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Research Article

RNAi knockdown of heat shock proteins affects thermotolerance of *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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Abstract: The Mediterranean flour moth, *Ephestia kueheniella* is one of the most severe pests in stores worldwide. Like many other stored product pests, chemical pesticides are often used to control this insect. As many of these chemicals negatively impact non-target species, safer alternative methods are needed. Temperature is one of the environmentally friendly control methods that can achieve the desired levels of control. Still, the insects' induction of cold and heat tolerance can decrease control efficacy. In the current study, we first investigated the susceptibility of all developmental stages of the Mediterranean flour moth to extreme heat and cold temperatures. Exposure to 44 °C for 80 minutes or -15 °C for 30 minutes caused significant mortalities in all developmental stages. Exposure to mild low or high temperatures induced cold or heat tolerance and reduced the mortality rate. Quantitative RT-PCR confirmed that heat or cold-inducible tolerance was accompanied by up-regulation of transcripts for two heat shock proteins, HSP70 and HSP90; transcript levels of HSP70 and HSP90 increased 3.8 and 4.3-fold after pre-exposure to 35°C for 30 min and 3.8 and 3.3-fold after pre-exposure to 10°C for 30 min compared to controls, respectively. Suppression of HSP70 and HSP90 transcripts using RNA interference (RNAi) significantly reduced heat and cold tolerance, indicating that these two proteins play a crucial role in the induction of heat and cold tolerance in the Mediterranean flour moth.

Keywords: HSP70, HSP90, RNA interference, Integrated pest management

Introduction

The Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae) is a polyphagous and destructive pest of stored products, with the larvae causing high levels of damage by consuming and contaminating our food (Jallouli *et al.*, 2013). Damage to stored products can occur directly, through larval feeding, and indirectly through contamination from the insects' faeces and exuviae (Nay, 2005). The primary control method of stored product pests, including the Mediterranean flour moth, is using synthetic chemical pesticides such as methyl bromide and phosphine (Stejskal *et al.*, 2015). Although these gaseous pesticides have proven effective, concerns have arisen about their negative impacts on the environment, non-target organisms, and the increasing incidence of resistance. As a result, researchers have been prompted to explore alternative methods that are both environmentally friendly and economically feasible (Stejskal et al., 2015; Izadi et al., 2019).

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Insects are poikilothermic organisms that can live in a limited range of temperatures. Outside these temperature ranges, the insects may be incapacitated and even killed (Bozinovic *et al.*, 2016; Trotta *et al.*, 2018). Because insects are quite susceptible to temperature changes in their living environments, using temperature to restrict pest populations is a promising, potentially effective, and inexpensive method to control stored product pest insects (Verberk *et al.*, 2016). Mason and Strait (1998) defined three insect temperature zones: optimum, suboptimum, and lethal. The optimum zone is the range of temperatures at which the insects develop and reproduce effectively. The suboptimum temperatures zone is the range of temperatures below or above the optimum zone where the survival and reproduction of the insects are reduced. In contrast, lethal temperatures are those above or below the suboptimum zone that will eventually kill the insects. Pretreatments of insects to suboptimum temperatures can lead to an increased survival rate after exposure to lethal temperatures (Yocum *et al.*, 1992; Boardman *et al.*, 2013; Boardman *et al.*, 2015; Kim *et al.*, 2017). This short-term enhanced thermotolerance following heat or cold pretreatments is due to the induced production of heat shock proteins (HSPs) (Kim *et al.*, 2017; Farahani *et al.*, 2020). The HSPs are highly conserved proteins that serve a variety of functions within cells but are particularly important in acting as molecular chaperones, helping nascent proteins fold correctly, and during periods of cellular stress, helping proteins refold, particularly following exposures to suboptimal temperatures (reviewed in King and MacRae 2014; Xu *et al.*, 2015; Tungjitwitayakul *et al.*, 2016). In insects, five HSP families have been defined, based on their molecular weights, and include the small heat shock proteins (sHSPs, with molecular weights ranging from 12 to 43 kDa), Hsp60, Hsp70, Hsp90, and HSP10 (King and MacRae, 2014; Nguyen *et al.*, 2016).

If high or low-temperature treatments are to be considered for possible control of the Mediterranean flour moth, assessing these insects' temperature tolerances will be important. It is anticipated that if lethal temperatures are to be applied to the insects, the heating or cooling process may be slow enough to enable the insects to adjust to the changing temperature through the production of HSPs, thereby reducing the temperature treatment's efficacy. Thus, the aim of this study was to assess the susceptibility of the Mediterranean flour moth at different developmental stages to low and high temperatures and to investigate the effect of pretreatment with moderate temperatures before exposure to lethal temperature on the enhancement of larvae survival rate. The role of HSPs in the insects' thermotolerance was also examined using RNA interference-mediated knockdown of two HSP genes' transcripts, and here, we demonstrate the effective use of RNAi in this species to evaluate genes' function. Recently, RNAi has been proposed as an alternative method for controlling pests (Mitter *et al.*, 2017; Liu *et al.*, 2020).

Materials and Methods

Insect Culture

The initial population of the Mediterranean flour moths, *E. kuehniella*, in different larval stages, was obtained from the biological control laboratory, Department of Plant Protection, Faculty of Agriculture, University of Tehran. The insect larvae were reared on a mixture consisting of 1 kg wheat flour, 55 g yeast, and 30 g wheat germ in plastic boxes (15 × 15 × 10 cm3) under the following conditions: 27 ± 1 °C, 40 ± 5% relative humidity, and a 16 h light/ 8 h dark (16L:8D) photoperiodic cycle (Marec *et al.*, 1999) for three generations.

Heat tolerance bioassays

Thirty unmated male and female adult moths were transferred into a plastic jar (15×30×10 cm) covered with a fine net for egg laying to obtain cohort insects. After 24 h, the adults were removed, and the eggs were transferred to the artificial diets in growth chambers with the conditions above. All experiments were conducted on 1-day-old insects.

To determine the susceptibility of the insects to high temperatures, different developmental stages

of *E. kuehniella* (eggs, larvae, pupae) were held into 9 cm Petri dishes, and adults were held in clear plastic cylinder bottles (3 cm diameter × 8 cm height). Insects were exposed to 44 ℃ for 1 h using a temperature-controlled incubator (Memmert, Germany) with 45 ± 1% humidity. Additionally, fifth instar larvae were exposed to different temperatures (38, 40, 42, 44, 46 and 48 °C) for 80 min or 44 °C for different exposure periods (0–110 min) to determine the lethal high temperature and lethal time exposure, respectively. After 2 h recovery at 25 ℃, mortality was recorded. Egg and pupal mortalities were determined at 25 ℃, respectively.

The effective temperature for heat tolerance induction was determined by exposing fifth-instar larvae to different temperatures (25, 30, 33, 37, and 40 °C) for 10 min and then 44 °C for 80 min. After determining the most effective temperature, the effective exposure time for the induction was measured by exposing fifth-instar larvae to different periods (0, 10, 15, 20, and 30 min) at 37 °C and 46 °C for 100 min. After heat treatment, the survival of individuals was determined after 2 h recovery at 25 °C.

The sexes were determined based on two spots on the back of the abdominal segments of males suggesting the presence of testes. The larval stages were determined by head capsule measurements.

Cold tolerance bioassays

The susceptibility of different developmental stages of *E. kuehniella* to a cold temperature was determined by exposing them to -15 ℃ for 30 min in a laboratory fridge (BFN22D 348, Emersun, Iran). The lethal low temperature and the lethal time exposure were measured by exposing fifth instar larvae to different temperatures (5, 0, -5, -10, -15 and -18 °C) for 30 min and -15 °C for different exposure periods (0–40 min), respectively. After 2 h recovery at 25 ℃, mortality was recorded. Egg and pupal mortalities were determined upon hatching and adult emergence at 25 °C. The effective temperature for cold tolerance induction was determined by exposing fifth-instar larvae to different temperatures (15, 10, 5, and 0 °C) for 10 min and then -15 ℃ for 30 min. The effective exposure time for the induction was measured by exposing fifth-instar larvae to different periods (0, 10, 15, 20, and 30 min) at 10 ℃ and -15 ℃ for 30 min. After the cold treatment, the survival of individuals was determined 2 h post-recovery at 25 ℃.

All treatments were performed in three replicates with 80 cohort individuals in each replicate.

RNA extraction and cDNA synthesis

According to the manufacturer's instruction, total RNA from 10 larvae was extracted using Trizol reagent (Invitrogen). The extracted RNA was treated with RNase-free Dnase (TaKaRa, Shinga, Japan). The absence of DNA contamination was confirmed by PCR using 18S ribosomal RNA primers (Forward: 5' CCT TTA ACG AGG ATC TAT TGG 3' and Reverse 5' ATA CTT GGC AAA TGC TTT CG 3'). cDNA was synthesized by reverse transcription using a cDNA synthesis kit (Takararr037a) (TaKaRa, Shinga, Japan) according to the manufacturer's instructions.

HSP gene cloning

Because the heat shock gene sequences in the Mediterranean flour moth were unknown, primers were designed using alignments of gene sequences from other insects, including *Chilo suppressalis*, *Bombyx mori*, *Pieris brassicae*, *Spodoptera exigua*, and *Helicoverpa armigera*. Degenerate primers were used to amplify ~550 bp gene fragments of the Mediterranean flour moth hsp70 and hsp90 genes are listed in Table 1.

The amplification products were resolved by gel electrophoresis, bands were excised from the gel, and a Expin Gel SV kit (GeneAll, Germany) was used to isolate the DNA.

The PCR products were ligated into a pTG19-T PCR Cloning Vector (Vivantis Technologies Sdn Bhd, Malaysia) using T4 DNA Ligase. The plasmids were used to transform *E. coli* DH5a cells using heat shock. Colonies with inserts were screened with blue/white X-gal selection under standard ampicillin conditions. Plasmid DNA was extracted from recombinant bacterial cells using the alkaline lysis method described by Sambrook & Russell (2001), and the DNA was sequenced from multiple (5-10) independent bacterial colonies.

Table 1 Primers used in current study.

Primers	Primer forward	Primer reverse	*PCR type*
HSP70	5'-AACCACTTCGTTCAGGAGT-3'	5'-TTGTTGTCCTTGGTCATGGC-3'	RT-PCR
HSP90	5'-CAGTTCGGTGTGGGTTTCTA-3'	5'-TTGTAGAAGTCGCCGTACTCC-3'	RT-PCR
T7-HSP70	5'-TAATACGACTCACTATAG AACCACTTCGTTCAGGAGT-3'	5'-TAATACGACTCACTATAG TTGTTGTCCTTGGTCATGGC-3'	(dsRNA synthesis)
T7-HSP90	5'-TAATACGACTCACTATAG CAGTTCGGTGTGGGTTTCTA-3'	5'-TAATACGACTCACTATAG TTGTAGAAGTCGCCGTACTCC-3'	(dsRNA synthesis)
T7-GFP	5'-TAATACGACTCACTATAG GTGGAGAGGGTGAAGG-3'	5'-TAATACGACTCACTATAG GGGCAGATTGTGTGGAC-3'	(dsRNA synthesis)
re- HSP70	5'-AACCACTTCGTTCAGGAGT-3'	5'-CTCCTCTGTCGCTCGGTAT-3'	RT-qPCR
re- HSP90	5'-CAGTTCGGTGTGGGTTTCTA-3'	5'-GAGGATGACAAGCCCAAGAT-3'	RT-qPCR
RL32	5'-TGGCACCACACCTTCTAC-3'	5'-CATGATCTGGGTCATCTTCT-3'	RT-qPCR

Sequence alignments and analyses

The sequences of the HSP70 and HSP90 cDNA were confirmed by a homology search of other HSP70 and HSP90 sequences known within the BLAST program available on the GenBank database of National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic trees were constructed using the neighbor-joining method with a Poisson correction model (1,000 bootstrap replications to check for repeatability of the results) using the MEGA 6.0 software.

Preparing dsRNA

Template DNA was amplified with the gene-specific primers that possessed the T7 RNA polymerase promoter sequence at the 5' end (5-TAATACGACTCACTATAG-3) (Table 1). The PCR products were prepared using the MEGAscript dsRNA synthesis kit (Ambion, Huntingdon, UK.) according to the manufacturer's instructions. The dsRNA fragments for both HSP70-dsRNA and HSP90-dsRNA, were approximately 500 bp. The concentration and quality of dsRNAs were assessed with a Nanodrop spectrophotometer.

RNA interference of HSP genes

RNAi bioassays were conducted by injecting 4 µL of the dsRNA (250 ng) solution into the hemocoel of 1-day-old fifth-instar larvae with a Hamilton microsyringe (Farahani *et al.*, 2020). A green fluorescent protein (GFP) dsRNA (dsRNA-GFP) was used as an exogenous control (negative control). Six replicates of 80 1-day-old fifth-instar larvae were conducted for each dsRNA treatment. The survival rate after dsRNA injection was more than 90%. After 24 h, the insects were subjected to 46 °C for 100 min or -15 °C for 30 min to determine the effect of heat and cold temperatures on survival, respectively. Then mortality was assessed 24 h after the thermal treatment.

Quantitative PCR (RT-qPCR)

RT-qPCR was employed to determine the expression of HSP70 and HSP90 in treated and non-treated Mediterranean flour moth using RealQ Plus 2x Master Mix Green (Ampliqon) on a Rotor-Gene® Q system (QIA-Gene). Gene-specific primers (Table 1) were designed using Primer Blast. A ribosomal protein gene, RL32, constitutively expressed in all tissues, was used as an internal control (Kumar *et al.*, 2015; Kim *et al.*, 2017). The PCR amplifications were performed with the following cycling conditions: one cycle at 95 °C (15 min), followed by 40 cycles of denaturation at 95 °C (15 s), annealing at 55 °C for 20s, and extension at 72 °C for 20 s. The $2^{-\Delta\Delta C_t}$ method was used to quantify the relative transcript level of target genes (Livak and Schmittgen 2001). A melt curve analysis assessed whether a single amplicon was produced in all RT-qPCR reactions. For each treatment, three replicates of 10 individuals were analyzed.

Statistical analysis

Means were compared using the least squared difference (LSD) tests of one-way ANOVA using SPSS software. Graphs were drawn using Excel 2013.

Results

Temperature tolerance bioassays

An initial assessment of different developmental stages of *E. kuehniella* to a high (44 °C) and a low (-15 °C) temperature revealed that only a small percentage of late instar larvae and pupae tolerated these extreme temperatures (Fig. 1); all other stages died after 1 h of heat or 30 min of cold. For the heat treatment, L5 larvae were the most robust, with 36% of the insects surviving the 44 °C exposure (Fig. 1a). For the cold treatment, L4, L5, and pupae showed very similar tolerances to the -15 °C treatment, with only about 10% survival (Fig. 1b). Overall, L5 larvae appeared most tolerant of heat and cold and were selected to assess variable exposure durations at these extreme temperatures. Maximal (100%) mortality was achieved after exposing L5 to 44 °C for 110 min or -15 °C for 40 min (Fig. 2a and Fig. 2b). Mortality was time-dependent with both the heat or cold treatments, with median lethal times of 54.9 min (95% CI: 52.7– 58.1 min) and 15.6 min (95% CI: 13.4– 17.7 min) for heat and cold treatments, respectively.

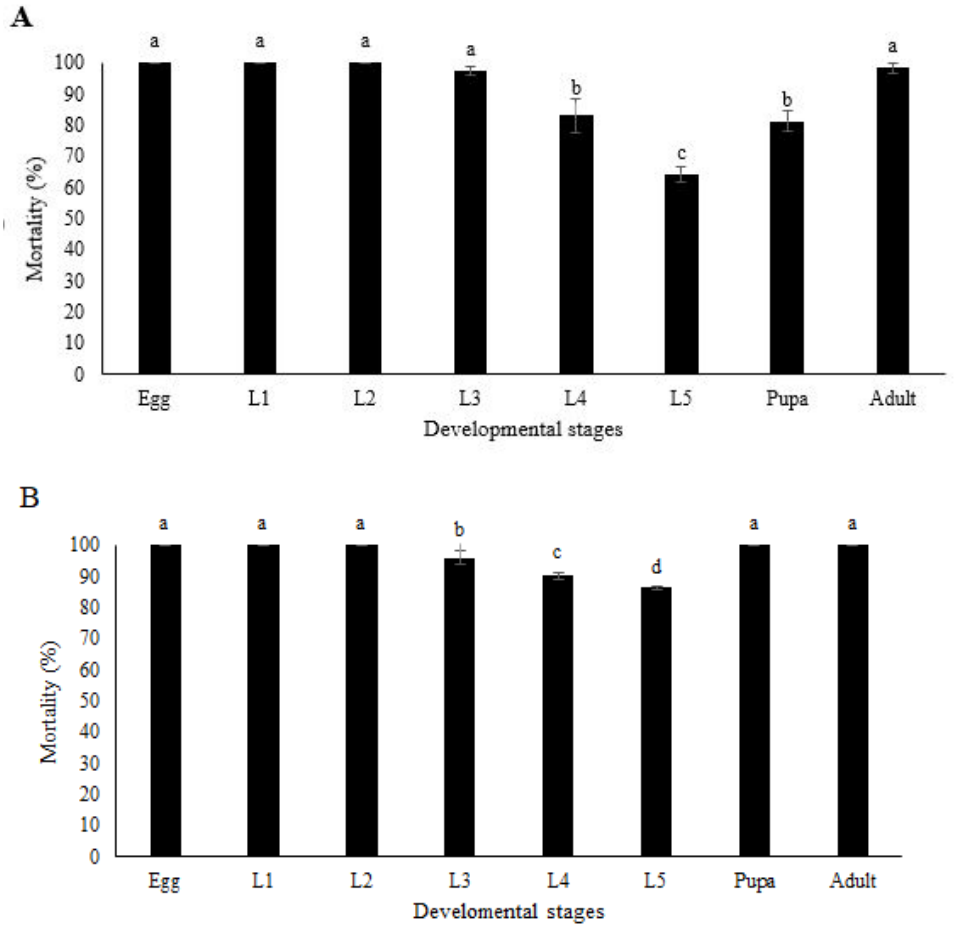

Figure 1 Susceptibility of different developmental stages to extreme temperatures. Insects were subjected to (a) heat: 44 °C for 1 hour or (b) cold: -15 °C for 30 min, and the percent dead was assessed. Values represent the means and standard errors for three replicates of 80 individuals of each developmental stage. Different letters denote significantly different values from one another (LSD).

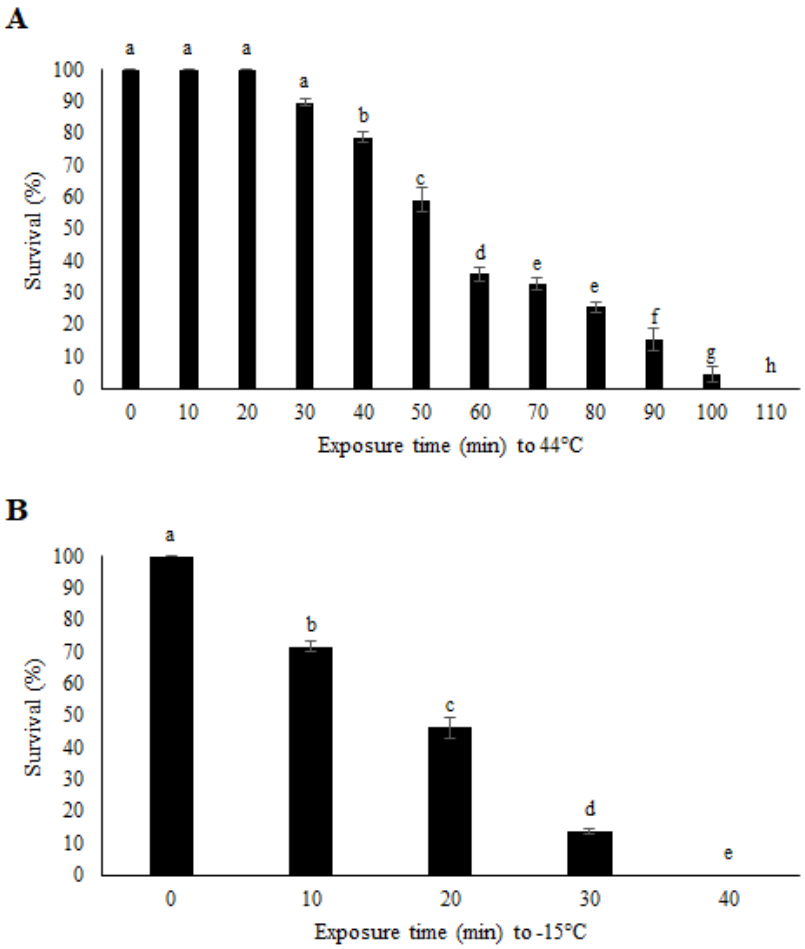

Figure 2 Effect of exposure times on mortality of *E. kuehniella* L5 larvae using (a) heat: 44 °C for 0-110 min or (b) cold: -15 °C for 0-40 min. The values represent the mean and standard errors for three replicates of 80 L5 larvae. Different letters denote significantly different values from one another (LSD).

Exposure of fifth instar larvae to different extreme low and high temperatures revealed that treatment at 48 °C for 80 min or -18 °C for 30 min led to 100% mortality (Fig. 3a and Fig. 3b). The median lethal temperatures of high and cold temperatures were 44.3 °C (95% CI: 44.1-44.6 °C) and -8.1 °C (95% CI: -6.9—9.2 °C), respectively.

Pre-exposure of fifth instar larvae to different moderately high and low temperatures statistically increased survival rates at the extreme temperatures; the pre-exposures at 35 °C or 10 °C for 10 min resulted in the highest survival rates of L5 larvae following their subsequent exposure to the lethal high (44 °C for 80 min) and low (-15 °C for 30 min) temperature treatments, respectively (Fig. 4a and Fig. 4b). We subjected the insects to these two pretreatment temperatures for variable durations based on these observations. We observed that heat or cold tolerances could be induced within as little as 10 min pretreatment (Fig. 5a and 5b). The highest survival rates were observed in insects that were pretreated at 35 °C for 30 min or 10 °C for 30 min, with survival rates of 50.25 ± 1.67 and 47.92 ± 2.31, respectively.

Heat shock genes identification

The HSP70 and HSP90 gene fragments from *E. kuehniella* were PCR-amplified from cDNA

using primers designed to anneal to conserved regions of other insect HSP70 and HSP90 genes. Analysis of the *E. kuehniella* putative HSP70 gene fragment sequence confirmed that it was similar to other insects' HSP70 genes. A phylogenetic analysis of the putative HSP70 gene sequence of the *E. kuehniella* and other insects likewise confirmed that the Mediterranean flour moth's gene is most similar to HSP70 genes of other lepidopterans, and in particular, to moths of the family Pyralidae (Fig. 6a). A phylogenetic analysis of HSP90 genes from several lepidopteran insects likewise confirmed that *E. kuehniella* gene's sequence is most similar to that of another lepidopteran species, *Galleria mellonella* (Fig. 6b). It is worth noting that the Mediterranean flour moth putative HSP70 gene fragment sequence was identical to that of *Cadra cautella* (almond moth). This identity was confirmed not to be due to sample contamination. Multiple biological replicates of cDNA were prepared from both species. While the HSP70 fragment was identical in these two species for each replicate, the HSP90 sequence, derived from the same cDNA samples, showed the same differences between *E. kuehniella* and *Cadra cautella*.

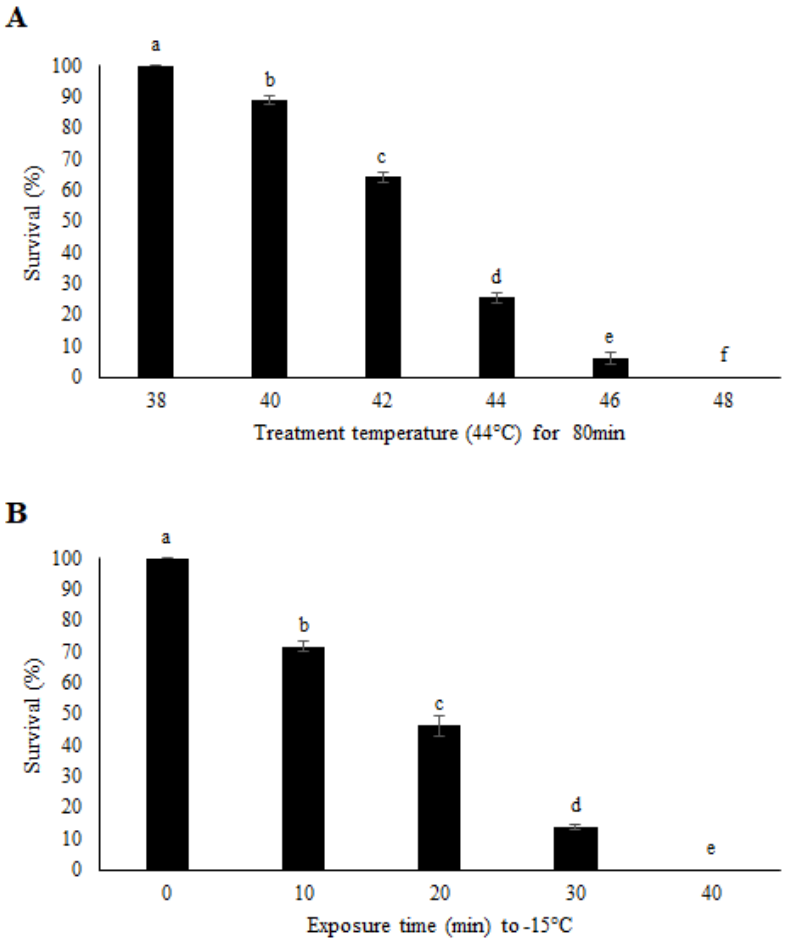

Figure 3 Effect of different (a) high or (b) low temperatures on mortality of *E. kuehniella* L5 larvae. Values represent the means and standard errors for three replicates of 80 individuals of each temperature. Different letters denote significantly different values from one another (LSD).

RNAi-mediated knockdown of HSP transcripts
Knockdown of the two HSP genes, HSP70 and HSP90, was achieved by injecting L5 larvae with gene-specific dsRNA, to determine the role of these HSPs in heat and cold tolerance induction. The mortality rate significantly increased 1.4 and 1.6-fold in dsHSP70- and dsHSP90- injected larvae compared to the negative controls (injected with non-specific dsGFP) in heat treatments (Fig 7a). In cold treatments, injection of dsHSP70 and dsHSP90 resulted in increased mortality rates 1.7 and 1.4-fold, compared with the dsGFP controls, respectively (Fig. 7b).

Injection of dsHSP70 and dsHSP90 into fifth instar larvae suppressed the expression level of each of these targeted genes. The HSP70 mRNA level was reduced significantly 2.8 and 1.4-fold compared to controls 24 h and 48 h after dsRNA injections, respectively (Fig. 8a). HSP90 expression levels were decreased significantly 1.6-fold and 1.2-fold relative to the controls 24 h after dsRNA injections (Fig. 8b).

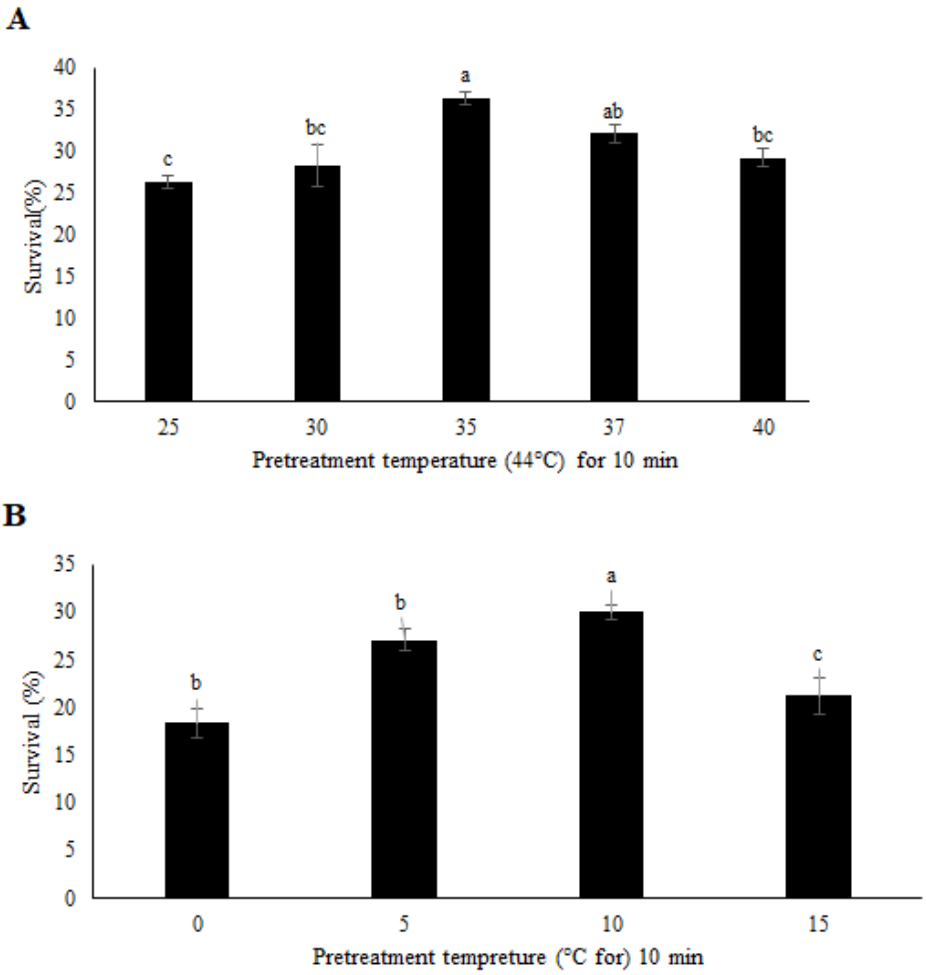

Figure 4 Induction of extreme temperature tolerance in *E. kuehniella* L5 larvae. Insects were pretreated for 10 min at various temperatures and then subjected to (a) 44 °C for 80 min or (b) -15 °C for 30 min. The values represent the means and standard errors for percent survival for the replicates of 80 individuals. Different letters denote significantly different values from one another (LSD).

The expression level of these two HSP genes was conducted using quantitative RT-PCR. These analyses confirmed that either heat or cold shock induced both newly identified HSP genes from the Mediterranean flour moth. Exposure to a moderately high temperature significantly increased the expression level of HSP70 and HSP90 3.8 and 4.3-fold compared to the controls (Fig. 8). Also, pretreatment with moderately cold temperature similarly increased the expression levels of HSP70 and HSP90 3.8 and 3.3-fold compared to the controls (Fig. 8).

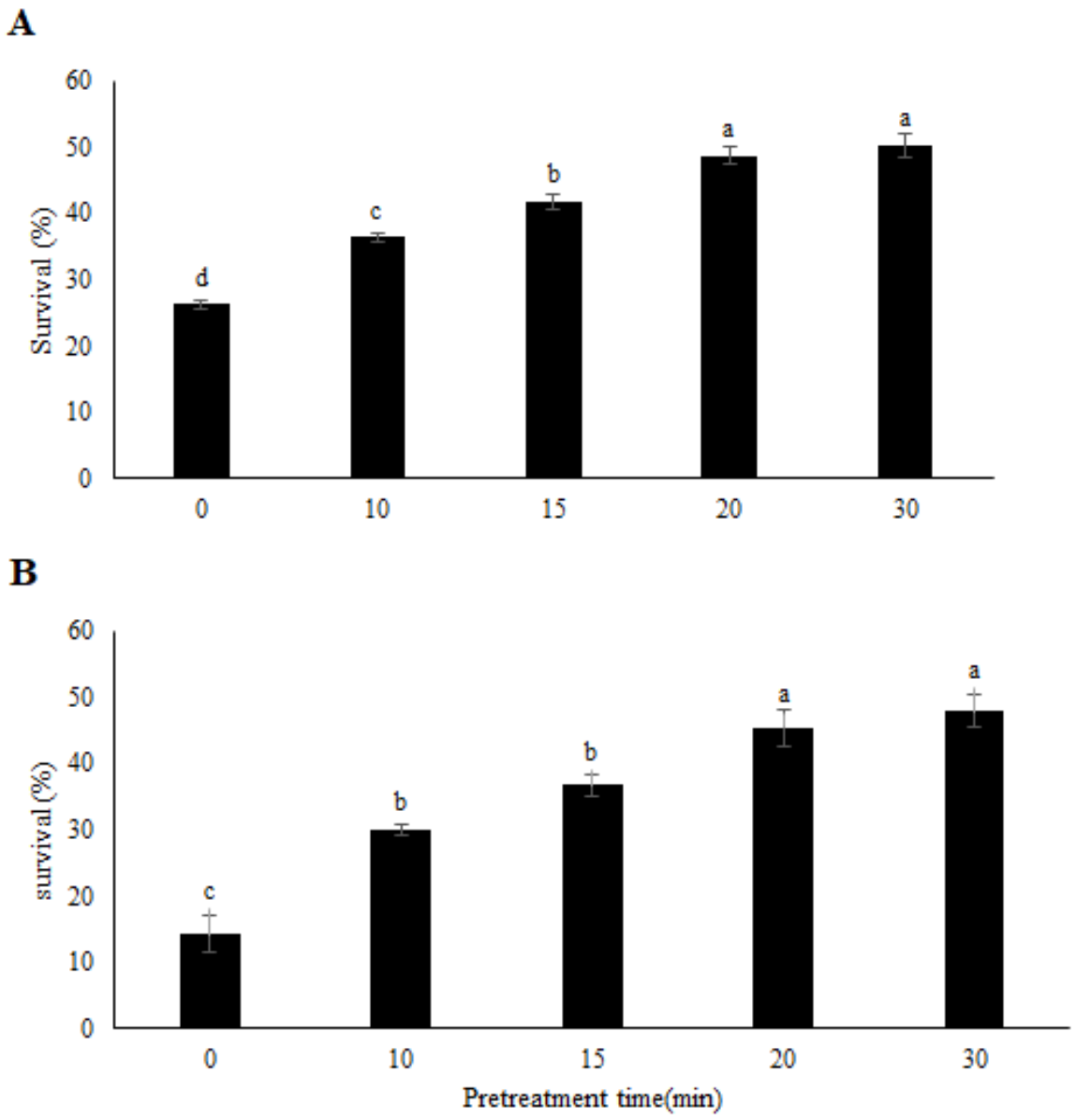

Figure 5 Induction of extreme temperature tolerance by varying durations of (a) 35 ºC pretreatments, subsequently treated to 44 °C for 80 min, or (b) 10 ºC pretreatments, subsequently treated to -15 °C for 30 min. The values represent the means and standard errors for percent survival for the replicates of 80 individuals. Different letters denote significantly different values from one another (LSD).

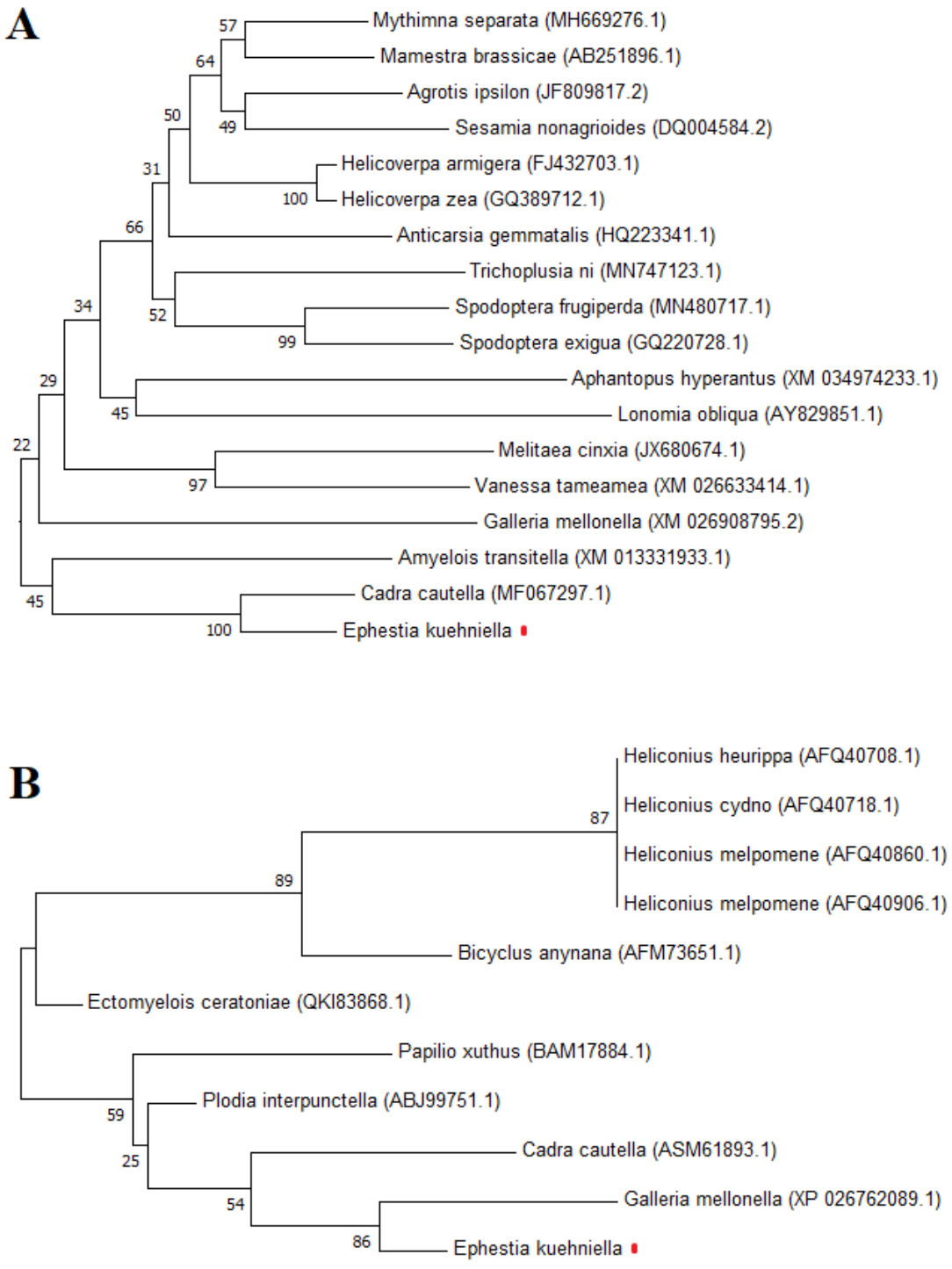

Figure 6 Phylogenetic relationship of sequences of HSP70 and HSP90 of *E. kuehniella* and other insects. (A) An unrooted neighbor-joining tree of HSP70 cDNA sequences was constructed with bootstrap support based on 10,000 replications shown at the relevant branch points. (B) Neighbor-joining tree of predicted protein sequences of HSP90 from the carob moth and other lepidopteran insects.

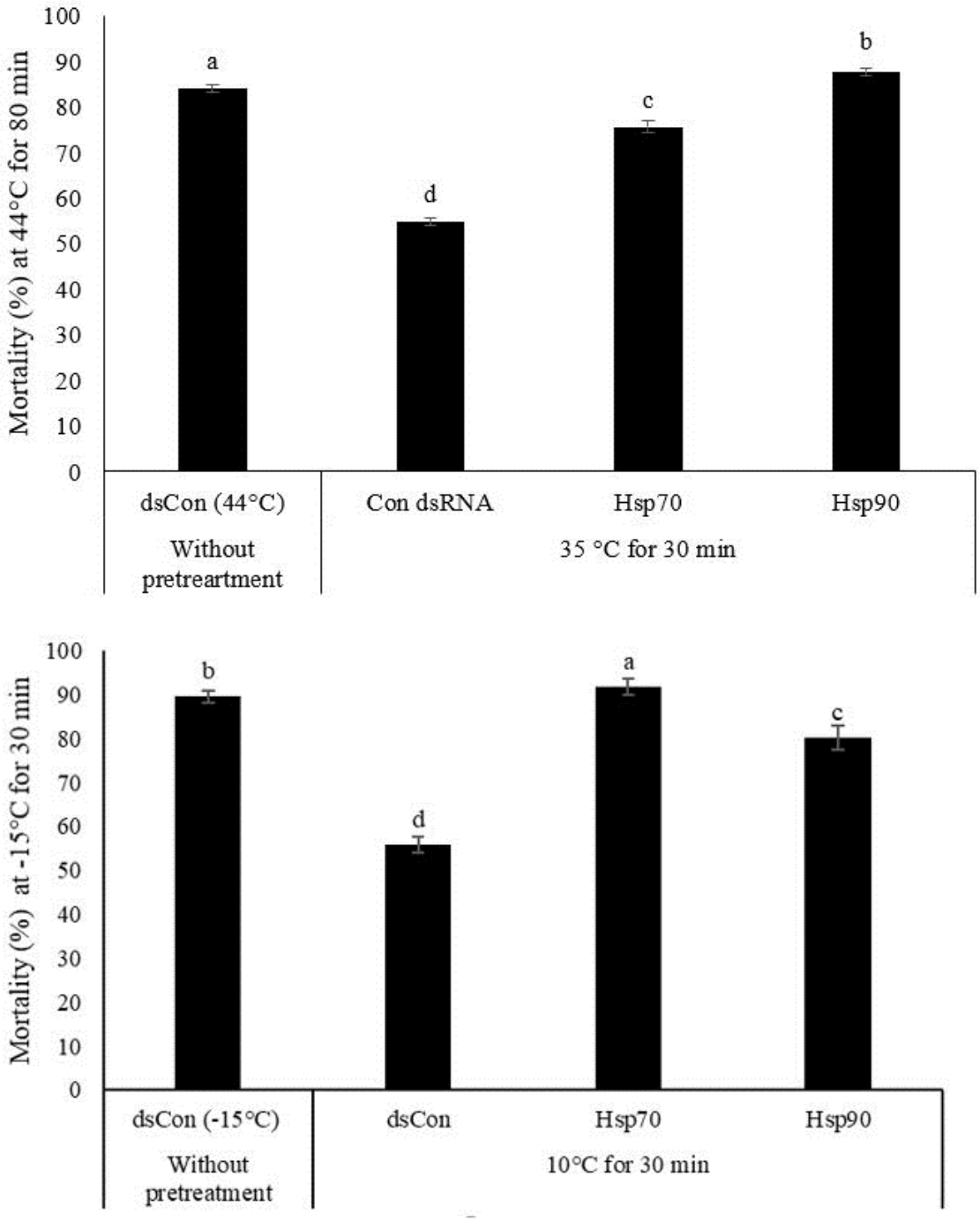

Figure 7 Effect of RNAi-mediated HSP genes transcript knockdown on mortality rates in *E. kuehniella* larvae subjected to (a) a moderate heat (35 °C for 30 min) or (b) a moderate cold (10 °C for 30 min) pretreatment, followed by exposure to a higher temperature treatment. Insects were injected with dsRNA targeting HSP70, HSP90, or GFP (negative control) genes' transcripts, allowed to recover for 24 h and then subjected to the temperature pretreatments. The values represent the means and standard errors for percent survival for the replicates of 80 individuals. Different letters denote significantly different values from one another (LSD).

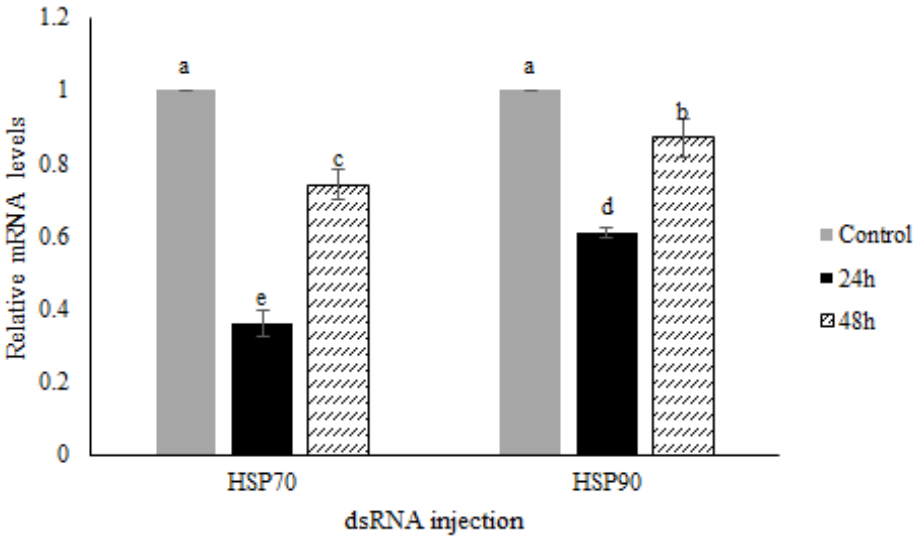

Figure 8 The extent of transcript knockdown following injection of L5 larvae with HSP70, HSP90, or GFP (negative control) dsRNAs. Following dsRNA injection, we allowed insects to recover 24 or 48 h before being subjected to RNA extraction and qRT-PCR analysis. Transcript levels were standardized relative to rl32 transcripts levels. The values represent the means and standard errors for three replicates of 10 individuals. Different letters denote significantly different values from one another (LSD).

Discussion

Insects live in environments with variable biotic and abiotic factors that affect their physiology, fitness and geographical range (Parkash and Ranga, 2013). Because they are poikilothermic organisms, the temperature is a significant factor that can restrict their activities and distribution (Lee, 2010). Generally, insects grow and reproduce within a limited range of temperatures, typically between 13 and 35 °C (Fields, 1992), and when confronted with temperatures outside of this range, they suffer from significant impacts on growth and increased mortality (Heinrich 1981). Due to their sensitivities to extreme temperature shifts, extreme temperature treatments have been and continue to be used as physical control methods for pest insects of stored products (Neven 2000). The Mediterranean flour moth is a pest insect of various stored foods. As chemical insecticides must be used sparingly in our foodstuffs, alternative pest control methods, such as heat or cold treatments, may prove useful. The durations and temperatures needed to control different pest insects will depend on the species and stage of development of the insects to be treated. In this study, the susceptibility of all developmental stages of the Mediterranean flour moth to high and low temperatures was investigated. We observed that heat treatment of 44 °C for 80 min or -15 °C for 30 min resulted in significant mortality of most developmental stages. The late larval instars (L4 and L5) were the most tolerant to these temperatures, with more extreme temperatures or longer durations of temperature stress required to kill this more robust insect stage. There are many reports about the differential tolerance of developmental insect stages to temperatures (Andreadis *et al.*, 2012; Kim *et al.*, 2017; Farahani *et al.*, 2020). These temperature tolerances are not atypical, as other lepidopteran pests have been observed to require similar temperature and duration to achieve effective control. Wang *et al.* (2015) reported that exposure to 47 °C or -7 °C for 2 h killed all larval instars of the noctuid moth *Xestia c-nigrum*. Likewise, in the carob moth (*Ectomyelois ceratoniae*), exposures to 46 °C for 60 or -15 °C for 30 min were sufficient to kill most developmental stages of the insect. Still, like the Mediterranean flour moth, the late larval

instars showed greater tolerance of the extreme temperatures relative to other larval stages (Farahani *et al.*, 2020). Andreadis *et al.* (2012) reported that pupae *of E. kuehniella* were the most cold-tolerant among all their developmental stages, so this stage had the highest mean lethal time (LTime50), and lowest mean lethal temperature ((LTemp50) among other ones.

It is unclear whether the greater tolerance to extreme temperatures of late instar larvae is due to as yet undetermined physical attributes of the larvae, or to more pronounced or varied molecular responses to the stress. Induction of tolerance to extreme temperatures by exposure to sublethal temperatures has been demonstrated in various insects, including other lepidopterans. For example, Kim *et al.* (2017) observed that pre-exposure of *P. interpunctella* larvae to moderately high temperatures induced heat tolerance and enhanced their survival rate. We observed a similar phenomenon in the Mediterranean flour moth larvae, where pre-exposure to sublethal hot or cold temperatures improved survival rates at more extreme temperatures. Inducible temperature stress tolerance is a phenomenon that has been observed in all organisms studied to date. It includes a range of molecular responses, including, but not limited to, the synthesis of HSPs. As molecular chaperones, HSPs play a key role in helping other stress-induced denatured or misfolded proteins refold following temperature stress events. Other protective mechanisms can also contribute to the viability of insects that have endured a moderate temperature pretreatment, such as increased production of water-soluble protein, triglycerides, glycerol and trehalose in the hemolymph (Ju *et al.*, 2014; Kim *et al.*, 2017). In our study, we focused on just the role of some HSPs. We observed that moderate heat and cold treatments of the Mediterranean flour moth larvae induced increased HSP70 and HSP90 gene expression. While many studies have observed that expression of HSP transcripts and/or HSP synthesis is often correlated with increased thermotolerance, confirmation that the HSPs are actively involved in conferring thermotolerance is not frequently assessed.

Here, we demonstrated that RNAi-mediated knockdown of either HSP70 or HSP90 transcripts reduced tolerance to lethal temperature stress, even when provided a sublethal temperature pretreatment. This loss of thermotolerance provides explicit evidence that each of these two HSPs has roles in conferring resistance to extreme temperatures, at least over the short term. The utility of RNAi as a molecular biology tool to provide evidence of gene function is well-recognized in many eukaryotic species. Still, RNAi has not been effective in some lepidopteran insects (Terenius *et al.*, 2011). In the Mediterranean flour moth, injection of dsRNA into the hemocoel effectively reduced the targeted genes' transcripts, demonstrating that this lepidopteran species is amenable to this useful molecular biology tool. At this stage, we have confirmed that each of these two HSPs is important in the induction of short-term thermotolerance in late-instar larvae, but further studies will be needed to assess the relative roles of each of these proteins in protecting different stages of the insect, to ascertain why late instar larvae are more tolerant than other stages of the insect's life cycle.

HSPs are typically considered as the first line of defense against temperature stressors in many insects. HSP70 and HSP90 proteins are often the most abundant HSPs in cells under stressful conditions (Lindquist and Craig, 1988), assisting with protein folding and relieving cellular damage during thermal stress (Clark and Worland, 2008; Schopf *et al.*, 2017). Increased transcription of these two classes of HSPs following temperature stress has been observed in a diverse range of insects in recent studies. For example, Guo and Feng (2018) observed that the transcript levels of HSP70 and HSP90 were up-regulated after heat shock treatments in grass thrips (*Anaphothrips obscurus*). Chang *et al.* (2018) noted that the expression level of HSP70 was significantly increased after exposure to cold stress in the dipteran pest, the celery leaf miner (*Liriomyza trifolii*). Gu *et al.* (2019) showed the HSP70 and

HSP90 transcript levels of the rice leaf roller *Cnaphalocrocis medinalis* (Lep.: Cramidae) significantly increased after exposure to moderately high temperatures. Like many other studies of induced temperature tolerance in insects, these studies provided correlative evidence that these HSPs are implicated in conferring thermotolerance. As more research groups use RNAi techniques in insects, we see more supporting evidence that HSPs are essential for recovery from temperature stresses. The role of HSPs in induced thermotolerance has now been validated using RNAi in a growing number of insects, representing many of the insect orders, including Coleoptera (beetles; Atungulu *et al.*, 2006), Diptera (flies; Rinehart *et al.*, 2007; Colinet *et al.*, 2010), Heteroptera (true bugs; Kostal and Tollarova-Borovansk, 2009), and Lepidoptera (moths; Kim *et al.*, 2017). Our research supports these studies' findings, highlighting the importance of these proteins in protecting the Mediterranean flour moth from heat and cold stress.

If temperature treatments are to be used as an alternative insect control technique for stored food products, care must be exercised to avoid damage to the food itself. Disruption of the pest insects' HSPs could permit moderate temperature treatments to be as effective as more extreme temperature treatments, thereby reducing the risk of damage to the foodstuffs. Given that the Mediterranean flour moth is one lepidopteran species sensitive to RNAi, future efforts to deliver dsRNAs in a higher throughput manner to this insect are worth exploring. Several advancements have been made in the development of microcarriers that can provide environmental stability of dsRNAs and improve cellular uptake in insects for large-scale dsRNA treatments (Joga *et al.*, 2016; Kumar *et al.*, 2019). With further improvements in dsRNA delivery technologies, it might be possible to couple species-specific knockdown of HSP genes in pest insects with moderate temperature treatments to control pest insects without adversely affecting non-target species.

In summary, understanding insects' tolerance to stress may also help us develop new, environmentally safer approaches to insect control, reducing our reliance on our currently available chemical insecticides.

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خاموشی ژن پروتئین‌های شوک حرارتی روی تحمل دمایی شبپره مدیترانه‌ای آرد *Ephestia kuehniella* (Lepidoptera: Pyrallidae) تأثیر می‌گذارد

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چکیده: شبپره مدیترانه‌ای آرد، *Ephestia kueheniella*، یکی از آفات مهم انباری در سراسر جهان است که همانند بسیاری از آفات محصولات انباری، استفاده از آفتکش‌های شیمیایی، روش رایج کنترل این‌گونه محسوب می‌شود. ازآنجاکه بسیاری از این مواد شیمیایی تأثیرات منفی بر گونه‌های غیرهدف دارند، یافتن روش‌های جایگزین ایمن‌تر مورد نیاز است. دما به‌عنوان یکی از روش های کنترل دوستدار محیطزیست می‌تواند سطوح مطلوب کنترل را فراهم آورد، اما القای تحمل سرمایی و گرمایی در حشرات می‌تواند منجر به کاهش کارایی این روش گردد. در مطالعه حاضر، ابتدا، حساسیت تمام مراحل رشدی شبپره مدیترانه‌ای آرد به دماهای شدید بالا و پایین مورد بررسی قرار گرفت. نتایج نشان داد قرارگیری حشرات در معرض دمای ۴۴ درجه سلسیوس به‌مدت ۸۰ دقیقه و یا ۱۵- درجه سلیسوس به‌مدت ۳۰ دقیقه باعث افزایش قابل‌توجهی در میزان مرگ‌ومیر تمام مراحل رشدی این‌گونه می‌گردد. قرارگیری حشرات در معرض دماهای معتدل پایین یا بالا باعث القای تحمل سرما یا گرما و در نتیجه کاهش میزان مرگ‌ومیر گردید. نتایج آزمون RT-PCR کمی تأیید کرد که تحمل القا شده به گرما یا سرما همراه با افزایش رونویسی دو پروتئین شوک حرارتی، HSP70 و HSP90 بوده است؛ سطوح رونویسی HSP70 و HSP90 پس از پیش تیمار حشرات در دمای ۳۵ درجه سلیسوس به‌مدت ۳۰ دقیقه به‌ترتیب ۳,۸ و ۴,۳ برابر و پس از پیش تیمار حشرات در دمای ۱۰ درجه سلیسوس به‌مدت ۳۰ دقیقه به‌ترتیب ۳,۸ و ۳,۳ برابر در مقایسه با گروه شاهد افزایش یافت. خاموشی دو ژن HSP70 و HSP90 با استفاده از تکنیک تداخل RNA (RNAi) باعث کاهش قابل‌توجهی در تحمل گرما و سرما گردید که این موضوع حاکی از نقش حیاتی این دو پروتیئن در القای تحمل گرما و سرما در شبپره مدیترانه‌ای آرد است.

واژگان کلیدی: HSP70, HSP90، تداخل RNA، مدیریت تلفیقی آفات