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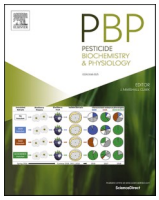

Simulated heat waves aggravate the immune response to sublethal acetamiprid exposure in the honey bee (*Apis mellifera*)

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ABSTRACT

Simultaneous exposure to thermal stress and agrochemicals represents a realistic yet understudied toxicological scenario for pollinators. Honey bees (*Apis mellifera*) increasingly face intense heat waves while foraging in pesticide-treated landscapes. Understanding the cumulative impact of these stressors is therefore critical for predicting pollinator health. We investigated the combined effects of simulated heat waves (exposed to 42 °C for two hours over three consecutive days) and chronic sublethal exposure to the neonicotinoid acetamiprid on honey bee immunity and physiology. By subjecting bees to a subsequent mechanical challenge, we assessed both their baseline physiological state and their capacity to mount an active immune defense under stress. Using a comprehensive panel of biomarkers – including antimicrobial peptides (AMPs: abaecin, apidaecin, defensin-1, hymenoptaecin), heat shock proteins, and oxidative stress markers - we evaluated the bees' immune and physiological resilience. Strikingly, while heat stress alone significantly reduced total hemocyte counts, indicating compromised cellular immunity, it did not trigger a humoral immune response. In contrast, combining heat stress with acetamiprid led to a significant overexpression of all tested AMP genes and elevated AMP concentrations in the hemolymph. Furthermore, this combined treatment upregulated *catalase* expression, signaling enhanced oxidative stress, and increased total hemolymph protein levels, suggesting altered physiological homeostasis. These results indicate that the interplay between thermal stress and pesticide exposure lowers the threshold for immune activation. We conclude that even sublethal pesticide doses can become immunologically burdensome during heat events. This stressor interaction risks physiological exhaustion and energy trade-offs, potentially compromising colony resilience against ubiquitous parasites in a warming world.

1. Introduction

The western honey bee (*Apis mellifera* L.) is a crucial pollinator that ensures the reproduction of numerous wild plants and crops (Khalifa et al., 2021). However, multiple stressors have contributed to a notable rise in colony losses in recent decades, particularly pathogens and parasites, pesticides, inadequate nutrition, and climate change (Goulson et al., 2015). Due to the critical role of honey bees in maintaining ecosystem stability and agricultural productivity, there is a need to understand how interacting stressors influence honey bee physiology and immune function.

To defend against external threats, honey bees rely on both social and individual immunity (Evans et al., 2006). Social immunity limits pathogen transmission through collective behavioral defenses (Evans and Spivak, 2010; Simone-Finstrom, 2017). The present study, however, focuses on individual immunity. At the individual level, honey bees rely exclusively on innate immunity, consisting of cellular and humoral components.

Cellular immunity is mediated by hemocytes (immune cells circulating in hemolymph), which perform phagocytosis, nodulation, and encapsulation (Negri et al., 2016). Changes in hemocyte abundance and activity are considered sensitive indicators of immune activation following infection or wounding. Hurychová et al. (2024) reported a significant increase in hemocyte count 4 h after injecting heat-killed

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bacteria into the bee's abdomen, which demonstrates rapid cellular immune responsiveness.

The main elements of humoral immunity are phenoloxidase, lysozymes, and antimicrobial peptides (AMPs). AMPs are small effector molecules that target bacteria, protozoa, fungi, and viruses. Four AMP families have been described in honey bees: apidaecin, abaecin, hymenoptaecin, and defensin (Danihlík et al., 2015; Larsen et al., 2019). These peptides are synthesized primarily in the fat body and released into the hemolymph upon immune stimulation (Larsen et al., 2019; Laughton et al., 2011). Because AMP expression rapidly reflects immune system modulation, these peptides are widely used as functional biomarkers of humoral immunity.

Immune function is highly sensitive to environmental stressors. Although heat stress has not traditionally been considered a major driver in honey bee colony losses, its relevance is increasing under current climate change scenarios. These shifts are characterized by more frequent, prolonged, and severe heat waves (Xu et al., 2018). While honey bees maintain brood nest temperature within a narrow range of 34–36 °C (Stabentheiner et al., 2021), extreme heat events may exceed the colony's thermoregulatory capacity. When this happens, it imposes significant physiological stress on the individuals within the hive. Furthermore, foragers are at even greater risk, as they experience direct exposure to peak ambient temperatures during flight. However, thermal tolerance varies among bee species, reflecting differences in heat resistance mechanisms (Kovac et al., 2014; Li et al., 2019).

Heat stress influences multiple aspects of honey bee biology, including growth, development, reproduction, and longevity, as well as their ability to perform physiological tasks such as foraging and pollination (Bordier et al., 2017a; Jones et al., 2005; McAfee et al., 2020; Medina et al., 2023; Smodiš Škerl and Gregorc, 2010). The immune system is likewise sensitive to elevated temperatures (Medina et al., 2020). Studies examining foragers exposed to heat stress across different temperatures and exposure durations have reported increased expression of *defensin*, *abaecin*, and *hymenoptaecin* with rising temperature and prolonged exposure (Li et al., 2022). Such upregulation may represent a compensatory response but could also impose energetic costs and contribute to immune system exhaustion under prolonged stress. In contrast, McKinstry et al. (2017) reported that heat stress reduced the expression of AMPs and increased the expression of heat shock proteins (HSPs), suggesting a potential trade-off or antagonistic interaction between immune activity and cellular stress responses.

Heat shock proteins maintain cellular protein homeostasis and are upregulated under stressful conditions such as elevated temperatures, exposure to pesticides, environmental toxins, pathogens, or oxidative stress. They function as molecular chaperones that stabilize proteins, assist in their proper refolding, and promote the degradation of irreversibly damaged proteins. This protective mechanism represents a fundamental component of the cellular stress response, ensuring protein stability and functional integrity (Abou-Shaara, 2024).

However, bees are rarely exposed to single stressors under field conditions (Kang et al., 2024). In addition to thermal stress, they are routinely exposed to insecticides during foraging, with residues commonly detected in nectar, pollen, and bee bread (Mahdavi et al., 2025). Within this group, neonicotinoid insecticides have attracted particular attention due to their extensive use and well-characterized neurotoxic mode of action (Jeschke et al., 2011). Neonicotinoids act systemically by binding to nicotinic acetylcholine receptors in the insect's nervous system, ultimately leading to paralysis and death (Blacquière et al., 2012).

Acetamiprid is currently the only neonicotinoid insecticide approved for outside use in the European Union. In contrast, several other neonicotinoids, including imidacloprid, thiamethoxam, and clothianidin, have been banned for outdoor application due to their adverse effects on non-target organisms, particularly pollinators. Despite acetamiprid's lower acute toxicity (Iwasa et al., 2004), experimental studies have demonstrated that sublethal exposure can induce measurable physiological and molecular effects, often without detectable impacts on survival (Badawy et al., 2015; Bordier et al., 2017b; Han et al., 2023; Shi et al., 2020; Shi et al., 2019).

At the molecular level, sublethal acetamiprid exposure is associated with changes in immune-related gene expression. Experimental evidence indicates that these responses are often peptide- and tissue-specific. For example, Han et al. (2023) reported that a 10-day exposure to 0.32 mg/L acetamiprid significantly upregulated *secapin* in the head and gut, while *apidaecin* was downregulated in the head and remained unchanged in the gut. Notably, no significant changes were detected for *defensin*, *hymenoptaecin*, or *abaecin* in that study. However, Christen et al. (2016) demonstrated that responses are also highly dependent on concentration and timing. Under short-term laboratory conditions, they observed that *apidaecin* was significantly downregulated only after 72 h, while *defensin-1* was upregulated at multiple time points, particularly at higher doses.

These findings suggest that insecticide-induced immune responses are highly context-dependent. Experimental variables such as dose, exposure duration, route of application, and developmental stage complicate direct comparisons across studies. While the lethal effects of neonicotinoids are well characterized, the sub-lethal physiological consequences occurring under thermal stress remain insufficiently understood. Investigating these interactions is essential to clarify how cumulative stressors impact bee health in fluctuating environments.

To our knowledge, the cumulative impact of heat waves and acetamiprid exposure on honey bee immunity remains unexplored. Here, we address this gap by examining how simulated heat waves (42 °C), alone and in combination with chronic sublethal acetamiprid exposure, affect cellular and humoral immune responses. By subjecting bees to a subsequent mechanical challenge, we assessed both their baseline physiological state and their capacity to mount an active immune defense under stress. We analyzed a comprehensive panel of biomarkers, including the gene expression and hemolymph concentrations of AMPs, HSPs, and oxidative stress markers. Our data reveal that the combined exposure to thermal and chemical stressors exerts an interactive negative effect on immune parameters, compromising the bees' resilience more severely than heat stress alone.

2. Materials and methods

2.1. Bee rearing and feeding

Honey bee colonies (*Apis mellifera carnica,* Pollm 1879*)* were kept at the apiary of Agricultural institute of Slovenia (GPS location: 46.061304, 14.518034), maintained according to standard beekeeping practice and regularly treated by oxalic and formic acid against *Varroa* mites (*Varroa destructor*). Six frames with capped brood (two per each hive) were selected approximately one to two days before uncapping, placed in frame cages, and kept in an incubator set at 34 °C. The next day, newly emerged bees younger than 24 h were equally divided between 36 clear plastic experimental cages, containing 50 individuals per cage (6 cages as biological replicates per experimental group). Cages were maintained in an incubator set at 28 °C and 60–80% humidity for 22 days (Williams et al., 2013).

2.2. Treatment

All cages were provided with a 6x6cm wax comb and ad libitum access to water, 50% sucrose solution, and a sugar patty enriched with protein (Medopip Plus). Food and water were replenished every 2–3 days. The trial started when the bees were three days old to allow for stabilization and adaptation to the cage environment, and concluded after 19 days. Cages were divided into three main experimental groups: control group, second group was exposed to heat stress, and the third group exposed to heat stress while being fed sublethal doses of insecticide (Fig. 1). The commercial formulation Mospilan® 20 SG (containing

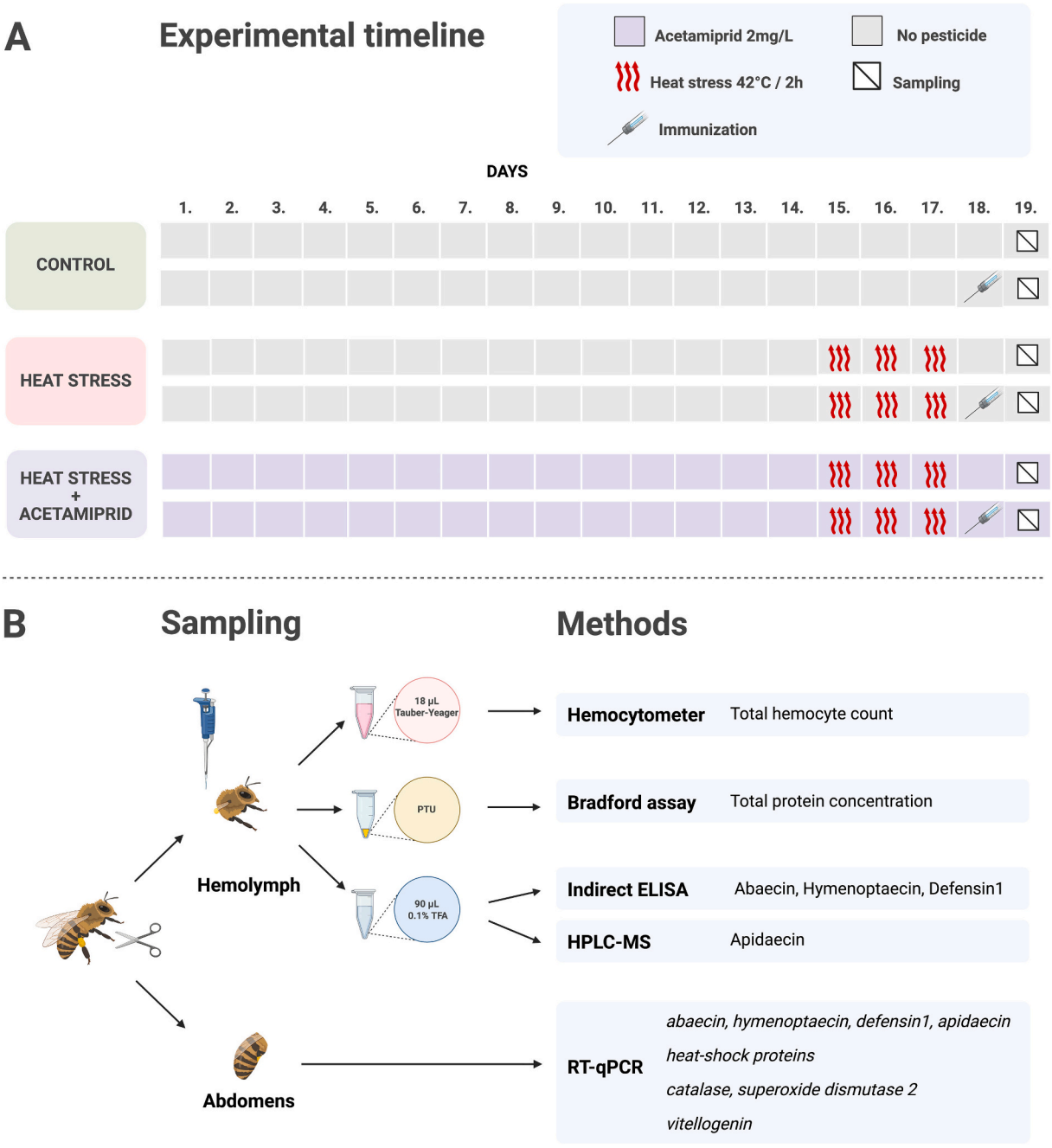

Fig. 1. Timeline showing treatment application and immune stimulation across the experiment (A) and sampling scheme illustrating hemolymph and abdomen collection, sample processing, and the methods used (B).

20% *w*/w acetamiprid; Nisso Chemical Europe GmbH, Germany), produced in 2022, was used for the experiment. Granules were dissolved in a sucrose solution to achieve a final concentration of 2 mg/L of active ingredient. This concentration represents an environmentally relevant high-end exposure scenario and serves as an established benchmark in sublethal bee toxicology for eliciting physiological shifts without increasing acute mortality (Christen et al., 2016; Shi et al., 2017; Wang et al., 2025; Schuhmann and Scheiner, 2025). Sugar solution containing acetamiprid was continuously administered to the designated groups throughout the experiment. Heat stress was applied on the 15th, 16th, and 17th day of the experiment. Assigned cages were moved to an incubator set at 42 °C for two hours each day at noon, to simulate the midday rise of temperature. The temperature of 42 °C was selected based on previous studies demonstrating that exposure within the 40–42 °C range reliably induces physiological heat-stress responses in honey bees without causing acute mortality (McKinstry et al., 2017; Li et al., 2022; McMenamin et al., 2020). On day 18, half of the cages from the experimental groups underwent an immune stimulus by mechanical injury. Bees were anesthetized with gaseous carbon dioxide and poked into the abdominal cavity with a Hamilton syringe, targeting the lateral side of the abdomen between 3rd and 4th tergite. On day 19, 24 h after the immune stimulus, all groups were sampled.

2.3. Sample collection

Hemolymph was sampled from the thoraces to avoid contamination from the digestive tract. The abdomen was cut off with scissors, and the thorax was gently squeezed. From the drop that appeared, 2 µL of

hemolymph per bee was collected with an automatic pipette (Hurychová et al., 2024). One pooled sample contained 10 μL of hemolymph from 5 bees. The hemolymph samples used for quantification of AMPs were diluted 10 times with 90 μL of 0.1% trifluoracetic acid (TFA). To determine total protein content, hemolymph samples were added to an Eppendorf tube containing a few crystals of phenylthiourea (PTU) to prevent coagulation and melanization (Fig. 1). Hemolymph samples were immediately put on ice, stored in the freezer at −80 °C, and later freeze-dried. Abdomens were immediately put on ice and stored at −80 °C until quantification of relative gene expression.

2.4. Total hemocyte count

To evaluate total hemocyte count, 2 μL of hemolymph was immediately mixed with 18 μL of Tauber-Yeager solution (4.65 g NaCl, 0.15 g KCl, 0.11 g $CaCl_2$, 5 mg Gentian violet, 0.125 mL acetic acid, 100 ml distilled water) (Tauber and Yeager, 1935), to obtain 10-fold dilution (Fig. 1). A drop of diluted hemolymph was applied on the Bürker-Türk hemocytometer and covered with a coverslip. Hemocytes were counted in four outer squares using a bright-field microscope at 100× magnification. The number of circulating hemocytes per μL was calculated in a total of 18 samples per treatment.

2.5. Quantification of relative gene expression

Isolation of RNA, cDNA synthesis, and qPCR were performed as described by Hurychová et al. (2024), adapted from Dostálková et al. (2020). Samples containing five abdomens each were added to a plastic bag (Bioreba, Reinach, Switzerland) with 1 mL of 5 M guanidinium-isothiocyanate lysis buffer containing 1% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and homogenized with a pestle. RNA was extracted from 300 μL of homogenate in GITC buffer, using NucleoSpin RNA Plus kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). The volume of 60 μL of total RNA in RNase-free water was obtained. To quantify the eluted RNA, Synergy HT microplate reader was used (BioTek, Bad Friedrichshall, Germany). To check for the presence of bands and RNA integrity, electrophoresis on 1.1% (*w/v*) agarose gel stained by GelRed (Biotium, Fremont, CA, USA) was made. Reverse transcription was performed with an iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA) to obtain 10 μL reaction volumes according to the instructions. The resulting 10 μL cDNA reactions were subsequently diluted in 70 μL of nuclease-free water prior to qPCR to ensure optimal template concentration. After transcription, quality control using endpoint PCR for the housekeeping gene *EF-1 alpha* was performed. RNA templates were used to ensure the samples were free of gDNA contamination. PCR reaction products were then tested by gel electrophoresis on 2.5–3% (w/v) agarose gel with detection by GelRed® Nucleic Acid Gel Stain, 10000× (Biotium, Fremont, CA, USA) and with a 50–1000 bp PCR Marker (Promega, Madison, WI, USA). Ct values for the housekeeping gene *EF-1 alpha* were measured by qPCR, and samples were diluted to obtain unified Ct values of 21. The efficiency of the qPCR reaction for each gene was also measured. In the next qPCR reaction, Ct values were measured for the genes of interest (GOI; *apidaecin*, *abaecin*, *defensin1*, *hymenoptaecin*, *heat shock factor*, *hsp90*, *hsp83*, *hsp70-ab*, *catalase*, *vitellogenin*, and *superoxide dismutase 2*) and for the housekeeping genes (HKG; *RPS5* and *EF-1α*), which served as reference genes. The relative expression of genes of interest to reference genes was calculated according to Pfaffl (Pfaffl, 2001) as shown in Hurychová et al. (2024). For each treatment, 18 samples were analyzed. Primer sequences and PCR cycler set up are summarized in Supplementary material S1.

2.6. Quantification of abaecin, defensin1, and hymenoptaecin using ELISA

Samples consisting of 10 μL of hemolymph collected from 5 bees were diluted with 90 μL of 0.1% trifluoroacetic acid (TFA), stored at −80 °C and subsequently freeze-dried. A total of 18 samples per treatment group were analyzed. Relative quantification of abaecin, defensin-1, and hymenoptaecin was conducted using an indirect ELISA method previously developed and optimized for honey bee hemolymph (Hurychová et al., 2024; Pinďáková et al., 2025). While absolute limits of detection are not established for this relative quantification approach, the high specificity of the custom-made primary antibodies against the target AMPs has been thoroughly validated by gel electrophoresis, Western Blot and mass spectrometry for our previous study Hurychová et al. (2024). The lyophilized samples were reconstituted in 650 μL of coating buffer and incubated for 15 min at 4 °C. After the incubation, the volume of 100 μL of diluted samples was immediately applied in duplicate to the Corning® 96-Well EIA/RIA Assay Microplate (Corning Inc., Somerville, MA, USA). Blanks (coating buffer), standards (peptide epitopes supplied by the antibody manufacturer), and negative controls (hemolymph from a freshly emerged bee) were also pipetted in duplicate for each peptide. The plate was incubated at 4 °C overnight for antigen binding. The following day, ELISA plates were washed three times with 1× washing buffer with 0.1% (*v*/v) Tween 20. The unspecific binding was prevented by adding 200 μL of 0.5% non-fat milk (Sigma-Alrich, St. Louis, MO, USA) and incubated for 2 h at 37 °C. After washing again for three times, the primary polyclonal rabbit antibodies (Clonestar Peptide Services, Brno, Czech Republic) were added to the wells. Antibodies were diluted in a 1× washing buffer as follows: 1:5000 for defensin1, 1:5000 for abaecin, and 1:500 for hymenoptaecin. After one hour of incubation at 37 °C, the plate was rinsed three times with a washing buffer. After washing, 100 μL of secondary antibody-peroxidase conjugate (goat anti-rabbit IgG (whole molecule)-peroxidase conjugate, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:3000 in 1× washing buffer with 0.1% (v/v) Tween 20 was added to the wells and the plates were incubated for one hour at 37 °C. After washing again four times with 1× washing buffer with 0.1% (v/v) Tween 20, 100 μL of the 2.4 mM tetramethylbenzidine substrate in phospho-citrate buffer with sodium perborate was added to the wells and the plates were incubated in the dark at 37 °C for one hour. The reaction was stopped by adding 50 μL of 0.5 M H_2SO_4 to each well. The absorbance was measured at 450 nm in a Synergy HT microplate reader (BioTek, Winooski, VT, USA).

2.7. Quantification of apidaecin in the hemolymph using LC-MS

Apidaecin was quantified from the samples containing 10 μL of hemolymph (5 bees per sample) and 90 μL of 0.1% trifluoroacetic acid (TFA). Samples were freeze-dried and reconstituted before measurement with 100 μL of 5% formic acid. The quantification of apidaecin via LC–MS/MS was performed according to a previously established and validated protocol (Danihlík et al., 2014). The method validation parameters, including the limit of detection (LOD), limit of quantification (LOQ), and recovery rates for the honey bee hemolymph matrix, are detailed in the aforementioned study, demonstrating high analytical sensitivity and reproducibility. UHPLC-MS analysis was done according to Dostálková et al. (2020) on a UHPLC-QTOF system Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Compact qTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with electrospray ionization. Isotopically [$^{13}C_6^{15}N_4$] labeled internal standard of apidaecin 1 A (purity >98%; Clonestar Peptide Services, Brno, Czech Republic) was used according to Danihlík et al. (2014).

2.8. Total proteins

Freeze-dried hemolymph samples from five bees, collected on PTU crystals, were dissolved in 50 μL of H_2O and diluted tenfold. The calibration curve was prepared using solutions of various concentrations of bovine serum albumin (BSA). The total protein content was then determined using the Bradford assay (Bradford, 1976). In triplicate, 5 μL

of sample or standard, 45 μL of H_2O, and 200 μL of the working Bradford reagent were pipetted to microtiter plates. Samples were incubated for 5 min, and the absorbance was measured at 595 nm using a Synergy H1 microplate reader.

2.9. Statistics

Statistical analyses were performed using JASP software version 0.19.3 (JASP Team, University of Amsterdam, Netherlands). Normality was assessed using the Shapiro-Wilk test, and to meet the assumptions of this test, a natural logarithmic transformation (ln) was applied to the data. A two-way ANOVA was utilized to evaluate the effects of treatment (control, heat stress, and heat stress combined with acetamiprid) and immunization status (non-immunized and immunized) across six experimental groups. Statistical results are reported as *F*-values with their degrees of freedom, *p*-values indicating significance, and ω^2 as an estimate of effect size describing how much variance is explained by each factor. Post hoc comparisons were performed using the Bonferroni correction, and all statistical tests were performed at a significance level of $\alpha = 0.05$. Box plots were constructed using JASP version 0.19.3 and display non-ln-transformed values. A heatmap of *Z*-scores was generated using Python libraries. The Z-scores were calculated from the logarithmized original data.

3. Results

3.1. Gene expression of antimicrobial peptides is upregulated by combined heat stress and acetamiprid exposure

To evaluate how heat stress and sublethal acetamiprid exposure affect honey bee immunity, we measured the expression of four antimicrobial peptide genes (*abaecin*, *apidaecin*, *defensin1*, and *hymenoptaecin*) in abdominal tissue.

Immune stimulation strongly increased the expression of all four AMPs (Fig. 2). Significant upregulation was observed for *abaecin* ($F_{1,102} = 158.40$, $p < 0.001$, $\omega^2 = 0.39$), *apidaecin* ($F_{1,102} = 119.86$, $p < 0.001$, $\omega^2 = 0.34$), *defensin1* ($F_{1,102} = 138.44$, $p < 0.001$, $\omega^2 = 0.39$), and *hymenoptaecin* ($F_{1,102} = 245.69$, $p < 0.001$, $\omega^2 = 0.53$), with this effect observed consistently in all three treatment groups (control, heat stress, heat stress + acetamiprid).

Treatment group also had a significant effect: *abaecin* ($F_{2,\ 102} = 60.06$, $p < 0.001$, $\omega^2 = 0.29$), *apidaecin* ($F_{2,\ 102} = 47.38$, $p < 0.001$, $\omega^2 = 0.26$), *defensin1* ($F_{2,\ 102} = 41.52$, $p < 0.001$, $\omega^2 = 0.23$), and *hymenoptaecin* ($F_{2,\ 102} = 38.94$, $p < 0.001$, $\omega^2 = 0.16$). Post hoc tests showed heat stress alone did not differ from control (all $p > 0.05$), whereas combined heat stress and acetamiprid treatment significantly increased expression compared to both control and heat stress-only groups (all $p < 0.001$). No significant interaction between immune stimulation and treatment was detected ($p > 0.05$).

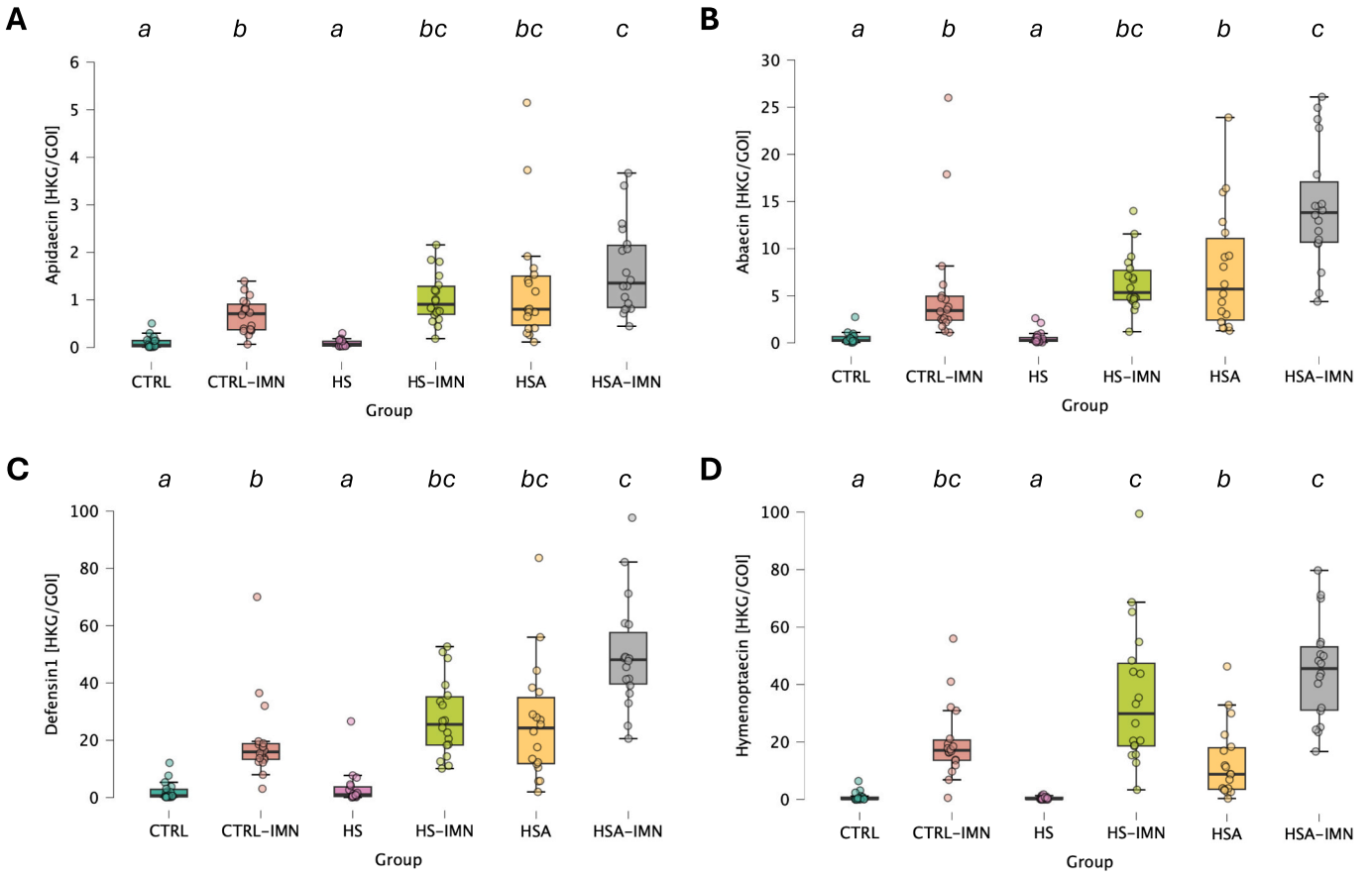

Fig. 2. Relative expression levels of selected genes of interest (GOI) - *abaecin* (A), *apidaecin* (B), *defensin*1 (C), and *hymenoptaecin* (D) - across six treatment groups: CTRL (control), CTRL-IMN (control + immunized), HS (heat stress), HS-IMN (heat stress + immunized), HSA (heat stress + acetamiprid), and HSA-IMN (heat stress + acetamiprid + immunized), each with $n = 18$. Gene expression was normalized to the reference genes *RPS-5* and *EF-1α* (housekeeping genes, HKG). Boxplots show the median, interquartile range, and data distribution, with dots representing individual values. Significant differences ($p < 0.05$) among treatment groups are indicated by different lowercase letters above the bars in the chart.

3.2. AMPs concentrations in hemolymph align with AMPs gene expression

AMPs levels in honey bee hemolymph followed patterns that closely mirrored the gene expression results. Immune stimulation significantly increased the level of all AMPs (all $p < 0.001$), and this effect was consistent across all treatment groups (Fig. 3).

Treatment group also had a significant effect on AMPs concentrations (all $p < 0.001$). As in the gene expression data, Bonferroni post-hoc comparisons showed no significant differences between the control and heat-stress-only groups ($p > 0.05$). In contrast, combined heat stress and acetamiprid exposure substantially elevated AMPs concentrations compared with both the control and heat-stress-only groups ($p < 0.001$ for all peptides).

Importantly, although the interaction between immune stimulation and treatment group was not significant overall ($p > 0.05$), heat stress amplified the immune response of abaecin and hymenoptaecin. Relative levels of abaecin and hymenoptaecin were significantly higher in immunized bees under heat stress compared to immunized controls (mean difference ± SE: 0.22 ± 0.09 for abaecin, $p = 0.024$; 0.29 ± 0.08 for hymenoptaecin, $p = 0.018$). Full ANOVA statistics for AMP concentrations are provided in Supplementary Material S3.

3.3. Correlation of gene expression and production of AMPs

We confirmed a tight correlation between transcription and translation in our experimental setup. A Pearson correlation analysis of ln-transformed data showed strong positive correlations between relative gene expression and peptide abundance for apidaecin ($r = 0.90$) and abaecin ($r = 0.84$), as well as moderate to strong correlations for defensin-1 ($r = 0.60$) and hymenoptaecin ($r = 0.57$) (all $p < 0.001$). Detailed statistical results and scatter plots are provided in Supplementary material S3.

3.4. Exposure to stressors elevates total protein concentration in the hemolymph and lowers total hemocyte count

Two-way ANOVA revealed a significant main effect of heat stress on total protein concentration in the hemolymph ($F_{2,98} = 25.95$, $p < 0.001$, $\omega^2 = 0.33$), with elevated levels observed in all heat stress groups compared to both untreated and immunized controls. The highest increase in total protein concentration was observed between the non-immunized control group and the non-immunized heat-stressed group. Immunization had no effect on total protein concentration and no significant interaction between treatment and immune challenge was detected (Fig. 4).

Total hemocyte count was not significantly affected by immune stimulation ($F_{1,88} = 1.96$, $p = 0.16$, $\omega^2 = 0.01$), although there was a tendency toward higher counts in immunized bees compared to non-immunized controls. In contrast, the treatment group had a significant effect on hemocyte levels ($F_{2,88} = 4.92$, $p = 0.009$, $\omega^2 = 0.09$). Bonferroni post hoc tests indicated that bees subjected to heat stress alone had significantly lower hemocyte counts compared to controls ($p = 0.013$), whereas the combined heat stress and acetamiprid-treated group did not differ significantly from either the control group ($p > 0.99$) or the heat stress-only group ($p = 0.066$). No significant interaction between treatment and immune stimulation was detected.

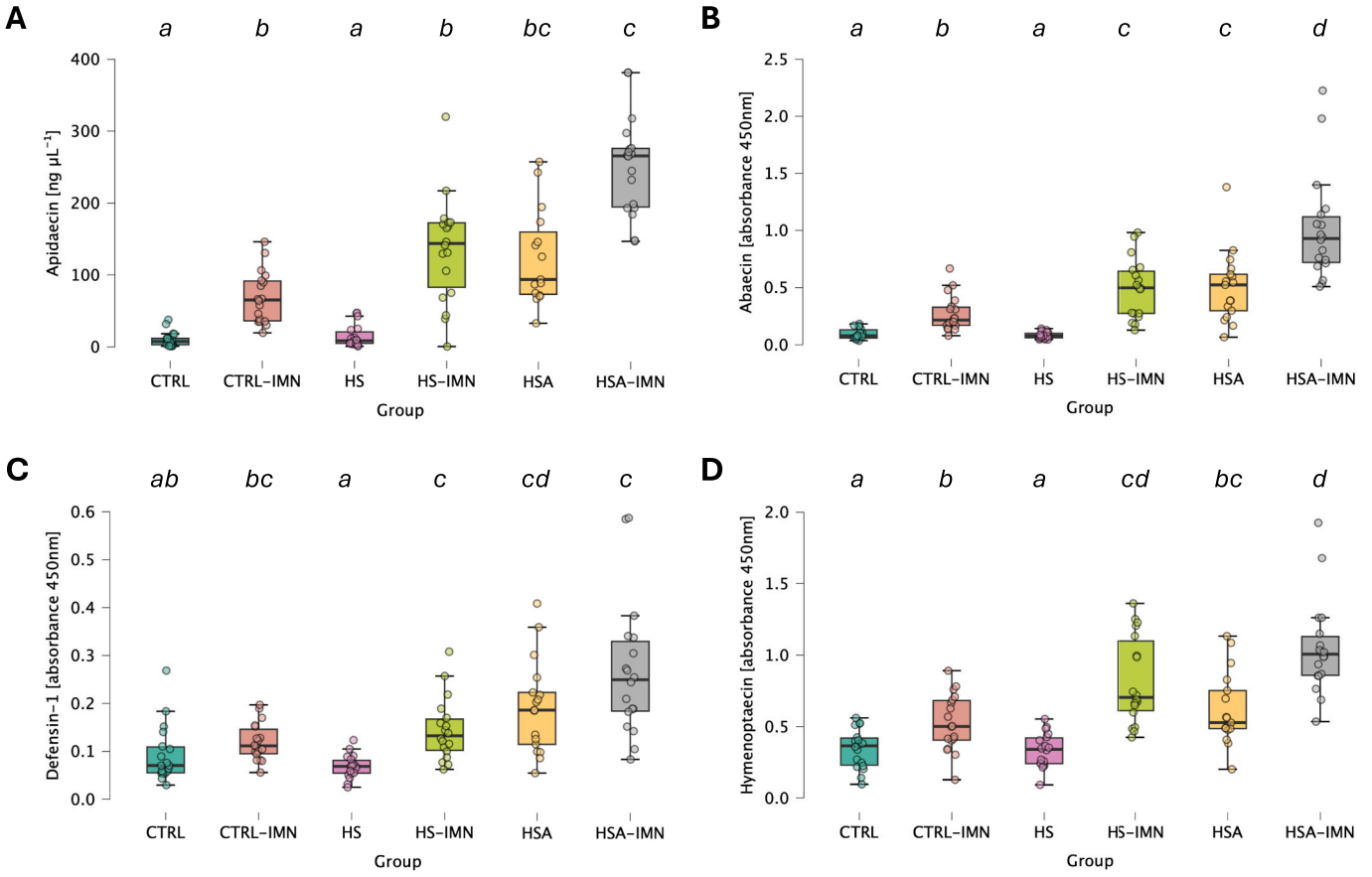

Fig. 3. Concentration of apidaecin (B) and relative quantification of three additional antimicrobial peptides - abaecin (A), defensin-1 (C), and hymenoptaecin (D) - in the hemolymph across six treatment groups: CTRL (control), CTRL-IMN (control + immunized), HS (heat stress), HS-IMN (heat stress + immunized), HSA (heat stress + acetamiprid), and HSA-IMN (heat stress + acetamiprid + immunized). Boxplots show the median, interquartile range, and data distribution, with dots representing individual values. Significant differences ($p < 0.05$) among treatment groups are indicated by different lowercase letters above the bars in the chart.

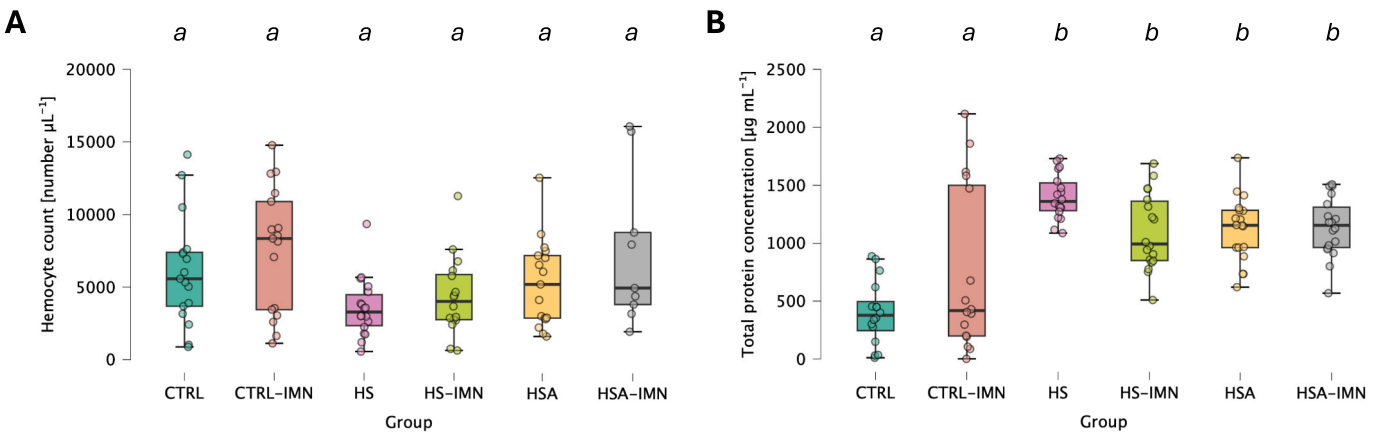

Fig. 4. Total hemocyte count (A) and total protein concentration (B) in the hemolymph, across six experimental groups: CTRL (control), CTRL-IMN (control + immunized), HS (heat stress), HS-IMN (heat stress + immunized), HSA (heat stress + acetamiprid), and HSA-IMN (heat stress + acetamiprid + immunized). Boxplots show the median, interquartile range, and data distribution, with dots representing individual values. Significant differences ($p < 0.05$) among treatment groups are indicated by different lowercase letters above the bars in the chart.

3.5. *Exposure to stressors alters gene expression of catalase, vitellogenin, and heat shock proteins*

We analyzed gene expressions of antioxidant enzymes, *vitellogenin*, and HSPs in honey bee abdomens. Expression of *catalase* was significantly influenced by treatment ($F_{2,102} = 8.41$, $p < 0.001$, $\omega^2 = 0.12$), with higher expression in the heat stress + acetamiprid group compared to both the control and heat stress-only group (Bonferroni $p = 0.002$ for both). Immunization slightly reduced *catalase* expression, but this effect was not statistically significant. Gene expression of *vitellogenin* was strongly reduced by immunization ($F_{1,96} = 72.45$, $p < 0.001$, $\omega^2 = 0.04$), while treatment had no significant effect. Among HSPs, *hsp90* was significantly affected by treatment ($F_{2,100} = 10.13$, $p < 0.001$, $\omega^2 = 0.14$), with lower expression in heat stress-only bees compared to

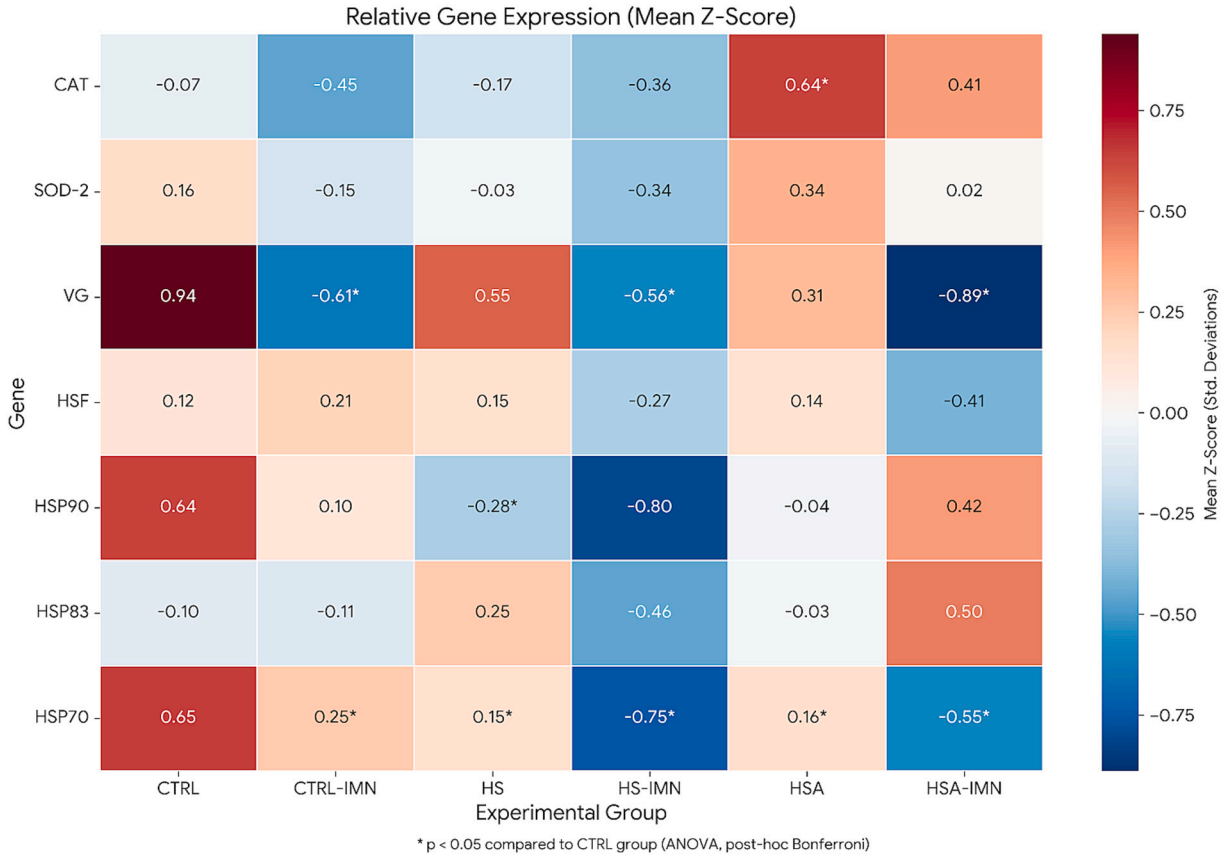

Fig. 5. Heatmap displaying the mean *Z*-scores of relative gene expression for catalase (CAT), superoxide dismutase 2 (SOD-2), vitellogenin (VG), heat shock factor (HSF), and heat shock proteins (hsp90, hsp83, hsp70-ab). The color scale represents the magnitude of deviation from the mean, ranging from deep blue (down-regulation) to deep red (upregulation). Asterisks (*) indicate statistically significant differences compared to the control group (CTRL) ($p < 0.05$) as determined by Two-Way ANOVA followed by Bonferroni post-hoc tests. See Supplementary Material S3 for full statistical tables. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

controls (Bonferroni $p < 0.001$), but immunization had no effect. In contrast, *hsp70-ab* was influenced by both immunization ($F_{1,99} = 14.61$, $p < 0.001$, $\omega^2 = 0.10$) and treatment ($F_{2,99} = 7.29$, $p = 0.001$, $\omega^2 = 0.07$), showing consistently lower expression in immunized bees and reduced levels in heat stress-only ($p = 0.002$) and heat stress + acetamiprid groups ($p = 0.011$) compared to controls. Gene expression of *superoxide dismutase 2*, *heat shock factor*, and *hsp83* were not significantly affected by either factor (all $p > 0.05$).

To visualize the regulatory patterns of stress- and immune-related genes across treatments, we standardized the relative expression data using *Z*-scores (Fig. 5). This analysis revealed contrasting expression profiles driven by immunization and chemical exposure. A consistent downregulation cluster was observed for *vitellogenin*, where expression levels dropped below the experimental mean (negative Z-scores) in all immunized groups, regardless of the thermal or chemical stress regime. *hsp70-ab* followed a similar trend, showing suppression particularly in the stressed immunized groups. In contrast, *catalase* expression displayed a treatment-specific pattern, showing distinct upregulation (positive Z-scores) in groups exposed to the combination of heat stress and acetamiprid (HSA and HSA-IMN). Notably, the Heat Stress + Immunization (HS-IMN) group exhibited a broad systemic suppression, with negative Z-scores recorded for every analyzed gene, suggesting a compromised transcriptional capacity under this specific dual-stress scenario.

3.6. Transcriptional co-regulation of stress-related genes

To investigate potential co-regulatory mechanisms under combined stress conditions, we performed a Pearson correlation analysis on ln-transformed gene expression data. The analysis revealed significant positive correlations within functional gene clusters. Specifically, we observed a moderate positive correlation between the antioxidant enzymes *catalase* and *sod-2* ($r = 0.48$, $p < 0.001$), suggesting a coordinated antioxidant response. Furthermore, a strong link was identified within the heat shock response pathway, particularly between the transcription factor *hsf* and its downstream target *hsp70-ab* ($r = 0.50$, $p < 0.001$), as well as between the chaperone *hsp90* and *hsp70-ab* ($r = 0.44$, $p < 0.001$). Gene expression of *vitellogenin* also showed a positive correlation with *hsp70-ab* ($r = 0.34$, $p < 0.001$), indicating a potential link between nutritional status and stress response capacity. For details see Supplementary material S4.

4. Discussion

Our study provides insight into how heat stress affects the immunity of honey bees, with particular attention to the combined effects of heat stress and exposure to Mospilan, a commercial insecticide containing acetamiprid as its active ingredient. The study primarily focuses on antimicrobial peptides, which represent a key component of the honey bee humoral immune response (Danihlík et al., 2014).

Studies have shown that levels of AMPs are affected not only by infections but also by response to environmental stressors and injury (Erler et al., 2011; Medina et al., 2020). Multiple studies have used mechanical injury as an immune challenge to stimulate the insect immune system (Bordier et al., 2017b; Dostálková et al., 2020; Siede et al., 2012). In our study, we assessed the immune response to stressors, both in the absence and presence of an immune challenge across three treatment groups; control, heat stress, and heat stress combined with acetamiprid. This allowed us to evaluate the capacity of honey bees to mount an immune response under different stress conditions. We confirmed that bees subjected to aseptic mechanical injury exhibited significantly increased gene expression and hemolymph concentrations of AMPs, regardless of the treatment group. Our findings therefore show that mechanical injury alone is sufficient to trigger a strong immune response.

We simulated midday heatwaves by exposing bees to 42 °C for 2 h on three consecutive days. We selected this temperature because 40–42 °C has been proven sufficiently high to induce a physiological response while avoiding lethal effects (Li et al., 2022; McMenamin et al., 2020). The exposure duration was chosen to mimic transient peak thermal conditions that foragers may encounter during extreme midday heat events. Previous work further indicates that even short-term heat exposures are biologically meaningful, as Li et al. (2022) demonstrated that a 1-h heat challenge is sufficient to induce measurable transcriptional responses.

In our experiment, heat stress did not significantly alter the gene expression or hemolymph concentrations of abaecin, apidaecin, hymenoptaecin, or defensin1. Similar results were previously described in bumblebees, where simulated heatwaves did not affect antimicrobial activity and phenoloxidase activity in the hemolymph (Tobin et al., 2024). However, McKinstry et al. (2017), reported significant downregulation of *abaecin*, *defensin1*, and *hymenoptaecin* gene expression in honey bee abdomens following a 4-h exposure to 45 °C. Li et al. (2019) observed the opposite trend; they found that the expression of *abaecin*, *defensin*, and *hymenoptaecin* increased with higher temperatures and longer exposure durations. Gene expression was notably upregulated even after just 1 h of heat exposure, with expression peaking at 45 °C. These contradictory results suggest that both the intensity and duration of heat exposure, as well as differences in experimental design, may influence the response of AMPs. In our study, however, there was one notable exception. Heat-stressed bees that were subsequently immunized exhibited significantly elevated hemolymph levels of abaecin and hymenoptaecin compared to immunized controls. These differences in response to immune stimulus can be explained by the possibility that prior heat exposure primes or enhances the immune response to mechanical injury for abaecin and hymenoptaecin. Such immune priming has been documented in insects, where moderate stress exposure can sometimes induce the immune system, which enhances the response to later stressors (Sheehan et al., 2020).

In this study, we also observed a substantial upregulation of all AMPs in groups subjected to combined heat stress and acetamiprid exposure compared to the control and heat stress-only group. The commercial formulation Mospilan (acetamiprid) was chosen for this study to account for the potential toxicity of the formulating reagents. Pesticide adjuvants have been shown to contribute to the increased toxicity of neonicotinoids in honey bees (Chen et al., 2019; DesJardins et al., 2023). Our design, therefore, reflects the realistic exposure scenario that honey bees face in agricultural environments, where they are typically exposed to complete insecticide formulations rather than pure active substances. To ensure our assay was sensitive enough to detect the interactive effects of these multiple stressors, we selected an acetamiprid concentration of 2 mg/L. This concentration represents an environmentally relevant sublethal dose that reflects the high-end spectrum of exposure in intensive agricultural landscapes. While mean residues in honey and nectar are often in the low-μg/L range, residue surveys show that acetamiprid and other plant protection products can occasionally reach the upper-μg/L to low-mg/L range in honey and pollen from intensively treated crops (Gaweł et al., 2019; Kędzierska-Matysek et al., 2022; Kasiotis et al., 2023; Zioga et al., 2020). Notably, this dose is directly comparable to other sublethal honey bee studies that have successfully employed acetamiprid doses ranging from 1 mg/L to 5 mg/L to investigate immune responses and metabolic pathways without increasing acute mortality (Christen et al., 2016; Shi et al., 2020; Wang et al., 2025; Schuhmann and Scheiner, 2025).

The pronounced upregulation of all AMPs observed in our combined treatment group is particularly noteworthy because, in existing literature, the response of the honey bee immune system to sublethal acetamiprid exposure is often inconsistent or entirely absent. For example, studies using even higher doses ranging from 5 to 25 mg/L found no significant impact on the gene expression of *hymenoptaecin* or *abaecin* (Shi et al., 2020). Similarly, field studies using the commercial formulation Mospilan reported no effect on the expression of *defensin-1* or *apidaecin* (Christen et al., 2016). Furthermore, experimental evidence

indicates that when responses do occur, they are frequently peptide- and tissue-specific. For instance, *apidaecin* was downregulated in the head while remaining unchanged in the gut (Han et al., 2023), or downregulated only after specific time intervals, whereas *defensin-1* was upregulated at multiple time points, particularly at the highest doses (Christen et al., 2016).

We therefore speculate that the pronounced AMPs upregulation in our combined treatment group likely reflects the interaction between the two stressors, where heat stress and Mospilan together created a cumulative burden sufficient to trigger a significant response of AMPs. This supports the hypothesis that multiple environmental stressors can interact jointly to compromise the immune system in honey bees (Collison et al., 2016; Kang et al., 2024). However, the cost of maintaining such immune defenses is highly energy demanding and could, if present long-term, divert resources from other vital processes (Schmid-Hempel, 2005).

In addition to the humoral immunity and AMPs, insects such as honey bees also rely on cellular immunity. Cellular immune responses are mediated by hemocytes, which perform functions such as phagocytosis, nodulation, and encapsulation, and are known to respond to environmental stressors. In our study, we observed a lower total hemocyte count in the hemolymph following heat stress-only treatment. Similarly, total hemocyte count has been significantly decreased after exposure to neonicotinoids (Brandt et al., 2016) and varroa mite infestations (Koleoglu et al., 2018). Total hemocyte count is an indirect measurement of cellular immunocompetence of honey bees, and lower numbers could imply that immune activation under heat stress occurs at a physiological cost.

Our results also show a higher total protein concentration in the hemolymph for all groups subjected to heat stress. Immune stimulus, however, had no effect on total protein concentration, which is in agreement with a study by Dostálková et al. (2020) where immunization had no effect on the nutrient composition of the hemolymph. Total protein concentration is a non-specific parameter that reflects physiological conditions, such as nutritional state, immune activation, and stress response. We hypothesized that the higher total protein concentration following heat stress might be explained by the upregulation of heat shock proteins; therefore, we measured gene expression of *heat shock factor*, *hsp83*, *hsp90*, and *hsp70-ab*.

Heat shock proteins maintain proper protein integrity and function, and are rapidly induced after exposure to elevated temperatures, oxidative stress, toxins, or pathogens (Abou-Shaara, 2024). Their transcription is activated by heat shock factors, which bind to the promoter of heat shock protein genes (Zhang et al., 2023). Multiple studies connected upregulation of heat shock proteins to heat stress and thermotolerance in honey bees (Alghamdi and Alattal, 2023; Kim et al., 2022; Li, 2024; McKinstry et al., 2017; McMenamin et al., 2020). However, we did not observe any differences in the expression of *heat shock factor* or *hsp83*. Notably, *hsp90* expression was downregulated following heat stress alone, and *hsp70-ab* expression was downregulated in both the heat stress and heat stress + acetamiprid treatments. Despite the observed downregulation of *hsp70* and *hsp90*, our correlation analysis revealed a strong positive relationship between the transcription factor *hsf* and its target *hsp70-ab* (Supplementary material S2, Fig. S1). This suggests that the regulatory signaling pathways remain functionally intact and tightly coupled, even when the overall expression output is suppressed.

The downregulation of *hsp90* and *hsp70-ab* observed 48 h post-exposure likely reflects a transition from an acute stress response to a recovery phase. We hypothesize that this result is due to a negative feedback loop. Once sufficient chaperone proteins have been synthesized to stabilize cellular damage, the cell suppresses further transcription to conserve metabolic energy (Morimoto, 1998). Although some studies have also reported on reduced gene expression of HSPs following heat stress (McMenamin et al., 2020), the predominant trend described in the literature indicates that heat stress elevates HSP gene expression (Erler et al., 2011; Li, 2024; McKinstry et al., 2017). A primary limitation of our study is that sampling was conducted 48 h after the final heat exposure, whereas most studies assess expression immediately. As reviewed by Banfi et al. (2025), the transcriptional dynamics of HSPs are generally characterized by a rapid onset, with expression levels frequently reaching their peak within a few hours of heat exposure. Consequently, our results provide a snapshot of the late-stage recovery and physiological stabilization phase rather than the acute induction spike. We acknowledge that without data from earlier time points, we cannot fully characterize the initial activation of the heat shock response. To better understand how honey bees respond to heat stress, future research should clarify how HSPs and AMPs gene expression changes at multiple time points after exposure.

In addition to heat shock proteins, we also measured the expression of genes involved in oxidative stress response and immune regulation, including *catalase*, *superoxide dismutase 2*, and *vitellogenin*, to get an understanding of how honey bees respond to environmental stressors beyond the immune response. When honey bees are exposed to environmental stressors like pesticides, elevated temperatures, physical injury, or infections, increased amounts of reactive oxygen species (ROS) are produced as a part of cellular stress responses. Similar to other aerobic organisms, insects developed a complex antioxidant defense system that consists of a complex array of both enzymatic and non-enzymatic components. Under normal physiological conditions, a balance is maintained between ROS production and antioxidants. However, this balance can be disrupted by diverse stress stimuli, resulting in oxidative stress when increased levels of ROS cause oxidative damage of proteins, lipids, and nucleic acids leading to metabolic disturbances, cell death and tissue injury (Weirich et al., 2002). Superoxide dismutases, catalase and various peroxidases belong to the key antioxidant enzymes in honey bees (Corona and Robinson, 2006; Gřešková et al., 2024).

In our study, *superoxide dismutase 2* gene expression remained stable across all treatment groups, implying that this antioxidant gene may be less responsive to the specific stressors tested. Similarly, no significant changes in SOD enzyme activity were found in the brains of acetamiprid-treated bees (Mackei et al., 2024). Interestingly, total SOD activity was upregulated in heads of bees exposed to medium but not by high doses of acetamiprid alone, whereas it was increased by high acetamiprid doses when combined with another pesticide tetraconazole (Wang et al., 2025). However, low and middle doses of combined pesticide treatment suppressed *sod1* gene expression in bee heads, suggesting different SOD isoforms might be involved in bee responses depending on the dose and type of pesticide used. The *sod2* gene encodes the Mn-SOD isoform located exclusively in mitochondria matrix. The stable expression of *sod2* in our study, therefore, indicates that the intracellular levels of superoxide radical, putatively induced by stress conditions, did not exceed the limits to trigger amplified antioxidant response mediated by SOD activity.

We observed that *catalase* gene expression was not significantly affected by heat stress or immunization, suggesting that these stressors do not strongly activate catalase-mediated antioxidant response. However, exposure to the neonicotinoid insecticide Mospilan led to a significant upregulation of *catalase* gene expression, which points to elevated levels of hydrogen peroxide induced during bee stress responses. Compared to peroxidases, other hydrogen-peroxide removing antioxidant enzymes, catalases are known to show relatively high Michaelis constant in millimolar range (Ward and Fallon, 2024; Ward and Fallon, 2024). This agrees with the catalase role in the detoxification of high bursts of hydrogen peroxide produced under stress conditions. Overall, our results support the use of catalase as a marker for insecticide-induced oxidative damage in honey bees (Balieira et al., 2018; Benchaâbane et al., 2022; Wang et al., 2025; Ye et al., 2025).

We propose that oxidative stress acts as the mechanistic driver behind the interaction between heat and acetamiprid on AMP expression. While heat stress alone was insufficient to sustain elevated antioxidant gene expression, the addition of acetamiprid triggered a

significant upregulation of *catalase* (Fig. 5). This implies that the combined treatment generated a load of ROS that exceeded the bees' buffering capacity, subsequently activating immune signaling pathways. The co-regulation of *catalase* and *sod-2* confirmed by our correlation analysis further supports the hypothesis of a coordinated, albeit overwhelmed, defense response against oxidative damage.

Vitellogenin, a multifunctional protein primarily known for its role in longevity, reproduction and as a nutrient storage protein, is also involved in the oxidative stress response. Previous studies have shown that vitellogenin levels increase in response to oxidative stress caused by abiotic factors, such as poor air quality (Mayack et al., 2023) simulated heat waves (Bordier et al., 2017a) and neonicotinoid insecticides (Sun et al., 2024). This suggests that the upregulation of vitellogenin helps honey bees to cope with oxidative stress. In our experiment, however, *vitellogenin* gene expression was not significantly affected by heat stress or Mospilan exposure. On the contrary, a significant downregulation of *vitellogenin* was observed in all immunized groups, suggesting that aseptic injury had the most pronounced effect on *vitellogenin* expression. Considering that immune stimulation significantly upregulated all AMPs, we see a negative correlation between *vitellogenin* and AMP levels in honey bees. We observed similar correlation previously in winter bees exposed to immune stimuli (Dostálková et al., 2020). The observed downregulation of *vitellogenin* in immunized bees, alongside the upregulation of antimicrobial peptides could suggest a biological trade-off between immune defense and longevity-related functions (Schmid-Hempel, 2005). This biological trade-off is visually underscored by the *Z*-score analysis (Fig. 5), which displays a striking inverse expression pattern: groups with the highest AMP upregulation exhibited the deepest downregulation of *vitellogenin*. Since vitellogenin is crucial for oxidative stress resistance and longevity, its depletion suggests that the metabolic cost of mounting a massive immune response to combined stressors compromises the bees' long-term survival prospects.

Taken together, this multi-biomarker approach demonstrates that the interaction between thermal stress and acetamiprid exposure fundamentally alters the honey bee's physiological landscape. While the immune system remained relatively quiescent under heat stress alone, the addition of a sublethal neonicotinoid dose effectively lowered the activation threshold, triggering a costly systemic response. This transition – from tolerance to an energetically expensive defense state characterized by AMP upregulation and vitellogenin depletion - highlights a critical vulnerability. It suggests that in a warming climate, the 'safe' limits for agrochemical exposure may shift, creating cumulative burdens that erode colony resilience in ways that standard single-stressor toxicity tests fail to predict.

The broader implications of these physiological shifts are significant, yet they must be weighed against the specific limitations of our experimental design. First, while laboratory cage conditions allow for precise control of stressors, they do not include colony-level defenses like social thermoregulation or collective immunity. Applying these results to natural colony settings should therefore be done with caution. Second, while 2 mg/L is a relevant high-end dose, the lack of a broader range of concentrations makes it difficult to map out exactly how the bees' physiology shifts across different levels of exposure. Third, because sampling took place 48 h after the final heat exposure, our data likely reflects a late-stage recovery and stabilization phase rather than the initial spike of rapidly responding genes like HSPs. Finally, our study used a specific set of biomarkers. While these markers highlight important immune and oxidative shifts, they do not cover every aspect of the honey bee's complex physiology. Future work using multiple sampling times and colony-level outcomes is needed to fully understand how these multiple stressors interact. In particular, testing these interactions in the presence of live pathogens would be a vital next step to confirm if these molecular changes result in higher colony mortality.

5. Conclusions

Our study provides experimental evidence that heat waves and sublethal pesticide exposure collectively alter the physiological state of honey bees. While heat stress alone did not trigger a systemic humoral immune response, its combination with acetamiprid caused a pronounced upregulation of antimicrobial peptides and antioxidant enzymes. This suggests that while bees may tolerate heat stress in isolation, the combined burden of a chemical stressor triggers a significant physiological shift not observed when heat stress is present alone. We conclude that current pesticide risk assessments, which typically test chemicals at optimal temperatures, may underestimate the cumulative physiological costs imposed on pollinators in a warming world. Specifically, the metabolic trade-offs evidenced by vitellogenin depletion could critically erode the colony's ability to withstand common biotic pressures, such as the pathologies caused by *Varroa destructor* infestation.

CRediT authorship contribution statement

Lenka Jerele: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Pavel Urva:** Methodology, Investigation. **Antonín Bednář:** Writing – review & editing, Investigation. **Jana Jemelková:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Marek Petřivalský:** Writing – review & editing, Supervision, Resources. **Jiří Danihlík:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Maja Ivana Smodiš Škerl:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Ethical statement

This work did not require ethical approval from a human subject or animal welfare committee.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Google Gemini Thinking 3 Pro in order to calculate *Z*-scores from raw data and generate Fig. 5 and Fig. S1 in Supplementary material S2. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2026.107082.

Data availability

The curated datasets, including all raw experimental measurements and comprehensive statistical summary tables supporting the findings of this study, have been deposited in the Zenodo public repository to ensure maximum transparency and accessibility. The data are publicly available under the DOI: https://doi.org/10.5281/zenodo.18800326

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