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Original paper

Prolonged heat stress during winter diapause downregulates gene expression of attacin and gloverin, two antimicrobial peptides in the European corn borer, *Ostrinia nubilalis* (Hübner)

Vanja TATIĆ[1], Jelena SPREMO[1], Iva UZELAC[1], Miloš AVRAMOV[1], Dragan GRČAK[2], Snežana GOŠIĆ-DONDO[2], Željko D POPOVIĆ[1*]

[1]*University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia*

[2]*Maize Research Institute, Zemun Polje, Slobodana Bajića 1, 11185 Belgrade, Serbia*

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Summary. Many insect species overcome unfavourable environmental conditions by entering diapause, a type of hypometabolic state of arrested development during which they become vulnerable and sensitive to microbial infections, especially in light of ongoing climate changes. In order to estimate how prolonged heat stress during the winter diapause of *Ostrinia nubilalis* (Hübner) affects the insect's immune system, diapausing larvae were reared from November 2018 until May 2019 in corn stalks under different thermal conditions – field, with ambient winter temperatures and laboratory, with above-average temperatures (12-18 °C). Changes in *O. nubilalis* immune response have been assessed at the transcriptional level, by analyzing the expression of genes encoding two major antimicrobial peptides, *attacin* and *gloverin*, using qPCR. Total RNA was isolated from whole-body homogenates of diapausing 5th instar larvae reared at different temperatures and non-diapausing larvae and pupae, as control groups. Relative gene expression was determined using *actin* as the reference gene. During early-diapause, the relative expression of *attacin* and *gloverin* was suppressed in diapausing larvae under both thermal regimes, in comparison to non-diapausing larvae and pupae. However, downregulation of gene transcription was prolonged and more profound in heat stressed diapausing larvae, in comparison to larvae from field conditions. Also, larvae resting in field conditions had higher transcriptional activity of both analyzed antimicrobial genes, especially from mid-diapause and onwards. Heat stressed larvae only exhibited a significant peak in gene expression during March, probably as a consequence of their increased immune response due to pathophysiological processes that led to high larval mortality and premature diapause termination in early April. Moreover, recorded transcriptional suppression in the early months of the resting state corresponds to general metabolic depression, probably as part of the diapausing program. The gradual increase in relative expression of both antimicrobial peptides during the latter months of diapause in larvae from field conditions can be correlated with the termination of their resting period and preparation of larval immune system for active development and subsequent metamorphosis.

Keywords: antimicrobial peptides, diapause, gene expression, heat stress, immunity, *Ostrinia*.

INTRODUCTION

The Intergovernmental Panel on Climate Change 5th Assessment Report (IPCC AR5) has noted that the average global atmosphere temperature has increased by more than 1 °C since the late 19th century. As current trends show no signs of slowing down, further increases reaching 1.5 °C could be expected between 2030 and 2052 (IPCC 2014). Furthermore, predicted temperatures for the end of 21st century, if current rates of carbon dioxide emissions maintain their levels, are 4.8 °C higher relative to surface temperatures measured from 1850 to 1900 (IPCC 2014).

Recent research confirms that climate change could have an impact on local extinctions across the globe, as species are

E-mail: zeljko.popovic@dbe.uns.ac.rs

unable to respond to sudden shifts in climate (Wiens 2016; Rafferty 2017). Insects in particular are at the greatest risk of species and range loss in the event that the Earth's global temperature rises by 2 °C (Warren et al. 2013). When it comes to pest insect populations, rising temperatures could trigger expansions of range, increased overwintering survival, an increased number of generations, and changes in their interactions with host plants (Liu et al. 2021; Skendžić et al. 2021). Thus, it is becoming more evident, especially in recent years, that the effects of global warming are farther reaching than previously assumed.

Insects, being poikilothermic organisms, depend on ambient temperatures not only for thermoregulation, but also for development, reproduction and overall survival (Bale 2002; Bale et al. 2002; Régnière et al. 2012). In order to combat natural seasonal changes and ensure survival and reproduction, many such organisms enter a hypometabolic state known as diapause. Diapause is characterized by decreased metabolic rates, arrested growth and development, and significant changes at the molecular level (Denlinger 2002; Koštál 2006; MacRae 2010; Nakamura et al. 2011, Popović et al. 2021). This type of dormancy can be obligatory, e.g. genetically encoded to occur at a specific life stage; or it can be facultative. Facultative diapause is strongly dictated by temperature fluctuations and photoperiodic cycles (Lees 1956).

Disordered environmental cycles, such as increasing winter temperatures, can lead to heat-induced stress responses, which can be manifested in many different ways. One pathway that is activated by high temperatures is aptly named the heat stress response and is mediated by heat shock proteins (HSPs) (Ritossa 1962). Another set of changes that can be brought on are changes in immune response (Catalán et al. 2012). Considering that organisms become highly vulnerable and prone to infections while in the resting state, the importance of the immune response becomes more evident. Species that are dependent only on innate immunity, including insects, employ different protective mechanisms, one of which is the production of antimicrobial peptides (AMPs).

Attacins, glycine-rich AMPs, were originally isolated from the haemolymph of the cecropia moth *Hyalophora cecropia*. Their activity is targeted against Gram-negative bacteria, specifically *Escherichia coli* (Engström et al. 1984). Attacins can be found in one of two isoforms, acidic or alkaline, with similar amino acid composition, but are encoded by different genes (Hultmark et al. 1983; Kockum et al. 1984). The mechanism of antimicrobial activity for these AMPs is based on increasing membrane permeability by inhibition of porine component synthesis (Carlsson et al. 1991).

Gloverins also belong to the glycine-rich group of AMPs and were initially isolated from the Glover's silkmoth *Hyalophora gloveri* (Axen et al. 1997). In aqueous solutions, gloverins have a disordered structure, while in a hydrophobic environment (e.g. the cell membrane) they undergo a conformational change and form an α-helix. The mechanism of activity for gloverins is considered to be similar to that of attacins (Axen et al. 1997).

During the resting state, overall metabolic activity in diapausing insects is lowered, which brings into question how such species protect themselves from microbial infections and maintain homeostasis. The aim of the present study was to further our understanding of how prolonged heat stress during winter impacts the immune system in an evolutionarily cold-adapted Lepidopteran species, *Ostrinia nubilalis* (Hübner). Thus, in light of ongoing and widespread climate changes that have already led to a rise in global temperatures, our results show that increased winter temperatures will have negative impacts on the physiology of cold-adapted insect species and may disturb their life cycles.

MATERIALS AND METHODS

Sample collection and experimental conditions

After entering diapause, *O. nubilalis* larvae were collected in November 2018 from maize fields of the Institute of field and vegetable crops in Novi Sad, Serbia (45°33′N, 19°84′E) and later separated into two groups. One (heat stressed) was kept in unusually high temperature conditions (12-18 °C), while the other (field) was allowed to go through diapause in field conditions, where temperature fluctuations followed a natural pattern. Further sampling was conducted every month from December 2018 to April 2019, from both the field and heat stressed groups, while the November group was used as a control (Table 1). Non-diapausing larvae and pupae were also used as control groups (Fig. 1). Diapausing pupae from the April heat stressed group were not collected due to their high mortality. Experimental groups were formed for every month, each being comprised of 5 biological replicates containing three larvae per replicate. Collected samples were frozen in liquid nitrogen and then stored at -80 °C until analysis.

RNA isolation, cDNA synthesis and qPCR analysis

Total RNA isolation was conducted using TRIzol Reagent, Invitrogen™ (Waltham, MA, USA), according to the manufacturer's instructions. RNA concentration was assessed by spectrophotometry using a BioSpec-nano spectrophotometer (Shimatzu, Japan). The integrity of RNA samples was checked by agarose electrophoresis, while visualization of S18 and S28 RNA bands was done under UV light with a BioDoc Analyzer (Biometra, Germany). Complementary DNA synthesis was performed using a High-Capacity

Table 1. Established experimental groups. Each experimental group was comprised of 5 biological replicates (pools), with each pool consisting of 3 randomly chosen larvae or pupae.

Active development	
ND – non-diapausing larvae	NDp – non-diapausing pupae
Diapause	
Field conditions	Heat stressed
Nc – November larvae (control)	Nc – November larvae (control)
DF – December larvae (field)	DH – December larvae (heat stress)
JF – January larvae (field)	JH – January larvae (heat stress)
FF – February larvae (field)	FH – February larvae (heat stress)
MF – March larvae (field)	MH – March larvae (heat stress)
AF – April larvae (field)	AH – April larvae (heat stress)
AFp – April pupae (field)	

cDNA Reverse Transcription kit from Applied Biosystems™ (Waltham, MA, USA) as follows. The 2X Reverse Transcriptase (RT) master mix was made using the following components: 2.0 µL of 10X RT buffer, 0.8 µL of 100 mM dNTP Mix, 2.0 µL of 10X RT random primers, 1.0 µL of MultiScribe™ Reverse Transcriptase and 4.2 µL of nuclease-free H_2O. In individual 0.2 mL tubes, the RT master mix was combined in equal parts with the RNA sample to a final volume of 20 µL. The thermal cycler conditions were optimized for the Applied Biosystems High Capacity cDNA Reverse Transcription Kit and include the following steps: 10 minutes at 25 °C, 120 minutes at 37 °C and 5 minutes at 85 °C.

Primer sequences for *attacin* and *gloverin* were acquired from BioTeZ Berlin Buch GmbH (Berlin, Germany) based on data from Chen et al. (2021) (Table 2). For later calculation of relative gene expression *actin* was used as a reference gene, and its primer sequences were obtained from Generi Biotech s.r.o. (Hradec Králové, Czech Republic).

Primer specificity and efficiency were determined using a two-step real time PCR program (Table 3) on CFX Connect™ Real-Time PCR Detection System by Bio-Rad (California, USA), while 2x AMPLIFYME SG Universal Mix by Blirt S.A. (Gdańsk, Poland) was used for the detection of amplicons, as follows. The qPCR master mix was prepared

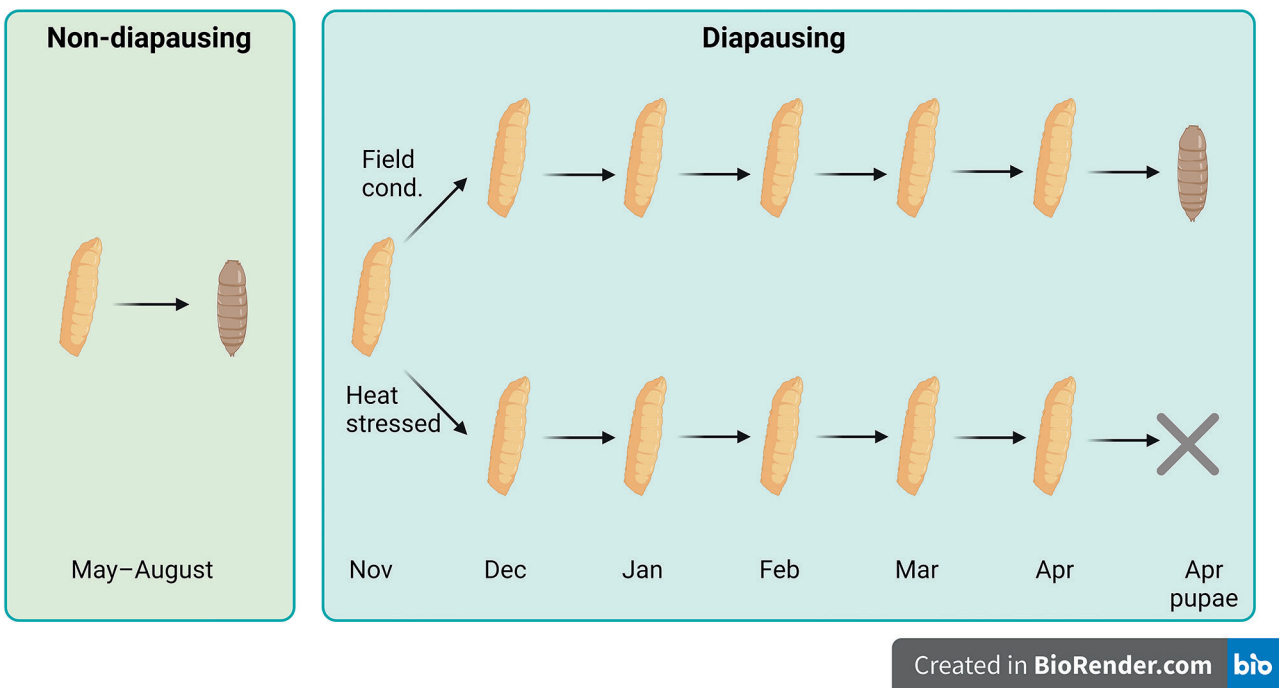

Fig. 1. Experimental design.

Table 2. Primers used for quantitative PCR analysis.

Gene	Primer sequence (5'–3')
attacin	F: CCTGGGTTGCCTGCTGGTG
	R: GCCAGTCCCTTAGTAATAGC
gloverin	F: GACCCAAAGACTGGAGACCTCAC
	R: GTGCTCGTAGCCCGCTTTG
actin	F: CAGAAGGAAATCACAGCTCTAGCC
	R: ATCGTACTCCTGTTTCGAGATCCA

by combining the following reaction reagents: 10 μL of the 2X Amplifyme SG mix, 0.6 μL of 10 μM forward and reverse primer each, 0.4 μL of 50X ROX solution and PCR-grade water until reaching a final volume of 20 μL. For determination of primer specificity and efficiency, the following cDNA dilutions were used: 1:1, 1:10, 1:100, 1:1000 and 1:10000.

Quantitative PCR analysis and relative quantification of gene expression were conducted using the previously described two-step real time PCR program (Table 3), on the CFX Connect™ Real-Time PCR Detection System by Bio-Rad (California, USA), while the 2x AMPLIFYME SG Universal Mix by Blirt S.A. (Gdańsk, Poland) was used for the detection of amplicons, as previously described. The concentration of the synthesized cDNA was brought to 12.5 ng/μL to be used for the quantitative PCR analysis.

Relative quantification of gene expression was calculated according to Ganger et al. (2017), while statistical analysis was done in Statistica 14.0.1. The statistical significance of determined values was tested using one-way ANOVA followed by post hoc Fisher's test, with a level of significance of $P < 0.05$.

RESULTS

The expression profile for *attacin* and *gloverin* in non-diapausing larvae significantly differs from that of both heat stressed and field acclimated diapausing larvae (Figs 2 and 3). In comparison to non-diapausing larve, diapausing larvae acclimated to field temperatures showed lower levels of expression only during early diapause. Subsequently, a significant increase in relative expression for both *attacin* and *gloverin* was observed during late-diapause. On the other hand, the heat stressed group had significantly lower levels of gene expression throughout the course of diapause, when compared to non-diapausing larvae.

Levels of expression for *attacin* (Fig. 2) were generally lower in the heat stressed group than in the field conditions group. Field reared larvae and pupae showed a significant rise in expression levels for *attacin* in February, and it continued rising in the following months. For the heat stressed larvae, however, the pattern of relative expression was lower than in field reared larvae, and was characterized by a significant dip in December, followed by a peak in relative expression in March, and a subsequent decrease in April.

For the *gloverin* gene (Fig. 3), from November to February, prolonged heat stress lead to greater downregulation of the relative expression of *gloverin* in comparison to field-acclimated groups. However, this transcriptional depression was followed by a significant peak in relative expression in March, and a dramatic decrease in April. In the field-acclimated larvae and their pupae, contrary to heat stressed diapausing larvae, the relative expression of *gloverin* was upregulated from January throughout the latter part of diapause.

DISCUSSION

All living organisms must interact with and adapt to stress-inducing conditions brought on by a changing environment. Considering that insects have had tremendous success in the colonization of various habitats, it could be said that they can tolerate many diverse stressors, such as temperature fluctuations (Denlinger 2002; Bale and Hayward 2010). However, changing environmental temperatures might have a significant effect on insect survival when heat stress is examined along with the immune response.

Results from the present study indicate that heat stress has a significant influence on the expression of selected genes involved in the immune response of cold-adapted overwintering larvae of *O. nubilalis*. Namely, prolonged heat stress was found to downregulate the expression of both *attacin* and *gloverin* genes throughout diapause, especially during the early months of this resting phase, in comparison to field-acclimated larvae and pupae. The relative expression of the two aforementioned AMPs increased during prolonged heat stress only in March-April, prior to high larval mortality that

Table 3. Two-step quantitative PCR program.

Step	Parameters		
	T (°C)	Time (sec)	Condition
1. Activation and initial denaturation	95	180	/
2. Denaturation	96	5	/
3. Primer binding, extension, detection	60	30	Go to step 2 and repeat 44 times
4. Melting curve	65-95	5	Increase temperature by 0.5 °C every 5 seconds

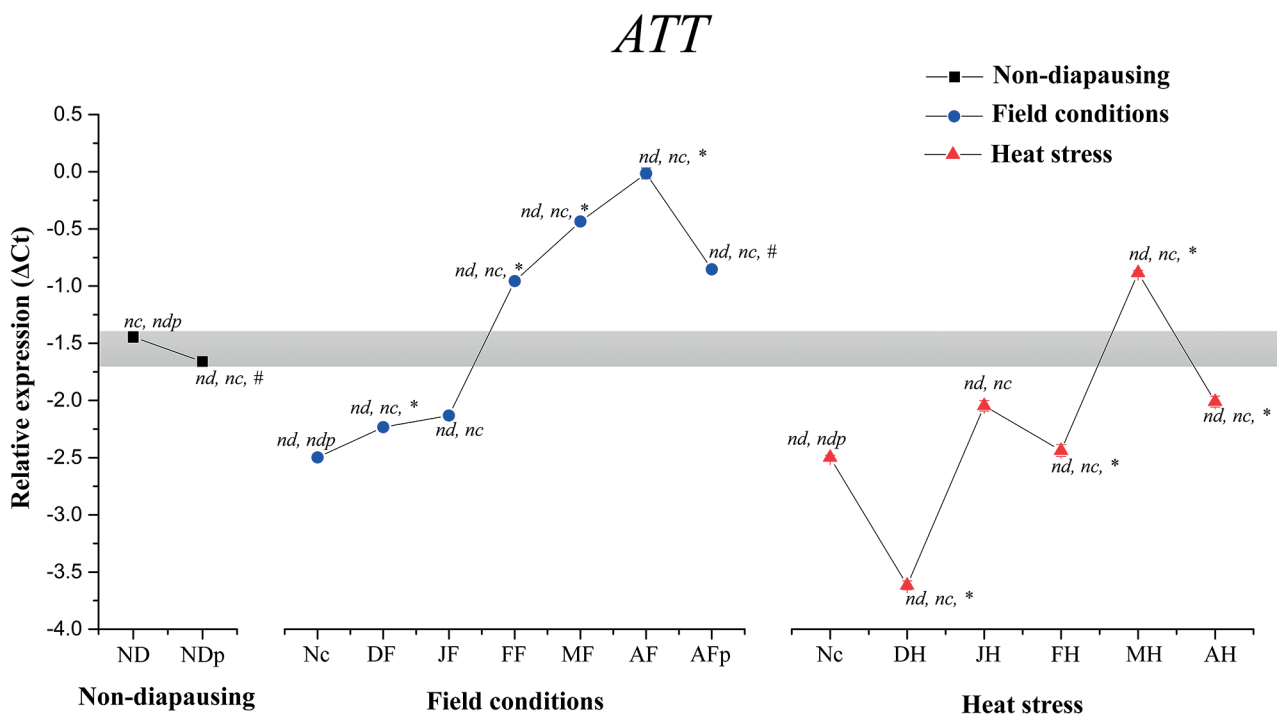

Fig. 2. Results of relative gene expression for *attacin*, shown in ΔCq. The statistical significance of determined values was tested using one-way ANOVA followed by post hoc Fisher's test, with a level of significance of $P < 0.05$. Statistically significant differences in expression levels are marked as: nd – significantly different from ND; nc – significantly different from Nc; ndp – statistically different from NDp; * – significant difference between the same months under different temperature conditions; # - significant difference between non-diapausing pupae and diapausing pupae.

led to unsuccessful termination of diapause and failure to pupate.

Contrary to our findings, in larvae of the wax moth *Galleria mellonela*, a short-term high temperature exposure prior to controlled infection resulted in increased expression of AMPs, as well as increased insect survival when compared to non-treated groups (Mowlds and Kavanagh 2008). Similarly, induction of heat shock in the aforementioned wax moth, followed by infection lead to increased survival of larvae by means of higher expression of AMP genes (Wojda and Taszłow 2013). On the other hand, in the absence of a microbiological challenge in the alfalfa leaf-cutting bee, *Megachile rotunda,* short term exposure to 35 °C increased the expression of selected components of the IMD and Toll signaling pathways that regulate AMP transcription, while exposure to 20 °C had an upregulating effect on the recognition elements of these pathways (Xu and James 2012). These results indicate the existence of specific cross-talk between immune system signaling and heat stress signaling. Acute heat stress, or mild temperature increases, might have beneficial effects on insects' immune system, as it primes the organism for future stressful conditions brought on by a potential infection (Xu and James 2012). Our findings in *O. nubilalis* show that even a nonsignificant temperature increase can have an effect on AMP expression, when that temperature increase coincides with a diapausing period which, in *O. nubilalis*, happens during cold winters, as this cold-adapted species relies on low temperatures for maintaining metabolic repression. The effects of prolonged heat stress, specifically during a resting phase that requires lower ambient temperatures and metabolic activity, can induce drastically different outcomes. For instance, larvae of *G. mellonella* failed to survive under prolonged heat-stress conditions, while short-term heat exposure was beneficial to their immune response and viability (Wojda and Taszłow 2013). Similarly, in this study, *O. nubilalis*, larvae that were under prolonged heat stress conditions had increased mortality in the latter part of diapause, failed to pupate and were unable to resume their life cycle. Thus, higher AMP expression recorded in March in this group can be correlated to above average temperatures that favor pathogen development and provoke larval immune response. Still, the drastic drop in expression observed in April is most likely caused by pathological processes in larvae that preceded their subsequent high mortality due to the lack of energy reserves, high oxidative stress and diverted metabolism (Popović et al. 2015, 2021).

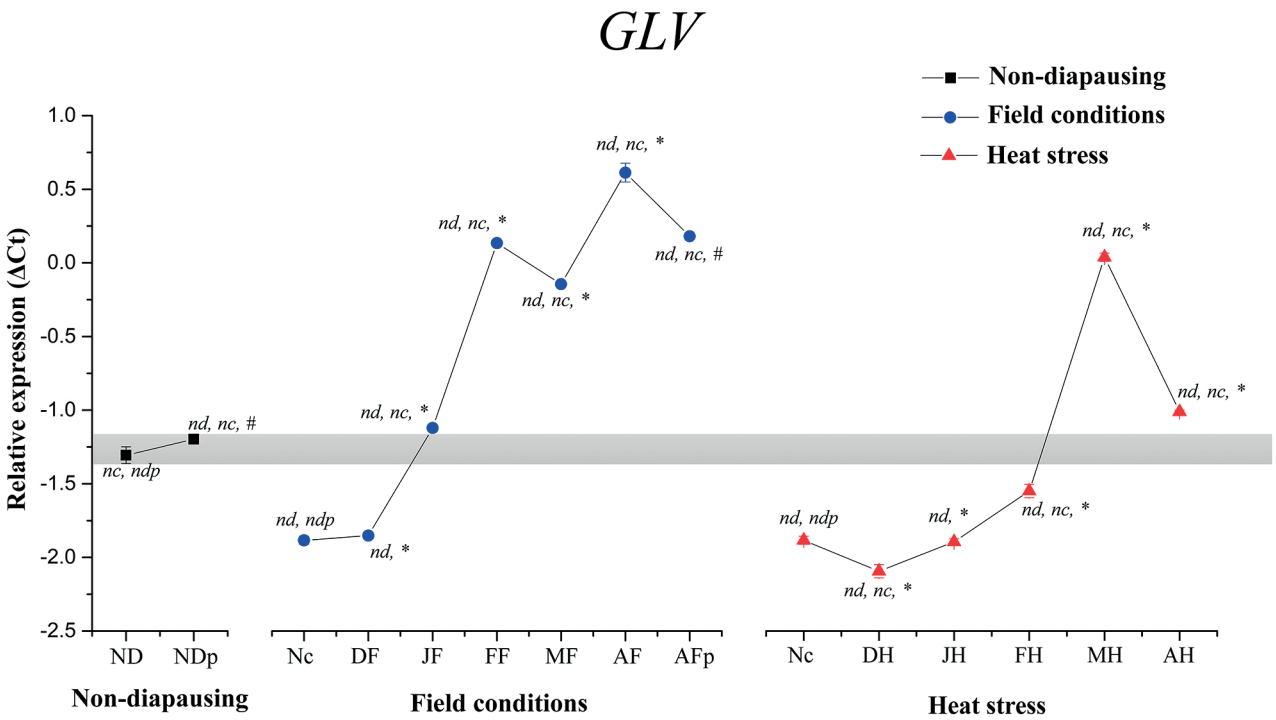

Fig. 3. Results of relative gene expression for *gloverin*, shown in ΔCq. The statistical significance of determined values was tested using one-way ANOVA followed by post hoc Fisher's test, with a level of significance of $P < 0.05$. Statistically significant differences in expression levels are marked as: nd – significantly different from ND; nc – significantly different from Nc; ndp – statistically different from NDp; * – significant difference between the same months under different temperature conditions; # - significant difference between non-diapausing pupae and diapausing pupae.

It is well-known that stress induced proteins play important roles in an organism's immune response (Catalán et al. 2012). Theories suggest that HSPs are released by damaged/infected cells and together with other signaling molecules act as a danger-damage signal – DAMP (danger-associated molecular pattern) (Matzinger 2002). Similar experiments on *O. nubilalis* larvae (unpublished) showed expression patterns of heat shock protein genes resembling those of *attacin* and *gloverin* found in the present study. To be specific, in heat stressed diapausing larvae, the expression of HSC70 and HSP90 genes follows a similar pattern to the expression of *gloverin* and *attacin,* respectively. In the case of *attacin* and *hsp90,* expression is upregulated in January but downregulated towards April, while *gloverin* and *hsc70* both show a significant increase in March, followed by a decrease in April. Based on this, we assume that HSP regulation by heat stress might be an important link for the development of immune defense in *O. nubilalis*. This assumption can be further supported with the fact that Toll-like receptor 4 (TLR4) recognizes both bacterial products, such as lipopolysaccharide (LPS), and HSP70s, endogenous cell products, as its native ligands. In a similar way, Toll-like receptor 2 (TLR2) has the ability to bind bacterial lipoproteins as well as HSP60 (Tsan and Gao, 2009). In the case of the housefly *Musca domestica*, an increase in HSP70 levels was shown after immune stimulation with *E. coli* and *Staphylococcus aureus* (Tang et al. 2012). Moreover, in the moth *G. mellonella*, an increase in the level of HSP90 in the fat body was found not only after exposure to raised ambient temperature, but also after immune stimulation under optimal temperature conditions (Wojda and Jakubowicz 2007). Also, larvae that were immune-stimulated at higher temperatures showed a significant resistance to infection, which is indicated by a higher level of AMP expression (Wojda and Jakubowicz 2007). Similarly, exposure of larvae to high temperatures before infection elevates the expression of AMP genes, which is accompanied by stronger immune defense, as well as a higher degree of survival (Wojda et al. 2009).

Taking all of these results into consideration, it is evident that prolonged heat stress, as opposed to mild heat shock, negatively affects the gene expression of two important antimicrobial peptides, attacin and gloverin, especially in early-diapause in the cold-adapted larvae of *O. nubilalis*. Previous studies on this species had shown the importance of metabolic suppression that has been shaped by both the internal diapausing program and low ambient temperatures,

such as those close to or below 0 °C (Popović et al. 2015, 2021; Uzelac et al. 2020). Therefore, in light of ongoing and widespread climate changes that have already led to an increase in global temperatures, we showed in this study that such ambient changes might have deleterious effects on the immune response of cold-adapted insect species and disrupt their life cycles. Although the present study was conducted on a pest insect species, the effects of global warming will have negative impacts on other insects and ectotherms that rely on cold winter periods during their resting phase. Thus, in the future, specific eco-physiologically based conservational strategies have to be designed and implemented in order to protect endangered biodiversity.

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