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Honey bee *Apis mellifera* L. responses to oxidative stress induced by pharmacological and pesticidal compounds

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The western honey bee, *Apis mellifera* L., is a eusocial insect that plays a significant role in ecosystem balances and the pollination of plants and food crops. Honey bees face multiple biotic and abiotic stressors, such as pathogens, diseases, chemical pesticides, and climate change, which all contribute to honey bee colony loss. This study investigated the impacts of multiple pharmacological and pesticidal molecules on honey bee survival and gene regulation responses. In an 11-day cage experiment, sublethal doses of tunicamycin, thapsigargin, metformin, paraquat, hydrogen peroxide, and imidacloprid were administered to newly emerged sister bees. Daily treatment consumption and mortality were recorded, as well as the transcription expression of 12 major genes (*AChE-2*, *Apisimin*, *Apidaecin*, *mrjp1*, *Sodq*, *cp450*, *SelT*, *SelK*, *Ire1*, *Xbp1*, *Derl-1*, and *Hsc70*), some of which are markers of oxidative and endoplasmic reticulum (ER) stresses in honey bees. At day 9 of the treatments, protein damage was quantified in caged bees. A Kaplan–Meier model indicated significant ($p < 0.001$) toxicological effects of paraquat, H_2O_2, and tunicamycin on bee survivorship compared with controls with better survival for other molecules. Post-ingestive aversion responses were recorded only for tunicamycin, hydrogen peroxide, and imidacloprid. Nonetheless, significantly higher protein damage on day 9 was identified only in bees exposed to paraquat and imidacloprid. Some antioxidant genes were significantly regulated vis-à-vis specific treatments. Our results reveal age-related regulation of other major genes with significant inter-gene positive correlations.

KEYWORDS

honey bee, oxidative stress, gene regulation, antioxidant gene, unfolded protein response

Introduction

The western honey bee, *Apis mellifera* L., is a eusocial insect that is arguably the most critical pollinator insect. It also provides important contributions to human life through its different hive products and pollination of food crops. Honey bees, including other non-*Apis* bees, pollinate 80% of the world's flowering plants and more than 90 different food crops (Rader et al., 2016), with a total estimated crop-value of $17 billion annually in the United States (Calderone, 2012). Besides their pollination services and agricultural importance, honey bees are ecologically vital in maintaining balanced ecosystems (Klein et al., 2007; Potts et al., 2010). Honey bee colonies face an assorted number of biotic and abiotic stressors, which include, but are not limited to, diseases, parasites, pathogens, and chemical pesticides, which have all led to significant losses during the last two decades (Vanengelsdorp et al., 2011; Steinhauer et al., 2014; Kulhanek et al., 2017; Li et al., 2018). Honey bee colony loss is a complex problem to tackle as such a phenomenon seems to be multifactorial and is the product of synergistic effects between various bee stressors (Alaux et al., 2010; Vidau et al., 2011; Aufauvre et al., 2012; Boncristiani et al., 2012; Doublet et al., 2015).

Honey bee biological processes and responses to stressors have been investigated in laboratory cage experiments, which provide a more controlled environment and limit the implication of other variables on the outcomes (Evans et al., 2009; Gregorc et al., 2018; Alburaki et al., 2019a; Alburaki et al., 2022). Previous studies have shown that nutritional stress and pathogenic infections (i.e., deformed wing virus, *Nosema ceranae*) have a severe impact on honey bee colony strength (Branchiccela et al., 2019). The first physiological step in the cascade of events induced by stressors is the manifestation of cellular oxidative stress. This results from a disturbance in the balance between producing reactive oxygen species (ROS), or free radicals, and antioxidant defenses (Di Conza and Ho, 2020), and any deficiency in antioxidant defenses can cause damage that occurs at various tissue levels in the organism. The second physiological response to stress in the cell is manifested at the endoplasmic reticulum (ER) level. The ER is a large, membrane-enclosed, cellular organelle found in all eukaryotes (Hirata et al., 2021). At this site, membranes are folded, proteins are secreted, lipids and sterols are synthesized, and free calcium is stored (Di Conza and Ho, 2020). If the stress persists and is not mitigated at the cellular level, an imbalance between the demand for protein folding and the capacity of the ER for protein folding can occur and lead to protein damage (Di Conza and Ho, 2020). Despite their difference in nature, stressors lead to physiological stress at the cell level, which in turn challenges the organism's homeostasis.

Honey bee colonies, as superorganisms, can maintain and return to homeostasis despite stressors such as parasite infestations or exposure to pesticides. Such a phenomenon is known as social resilience (Ulgezen et al., 2021), which ensures the survival of the honey bee population as a whole, especially in feral colonies. Such attributes, well manifested by feral colonies, could be harnessed and applied to managed colonies, which may help limit the constant losses and improve beekeeping management. Aside from managed honey bee populations, anthropogenic stress has been linked to the decline of feral honey bees (Siviter et al., 2023). More specifically, in urban landscapes and home backyards, pesticides are applied for pest management control, affecting both managed and feral honey bees.

Gene regulation in honey bees operates in a complex manner to alleviate the effect of multiple biotic and abiotic stressors affecting their survival. Some genes have been linked to specific stressors while others are still under investigation. For instance, major royal jelly protein 1 (*mrjp1*) plays pivotal roles in honey bee nutrition and larvae development (Srisuparbh et al., 2003; Li et al., 2021), and *Xbp1* and *IRE1* are transcription factors that regulate the expression of genes important to the proper functioning of the immune system and in the cellular stress response (Johnston et al., 2016; Adames et al., 2020). Sodesque (*Sodq*) catalyzes the conversion of superoxide into oxygen and hydrogen peroxide, thus controlling the levels of various reactive oxygen species (Wang et al., 2018). Two selenoproteins, T and K, are closely related to both ER stress and redox stress. *SelT* creates thioredoxin reductase-like oxidoreductase activity and modulates the contraction processes through the regulation of calcium release (Alburaki et al., 2019b; Pothion et al., 2020), while *SelK* is involved in ER-associated degradation of soluble glycosylated proteins and plays a role in the protection of cells from ER stress-induced apoptosis (Alburaki et al., 2019b; Xia et al., 2022). *Apismin* and *apideacin* are both thought to play roles in nutrition and pathogen infections and are considered anti-microbial peptide (AMP) genes (Casteels et al., 1989; Shen et al., 2007). Cytochrome P450 (*Cp450*) codes for enzymes are membrane-bound hemoproteins that play a pivotal role in the detoxification of xenobiotics, cellular metabolism, and homeostasis (Zhang et al., 2018).

Oxidative stress on caged honey bees was induced in the current study using six different abiotic stressors: two pesticides (imidacloprid and paraquat) and four pharmacological compounds (tunicamycin, thapsigargin, metformin, and hydrogen peroxide). Imidacloprid is a neonicotinoid insecticide highly toxic for bees and widely used in agriculture for pest management. This molecule acts on several types of post-synaptic nicotinic acetylcholine receptors in the insect's nervous system and binds to the nicotinic receptor, causing the neuron to fail to propagate any signal. Acetylcholinesterase is a cholinergic enzyme primarily found in post-synaptic neuromuscular junctions such as muscles and nerves, and it immediately hydrolyzes acetylcholine, which is a naturally occurring neurotransmitter, into acetic acid and choline (Dvir et al., 2010). The prime role of acetylcholinesterase is to terminate neuronal transmission and signaling between synapses to prevent acetylcholine dispersal and activation of nearby receptors (Smulders et al., 2004; Dvir et al., 2010). The sustained activation of the receptor results from the inability of acetylcholinesterase to break down the pesticide, an irreversible process that induces excessive ROS production (Dvir et al., 2010; Nicodemo et al., 2014; Alburaki et al., 2019a). Paraquat catalyzes the formation of ROS through accepting electrons from photosystem I and transferring them to molecular oxygen (Kennedy et al., 2021). Tunicamycin inhibits N-linked glycosylation, which induces ER stress (Hirata et al., 2021), and thapsigargin is known to inhibit SERCA (sarco endoplasmic reticulum Ca^{2+} ATPase). This sets off an unfolded protein response

(UPR); if protein misfolding is not resolved, it will induce ER stress (Quynh Doan and Christensen, 2015). Metformin reduces redox stress but is reported to induce distinct ER stress pathways in cardiomyocytes (Pernicova and Korbonits, 2014). Hydrogen peroxide works by producing destructive hydroxyl free radicals that can attack membrane lipids, DNA, and other essential cell components (Brudzynski, 2020).

In this study, we determined the toxicological effects of sublethal doses of pharmacological inducers and pesticides in honey bees as well as the transcriptional response and protein damage caused by oxidative and ER stresses.

2 Materials and methods

2.1 Laboratory bioassay design

This experiment was conducted at the individual honey bee level with six treatments (tunicamycin, thapsigargin, paraquat, hydrogen peroxide, imidacloprid, and metformin) and two controls (control-H_2O, which consisted of 1 : 1 sugar and water for the first five treatments, and control-PBS, made of 1 : 1 sugar syrup with PBS (1× phosphate-buffered saline, pH 7.4) for the metformin treatment as it is not soluble in water and was dissolved in PBS). Eight hundred 1-day-old sister bees were emerged in an incubator (35°C, RH 55%) and randomly distributed into eight different cages. Sister bees were obtained from a single Italian colony genetically typed as *Apis mellifera ligustica* carrying the mtDNA haplotype C1. The cages used in this study were specifically designed for feeding experiments and are fully described by Gregorc et al. (2017). Sublethal concentration treatments were chosen based on available toxicological data at the ECOTOX database (US Environmental Protection Agency EPA) as well as previous investigations to enable data comparison. Tunicamycin was administered at 19,600 ppb; thapsigargin, 195 ppb; metformin, 129,000 ppb; paraquat, 1,000 ppb; hydrogen peroxide, 4,000 ppb; and imidacloprid, 20 ppb.

The experiment lasted 13 days and consisted of two phases: the first, a 2-day acclimation period in which bees were allowed to adjust to cage conditions (day −2 to day 0/no data reported), and, secondly, treatment period, in which bees were investigated for effects of exposure on survival and at the molecular level at two time points during an 11-day exposure period. During the acclimation period, bees in cages were fed 1 : 1 sugar syrup without additional treatment. At the start of phase 2 (i.e., day 0), cages were randomly assigned to the eight treatments, which were administered *ad libitum* using 20-mL syringes through sugar syrup. The sugar syrup consumption was recorded daily by weighing the syringes using a sensitive scale (± 0.01 g) and calculating read differentials. Dead bees were collected daily and counted for each cage. Nine bees from each treatment were sampled at day 5 and 9 for molecular analyses. Bees were frozen on dry ice and immediately stored at −80°C for further molecular analyses.

2.2 RNA extraction

RNA was extracted from individual bees using whole-body tissue. For each treatment and date of collection, RNA was extracted from three bees using the Trizol extraction method (Chomczynski, 1993) with some modifications to the original protocol. The individual whole bee body was put into 1 mL of Trizol and crushed using sterile pestles. The lysate was pipetted up and down several times to homogenize the bee tissues and then sonicated (Bioruptor® Pico, Diagenode) for 10 cycles consisting of a 30-s pulse and a 30-s rest at 4°C. The samples were transferred to a rocker for 10 min at room temperature and then centrifuged at ~15,000 *g* for 10 min at 4°C. Resulting supernatants were transferred to fresh RNase-free tubes and incubated for 5 min at room temperature to permit complete dissociation of the nucleoproteins. Two hundred microliters of chloroform were added to the tubes and vortexed aggressively. Samples were then incubated on a rocker at 4°C for 10 min before being centrifuged at 15,000 *g* at 4°C for 20 min. The resulting aqueous phase was transferred to a new tube. Six hundred microliters of isopropanol were added to the samples, vortexed, and then incubated for 10 min at −20°C. RNA was precipitated by centrifugation at 15,000 *g* for 15 min at 4°C. The supernatant was discarded, and the RNA pellet was washed with 600 µL of 70% ethanol and was pipetted up and down three times and centrifuged (15,000 *g*) for 5 min at 4°C. The supernatant was discarded, and the pellet was air dried for 5 min and then resuspended in 50 µL of molecular-grade RNase-free water. RNA extracts were subsequently inspected using a NanoDrop ND 1000 spectrophotometer (Thermo Scientific) to determine the RNA quantity and quality and stored at −80°C.

2.3 Transcriptional analysis

Gene expressions of 12 major antioxidant and developmental genes were evaluated from three whole honey bee samples taken on day 5 and day 9 (*AChE-2*, *Apisimin*, *Apidaecin*, *mrjp1*, *Sodq*, *cp450*, *SelT*, *SelK*, *Ire1*, *Xbp1*, *Derl-1*, *Hsc70*). The cDNA was produced from total RNA using Bio-Rad's iScript™ Kit following the manufacturer's protocol. Previously published primers were confirmed on their respective targets and used in this study (Table 1). The 12 target genes were normalized against two housekeeping genes (i.e., glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein S5) known for their stability in honey bee tissues (Scharlaken et al., 2008; Alburaki et al., 2017). All RT-qPCR runs consisted of three biological replicates per treatment and time combination, and each biological sample was run with three technical replicates per gene using the following cycling protocol: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and a final melting step of 95°C for 10 s and a 0.5°C increment for 5 s, from 65°C to 95°C. All qPCR plates were normalized using an inter-plate calibrator that was run on each plate and the two housekeeping genes (Table 1). The gene analysis was conducted using relative normalized

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TABLE 1 List of the target genes analyzed in this study.

Gene	Name	Forward/reverse (5′–3′)	Size bp	NCBI Accession
Target genes				
AChE-2	Acetylcholinesterase 2	GACGCGAAGACCATATCCGT TCTGTGTCCTTGAAGTCCGC	140	NM_001040230.1
Apis	Apisimin	TAGCTGCCTTCTGCGTAGCC CACGTTCGATTCGCCTTTGACAC	80	NM_001011582.1
Apid	Apidaecin	GGAATACCAACCTAGATCCGCCTA TATTGCCGGGTTCAGCTTT	162	NM_001011613.1
mrjp1	Major royal jelly protein 1	TGACCAATGGCATGATAAGATTTT GACCACCATCACCGACCT	98	NM_001011579.1
Sodq	Sodesque	ATTTACCGCTTAGATGTTATGTAGGAG AGCACACGCAAATTTATTACTTCCA	179	XM_006558333.2
cp450	Cytochrome P450 305 a1	CAGGTGATATATTGGCAAAAGCTACGA TGTGGCCCTTTACCAGGAAC	94	XM_623618.6
SelT	Selenoprotein T	GACAGCCACCAGCATCATTG TGGACCACACAGGAACATCAT	150	XM_623426.6
SelK	Selenoprotein K	TGGAAGCGTTTTATGTGGTACT ATCTACGAGTTGGTGGACGTG	182	NM_001278332.1
Ire1	Inositol-requiring enzyme 1	GGGCAAAAATTGGTTCAGTCAA CAGCTACGGATCGTCCATCA	320	XM_006565606.3
Xbp1	X-box-binding protein 1	GGAACAACACAGCTGGCATC TTTTGATGTCTGCCCCACCA	275	XM_392383.7
Derl-1	Derlin 1	TCCATGGGTATTGTTTGCGT CGTCCCCAATTATGACCACCA	267	XM_001122972.5
Hsc70	Heat shock 70-kDa protein cognate 3	TCGAAGTCGTGCTGAGAGTA CCATCAGCAGTAAATGCCACA	350	NM_001160052.1
Housekeeping genes				
RpS5	Ribosomal protein S5	AATTATTTGGTCGCTGGAATTG TAACGTCCAGCAGAATGTGGTA	115	XM_006570237.3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TACCGCTTTCTGCCCTTCAA GCACCGAACTCAATGGAAGC	142	XM_393605.7

The primer sequence for each gene and amplicon size is given, as well as the NCBI accession number. Two housekeeping genes known for their stability across honey bee tissues were used to standardize the qPCR dataset.

expression calculated via the ΔΔCt values through the Bio-Rad Maestro software. The datasets were subsequently transferred to the R environment (Team, 2011) for statistical analysis and figure generation.

2.4 Quantification of protein damage

To quantify potential post-transcriptional damage caused by the various administered treatments, a protein carbonyl content assay was conducted on treatments of the last sampling date (day 9). Protein was extracted from individual bees in triplicate using a protein extraction buffer consisting of 20 mM Tris-HCl pH 8.0, 30 mM NaCl, and 10% glycerol. The tissues were crushed using a pestle and sonicated using a Bioruptor Pico (Diagenode) sonication device for 10 cycles of a 30-s pulse and a 30-s rest at 4°C. Homogenates were centrifuged at 5,000 *g* for 10 min at 4°C and the supernatants were collected. The protein carbonyl contents in studied samples were estimated using a Sigma-Aldrich kit (MO, USA), as described in the manufacturer's protocol.

2.5 Statistical analysis

The cage experiment was conducted at the individual bee level, with eight treatments, three biological replicates per treatment, and three technical replicates for each gene. All statistical analyses of this study were carried out in the R environment (Team, 2011) using RStudio version 2022.12.0 + 353. First, each dataset was tested for normality using the Shapiro test. Syrup consumption, recorded daily, was calculated at the cage level for each of the eight studied groups. An ANOVA was conducted at a 95% confidential interval

on data normality distributed with three levels of significance ($p < 0.05$*, $p < 0.001$**, $p < 0.001$**). A Kruskal–Wallis rank test, a non-parametric test, was used on data that failed the normality test in which multiple comparisons and *p*-values were adjusted with the Benjamini–Hochberg method using the "FSA" Library.

Survival probability and cumulative hazard were calculated for each treatment group by the Kaplan–Meier survival probability model in R using three packages: "dplyr," "survival," and "survminer." Figures were generated in the same environment utilizing three main libraries: "ggplot2," "doby," and "plyr." Gene regulation was displayed as relative normalized expression per date and overall averages. The heatmaps were generated using the "pheatmap" library either by date (days 5 and 9) or by overall expression of each studied gene. Correlation in the gene regulation between treatments was performed using the R libraries "PerformanceAnalytics" and "corrplot" with an intermediary level of significance ($p < 0.01$). A principal co-ordinate analysis (PCA) was carried out using the "factoextra" library to estimate the expression of each variable on a three-dimensional scale and treatment group similarity. Box and whisker plots were constructed to visualize the data and display the median, first and third quartiles, and both maximum and minimum values for each condition.

3 Results

3.1 Toxicity of stressors

The Kaplan–Meier model showed significant differences ($p < 0.001$) in survival rates among administered stressors (Figure 1). The lowest level of mortality during the 11-day experiment was recorded in cages exposed to the control-PBS treatment while the lowest survival rate was observed in bees exposed to paraquat. Paraquat induced early mortality, starting at day 3 post-administration followed by exposure to hydrogen peroxide (H_2O_2), which led to 100% mortality at day 9. Metformin and imidacloprid had significantly greater survival rates than the control, while tunicamycin led to a lower survival rate than the control. Caged bees exposed to both thapsigargin and control bees had similar survival rates. The cumulative hazard of paraquat was chronic: it started early at day 3 but did not lead to total mortality. However, hydrogen peroxide's cumulative hazard sharply increased at day 8, causing complete mortality of the caged bees.

For syrup consumption, caged bees consumed significantly ($p < 0.001$) lower amounts of tunicamycin, H_2O_2, and imidacloprid than all other treatment compounds, including both controls (Figure 2A). No significant differences were found in the amount of syrup consumed among thapsigargin, metformin, paraquat, and both controls. The accumulative consumption graph confirms this finding over the 11-day experiment (Figure 2B).

3.2 Transcriptional analysis and ER stress

Honey bee responses to stresses induced by pharmacological molecules and agricultural pesticides were evaluated by identifying the transcript level of genes involved in the mitigation of oxidative and ER stress. *AChE-2* was significantly ($p < 0.001$) upregulated by day 9 compared with day 5 irrespective of the treatments, while both *apisimin* and *apidaecin* showed no effect of time or treatment on their regulation, except a higher ($p < 0.05$) regulation of *apisimin* at day 5 for metformin than for imidacloprid (Figure 3). Treatments did not affect the regulation of *mrjp1*, however, an overall significant

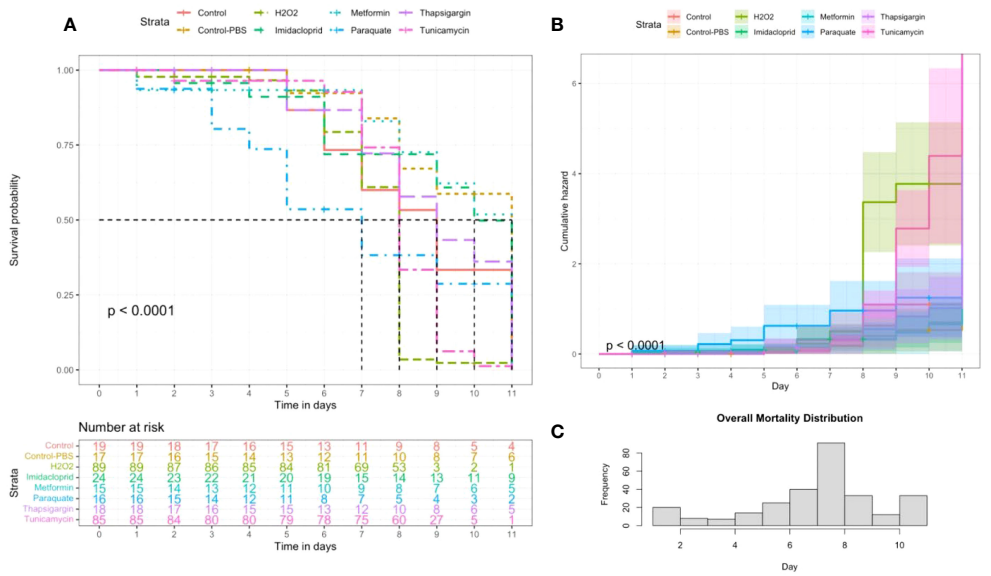

FIGURE 1
Kaplan–Meier survival probability **(A)** and cumulative hazard **(B)** models conducted on the honey bee groups subjected to six different treatments and two controls. The number of honey bees at risk was estimated through the same model for each treatment. Distribution of overall mortality throughout the day during an experiment **(C)**. Significant mortality differences among treatments.

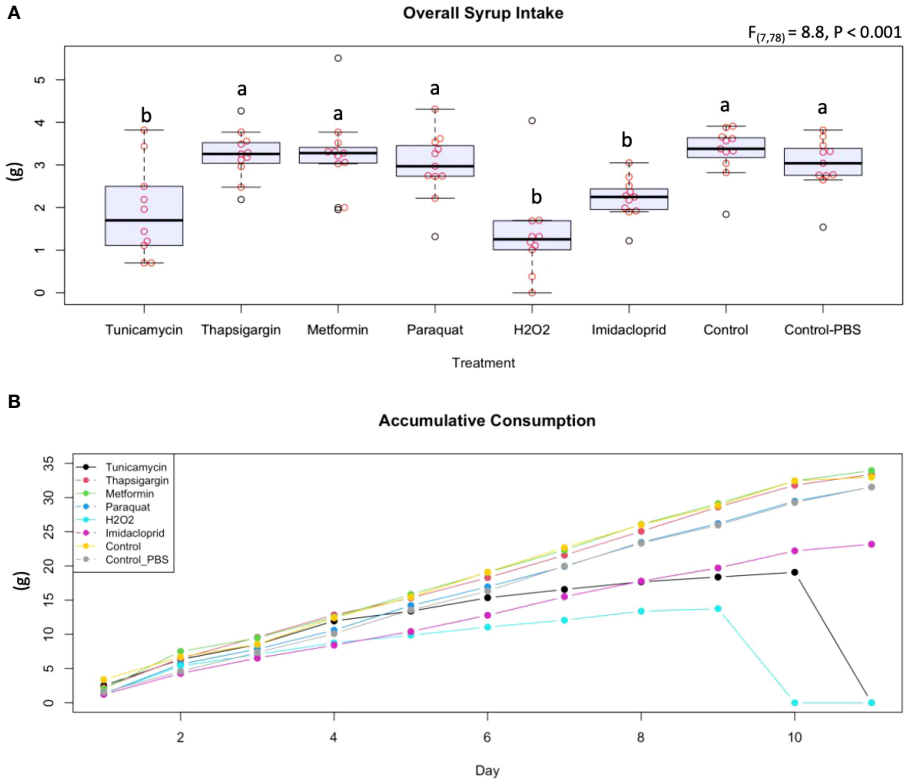

FIGURE 2
Average *at libitum* syrup intake **(A)** and accumulative syrup intake **(B)** displayed by treatment. The accumulative consumption of the eight administered treatments and controls (tunicamycin, thapsigargin, melformin, paraquat, hydrogen peroxide, imidacloprid, control, and control in PBS buffer) are shown in a longitudinal manner. The boxplots with different alphabetical letters are statistically significant.

($p < 0.05$) drop in its regulation was observed at day 9 (Figure 4). A similar finding was recorded for the regulation of *P450*, with significant ($p < 0.05$) upregulation at day 9. Regulation of the *Sodq* gene, however, was not affected by time, and was significantly higher ($p < 0.05$) for imidacloprid at day 5 than for tunicamycin, and significantly upregulated for the control compared with tunicamycin at day 9 (Figure 4). Neither of the studied selenoprotein genes (*SelT* and *SelK*) were affected by the treatments, similar to *Ire1* (Figure 5). Nonetheless, each of these three genes changed regulation vis-à-vis time. *SelT* and *Ire1* were downregulated by day 9, while *SelK* was upregulated by day 9 (Figure 5). *Xbp1* was significantly ($p < 0.05$) upregulated for metformin and thapsigargin compared with imidacloprid at day 5 with a significant ($p < 0.001$) overall downregulation at day 9 irrespective of the treatments (Figure 6). The regulation of both *Derl-1* and *Hsc70* was not affected by the treatments (Figure 6).

3.3 Gene interaction and correlation

The study of the overall gene regulation displayed as heatmaps revealed three different sets of genes: (1) genes that were upregulated (*Ire1, AChE-2*, and *Cp450*); (2) genes that were downregulated (*SelK, SelT, Apisimin*, and *mrjp1*); and (3) genes with mixed regulation (*Apideacin, Sodq, Hsc70, Xbp1*, and *Derl-1*) (Figure 7A). The heatmap dendrogram distinguished four genes that exhibited the closest similarity in their overall regulation across treatments (CONT, PAR, MET, THA) (Figure 7A). However, this pattern of regulation was not constant and differed by dates. For instance, at day 5, the highest expressed gene among all treatments and genes was *Ire1*, while at day 9 upregulation was observed for *Apid*, *Sodq*, *AChE-2*, and *Cp450* (Figure 7B). The correlation analysis conducted on the overall and date-by-date gene regulation and treatments reveals significant ($p < 0.01$) positive correlations within each date only (Figure 8). No significant negative correlations among genes were found at any time point.

3.4 Principal component analysis

The PCA conducted on the regulation of all studied genes revealed that 40.7% of the variables were expressed in *Dim1*, 27.8% in *Dim2*, and 12.4% in *Dim3* (Figure 9). According to the estimated number of clusters (K = 3 – 4) and the visual distribution of the individual variables (treatments), the PCA grouped four major groups in *Dim1* (40.7%) and *Dim2* (27.8%): (1) HYD and IMID, (2) CONT and CONT1, (3) MET and THA, and (4) PAR and TUN (Figure 9A). However, on *Dim1* (40.7%) and *Dim 3* (12.4%), treatments grouped into only three clusters: (1) HYD, CONT1, and IMID, (2) CONT, and (3) TUN, THA, PAR, and MET (Figure 9A).

Concerning the variable behavior (regulation of the genes) regarding the treatments, the PCA displayed by "variables"

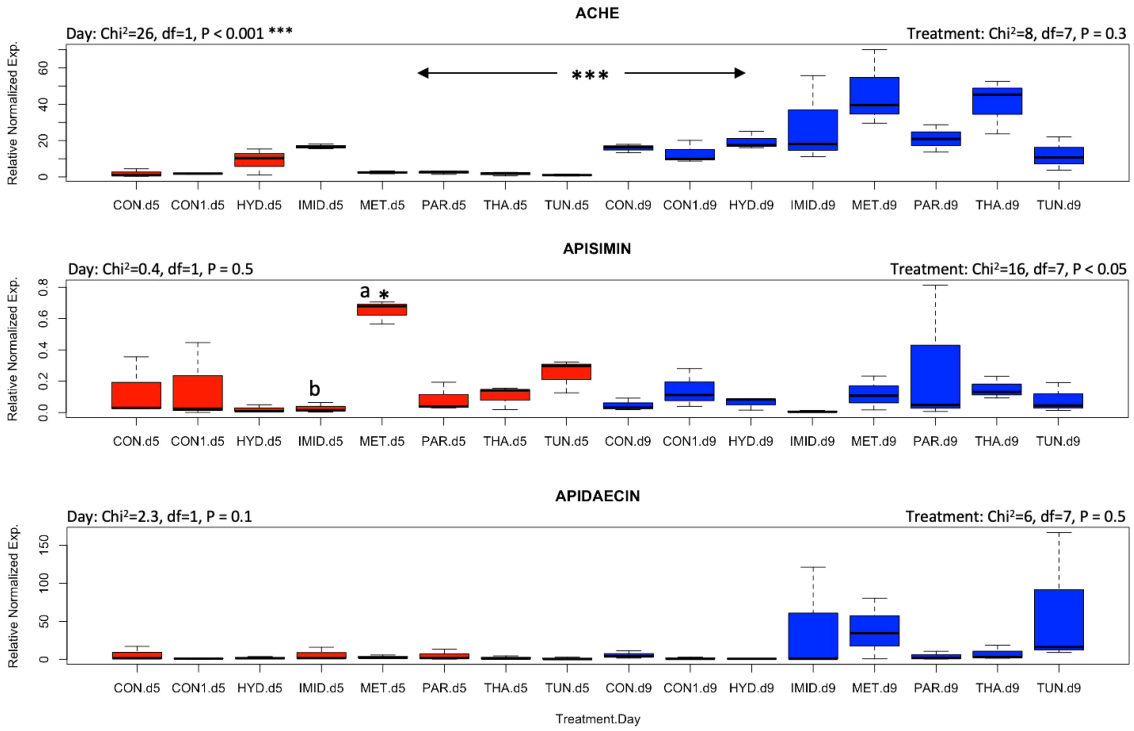

FIGURE 3
Overall gene expression of *AChE*, *apisimin*, and *apidaecin* across the eight treatments and days of exposure (day 5: red boxplots; day 9: blue boxplots). A non-parametric Kruskal–Wallis test was conducted at a 95% confidential interval with three levels of significance ($p < 0.05$*, and $p < 0.001$***) to determine the statistical differences among the treatments and dates. The boxplots with different alphabetical letters are statistically significant.

showed two sets of genes with opposing function and regulation vis-à-vis the treatments: (1) *cp450* and *Sodq*, and (2) *Ire1, Apis, SelT, SelK, Apid, Xbp1, Derl-1*, and *Hsc70. Mrjp1*, and *AChE-2* exhibited neutral regulation (Figure 9B).

3.5 Oxidative stress and protein damage

The protein carbonyl contents assay was conducted on caged bees sampled at day 9 of the treatment. The highest carbonyl content was identified in bees fed paraquat and imidacloprid, which contained significantly ($p < 0.001$) higher levels of protein damage than all other treatments. The control_H2O, metformin, thapsigargin, and tunicamycin did not statistically differ in their carbonyl content (Figure 10). The significantly lowest protein damage was identified in the control syrup containing PBS buffer (control_PBS) (Figure 10).

4 Discussion

This study focused on the link between sublethal doses of pharmacological inducers and agricultural pesticides on honey bee gene regulation and oxidative stress. The eight treatments and controls used were a combination of known oxidative stress inducers in living organisms such as paraquat (herbicide), imidacloprid (neonicotinoid insecticide), and hydrogen peroxide (cellular byproduct of oxidative stress), as well as newly tested pharmacological compounds and antibiotics. Oxidative (or redox) stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to detoxify the reactive intermediates or repair the resulting damage (Pizzino et al., 2017). This imbalance can lead to ER stress, which occurs when proteins are not properly folded or conformed (Yamamoto and Ichikawa, 2019). Due to their short-lived workers, social insects, such as honey bees, are excellent models for studying oxidative stress and its relation to senescence (Kramer et al., 2021). ER stress is also used to study diabetes through pancreatic β-cell destruction in humans by looking at insulin-producing cells found in drosophila (Katsube et al., 2019).

From a toxicological standpoint, our data showed that paraquat was the most chronically toxic molecule, although it was administered at sublethal concentrations (Figure 1). Paraquat catalyzes the formation of ROS through accepting electrons from photosystem I and transferring them to molecular oxygen (Kennedy et al., 2021). Our transcriptional results did not reveal a specific gene response to alleviate the effect of paraquat on honey bees; however, the highest and most significant protein damage was recorded in honey bees when fed this treatment (Figure 10). Moreover, the closest overall gene regulation similarity to paraquat was found in honey bees exposed to tunicamycin, according to the PCA analysis (Figure 9A). This same result has

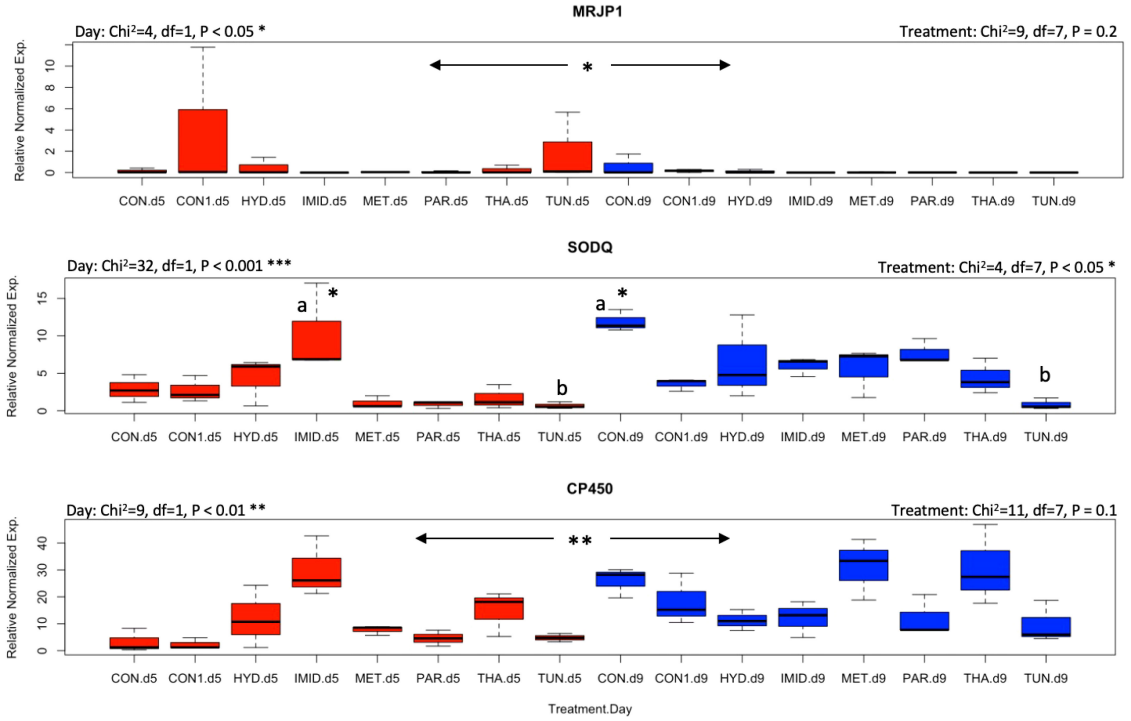

FIGURE 4
Overall gene expression of *mrjp1*, *Sodq*, and *cp450* across the eight treatments and studied dates (day 5: red boxplots and day 9: blue boxplots). A non-parametric Kruskal–Wallis test was conducted at a 95% confidential interval with three levels of significance ($p < 0.05$*, $p < 0.001$**, and $p < 0.001$***) to determine the statistical differences among the treatments and dates. The boxplots with different alphabetical letters are statistically significant.

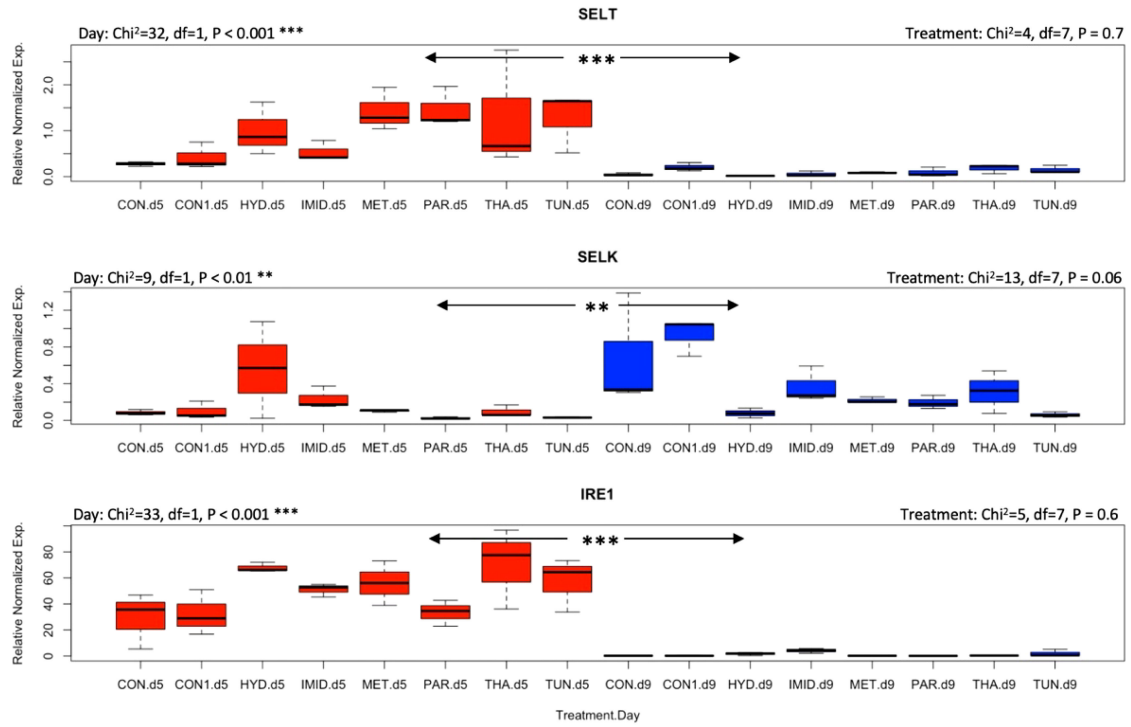

FIGURE 5
Overall gene expression of *SelT*, *SelK*, and *Ire1* across the eight treatments and studied dates (day 5: red boxplots; day 9: blue boxplots). A non-parametric Kruskal–Wallis test was conducted at a 95% confidential interval with three levels of significance ($p < 0.001$**, and $p < 0.001$***) to assess the statistical differences among the treatments and dates.

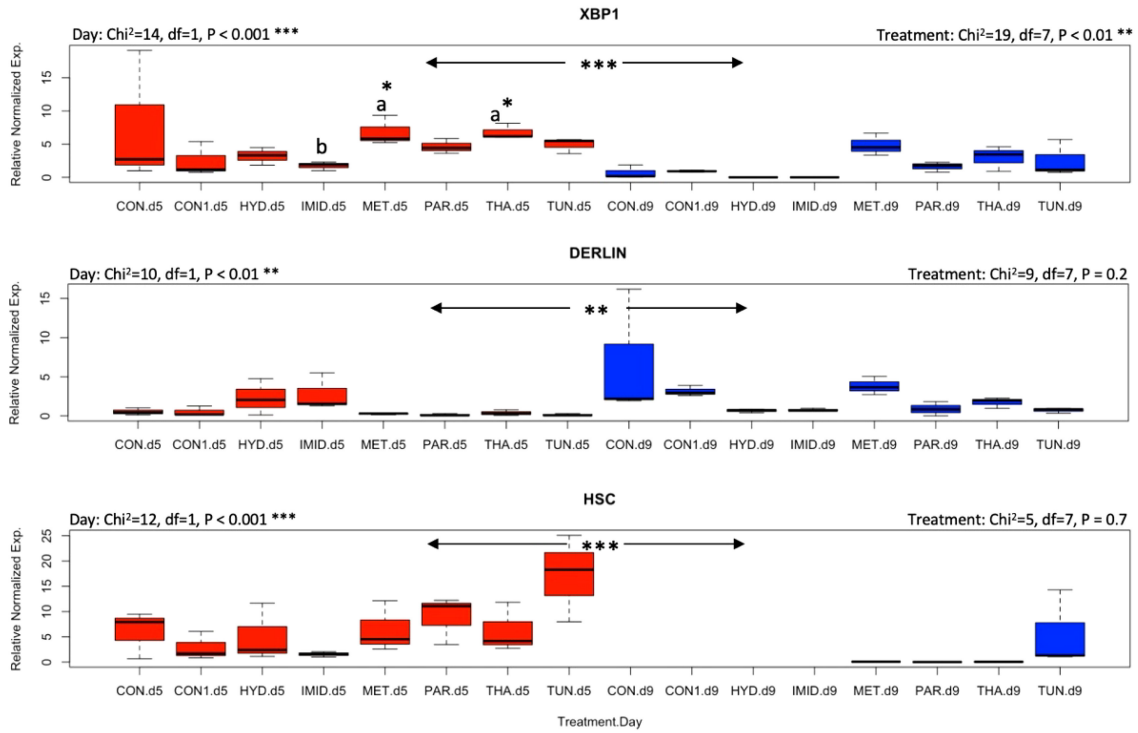

FIGURE 6
Overall gene expression of *Xbp1*, *Derl-1*, and *Hsc70* across the eight treatments and studied dates (day 5: red boxplots; day 9: blue boxplots). A non-parametric Kruskal–Wallis test was conducted at a 95% confidential interval with three levels of significance ($p < 0.05$*, $p < 0.001$**, and $p < 0.001$***) to assess the statistical differences among the treatments and dates. The boxplots with different alphabetical letters are statistically significant.

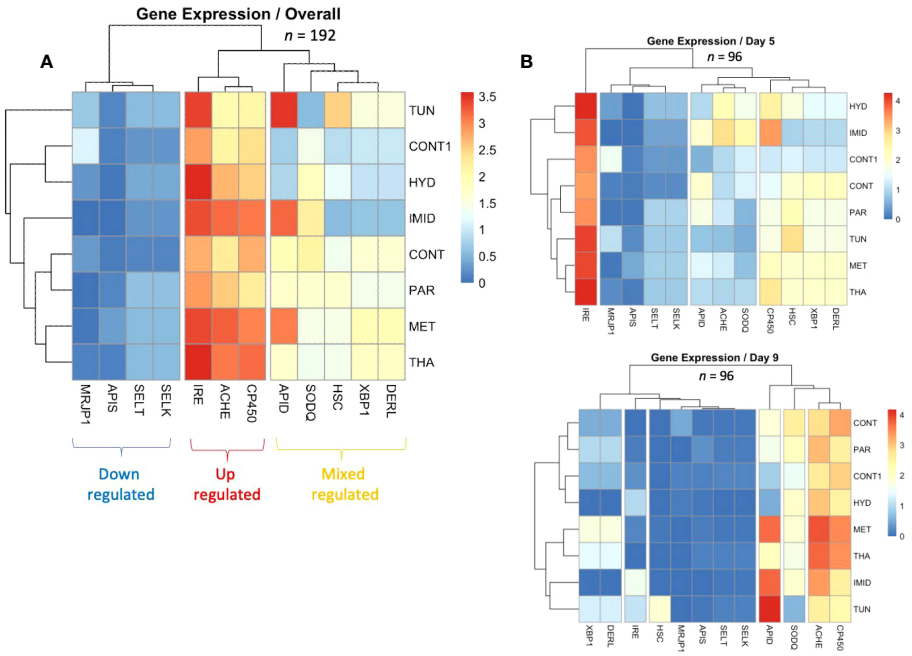

FIGURE 7
Heatmaps conducted on the overall regulation of the 12 studied genes in the eight different treatments **(A)** as well as their regulation per date **(B)** (days 5 and 9). The analysis of the overall gene regulation distinguished three major gene clusters showing upregulation (*Ire1*, *AChE-2*, and *Cp450*), downregulation (*SelK*, *SelT*, *Apis*, and *mrjp1*), and mixed regulation of genes (*Apis*, *Sodq*, *Hsc70*, *Xbp1*, and *Derl-1*).

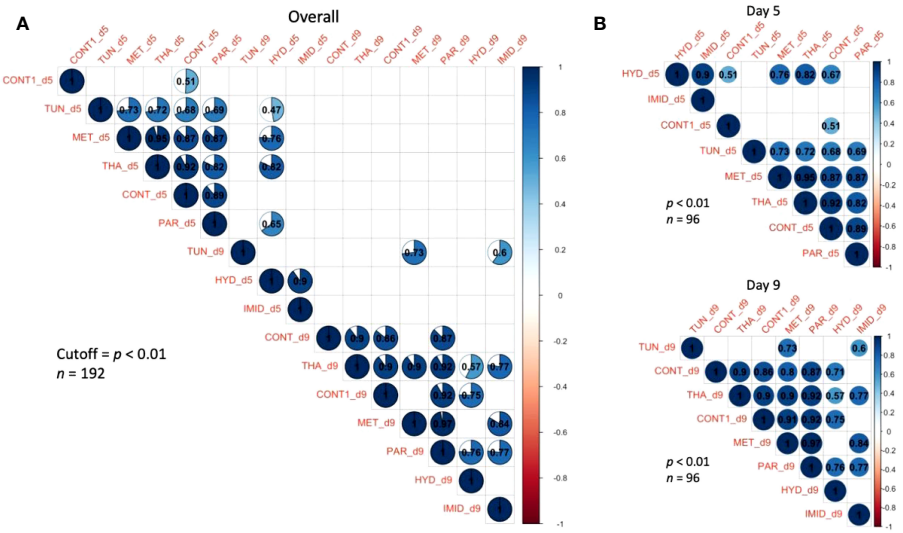

FIGURE 8
Correlation matrices of the gene regulation conducted on the eight studied treatments and displayed by overall **(A)** and date-by-date expressions **(B)**. A correlation analysis was conducted at an intermediary level of significance ($p < 0.01$). Correlation *R*-values are given in each circle and blank squares represent non-significant correlations at the cut-off level of $p < 0.01$.

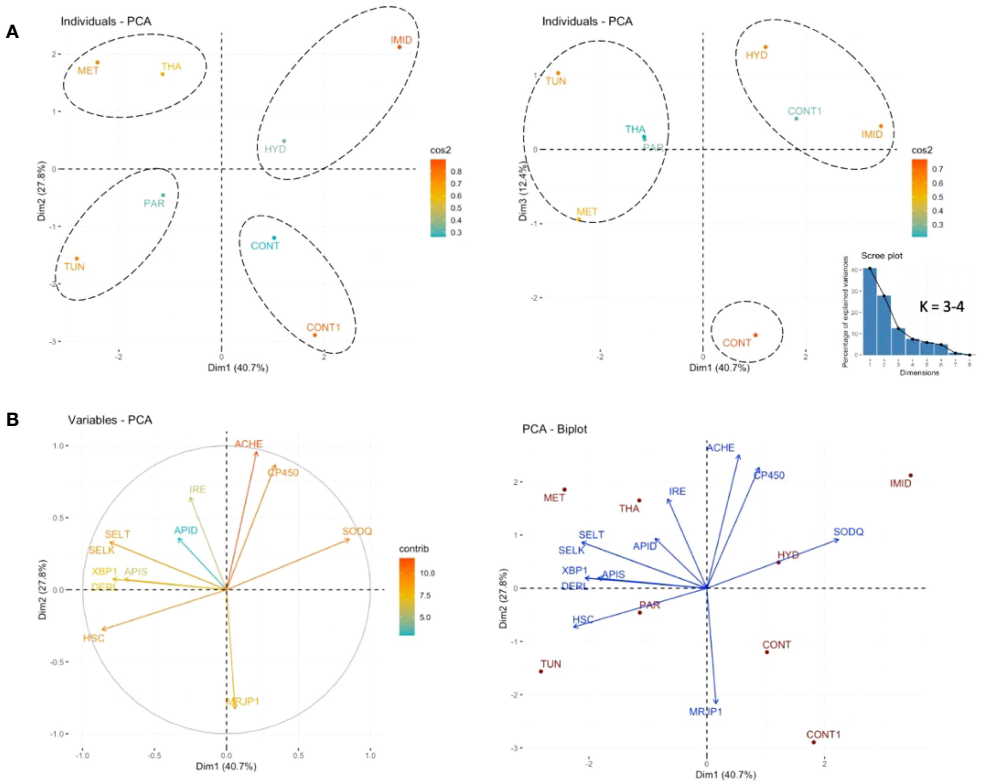

FIGURE 9
A PCA conducted on the overall regulation of the 12 studied genes in eight treatments. The percentages of the individual variables expressed on components 1 and 2 and 1 and 3 **(A)** are graphically visualized in a three-dimensional space. The expression and direction of each variable (genes) in *Dim1* and *Dim2* are given along with the PCA biplot of both expressed variables and treatments on *Dim1* and *Dim2* **(B)**. The scree plot shows the mathematically calculated number of estimated groups (K).

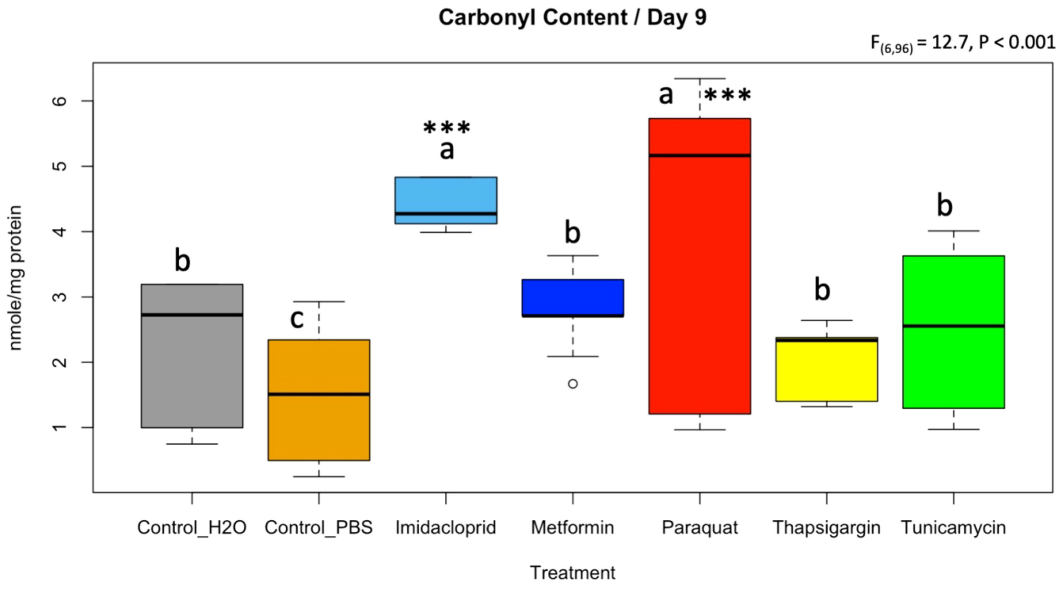

FIGURE 10
Level of protein damage quantified by a protein carbonyl content assay on honey bee samples at day 9 subjected to different treatments. An ANOVA was conducted at a 95% confidential interval with three levels of significance ($p < 0.001$***) to assess statistical differences among treatments. The boxplots with different alphabetical letters are statistically significant.

been shown to work synergistically with each other in *C. elegans* studies when also exposed to ER stress (Taylor et al., 2021). Interestingly, this same result was also seen in an experiment in which drosophila were fed paraquat, H_2O_2, and tunicamycin; it was seen that many genes modulated oxidative stress resistance when they were transcriptionally affected by oxidative stress from these treatments (Girardot et al., 2004). Tunicamycin is an antibiotic that inhibits N-linked glycosylation, inducing ER stress (Yamamoto and Ichikawa, 2019). Exposure to tunicamycin nonetheless produced only half (~2.5 mole/mg) of the carbonyl content recorded for paraquat (~5.1 mole/mg) (Figure 10). Despite honey bees consuming this antibiotic less than other molecules ($p < 0.001$; Figure 2A), tunicamycin exhibited acute toxicity at day 8 (Figure 1) with induced transcriptional response for *Hsc70* at day 9, Figure 6. Heat shock 70-kDa protein plays important roles in normal cellular function and homeostasis. For instance, Hsp70s function as molecular chaperones, assisting in protein synthesis, folding, assembly, trafficking between cellular compartments, and degradation (Sarioglu-Bozkurt et al., 2022). It is conceivable that its higher regulation at day 5 alleviated the rate of protein damage identified at day 9 for tunicamycin, which was one of the most toxic molecules tested (Figure 1). It can also be further proven by looking at the elevated expression of *IRE1*, as that could cause an upregulation of *Hsp70* to deal with the increased ER stress, as shown in a different study (Liu and Chang, 2008).

In honey bees, exposure to neonicotinoids has been well linked to elevated expressions of *AChE-2* (Badiou-Beneteau et al., 2012; Boily et al., 2013; Alburaki et al., 2015; Alburaki et al., 2023). Interestingly, our data confirmed previous findings on exposure to imidacloprid in particular. Avoidance of sugar syrup tainted with imidacloprid ($p < 0.001$; Figure 2), also recorded in this current study for tunicamycin and H_2O_2, was reported in previous investigations, as well as significantly higher protein damage in caged bees exposed to this insecticide (Alburaki et al., 2019a; Alburaki et al., 2022). Expressions of *AChE-2* were induced in bees exposed to imidacloprid at day 5. Still, it remained statistically non-significant compared with the control (Figure 3). However, this gene was significantly upregulated in all treatments at day 9 (Figure 3), signaling the potential occurrence of caging stress reported in a previous study (Alburaki et al., 2019a).

Apisimin was upregulated in a single occurrence in the case of bees exposed to metformin compared with honey bees exposed to imidacloprid (Figure 3). Similar to *apidaecin*, both genes are described as playing an important role in honey bee nutrition and are also antimicrobial peptides (AMPs) (Casteels et al., 1989; Srisuparbh et al., 2003; Shen et al., 2007). *Apidaecin* showed no regulation of any kind in our study (Figure 3). Although they are associated with detoxification properties (Lv et al., 2023), it is possible that the treatments did not reach lethal levels, preventing the detoxification process. This is further proven from *mrjp1* also not regulating at either day 5 or day 9 and *Hsp70* having no regulation at day 9. As royal jelly is important for development and *Hsp70* would show abundant changes when bees would experience unusual situations of stress (Oliveira et al., 2022), such as being put inside a cage to live with no room to fly properly, which is in itself a type of stress. Hydrogen peroxide, which works by producing destructive hydroxyl free radicals that can attack membrane lipids, DNA, and other essential cell components (Brudzynski, 2020), was avoided by bees (Figure 2A) and led to significant early toxicity when administered at 4,000 ppb (Figure 1). The closest similarity of the overall gene response to H_2O_2 according to the PCA was that of imidacloprid (Figure 9A).

Furthermore, our transcriptional data revealed crucial data related to genes potentially linked to the behavioral caste development in honey bees (Figures 4–6). A set of genes was significantly regulated based on the factor "Time" or "Age of the honey bees" irrespective of the treatments administered. This set of genes included *mrjp1*, *Cp450*, *SelT*, *SelK*, *Derl-1*, *Ire1*, and *Hsc70*. The first gene (*mrjp1*), which is well known to downregulate in foragers compared with nurse bees, has already been described as a physiological marker for behavioral development (Corona et al., 2023). The other genes require further investigations pertaining to their age-related regulation in honey bees.

In conclusion, tunicamycin, hydrogen peroxide, and imidacloprid treatments showed signs of disinclination in post-ingestion as well as a pharmacological enhancement in bee survivorship compared with the honey bees fed a sugar syrup control. While a few antioxidant genes were significantly regulated regarding the different treatments, our results reveal age-related regulation of other major genes with significant inter-gene positive correlations. Lastly, significant protein damage in the honey bee was observed in the treatments of paraquat and imidacloprid when administered *ad libitum* for 11 days.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Ethics statement

Ethics review and approval were not required for the study on animals in accordance with the local legislation and institutional requirements.

Author contributions

FT: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft. MG: Data curation, Funding acquisition, Resources, Writing – review and editing. JA: Conceptualization, Funding acquisition, Investigation, Resources, Writing – review and editing. SK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing – review and editing. MA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CM declared a shared parent affiliation with the authors MG, JA, and MA, to the handling editor at the time of review.

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