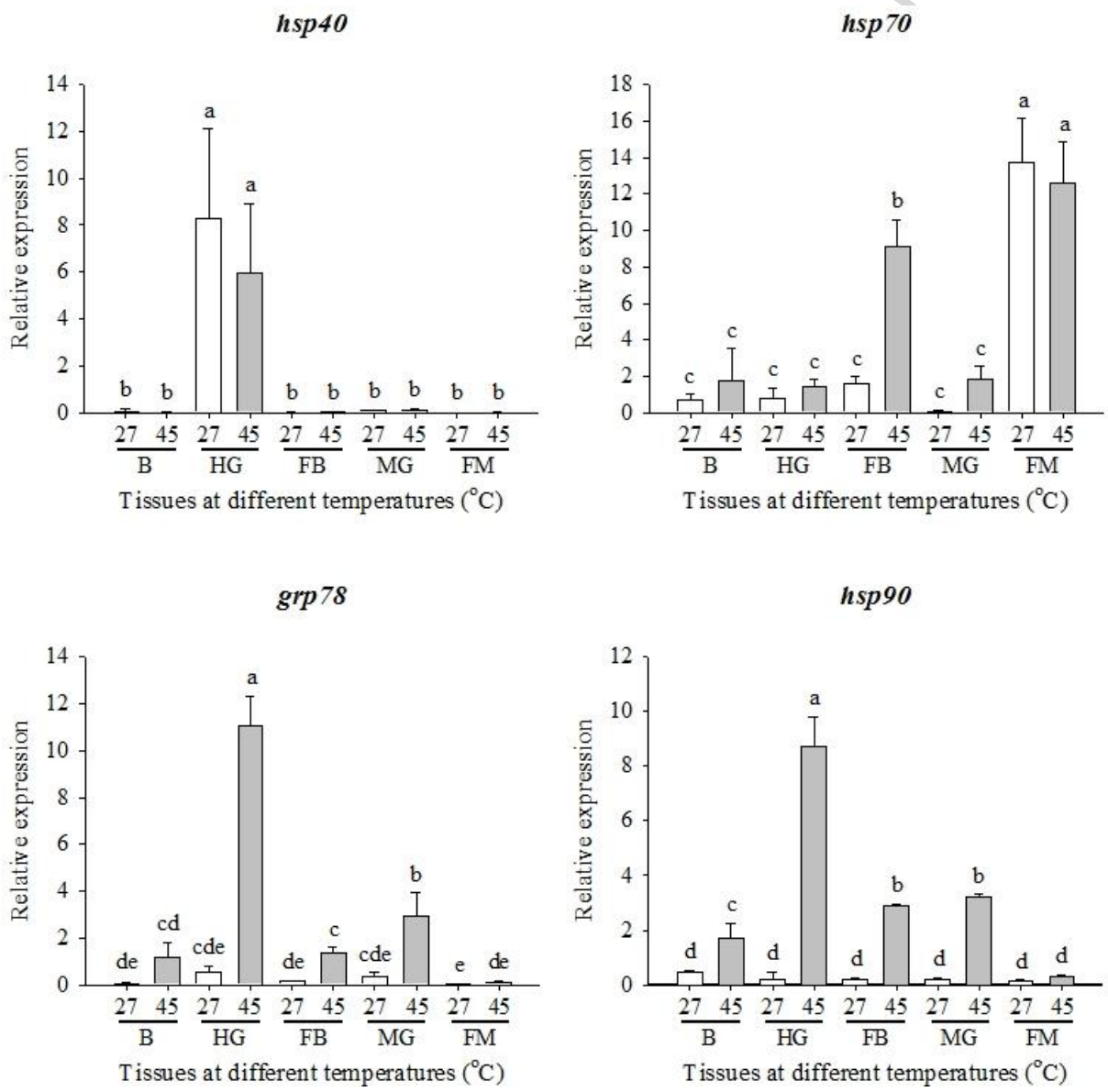


Fig. 3.

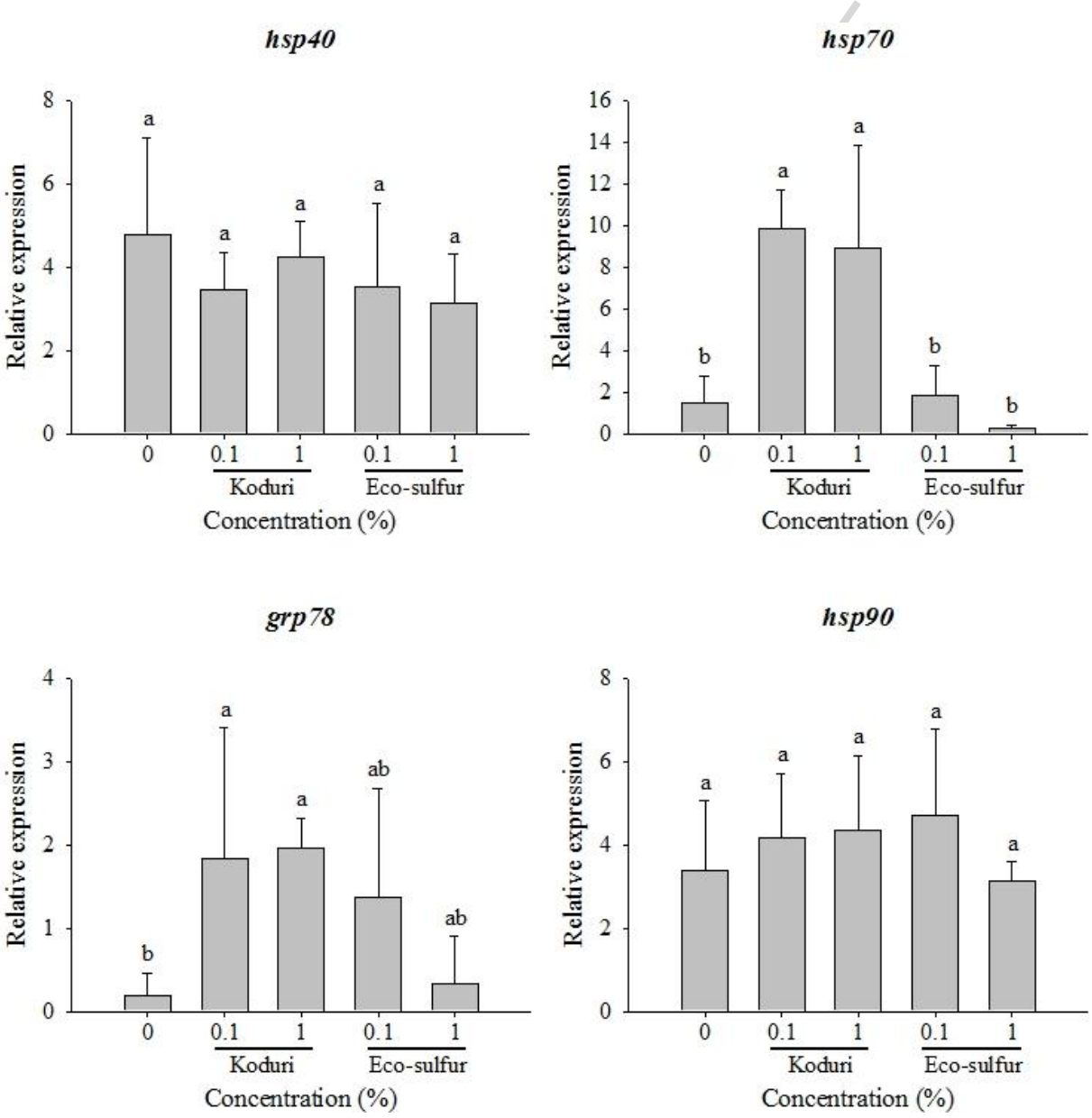


Fig. 4.

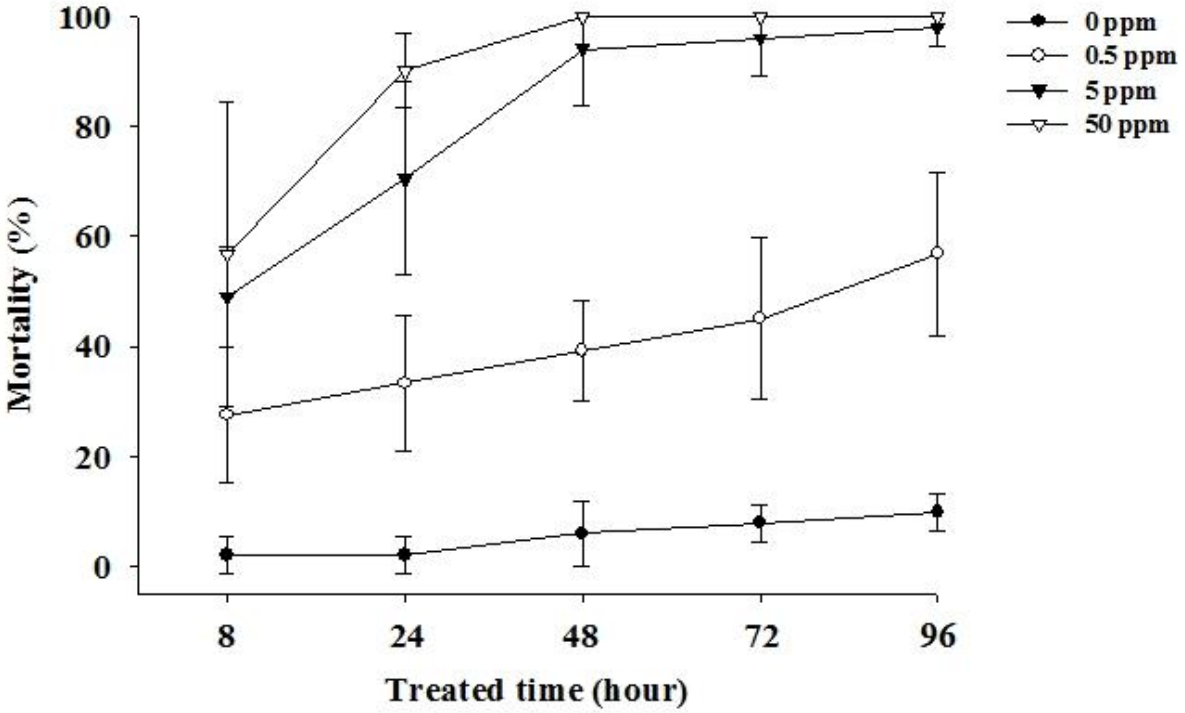
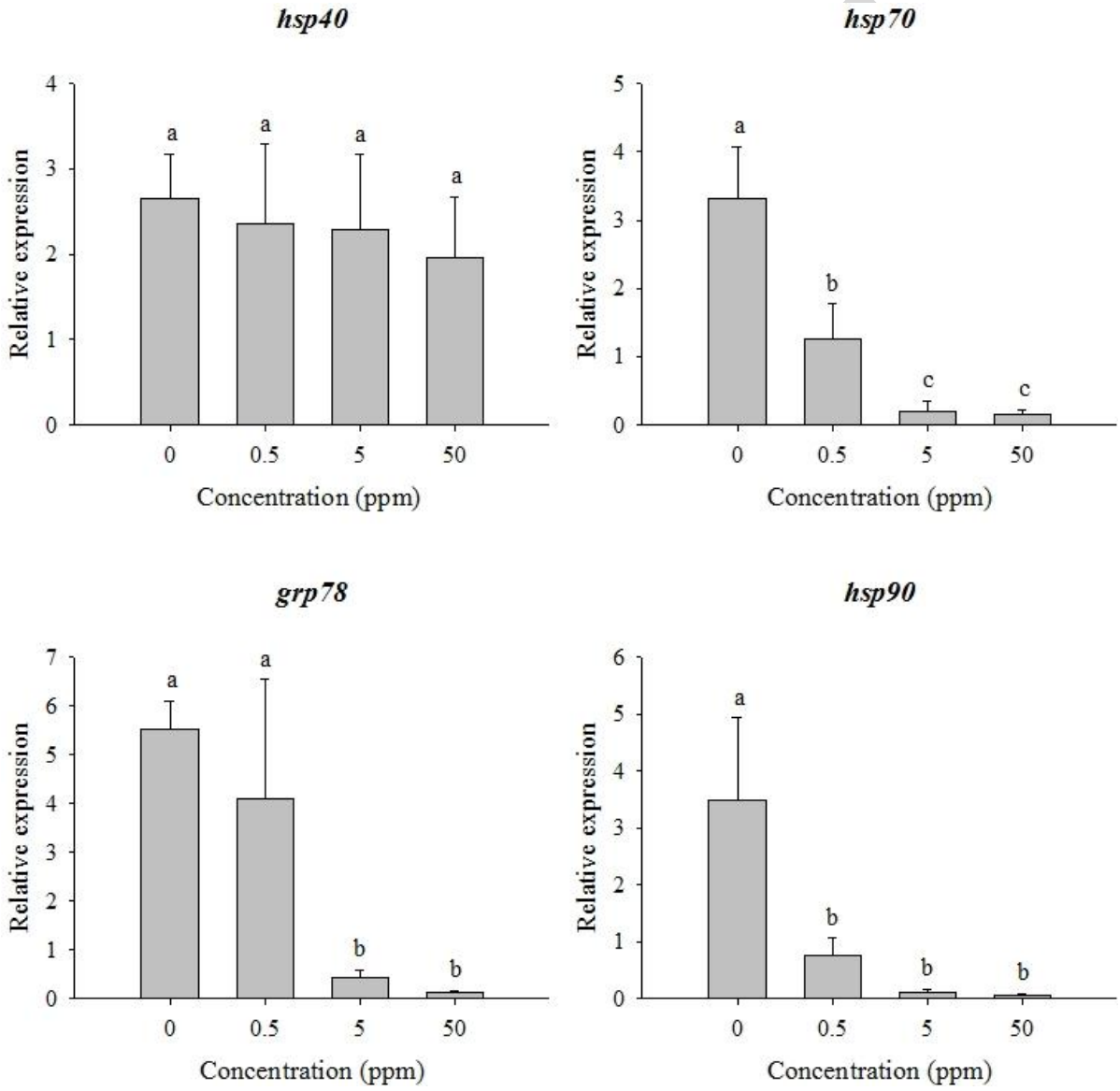
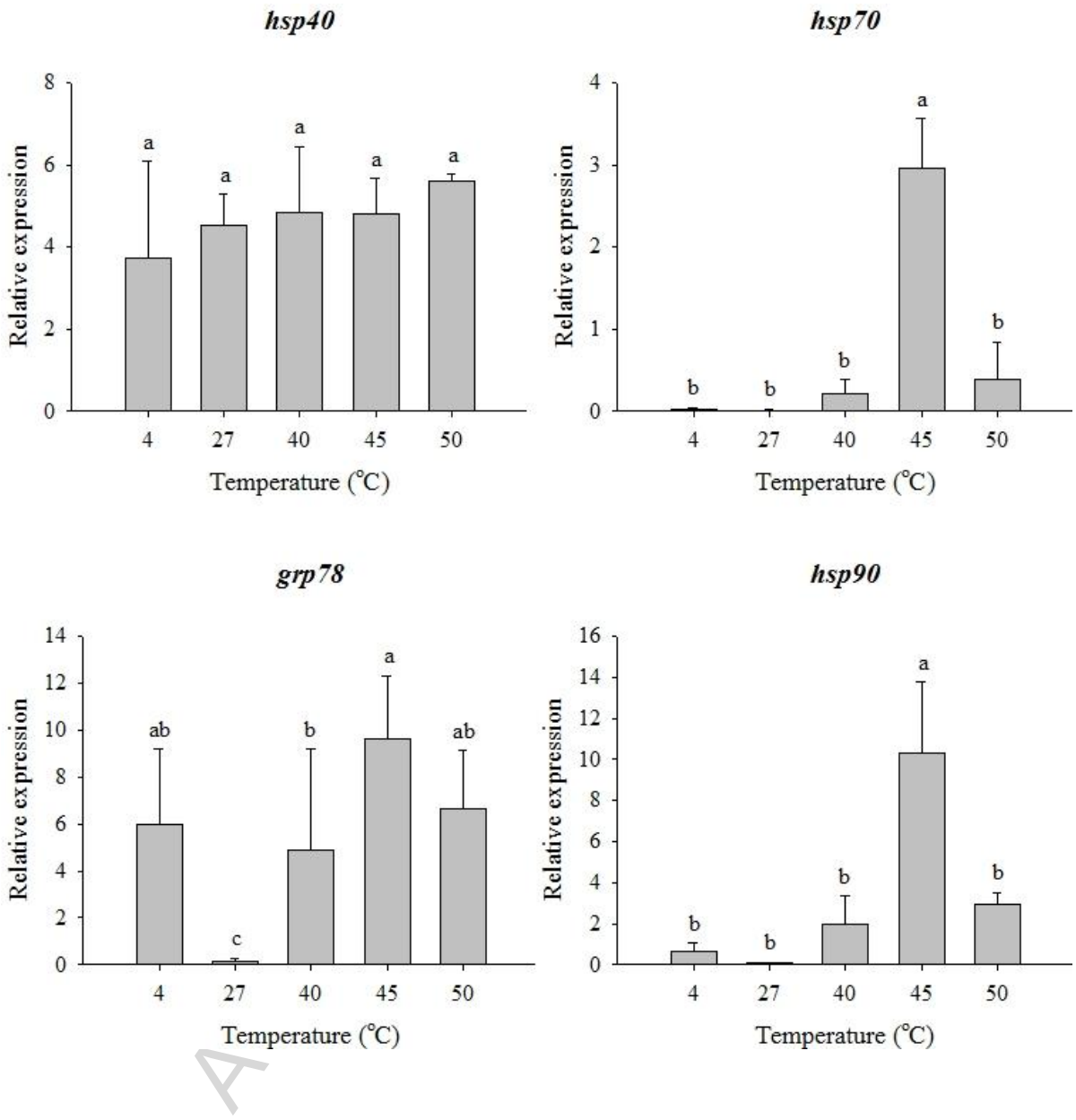


Fig. 5.





Graphical abstract

Highlights

►Four *hsp* levels were monitored as biomarkers of stress responses of temperature, thinning agents and imidacloprid from the honey bee, *Apis mellifera*. ► Honey bee responses to each *hsp* are differential and highly specific to different stresses. ► Unique expression profiles of each *hsp* can be used as valuable tools for monitoring the susceptibility of honey bees to various environmental impacts.