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Effect of thermal stress on the immune responses of *Chilo suppressalis* walker (Lepidoptera: Crambidae) to *Beauveria bassiana*

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ABSTRACT

Temperature is one of the important environmental elements affecting ecological fitness of insects through alterations in physiological systems. In the current study, a comparison was made on the cellular and humoral immune responses of the *Chilo suppressalis* larvae exposed to thermal stress (34 °C) and optimal rearing temperature (24 °C). Although total hemocyte count increased in the injected larvae by *Beauveria bassiana*, elevation of hemocyte numbers was significantly different in the larvae exposed to 34 °C for a short-time period compared to long-term exposure and control. A similar trend was observed in plasmatocyte and granulocyte counts as well as phenoloxidase activity. Gene expression of some antimicrobial peptides, including attacin1, attacin2, cecropin1, cecropin2, defensin, gallerimycin, lysozyme and prophenoloxidase-activating proteinase-3, was compared in the larvae exposed to thermal regimes and injection challenges. In all cases, expression of the target genes was relatively higher in the larvae injected by *B. bassiana* and short-term exposure at 34 °C. The present results confirmed that *C. suppressalis* could modulate the immune system in response to different thermal stress conditions mainly over a short period.

1. Introduction

Insects, similar to other organisms, during most of their lifetime, simultaneously encounter a vast array of stressful stimuli that may threaten their survival (Williams et al., 2015). Pathogens (such as fungi, viruses, bacteria, and protozoan/metazoan parasite) and fluctuation in environmental factors like temperature are among the most important stressors simultaneously or consecutively experienced by insects, which may have disruptive effects on their biological and physiological fitness; therefore, insects have evolved a set of behavioral, physical and immunological barriers to deal with the effects of these threats (Kaunisto et al., 2016; Wojda, 2017). Behavioral defence (e.g. biting, acquiring genetic resistance through selection of suitable mates, committing suicide to favor kin survival, or developing a fever response) are the first line of defence in insects minimizing or eliminating negative effects of parasites and pathogens (Greeney et al., 2012). The cuticle and epidermis are the second lines of defense forming an efficient protective barrier over external surface extending into the trachea, foregut, and hindgut (Gillespie and Kanost, 1997). Nevertheless, many pathogens and parasites are able to breach these barriers; therefore, insects must also employ their innate immune systems as the final line of their defense. The innate immune system is comprised of cellular and humoral responses, both of them are mediated through various signaling pathways (Lavine and Strand, 2002). Humoral defenses contain antimicrobial molecules involved in melanin formation, coagulation and toxicity against pathogens, while cellular responses refer to hemocyte mediated processes such as phagocytosis, encapsulation, and nodulation (Strand, 2008). Environmental conditions like ambient temperature profoundly affect the performance of the insect immune system (Le Moullac and Haffner, 2000; Adamo, 2004; Mydlarz et al., 2006; De Block and Stoks, 2008).

Since insects are poikilotherm organisms, and their immune system relies on temperature-dependent cellular and enzymatic activities, fluctuations in temperature will directly affect immune activity simply based on thermodynamics (Murdock et al., 2012). Early studies have demonstrated that warmer temperatures increase various immune responses (e.g. Ouedraogo et al., 2002; Ouedraogo et al., 2003; Zibaee et al., 2009; Catalán et al., 2012a, 2012b; Wojda and Taszłow, 2013a, 2013b). In fact, the thermoregulatory set-point known as behavioral fever makes a key contribution to immune resistance of both vertebrate and invertebrate toward infection (Kluger, 1979). In particular, the immune system appears to be tightly – albeit complexly – linked to stress response in insects, with both immuno-suppressive and immuno-enhancing effects (Adamo, 2014, 2016). For example, since stress and

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immune responses share resources such as lipid transport proteins, activation of the flight-or-fight stress response can suppress immunity via resource-based trade-offs, although it may lead to genetic variation in traits under natural selection (Thomas and Blanford, 2003; Cotter et al., 2004; Adamo et al., 2008). Conversely, disease resistance can be enhanced as a by-product of stress response, whereby intracellular stress responses that lead to cellular protective mechanisms (e.g. expression of heat shock proteins and detoxification enzymes) indirectly improve disease resistance and improve reproductive performance of the immune-challanged insects exposed to higher temperatures (Ballabeni et al., 1995; Davies et al., 2012, 2014).

Temperature and infection can separately or combinedly affect the physiological process in insects. Moreover, acceleration of climate warming emphasizes the need for a full understanding of its consequences. In a global view, climate warming leads to extreme meteorological events changing ecosystems, food webs, habitat and biodiversity of organisms. In agriculture, climate warming elevated temperatures and amount of carbon dioxide in atmosphere, which subsequently increases photosynthetic rates plant productivity; however, such a phenomenon depends on earth regions and type of plants encountering higher temperatures (McMahon et al., 2010). To investigate it in detail, temperature and other environmental factors may affect growth and reproduction of plants totally known as phenology. Since herbivorous insects synchronized their activity with phenology of hosts, any changes may lead to insect distribution, emergens and food-interaction with plants (Taylor et al., 2018). Insects, through the detoxification, antioxidant and immun systems reduce risks of these stresses; however, it should be pointed out that high temperature in infected insects by microorganisms positively affects their growth and survival through efficient immune defence compared to other environmental variables (Fuller et al., 2011). In our previous study, we indicated that exposure of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae to 34 °C increased antioxidative responses through enzymatic and non-enzymatic components (Shamakhi et al., 2018a). It was also found that the combined effects of thermal stress and fungal infection altered antioxidant system and lipid peroxidation of *C. suppressalis*. Our results indicated the higher concentrations of oxidative agents and subsequently antioxidant activities in the larvae exposed simultaneously to thermal stress and *B. bassiana* infection compared to thermal stress alone (Shamakhi et al., 2018b). In the present study, the immune responses of *C. suppressalis* larvae to the two stress factors, temperature and infection by *B. bassiana* were investigated through short- and long-term exposure to elucidate capability of immune responses. In this regard, total and differentiated hemocyte counts, phenoloxidase activity and gene expression of some antimicrobial agents were compared to control larvae after exposure to thermal regimes.

2. Materials and methods

2.1. Insects rearing

The egg patches of *C. suppressalis* were collected from rice fields of Amol (located in the north of Iran) during August 2017. The eggs were kept in a growth chamber at 24 ± 2 °C, 16:8 h (L:D) and 85% of relative humidity. After hatching, newly larvae were transferred to the test tubes (20 × 15 cm) and fed on rice seedlings (Shamakhi et al., 2018a). Laboratory conditions and quality of food were monitored every day and the old cutting stems were replaced by fresh ones. Rearing was prolonged for at least five generations based on the method of Zibaee et al. (2009).

2.2. Culture of Beauveria bassiana

B. bassiana (Isolates: BB_3 from Fashand, Iran), was grown on Potato Dextrose Agar in 8-cm diameter Petri dishes at 25 ± 2 °C and 70 ± 2% R.H. After 14 days of incubation, a suspension of conidia containing 1×10^4 spores/mL were prepared in 0.01% of Tween 80 (Sigma-Aldrich, USA) using hemocytometer.

2.3. Larval treatment

The two thermal treatments were considered for heat shock experiment. The larvae were divided into three separate groups and exposed on incubators under identical conditions (See below). Larvae in the first and second groups were placed in the test tubes immediately after hatching, reared separately on optimal (24 °C) and stress temperatures (34 °C) until they grew to the fifth larval instars (Shamakhi et al., 2018b). After 24 h of fifth instar emergence (Under given thermal regime), they were injected with *B. bassiana*. Again first group was placed at 24 °C and the second one was kept at 34 °C. After two time intervals of 24 and 72 h, desired immune responses were determined to show the combined effects of long-term thermal stress and fungal injection. The third group was the larvae reared on optimal temperature (24 °C), once they were reached to fifth larval instar they were injected by fungal spores but now they were exposed to stress temperature (34 °C) to show the combined effects of short-term thermal stress and *B. bassiana*. Similar to two earlier groups, immune challenges were determined after 24 and 72 h in which the larvae were kept at 34 °C. Each of the above groups was divided into three subgroups; The first subgroup includes intact larvae (referring to no injection), but second and third subgroups were injected laterally into their third thoracic segment with 1 µL of Tween-80 (0.01%) solution and a concentration of 10^4 spores ml^{-1} of the *B. bassiana* (Isolate BB_3) prepared in tween 80 (0.01%). Fifty larvae were used in each group and the whole experiment was done by three replicates. A schematic diagram of experimental procedure has been illustrated in Fig. 1.

2.4. Hemolymph collection and hemocyte counts

Hemolymph of the larvae exposed to experimental treatments was collected using capillary tubes (Sigma-Aldrich, London, England) by piercing the first abdominal proleg with a sterile needle. The collected Hemolymph was immediately diluted in an anticoagulant solution (0.01 M, ethylenediamine tetraacetic acid; 0.1 M, glucose; 0.062 M, NaCl; 0.026 M, citric acid; pH 4.6) as described by Azambuja et al. (1991). Then, 100 µL of the hemolymph was pipetted onto a hemocytometer and the numbers of total and differentiated hemocytes including plasmatocytes and granulocytes were counted by direct observation under light microscopy.

2.5. Assay of phenoloxidase activity

The activity of phenoloxidase were assayed in all treatments according to the method described by Wilson et al. (2002). Briefly, 10 µL of hemolymph was transferred to a plastic tube (1.5 mL), then 100 µL of ice-cold phosphate buffered saline (20 mM, pH 7) was added and the samples were frozen to disrupt hemocytes. To determine phenoloxidase (PO) activity in the defrosted solution, samples were poured into each well of a plate containing 20 mL of 10-mM l-dopa (3,4- dihydroxyphenylalanine) as a substrate. After 5 min of incubation at room temperature, the absorbance was measured at 492 nm.

2.6. Protein assey

The amount of total protein was assayed by the method of Lowry et al. (1951) using a commercial kit manufactured by ZiestChem company (Tehran, Iran).

2.7. Expression of antimicrobial peptides

2.7.1. Isolation of total bodies and RNA extraction

At first, the larvae of *C. suppressalis* were treated with thermal stress

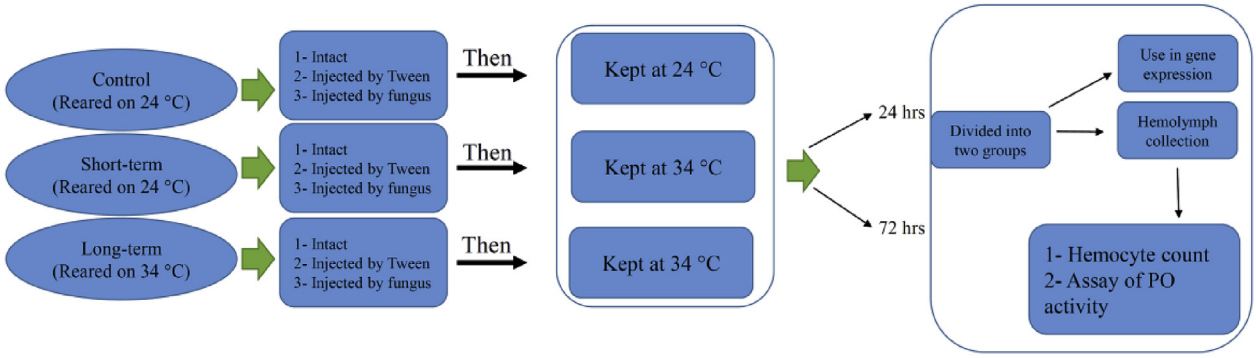

Fig. 1. Overview of the experimental design showing the effect of temperature on immunity of *Chilo suppressalis*. The larvae were divided into three separate groups in which the larvae of the first and second groups initially were reared on 24 and 34 °C to fifth larval instars. After 24 h of the current larval instar, they were injected with *Beauveria bassiana* but the first group was exposed to 24 °C but the second group was kept at 34 °C. After 24 and 72 h post-injection, immune factors were evaluated to show Long-term effect. The third group was the larvae reared on optimal temperature (24 °C), once they were reached to fifth larval instar they were injected by fungal spores but now they were exposed to stress temperature (34 °C) to show the combined effects of short-term thermal stress and *B. bassiana*. Similar to two earlier groups, immune challenges were determined after 24 and 72 h in which the larvae were kept at 34 °C. Each of the above groups was divided into three subgroups. The first subgroup includes intact larvae (referring to no injection), but second and third subgroups were injected laterally into their third thoracic segment with 1 µL of Tween-80 (0.01%) solution and a concentration of 10^4 spores ml-1 of *B. bassiana* (Isolate BB3).

and *B. bassiana* simultaneity as described earlier. Then, the larvae were randomly selected and rapidly frozen in 500 µL of DEPC-treated (diethylpyrocarbonate) water to onst of the procedures for immune-related gene expressions in response to the combined effects of thermal stress and *B. bassiana*. Total RNA was extracted from the whole larval body using guanidine-phenol solution (RNX-Plus, SinaClon Co., Tehran, Iran: Cat. No.: RN7713C). Based on instruction, 800 µL of ice-cold RNX-Plus solution was added to the tubes containing homogenized samples, vortexed for 5–10 s and incubated at room temperature for 5 min. Then, 200 µL of chloroform was added to the mixture and shaked well for 15 s prior to incubation on ice for 15 min. Tubes were centrifuged for 15 min at 12,000 rpm and 4 °C. Aqueous phase was transferred to a new eppendorf tube, an equal volume of isopropanol was added, gently mixed, and incubated on ice for 15 min. The current mixture was re-centrifuged at the same condition, supernatant was removed, 1 mL of 75% ethanol was added and tubes were centrifuged at 7500 rpm for 8 min at 4 °C. The supernatant was removed and the pellets were dried at room temperature for 3 min. Finally, the pellets were dissolved in 50 µL of DEPC (diethylpyrocarbonate)-treated water.

2.7.2. cDNA synthesis

cDNA was synthesized using a Thermo Scientific RevertAid First Strand Kit (Fermentase Co.). Synthesis of the first-strand cDNA was done in a total volume of 20 µL containing 4 µL of 5× reaction buffer for reverse transcriptase, 1 mL (20 U/µl) of RiboLock RNase Inhibitor, 2 mL (10 Mm) of dNTP Mix, 1 mL (200 U/ll) of RevertAid H Minus Reverse Transcriptase, 1 µL of Oligo-dT primers, 11 µL of DEPC-treated water, and 1 µL of the extracted RNA of control and treated larvae. The tubes were incubated for 60 min at 42 °C and the reaction was stopped at 70 °C for 10 min. The second strand cDNA synthesis was achieved by the reverse transcription product prior to being stored at −20 °C.

2.7.3. qRT-PCR

Gene expression was done through quantitative real-time PCR using Maxima SYBR Green/ROX Kit (FERMENTAS Co). The three technical replicates of amplification were considered using a 13.5 µL of reaction volume including 6.25 µL of the SYBR Green PCR Master Mix, 1 µL of each primer (10 mM) (Table 1, Wu et al., 2013), 3.3 µL of the Nuclease-Free water and 2 µL of diluted cDNA from all treatments, separately. The real-time qPCR condition was: 95 °C for 3 min, 45 × (95 °C for 5 s, 60 °C) for 34 s and finally of 60 °C for 5 s. Quantification of gene expression was done using the LightCycler® analysis software. The relative quantification of gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Data of temperature-dependent changes in immunity of *C. suppressalis* larvae were checked for normality using Kolmogorov–Smirnov test. To include both variables, rearing temperature and immune challenge, a two-way analysis of variance ANOVA (PROC GLM, SAS Institute, 2007) was run to analysis the effects of thermal stress (Control, Short-term 34 and Long-term 34) and injections (Intact, Tween and B3) on the immune responses of *C. Suppressalis* at time intervals of 24 h and 72 h via Tukey Honestly Significant Difference HSD multiple comparison (Factorial) ($P \leq 0.05$). Exceptionally, a one-way analysis via Tukey test at probability less than 0.05 was used to analyse the effect of thermal regimes on gene expressions of immune factors.

3. Result

3.1. Effect of thermal stress on hemocyte counts

In the current study, thermal stress mixed with *B. bassiana* injection showed the significant effects on the total and differentiated hemocyte numbers, phenoloxidase activity and gene expression of antimicrobial agents of *C. suppressalis* larvae (Figs. 2–10). Although the total number of hemocytes in the larvae injected with *B. bassiana* was significantly higher than the intact and the injected ones with Tween 80 in both periods of 24 and 72 h, but exposing the larvae at 34 °C for the short-term and the long-term periods intensified such an increase so that the total number of hemocyte in the larvae exposed to short-term 34 °C was 1.5- and 2-fold higher than the long-term treatment and the larvae reared at 24 °C, respectively (Fig. 2). Similar trend was observed in the numbers of plasmatocytes and granulocytes in which the injected larvae with *B. bassiana* exposed to short-term thermal stress showed the highest counts compared to long-term exposure and control groups at 24 and 72 h after injection (Figs. 3 and 4).

3.2. Effect of thermal stress on phenoloxidase activity

The activity of phenoloxidase in the larvae of *C suppressalis* significantly affected by simultaneous injection of *B. bassiana* and thermal stress (Fig. 5). The highest activity of phenoloxidase was recorded in the larvae exposed to short-term 34 °C following 24 h after fungal injection while the enzymatic activity showed no significant differences between the injected larvae exposed to 24 °C (control) and the long-term exposure of thermal stress (Fig. 5). At 72 h after fungal injection, the highest activity of the enzyme was found in the larvae injected by

Table 1
The primers used in this study.

Reverce primer	Forward primer	Name of gene[a]
GATACTGAGAGCCCGTGACC	GCACAGCCAGAATCATAACG	Attacin 1
CGCTGACCTGATCCCTGTAT	CTGGTGGTATAACGGCGACT	Attacin 2
TGAGTATTCTCTTTGGCATT	TCTTCAAGAAAATCGAGAAG	Cecropin 1
AAATTCAACGTCCCTTCACG	TTGTTTTTCGTGTTCGCTTG	Cecropin 2
CGCAAAGGCCATAGGAATAG	GCGCGTAATACCGTTTGTCT	Defencin
ATACAGGCGCATCCGTTAAG	AATACCCGGTGCACACAAAC	Gallerimycin
CTGGCAATGCGAAGCTAAA	GGGACCCGTTACTGTTGGT	Lysozyme
TCAGGGCTGTACTGCTGATG	AATTAGGCACACCGAGCAAC	Prophenoloxidase-activating proteinase-3
CAAAGGGCAGGGACGTAATCAAC	TCGAGCCGCACGAGATTGAGCA	18S rRNA

[a] Wu S–F, Sun F-D, Qi Y-X, Yao Y, Fang Q et al. (2013) Parasitization by Cotesia chilonis Influences Gene Expression in Fatbody and Hemocytes of *Chilo suppressalis*. PLoS ONE 8 (9): e74309. https://doi.org/10.1371/journal.pone.0074309.

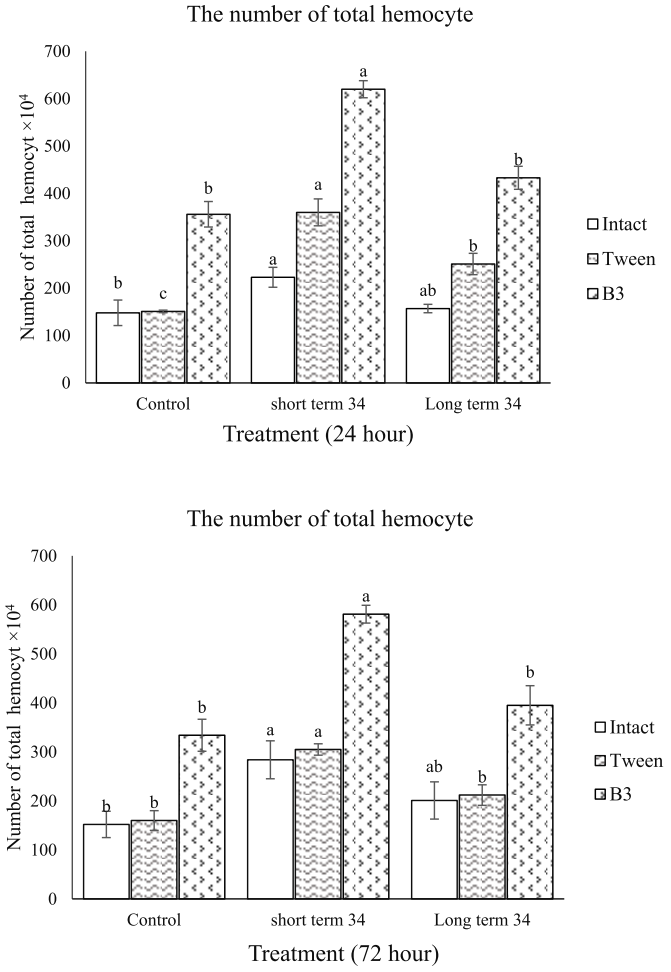

Fig. 2. Effect of thermal stress on total hemocyte count in the 5th instar larvae of *Chilo suppressalis* at the two time intervals of 24 and 72 h following exposure of thermal stress and injection treatments of intact, Tween and *Beauveria bassiana*-injected larvae. Data analyses have been done among control temperature (24 °C), short-term and long-term exposures at 34 °C. A two-way analysis of variance ANOVA was run to analysis via Tukey Honestly Significant Difference HSD multiple comparison (Factorial) ($P \leq 0.05$) which were marked by different letters.

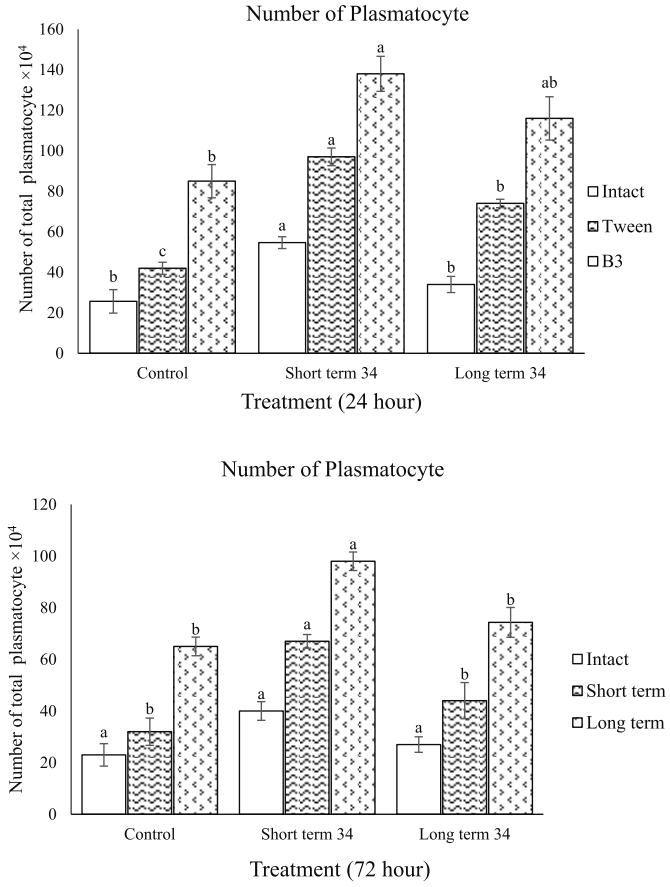

Fig. 3. Effect of thermal stress on plasmatocyte count in the 5th instar larvae of *Chilo suppressalis* at the two time intervals of 24 and 72 h following exposure of thermal stress and injection treatments of intact, Tween and *Beauveria bassiana*-injected larvae. Data analyses have been done among control temperature (24 °C), short-term and long-term exposures at 34 °C. A two-way analysis of variance ANOVA was run to analysis via Tukey Honestly Significant Difference HSD multiple comparison (Factorial) ($P \leq 0.05$) which were marked by different letters.

Tween-80 and *B. bassiana* both exposed to short-term exposure (Fig. 5).

3.3. Effect of thermal stress on expression of humoral components

Fig. 6 shows quality of the synthesized cDNA using specific primers related to humoral components of *C. suppressalis* larvae. It was shown that the size of PCR products was almost 200 bp based on the original reference (Wu et al., 2013; personal communication). Figs. 7–9 show the relative quantity of the genes involved in humoral immunity of the larvae within all treatments. The highest expression of attacin 1 was found in the larvae injected by *B. bassiana* compared to other two treatments so that the relative quantity was found 15- and 3.8-fold in short- and long-term thermal exposures (Fig. 7). Similar trend was recorded in case of attacin 2 by relative gene expression of 14- and 1.7-fold in short- and long-term thermal exposures (Fig. 7). Both cecropins 1 and 2 showed the highest expression levels in the larvae injected by *B. bassiana* although the quantifications were recorded 4.6- and 1.8-fold

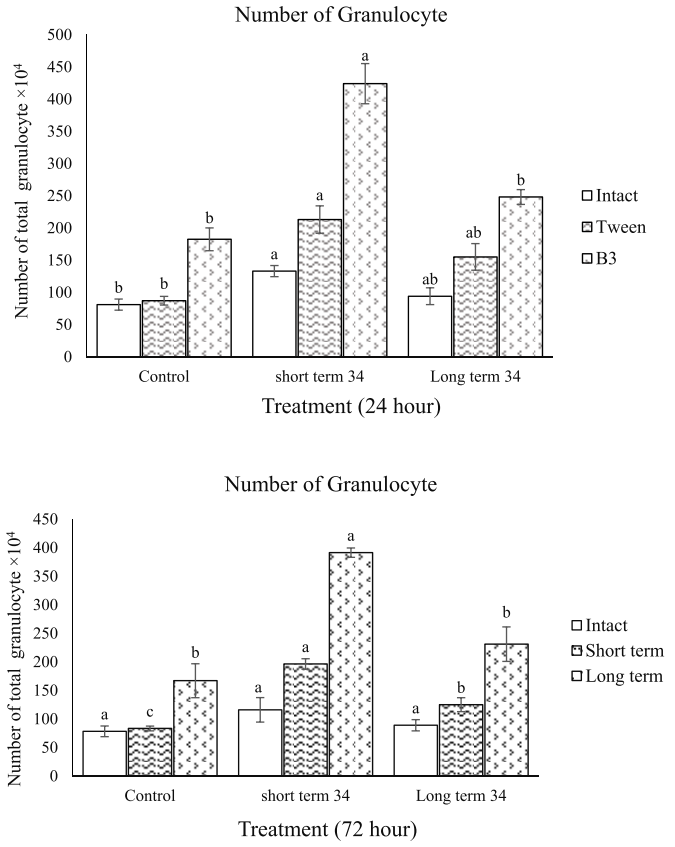

Fig. 4. Effect of thermal stress on granulocyte count in the 5th instar larvae of *Chilo suppressalis* at the two time intervals of 24 and 72 h following exposure of thermal stress and injection treatments of intact, Tween and *Beauveria bassiana*-injected larvae. Data analyses have been done among control temperature (24 °C), short-term and long-term exposures at 34 °C. A two-way analysis of variance ANOVA was run to analysis via Tukey Honestly Significant Difference HSD multiple comparison (Factorial) ($P \leq 0.05$) which were marked by different letters.

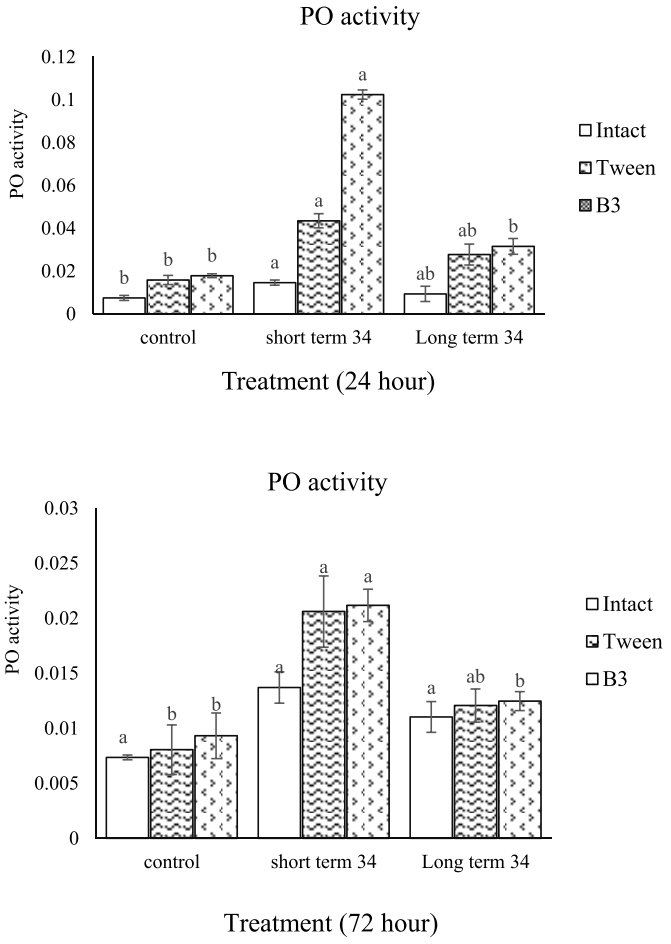

Fig. 5. Effect of thermal stress on phenoloxidase activity in the 5th instar larvae of *Chilo suppressalis* at the two time intervals of 24 and 72 h following exposure of thermal stress and injection treatments of intact, Tween and *Beauveria bassiana*-injected larvae. Data analyses have been done among control temperature (24 °C), short-term and long-term exposures at 34 °C. A two-way analysis of variance ANOVA was run to analysis via Tukey Honestly Significant Difference HSD multiple comparison (Factorial) ($P \leq 0.05$) which were marked by different letters.

for cecropin 1 as well as 6.4- and 2.1-fold for cecropin 2 at short- and long-term exposures, respectively (Fig. 8). Defensin showed the relative quantities of 3.9- and 2.9-fold compared to control at short- and long-term exposures while the expression level was recorded 32- and 6.7-fold for galleroimycin at the given exposures, respectively (Fig. 9). The expression levels of lysozyme and prophenoloxidase-activating proteinase-3 were found to be 9.1- and 4.3-fold as well as 25.3- and 5.4-fold compared to control at short- and long-term exposures, respectively (Fig. 10).

4. Discussion

Microbial infection are commonly fatal for organisms, but some studies indicate that insects clearly demonstrate that periodic heat pulses during infection may partially alleviate the damage of infectious injury (Ayres and Schneider, 2009; Xu and James, 2012; Catalán et al., 2012a, 2012b; Wojda and Taszłow, 2013a, 2013b). The changes in the immune responses associated with protective effects of short and long-term heat pulses are almost unknown, but relevant studies through extreme temperature as a stressor factor will provide information on how stress affects infection tolerance and alters ability of an organism to survive after infection (Ayres and Schneider, 2009). In the current study, the effects of short- and long-term thermal stress were investigated on the immune responses of *C. suppressalis*. The findings demonstrated that exposing *C. suppressalis* larvae to short-term thermal stress led to the higher total and differentiated hemocyte (plasmatocytes and granulocytes) counts compared to control larvae. Similar studies reported a correlation between thermal stress and immune responses of insects against entomopathogens. For example, Mowlds and Kavanagh (2008) showed that incubation of *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae at 24 and 37 °C for 24 h prior to infection by *Candida albicans* led to an increase in hemocyte count and higher gene expression of antimicrobial peptides. In another study on *Danaus chrysippus* L. (Lepidoptera: Nymphalidae) larvae, the authors demonstrated that cold exposure caused a significant decrease in the number of hemocytes, however, heating elicited an increase in circulating cells (Pandey et al., 2008). Zibaee et al. (2009) reported that the total and differentiated hemocyte counts in *Eurygaster integriceps* Puton (Heteroptera: Scutelleridae) increased with elevation of temperature so that the higher numbers were observed at 30 and 40 °C compared to 20 °C. Pandey et al. (2010) revealed that thermal stress caused a significant variation in the hemocytic immune responses of tropical tasar silkworm, *Antheraea mylitta* (Drury) (Lepidoptera: Saturniidae). Ghasemi et al. (2013) reported that chilling (4 °C) led to a significant reduction in total hemocyte count, while high temperature (40 °C) led to a higher number of hemocytes in *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). Exposure of *G. mellonella* to mild physical or thermal stress prior to infection with *Aspergillus fumigatus* increased hemocyte density after 24 h, but a decline was absorved after 48 and

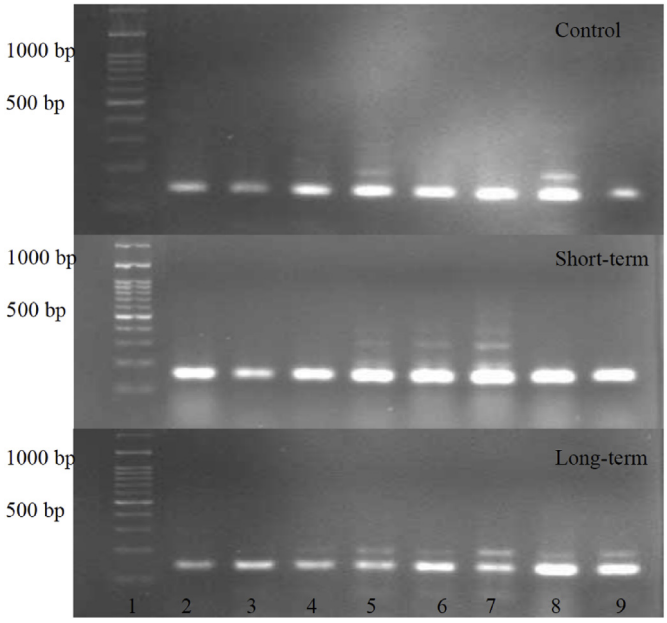

Fig. 6. PCR products from total mRNAs of *C. suppressalis* larvae exposed to control (24 °C) and stress temperature (34 °C) for both short- and long-term regimes. Lane 1: Molecular ladder, Lane 2: Attacin1, Lane 3: Attacin2, Lane 4: Cecropin1, Lane 5: Cecropin2, Lane 6: Defencin, Lane 7: Gallerimycin, Lane 8: Lusozyme, Lane 9: Prophenoloxidase-activating proteinase-3 (PAP).

72 h (Browne et al., 2014). Hemocytes are the major components in the cellular immune reactions of insects, therefore, variation in their relative abundance affects the ability of the immune system in subsequent reactions such as coagulation, nodulation, encapsulation and PO activity (Bergin et al., 2003). Generally, total and differentiated hemocyte counts rapidly change in reaction to stress, wounding or infection by microbial agents (Gillespie et al., 2000; Mowlds and Kavanagh, 2008; Pandey et al., 2010). Chronic exposure to stress such as adverse environmental conditions and infection increased the level of stress hormones [e.g. biogenic amine octopamine (OA) and adipokinetic hormone (AKH)], leading to increase of hemolymph lipid concentration (Adamo and Baker, 2011; Mowlds and Kavanagh, 2008). Since both immune and stress response are energy-counsoming processes, insect release octopamine to increase availability of energy-rich compounds. In addition, it has been indicated that Octopamine enhances phagocytosis by insect hemocytes through interacting with octopamine receptors (OARs) located on insect immune cells (Kim et al., 2009; Kim and Kim, 2010). In fact, oxidative stress following thermal and infection stress enhances infection tolerance by activating intracellular stress responses (Janssens and Stoks, 2014; 2014). Thus, the higher count of hemocytes found in our study may be attributed to the higher production of stress hormones and oxidative stress during thermal stress and fungal infection. Another reason for the higher number of hemocytes in short-term exposure might be due to the enhancing effect of elevated temperature on cell division. Furthermore, cell cycle is divided into three periods: interphase consisting G1, S phase and G2, mitosis phase and the final phase, cytokinesis, where the new cell is completely divided. In many cases, it was found that the time was taken for cells to divide decreases in response to higher temperature (Francis and Barlow, 1988). Thus, it is concluded that increase in the total hemocyte count of *C. suppressalis* larvae exposed to high temperature may be attributed to the more mitotic rate of hemocytes and increases hemocyte proliferation within hemopoietic organs.

Phenoloxidase (POs) is a key molecule in insect immunity, which is involved in conversion of phenols to quinones. The subsequent production of melanin contributes to wound healing, accompanies hemolymph coagulation, and stimulates humoral and cellular response (Gorman et al., 2007; Kanost and Gorman, 2008; Beckage, 2008). Catalán et al. (2012a) reported higher PO activity in the treated larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) at 30 °C compared to

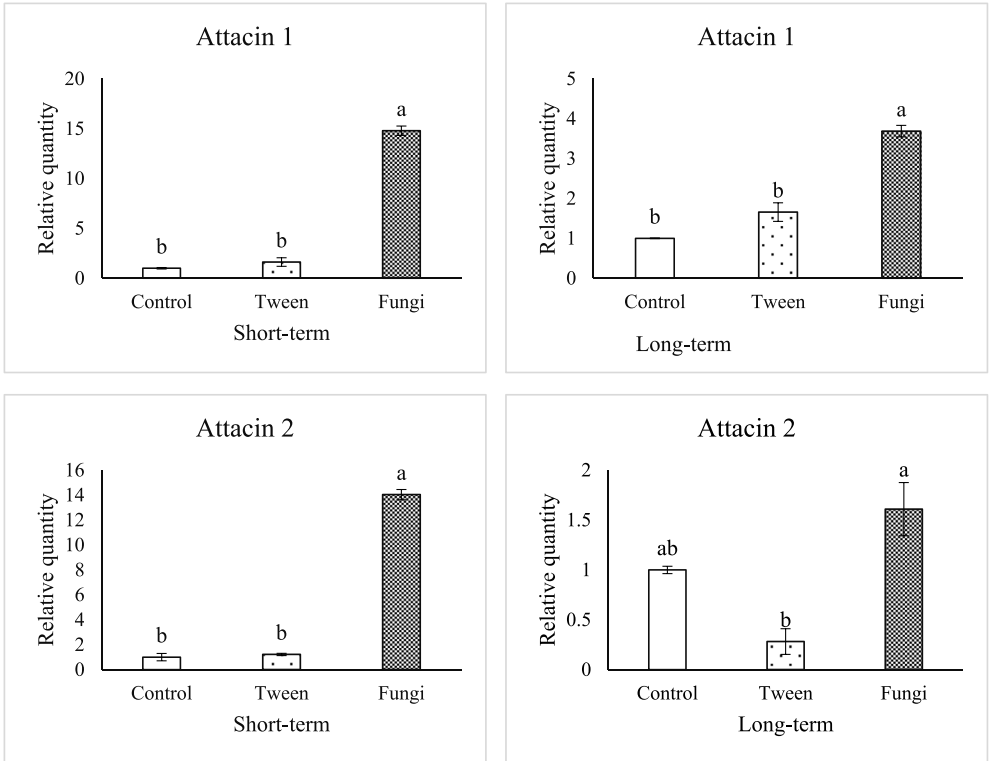

Fig. 7. Expression level of Attacin 1 and 2 in the larvae of *Chilo suppressalis* following exposure of thermal stress and *Beauveria bassiana*. A one analysis of variance ANOVA was run to analysis via Tukey test ($P \leq 0.05$) which were marked by different letters.

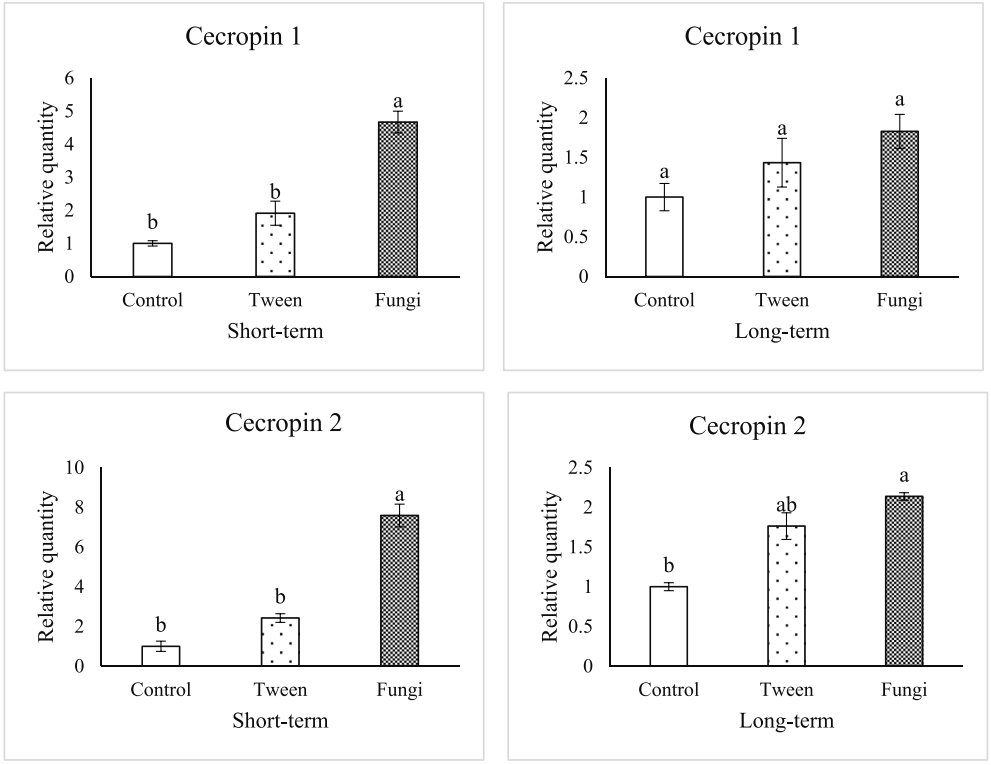

Fig. 8. Expression level of Cecropin 1 and 2 in the larvae of *Chilo suppressalis* following exposure of thermal stress and *Beauveria bassiana*. A one analysis of variance ANOVA was run to analysis via Tukey test ($P \leq 0.05$) which were marked by different letters.

10 and 20 °C. Furthermore, Catalán et al. (2012b) showed that mealworms injected with low concentrations of lipopolysaccharide and exposed to higher temperatures positively affected immunity by enhancing antimicrobial responses and phenoloxidase activity. Bozinovic et al. (2013) demonstrated that acclimatization of mealworms to temperature variations improved immune competence by enhancing humoral responses, while acute exposure to temperature led to negative impacts. Browne et al. (2014) also reported that heat-shock stress could

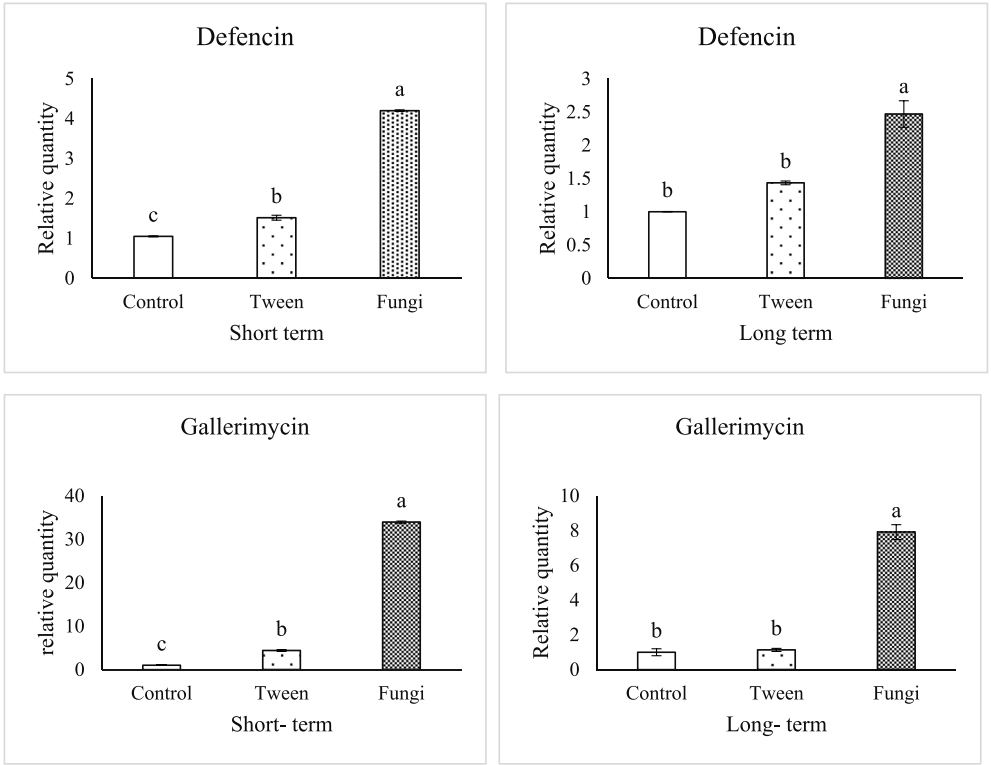

Fig. 9. Expression level of Defencin and Gallerimycin in the larvae of *Chilo suppressalis* following exposure of thermal stress and *Beauveria bassiana*. A one analysis of variance ANOVA was run to analysis via Tukey test ($P \leq 0.05$) which were marked by different letters.

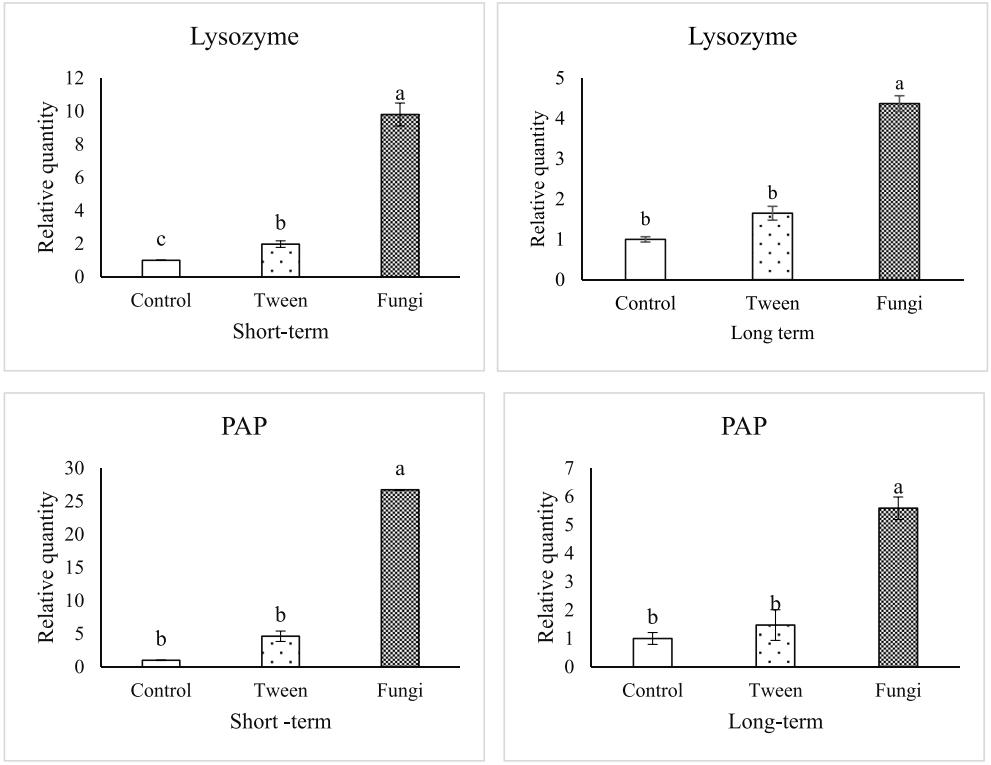

Fig. 10. Expression level of Lysozyme and Prophenoloxidase-activating proteinase-3 (PAP) in the larvae of *Chilo suppressalis* following exposure of thermal stress and *Beauveria bassiana*. A one analysis of variance ANOVA was run to analysis via Tukey test ($P \leq 0.05$) which were marked by different letters.

increase PO activity of *G. mellonella* larvae to infection by *Aspergillus fumigatus*. Since PO is synthesized and stored within hemocytes, increased activity of PO may be attributed to the higher number of hemocytes during thermal stress and fungal injection as reported in our study. Morever, a study indicated that AKH could increase PO activity in *Locusta migratoria* L. (Orthoptera: Acrididae) (Goldsworthy et al., 2002). Thus, we hypothesized that increase of AKH synthesise and number of hemocytes might be the two possible causes of increasing PO activity in *C. suppressalis* larvae exposed to thermal stress.

Antimicrobial peptides (AMPs) are a group of immune response peptides produced by hemocytes and epithelial cells of both epidermis and intestinal tract (Davies et al., 2012, 2014; McGettigan et al., 2005). AMPs are used as a second line of defense owing to their delayed activity as a requirement of RNA synthesis (Haine et al., 2008). Attacins, cecropins and defensins are the antimicrobial peptides (AMPs) induced in many species of insects in response to infection or wounding (Bullet and Stocklin, 2005; Liu et al., 2013). These polypeptides are active against gram-positive and gram-negative bacteria, but there are some reports indicating their higher concentrations in insects infected with fungi (Bullet and Stocklin, 2005; Magalhaes et al., 2008; Wu et al., 2013). Gallerymicine is a cystein-rich peptide exhibiting activity against yeast and various filamentous fungi but not bacteria (Wu et al., 2013). Our results revealed that entomopathogenic fungi *B. bassiana* significantly affected gene expressions of selected antimicrobial peptides (like attacin 1, attacin 2, cecropin 1, cecropin 2, defensin and gallerymicine) in *C. suppressalis*. It has been shown that expression of attacin, cecropin and defensin is upregulated in *C. suppressalis* parasitized by *Cotesia chilonis* (Wu et al., 2013). Other studies on *G. mellonella*, *A. pernyi*, *Xanthogaleruca luteola* Muller (Coleoptera: Chrysomelidae) and *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) have shown similar elevation after treatments by microbial agents (Schuhmann et al., 2003; Mak et al., 2010; Liu et al., 2013; Moghaddam et al., 2016). Moreover, our results indicated that thermal stress mainly short-term exposure significantly increased gene expression of all the selected antimicrobial peptides compared to long-term exposure. Wojda and Jakubowicz (2007) demonstrated that larvae of *G. mellonella* injected by yeast showed a developed defence response in the larvae reared at 38 °C compared to those kept at 28 °C. Wojda and Taszłow (2013a, 2013b) also noticed the effect of increased temperature on gene expression of antimicrobial peptides in which the authors reported a positive correlation between higher temperature and increased expression of antimicrobial peptide (cecropin, gallerimycin and galiomycin). Wojda et al. (2009) showed that larvae of *G. mellonella* simultaneously injected by *B. bassiana* and exposed to thermal stress had the higher gene expression of gallerimycin compared to control larvae. Vertyporokh et al. (2015) also demonstrated when *G. mellonella* larvae were infected with the entomopathogenic fungus *B. bassiana* and exposed to short-term heat shock, the expression of gallerimycin was higher than that in non-shocked counterparts (Vertyporokh et al., 2015). These stressful conditions stimulate insects to undergo physiological changes in order to adjust to new situations. Our findings may indicate that the signaling pathways regulated by short-term thermal stress and *B. bassiana* infection lead to enhance the "danger signal", which together with non-self recognition, co-stimulates the immune responses more effective than only microbial infection (Wojda and Taszłow, 2013a, 2013b).

Synthesis of lysozymes and activation of prophenoloxidase (proPO) system are the other key components of humoral responses. Lysozymes are normally present in hemolymph, and together with other bactericidal factors, lysozyme is often strongly induced when the insect is infected (Masschalck et al., 2002; Wojda et al., 2009). We observed increased gene expressions of lysozyme in *C. suppressalis* larvae infected by *B. bassiana* after exposure on short-term thermal stress. These results demonstrate the role of lysozyme against fungal infection in *C. suppressalis* larvae. There are some reports indicating the antibacterial and antifungal activity of lysozyme (During et al., 1999; Mak et al., 2010). Wojda et al. (2009) and Vertyporokh et al. (2015) demonstrated that thermal stress can increase activity of lysozyme in *G. mellonella* to infected with an entomopathogenic fungi, *B. bassiana*. The higher gene expression of lysozyme after infection or thermal stress may be in

response to other immune elements like cascade of extracellular proteases and hemolymph proteins (Bidochka and Khachatourians, 1987; Wojda et al., 2009). PO activation system plays a crucial role in immunity of insect against pathogens. Prophenoloxidase-activating protease (proPAP), which is the direct activator of prophenoloxidase (proPO), is an initial and important step to activate POs (Jiang et al., 1998). In the present study, injection of *C. suppressalis* larvae with *B. bassiana* and exposure to short-term thermal stress significantly increased expression level of prPAP3 compared to other treatments. Wang and Jiang (2017) reported that ingection of *Alcaligenes faecalis* or - *E. coli* bacteria via their peptidoglycans led to activation of proPO and melanization in *Manduca sexta*. In addition, the transcription level of proPAPs was already noticed to be increased in the fat body or hemolymph of insects after infection of parasitoides (Etebari et al., 2011; Wu et al., 2013).

5. Conclusions

The current study results showed that temperature and contamination of a microbial agent could significantly increase cellular and humoral immune responses of *C. suppressalis* larvae. More importantly, the type of exposure to temperature as the short- and long-term periods also have different effects so that exposure to short-term thermal stress or sudden increase of temperature may have a more severe effect on the immune responses of *C. suppressalis* larvae. Furthermore, the milder immune responses of larvae at long-term exposure unity can be related to some kinds of larval adaptability to thermal stress. However, these results can indicate survival of *C. suppressalis* larvae in the warm summer of northern Iran and the inability of microbial control through *B. bassiana* spray in this period. Such interactions may contribute to geographical distribution of organisms mainly insects throughout globe through tolerance, physiological capacities and plasticity as well as physiological processes underlying distribution prediction. The current speculation may be one of the reasons for the high prevalence and annual outbreaks of *C. suppressalis* in almost all paddy fields of northern Iran.

Disclosure statement

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Appendix A. Supplementary data

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