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Diverse alterations and correlations in antioxidant gene expression in honeybee (*Apis mellifera*) hemocytes interacting with microbial pathogen-associated molecular patterns and pesticide cocktails

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ABSTRACT

Imidacloprid and amitraz, two common pesticides, affect honeybee health, behavior, and pathogen resistance. Understanding multi-risk exposures is vital to explaining their role in hive performance decline and colony health. This study assessed antioxidant system genes to evaluate pesticide impacts on immune response and enzymes responsible for hydrogen peroxide (H_2O_2) production. Honeybee hemocytes were exposed to imidacloprid and amitraz at various concentrations, alongside pathogen-associated molecular patterns (PAMPs) like zymosan A (ZYM), lipopolysaccharide (LPS), and peptidoglycan (PGN). Imidacloprid primarily affects mitochondrial components like mitochondrial superoxide dismutase (MnSOD), while amitraz decreases the expression of both mitochondrial and cytosolic genes (MnSOD, DUOX, and CuZnSOD). PAMPs modulate antioxidant responses, with LPS showing the strongest impact. DUOX and the phenoloxidase system are consistently linked. While AmPPO (prophenoloxidase) involved in melanization is less affected, pesticides significantly disrupt H_2O_2 production and antioxidant defenses, complicating risk assessment robustness.

1. Introduction

Anthropogenic activity has been the driving force of many environmental deteriorations associated with pollution, land use, over-exploitation of resources, and altering the balance of the ecosystem (Anon, 2021; Edo et al., 2024; Gosselin and Callois, 2018; Mahmoud and Gan, 2018; Prakash and Verma, 2022; Sukkar et al., 2025; Gosselin and Callois, 2021). The application of pesticides is a major concern in environmental studies due to its impact on the environment and its role in reducing species survivability (Tudi et al., 2021).

The application of pesticides in agriculture usually affects non-target organisms such as honeybees (Zaller and Brühl, 2019; Iwasaki and Hogendoorn, 2021; Krupke et al., 2017). Honeybees play a critical role in pollination and honey production, contributing significantly to agricultural crop yields and biodiversity (Hung et al., 2018; Gallai et al., 2009; Khalifa et al., 2021; Champetier, Sumner, and Wilen, 2015). Exposure to multiple risk factors makes honeybees more susceptible to health deterioration, diseases, pest infestations, and, ultimately, colony collapse. Colony collapse disorder was identified in 2007 due to high colony losses in the USA and Europe caused by multiple risk factors (vanEngelsdorp et al., 2009).

Neonicotinoids are systemic insecticides that dominated the pesticide market in 2010 with the highest use of imidacloprid. Imidacloprid was meant to be used to treat infestations of sucking insects such as aphids (Bass and Field, 2018). However, honeybees are unintentionally exposed to imidacloprid, drastically affecting their behavior, immunity, and overall survival (Lambin et al., 2001; Balieira et al., 2018; Brandt et al., 2016; Nicodemo et al., 2014; Pal et al., 2022; Chen et al., 2021). In addition to neonicotinoids, honeybee hives are frequently treated with amitraz-based pesticides, primarily to control infestations of the parasitic mite *Varroa destructor* (Filazi and Yurdakok-Dikmen, 2018). Although amitraz is not designed to target honeybees, studies have revealed its sub-lethal effects, including reduced metabolic activity and impaired development (O'Neal et al., 2017; Hillier et al., 2013; Vandenberg and Shimanuki, 1990). Honeybees lack the enzymes required to efficiently metabolize amitraz into its active forms, which should make

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the compound less toxic to them (Kita et al., 2017). However, research suggests that amitraz may interact synergistically with other pesticides, amplifying the toxicity and compounding its effects on honeybee health (Dai et al., 2018; Shojaei et al., 2018; Sukkar et al., 2023a, 2024).

The physiological consequences of pesticide exposure are closely tied to honeybee immune responses. As invertebrates, honeybees rely solely on innate immunity to combat infections and other threats (Beutler, 2004). One of the primary immune responses involves the production of reactive oxygen species (ROS). ROS, such as hydrogen peroxide (H_2O_2), play a dual role in honeybee immunity, functioning as signaling molecules and antimicrobial agents that eliminate pathogens (Krautz et al., 2014). However, excessive ROS production can lead to oxidative stress, damaging cellular components like DNA, proteins, and lipids. To prevent this, organisms have evolved a robust antioxidant defense system that includes enzymes such as superoxide dismutase (SOD) (Sheng et al., 2014).

Superoxide dismutase (SOD) is a critical enzyme that catalyzes the conversion of superoxide radicals into hydrogen peroxide, a less reactive ROS (Sheng et al., 2014). In honeybees, SOD exists in two primary forms: CuZnSOD (SOD1), which is localized in the cytosol, and MnSOD (SOD2), found in mitochondria (Corona and Robinson, 2006). Both forms are essential for maintaining cellular homeostasis and protecting against oxidative damage. However, pesticide exposure can alter SOD expression and activity, potentially impairing the honeybee's ability to regulate ROS levels and defend against pathogens effectively (Murawska et al., 2021). In addition to SOD, ROS production is influenced by the activity of the Dual oxidase (DUOX) system, a key component of the insect immune response. DUOX is a member of the NADPH oxidase (NOX) enzyme family and plays a central role in generating ROS during immune activation (Gandara and Oliveira, 2023). DUOX enzymes are embedded in the cell membrane, where they catalyze the production of hydrogen peroxide. Beyond ROS production, DUOX is also implicated in mitochondrial functions, including calcium flux regulation. Disruptions in DUOX expression or activity can lead to mitochondrial dysregulation, affecting energy production, apoptosis, and other vital cellular processes. Pesticide exposure has been shown to modulate DUOX activity, potentially exacerbating oxidative stress and immune dysfunction in honeybees.

Another critical immune mechanism in honeybees involves the phenoloxidase cascade, which contributes to pathogen defense through melanin production (Eleftherianos et al., 2021; González-Santoyo and Córdoba-Aguilar, 2012). In vertebrates, melanin is primarily associated with pigmentation. However, melanin plays a defensive role in insects by encapsulating and isolating invading pathogens (Eleftherianos et al., 2021). The precursor enzyme, prophenoloxidase (AmPPO), is activated during immune responses, leading to melanin synthesis (González-Santoyo and Córdoba-Aguilar, 2012). The phenoloxidase cascade works with other immune components, including DUOX and SOD, to mount an effective defense against microbial invaders. However, pesticide exposure may impair this cascade, further weakening honeybee immunity.

Together, SODs, DUOX, and prophenoloxidase form the cornerstone of the honeybee's oxidative immune defense. These regulatory systems are crucial for balancing ROS production and mitigating the damage caused by oxidative stress. Any disruption to these pathways, whether due to pesticides, pathogens, or environmental stressors, can compromise honeybee health and increase the likelihood of colony collapse.

In this study, we measure the effect of imidacloprid and amitraz on the antioxidant system in honeybee hemocytes exposed to different microbial PAMPs including zymosan A (fungus), lipopolysaccharide (gram-negative bacteria), and peptidoglycan (gram-positive bacteria) to simulate microbial interactions and to understand the effect of pesticides on immune responses when the immune system is activated.

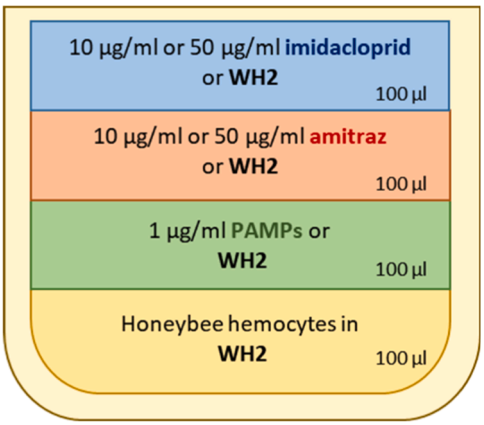

Fig. 1. Representation of treatment mixtures with honeybee hemocytes.

2. Materials and methods

2.1. Sample collection and hemolymph extraction

Honeybee hives were maintained at IUT Thionville-Yutz site located in Yutz, France. In the spring, 5th instar larvae of the Western Honeybee (*Apis mellifera*; Buckfast breed) were collected from brood frames and transported to an incubator set at 32 °C and maintained high humidity until hemolymph extraction. The frames sections were set in vertical position in the incubator. This allows the larvae to escape their cells and be easily collected without risk of injury from manual extraction. Larvae that remained in the frame were extracted via a plastic grafting tool. Hemolymph of the larvae was extracted under sterile hood by gently sterilizing the posterior end of the larvae with 70 % ethanol. The larvae were then punctured with a sterile needle, and hemolymph was quickly collected using a pipette before being pooled in 500 µl of WH2 medium (Hunter, 2010). The number of larvae extracted was triple the number of treatment conditions.

2.2. Pesticide and immune stimulator treatments

All chemicals were purchased from Sigma-Aldrich™. Stock solutions were prepared. Amitraz was dissolved in hexane to 10 mg/ml then diluted to 40 µg/ml and 200 µg/ml in WH2 medium with the evaporation of hexane taken to consideration. Imidacloprid was prepared the same manner as amitraz but with phosphate buffer saline (PBS) instead of hexane. The immune stimulators; lipopolysaccharide, peptidoglycan, and zymosan A were dissolved in PBS to 1 mg/ml stock solutions. Treatments were performed in 24-well TPP™ cell culture plates.

Pesticide treatments include 10 µg/ml imidacloprid (I10), 50 µg/ml imidacloprid (I50), 10 µg/ml imidacloprid (A10), 50 µg/ml amitraz (A50), 10 µg/ml imidacloprid + 10 µg/ml imidacloprid (I10-A10), 10 µg/ml imidacloprid + 50 µg/ml imidacloprid (I10-A50), and 50 µg/ml imidacloprid + 10 µg/ml imidacloprid (I50-A10). All pesticide exposure were coupled with 1 µg/ml of each PAMP or WH2 medium. A total of 36 treatment conditions and combinations were obtained. The preparation of the treatment combinations is illustrated in Fig. 1. Plates were sealed with UV-sterilized sealing tape and placed in an incubator at 20 °C for 18 hours.

2.3. RNA extraction and cDNA synthesis

When incubation was terminated, plates were centrifuged at 5000 *g* for 5 minutes then total RNA was extracted using TRIzol extraction solution (product: 15596018, Thermofisher™) following the manufacturer's protocol (MAN0001271). The supernatant was removed from each well and then 400 µl of TRIzol was added before moving to the next well. Plates were incubated for 5 minutes with TRIzol then the content

Table 1
Primer sequences and their respective melting temperatures used in qPCR analysis.

Gene target	Function/role	Primer pairs	Melting temperature (Tm)	Gene ID	Source
rp49	Housekeeping gene	F: 5'-CGTCATATGTTGCCAACTGGT−3' R: 5'-TTGAGCACGTTCAACAATGG−3'	58 °C	AF441189	(Lourenço et al., 2008)
CuZnsod	Cytoplasmic SOD production	F: 5'-AGTGTGCGTTCTTCAGGGTG−3' R: 5'-GGTTGAAATGTGCACCAGCA−3'	60 °C	NM_001178027.1	This study
Mnsod	Mitochondrial SOD production	F: 5'-GGTGGTGGTCATTTGAATCATTC−3' R: 5'-AAGAAGTGCAGCGTCTGGTTTAC−3'	60 °C	AY329356	(Corona and Robinson, 2006)
duox	Hydrogen peroxide production	F: 5'-CCCTAATAGCCCTCGTGAAG−3' R: 5'-GAGCATTCTCATAACGTGTG−3'	55 °C	XM_026439464.1	(Bartling et al., 2021)
AmPPO	Melanization	F: 5'-AGATGGCATGCATTTGTTGA−3' R: 5'-CCACGCTCGTCTTCTTTAGG−3'	60 °C	GB18313	(Evans, 2006)

of each well was pipetted up and down then transferred to a corresponding 2 ml Eppendorf tube. 80 µl of chloroform was added to each tube, vortexed, and then centrifuged at 12,000 *g* for 10 minutes at 4 °C. The obtained supernatant was transferred to a new tube and 200 µl of ice-cold isopropanol was added to each sample. The tubes were incubated for 10 minutes on ice then centrifuged at 12,000 *g* for 10 minutes to obtain the RNA pellet. All the supernatant was carefully removed and 400 µl of ice-cold 75 % ethanol was added to each tube then incubated for 5 minutes. Following incubation, the tubes were centrifuged at 7500 *g* for 5 minutes before removing the ethanol. The tubes were left to dry for 5 minutes then 50 µl of ultra-pure water was added to each tube. RNA samples were frozen at −80 °C and the quality of aliquots was checked by spectrophotometer. Synthesis of cDNA from the isolated total RNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (K1632, Thermofisher™. A maximum of 9 µl RNA (500 ng) were transferred into PCR tubes. If less volume was taken from RNA, RNase-free ultra-pure water was used to complete the volume to 9 µl. Then 1 µl of oligo (dT)18 primer was added followed by 4 µl of 5 × reaction buffer, 1 µl of RiboLock RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix, and 1 µl of RevertAid H minus M-Mul V reverse transcriptase (200 U/µl). Sample tubes were incubated in an ICycler thermocycler (Bio-Rad™) at 60 °C for 42 min and then heated to 70° C for 5 min. The samples were held at 4°C before removal from the thermocycler. Sample concentration and purity were quantified by BioSpec Nano spectrophotometer (Shimadzu corps™). Ultra-pure water was added to the samples to reach a 10 times dilution then stored at −80°C until usage.

2.4. Real-time polymerase chain reaction (PCR)

Gene expression analysis was performed by iCycler MyiQ™2 Two-

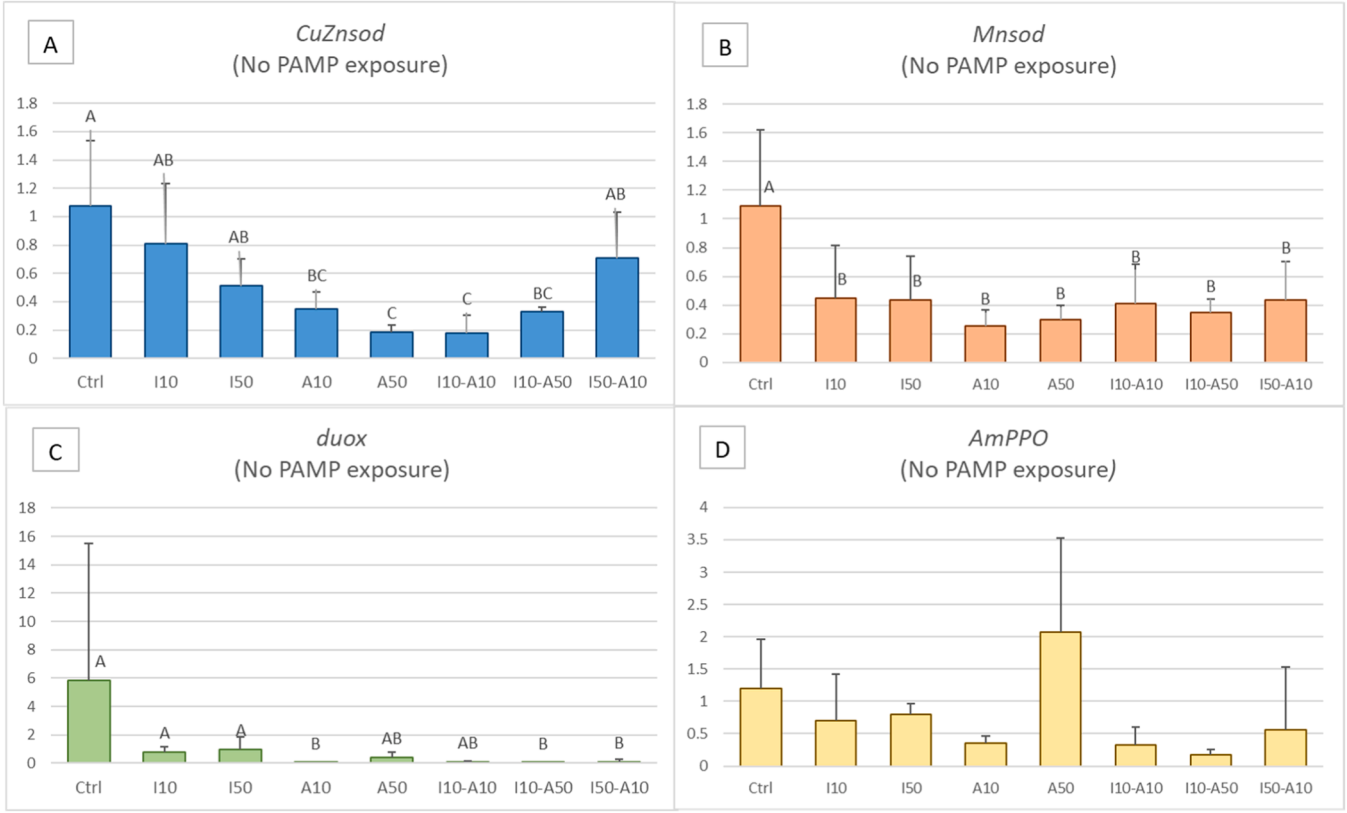

Fig. 2. Gene expression of honeybee antioxidant system genes without immune stimulation. Honeybee hemocytes exposed to 10 µg/ml imidacloprid (I10), 50 µg/ml imidacloprid (I50), 10 µg/ml amitraz (A10), 50 µg/ml amitraz (A50), 10 µg/ml imidacloprid + 10 µg/ml amitraz (I10 +A10), 10 µg/ml imidacloprid + 50 µg/ml amitraz (I10 +A50), and 50 µg/ml imidacloprid + 10 µg/ml amitraz (I50 +A10). The graphs include gene expression analysis of cytosolic superoxide dismutase (CuZnsod; graph A), mitochondrial superoxide dismutase (Mnsod; graph B), duox (graph C), and prophenoloxidase (AmPPO; graph D). None of the pesticide treatments were exposed to any immune stimulator. Different letters are used to refer to significantly different gene expressions. Error bars represent standard deviations ($n = 3$, $p < 0.05$).

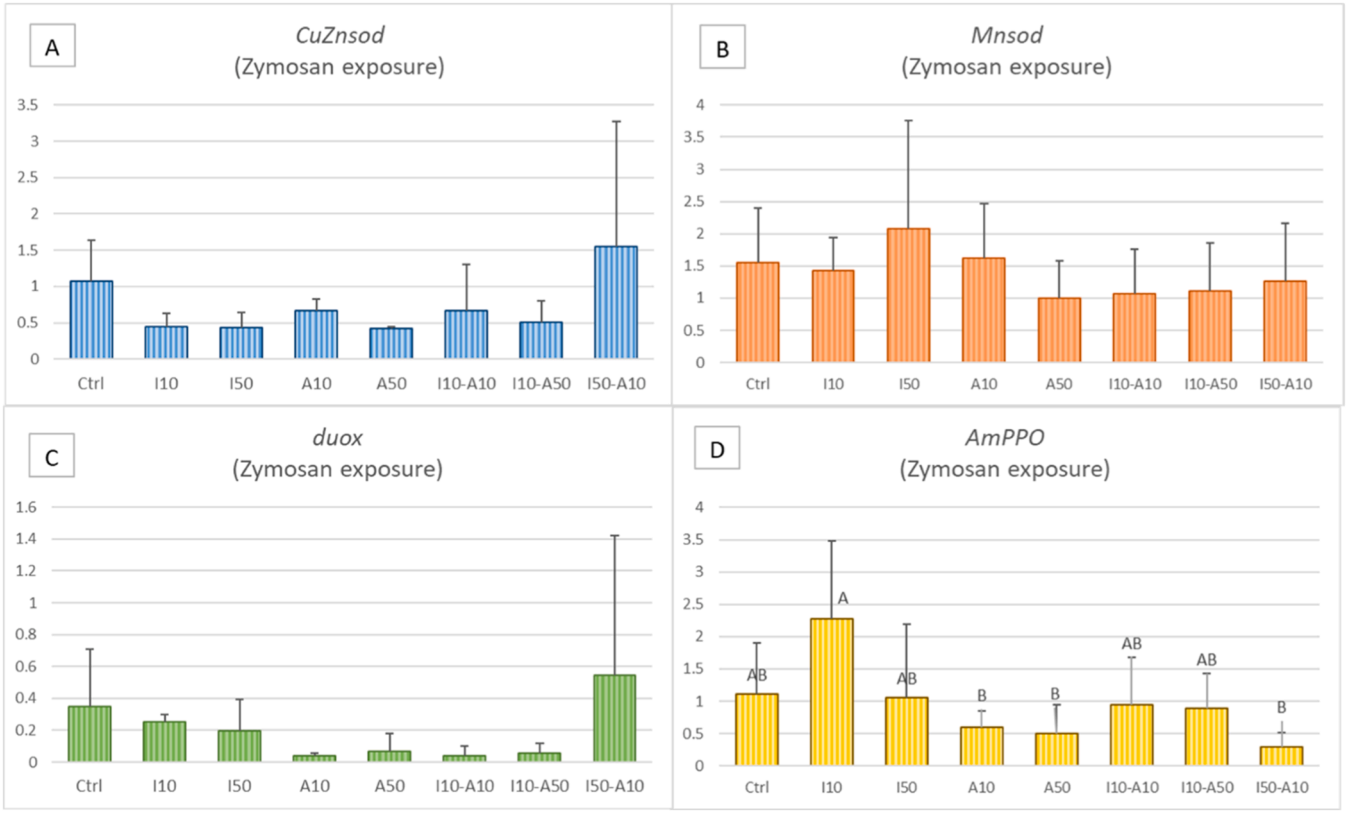

Fig. 3. Gene expression of honeybee antioxidant system genes with zymosan immune stimulation. Honeybee hemocytes exposed to 10 µg/ml imidacloprid (I10), 50 µg/ml imidacloprid (I50), 10 µg/ml amitraz (A10), 50 µg/ml amitraz (A50), 10 µg/ml imidacloprid + 10 µg/ml amitraz (I10 +A10), 10 µg/ml imidacloprid + 50 µg/ml amitraz (I10 +A50), and 50 µg/ml imidacloprid + 10 µg/ml amitraz (I50 +A10). The graphs include gene expression analysis of cytosolic superoxide dismutase (CuZnsod; graph A), mitochondrial superoxide dismutase (Mnsod; graph B), duox (graph C), and prophenoloxidase (AmPPO; graph D). All pesticide treatments were exposed to 1 µg/ml zymosan A. Different letters are used to refer to significantly different gene expressions. Error bars represent standard deviations ($n = 3$, $p < 0.05$).

color Real-Time Detection System (Bio-Rad™) in Hard-Shell High-Profile Semi-skirted 96-Well PCR Plates. Reaction mixtures contained 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix, 0.5 µM of forward and reverse primers (final concentration), and 300 ng of cDNA, and ultra-pure H2O was added to a total volume of 20 µl. Reaction cycles set: 1 × (30 s at 95°C); 45 × (10 s at 95°C, 30 s at Tm, 30 s at 72°C) followed by melt curve analysis increasing temperature from 55 to 95°C. Two technical replicates were used to verify repeatability. The sequences of forward and reverse primers are as indicated in Table 1 with their respective melting temperatures.

2.5. Data treatment and statistical analysis

Data of gene expression analysis was treated according to the Livak method (Livak and Schmittgen, 2001). After treatment, data were checked for normality by a Shapiro–Wilk test followed by Bartlett's test for homogeneity of variance. Non-normal data were transformed and normalized before performing a two-way ANOVA and a Duncan post-hoc to determine significant differences between groups. Data that did not satisfy normality or homogeneity of variances were treated with Kruskal-Wallis non-parametric test coupled with Dun post-hoc at 95 % confidence. All statistical analysis was performed by Addinsoft® XlSTAT™ 2019 3.2.

A principal component analysis (PCA) and factor map analysis were carried out to determine the correlation between the expression of different genes. The correlation was checked for each treatment within zymosan groups or for the whole groups by Spearman (n) test at a 95 % confidence

3. Results and discussion

3.1. Effect of pesticides and PAMPs on antioxidant genes expressions

The effect of pesticides on the gene expression of cytosolic SOD (*sod1*) or *CuZnsod* is apparent in Fig. 2A. The levels of *CuZnsod* decreased with 10 and 50 µg/ml imidacloprid but no significance was observed. However, a dose-dependent significant decrease was observed with amitraz exposures (A10) and (A50). The highest concentration of amitraz even results in significantly less *CuZnsod* gene expression compared to both imidacloprid single exposures; I10 and I50. The pesticide combinations I10-A10 and I10-A50 displayed a significant decrease compared to the control treatment but the I50-A10 combination was not significantly different compared to the control. This suggests that amitraz alters the cytosolic production of hydrogen peroxide while imidacloprid has a limited effect. In addition, the interaction between amitraz and imidacloprid does not show synergism. In fact, imidacloprid shows a dose-dependent antagonism with amitraz.

As for the mitochondrial SOD (*sod2*) or *Mnsod*, all pesticide treatments in single exposures or combinations resulted in a significant decrease in *Mnsod* gene expression compared to the control without pesticide treatment (Fig. 2B). There was no significant difference between pesticide treatments regarding *Mnsod* expression. The expression of *Mnsod* was previously shown in another experiment when imidacloprid was coupled with coumaphos while *CuZnsod* was not affected by the same treatments (Gregorc et al., 2018). This indicates that pesticides mainly affected the mitochondrial SOD expression and its associated hydrogen peroxide production. In addition, the effect of imidacloprid can be mainly observed in co-exposures rather than single exposures of

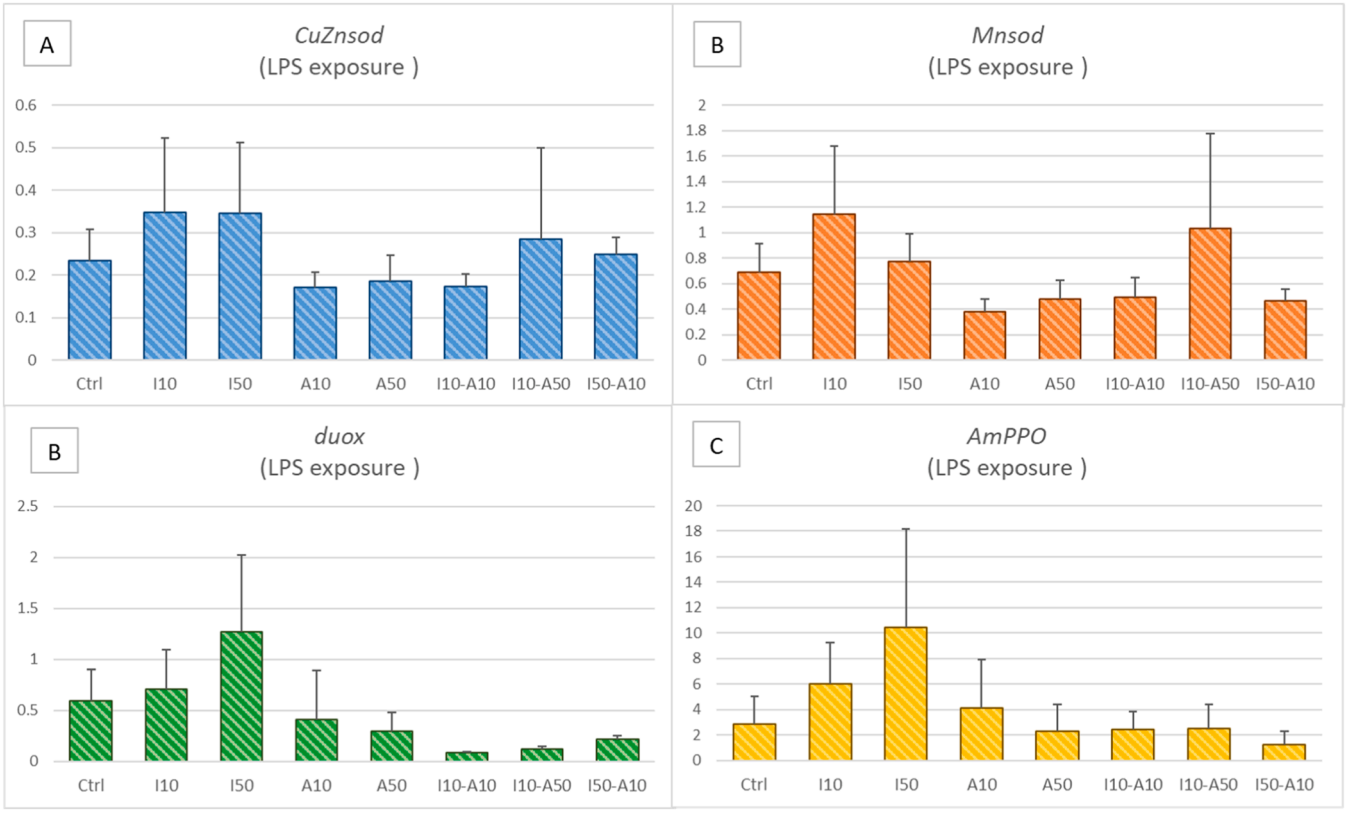

Fig. 4. Gene expression of honeybee antioxidant system genes with lipopolysaccharide immune stimulation. Honeybee hemocytes exposed to 10 μg/ml imidacloprid (I10), 50 μg/ml imidacloprid (I50), 10 μg/ml amitraz (A10), 50 μg/ml amitraz (A50), 10 μg/ml imidacloprid + 10 μg/ml amitraz (I10 +A10), 10 μg/ml imidacloprid + 50 μg/ml amitraz (I10 +A50), and 50 μg/ml imidacloprid + 10 μg/ml amitraz (I50 +A10). The graphs include gene expression analysis of cytosolic superoxide dismutase (CuZnsod; graph A), mitochondrial superoxide dismutase (Mnsod; graph B), duox (graph C), and prophenoloxidase (AmPPO; graph D). All pesticide treatments were exposed to 1 μg/ml lipopolysaccharide (LPS). Error bars represent standard deviations ($n = 3$, $p < 0.05$).

pesticides. A decrease in the expression of *duox* (Fig. 2C) is observed with pesticide treatments but significance was only shown to be associated with A10, I10-A50, and I50-A10 but not with I10-A10 treatments compared to the control and the imidacloprid single exposures. This indicates that the effect of amitraz is more potent on *Mnsod* than imidacloprid alone. Interestingly, the higher concentration of amitraz did not show a significant difference, suggesting the possibility of concentration-specific responses.

The expression of *AmPPO* did not vary significantly between treatments yet the lowest expression was observed in the pesticide mixtures 10I-10A and I10-A50 (Fig. 2D). However, the effect of imidacloprid is observed to be variable depending on the development stage (Chen et al., 2021) and this complexity could extend to the larval level as well as the interaction with PAMPs. In other terms, specific interactions may be observed but anticipation of the effect of pesticides on prophenoloxidase production and ultimately melanization cannot be generalized.

Both imidacloprid and amitraz appear to affect mainly the mitochondrial components such as MnSOD. Imidacloprid was previously observed to inhibit mitochondrial state 3 respiration, reducing adenosine triphosphate (ATP) production, mainly in the head (Nicodemo et al., 2014). The effect of amitraz exceeds that of imidacloprid, as the expression of both *CuZnsod* and *duox* is significantly reduced only in the presence of amitraz, whether as a single exposure or in combination with imidacloprid. In contrast, imidacloprid alone did not significantly reduce gene expression. In a previous study, we observed that short-term exposure (3 hours) to similar treatments led to reduced extracellular H_2O_2 production, which was more significantly decreased by imidacloprid than by amitraz (Sukkar et al., 2023b). However, we have to consider that oxidative response and H_2O_2 could be either beneficial or deleterious depending on the cellular compartments its localized. For instance, Yazlovytska et al. (2023) found that increased level of oxidative stress biomarkers was beneficial for honeybee survival and immunity through enhancing lysozyme activity with certain diets.

The observations generally displayed different results when applying the zymosan PAMP as an immune stimulator with pesticide treatments Fig. 3. The expression of *CuZnsod* (Fig. 3A) and *duox* (Fig. 3B) was lower in all pesticide treatments except I50-A10 but none were significantly different. As for *Mnsod* (Fig. 3C), no variation was apparent between treatments. The expression of *AmPPO* was not significantly different from the control (Fig. 3D).

However, the lowest expressions were observed with amitraz single exposures A10 and A50 and the pesticide combination I50-A10. The graphs in Fig. 3 display contrasting significance compared to those in Fig. 2**.** To simplify, *CuZnsod*, *Mnsod,* and *duox* expressions showed significant differences between treatments in Fig. 2 but not *AmPPO*. In Fig. 3, only *AmPPO* expression showed significance. This observation agrees with the previously mentioned ability of zymosan to redirect the immune responses via modulating their associated pathways (Sukkar et al., 2024).

A synergistic effect between fungal interaction and pesticides on honeybee health including, development survival and immunity was observed when bees were exposed to *Nosema ceranae* and fenpyroximate (Zheng et al., 2024). The interaction also resulted in the disruption of the antioxidant system by increasing the expression of superoxide dismutase. In contrast, our results showed an antagonism between the effect of fungal interaction and imidacloprid or amitraz. The response of honeybee SOD gene expression is variable with pesticides but appears to be associated with an increase when there is fungal interaction such as the microsporidian *Nosema spp.* or the PAMP, zymosan A representing fungi.

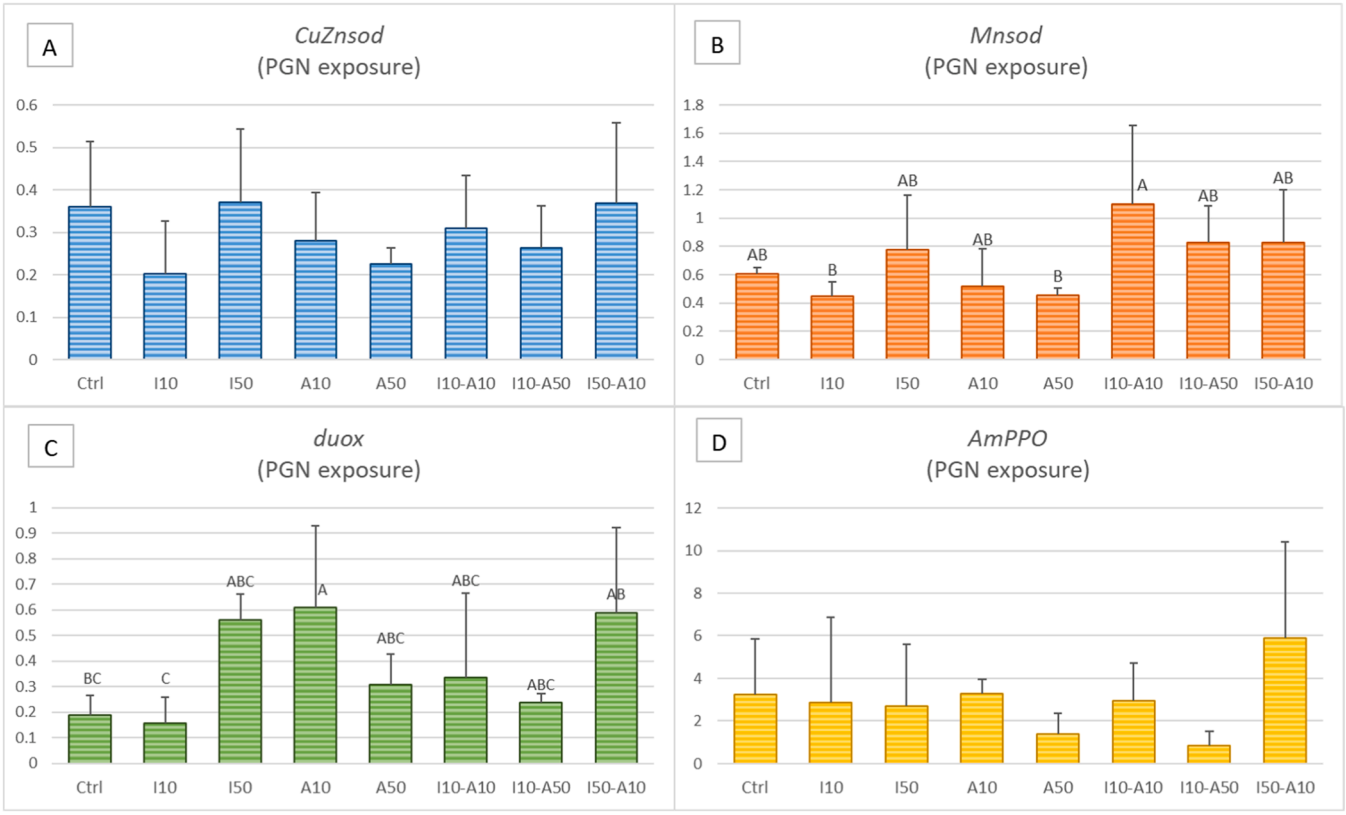

Fig. 5. Gene expression of honeybee antioxidant system genes with peptidoglycan immune stimulation. Honeybee hemocytes exposed to 10 μg/ml imidacloprid (I10), 50 μg/ml imidacloprid (I50), 10 μg/ml amitraz (A10), 50 μg/ml amitraz (A50), 10 μg/ml imidacloprid + 10 μg/ml amitraz (I10 +A10), 10 μg/ml imidacloprid + 50 μg/ml amitraz (I10 +A50), and 50 μg/ml imidacloprid + 10 μg/ml amitraz (I50 +A10). The graphs include gene expression analysis of cytosolic superoxide dismutase (CuZnsod; graph A), mitochondrial superoxide dismutase (Mnsod; graph B), duox (graph C), and prophenoloxidase (AmPPO; graph D). All pesticide treatments were exposed to 1 μg/ml peptidoglycan (PGN). Different letters are used to refer to significantly different gene expressions. Error bars represent standard deviations ($n = 3$, $p < 0.05$).

However, the context of which the interaction between honeybee, pesticide, and pathogen is of high importance as well. As we are studying the effects of general microbial interactions, the presence of microbial effectors should also be included. For instance, honeybees exposed to imidacloprid displayed increased levels of *Nosema* (Pettis et al., 2012).

According to Fig. 4**,** exposure to LPS does not induce any significant change in *CuZnsod, Mnsod, duox,* or *AmPPO* gene expression. However, the pesticide cocktails I10-A10, I10-A50, and I50-A10 resulted in the lowest expression of *duox* and *AmPPO* with LPS (Fig. 4C and D) contrary to single exposures of imidacloprid and amitraz. We can also observe similar pattern in the change of *CuZnsod* and *Mnsod* expressions where I10 showed the highest expressions while the lowest were with amitraz single exposure the mixture I10-A10. The case of pattern similarity is also true for *duox* and *AmPPO* where their expression increases with increasing imidacloprid concentrations and decrease with increasing amitraz concentrations while the lowest expressions are observed with pesticide mixtures. The exposure of LPS may counter the negative effect of pesticides on the antioxidant gene expression in honeybee hemocytes with less effect on pesticide combinations as observed. We note that the induction of *AmPPO* via LPS exposure is generally at least 2-fold higher (Fig. 4D) when compared to its expression without LPS (Fig. 2D). That is not to say that LPS does not affect *AmPPO* expression generally. When larval honeybee hemocytes where exposed to LPS with increasing concentrations of imidacloprid, a dose-dependent decrease in extracellular H_2O_2 production was observed (Walderdorff et al., 2018) indicating that such decrease in reactive oxygen species used for defense against pathogens in independent of SOD gene expression since our results indicate no significant change.

Gram-negative bacteria that infect honeybees such as *Serratia marcescens* are not usually a main concern to honeybee health and is mainly found infect worker bees (Raymann et al., 2018). The resistance of honeybees to *S. marcescens* is connected to competition with honeybee gut microbiota (Steele et al., 2021). However, our results indicate that the honeybee antioxidant system concerning SODs, DUOX, and AmPPO respond favorably to LPS when exposed to risk factor such as pesticides. This could be an evolutionary adaptation in honeybees to gram-negative bactria such as those associated with gut microbiota (Steele and Moran, 2021; Loncaric et al., 2011). It should also be considered that our results are associated with hemocytes at the larval developmental stage with could also influence the results. In fact, the majority of bacterial clusters in honeybee guts are gram negative (Moran, 2015) explaining the response to LPS in such a manner as presented in our results.

Exposing honeybee hemocytes to peptidoglycan (Fig. 5) resulted in no significant change in *CuZnsod* (Fig. 5A), indicating that PGN can ameliorate the effect of imidacloprid and amitraz on the gene expression of *CuZnsod* when compared to the absence of PAMPs (Fig. 2A). In addition, with PGN the highest relative gene expression is less than 0.4 of that of the control treatment without PAMPs (Fig. 2A). This implied that PGN overall decreases the production of cytosolic SOD. Still, the effect of pesticides with PGN is not synergetic and may have a limited effect when PGN is present. The expression of *Mnsod* is not significantly different compared to the no-pesticide control (Fig. 5B) yet the pesticide mixture I10-A10 gives a significantly higher gene expression than I10 and A50 single exposure. The highest expressions are shown in the pesticide mixtures. The expression of *doux* is generally increased (Fig. 5**C**) but only A10 showed a significant increase compared to the control. The lowest expression was with the I10 treatment and it was significantly lower than A10 and the I50-a10 mixture.

Table 2
Correlation matrices of antioxidant genes in honeybee hemocytes exposed to pesticides and immune stimulators. The table includes the general correlation matrix of all treatments and the correlations fractioned by each group of immune stimulators/PAMP. Numbers in bold refer to significant correlations.

General correlation matrix				
Transcript	*CuZnsod*	*Mnsod*	*duox*	*AmPPO*
CuZnsod	**1**	**0.453**	−0.061	**−0.248**
Mnsod	**0.453**	**1**	0.152	**0.259**
duox	−0.061	0.152	**1**	**0.582**
AmPPO	**−0.248**	**0.259**	**0.582**	**1**
Control group correlation matrix				
Transcript	*CuZnsod*	*Mnsod*	*duox*	*AmPPO*
CuZnsod	**1**	0.213	0.303	0.080
Mnsod	0.213	**1**	0.349	0.364
duox	0.303	0.349	**1**	**0.550**
AmPPO	0.080	0.364	**0.550**	**1**
ZYM group correlation matrix				
Transcript	*CuZnsod*	*Mnsod*	*duox*	*AmPPO*
CuZnsod	**1**	0.303	−0.199	−0.221
Mnsod	0.303	**1**	0.100	0.141
duox	−0.199	0.100	**1**	**0.603**
AmPPO	−0.221	0.141	**0.603**	**1**
LPS correlation matrix				
Transcript	*CuZnsod*	*Mnsod*	*duox*	*AmPPO*
CuZnsod	**1**	**0.572**	0.277	0.270
Mnsod	**0.572**	**1**	**0.513**	**0.719**
duox	0.277	**0.513**	**1**	**0.604**
AmPPO	0.270	**0.719**	**0.604**	**1**
PGN correlation matrix				
Transcript	*CuZnsod*	*Mnsod*	*duox*	*AmPPO*
CuZnsod	**1**	**0.411**	0.181	−0.032
Mnsod	**0.411**	**1**	0.289	0.340
duox	0.181	0.289	**1**	**0.579**
AmPPO	−0.032	0.340	**0.579**	**1**

As for *AmPPO*, the exposure to PGN doesn't vary in terms of pesticide interaction between pesticide treatments (Fig. 5D) or compared to pesticide exposure without PAMPs (Fig. 2D). However, the gene expression (Fig. 5D) is more than 2-fold more than the no-PAMPs control treatment in (Fig. 2D) indicating that PGN can generally induce melanization but its effect is not altered significantly by pesticide exposure. Our study concerning honeybee larvae shows the variation of gene expression between PAMPs exposures. However, the variation shifts and changes with different casts in honeybees as Byhrø et al. (2019) demonstrated between nurse bees and forager bees regarding the production of antimicrobial peptides.

3.2. Principal component analysis and correlations of antioxidant genes

Evaluating correlations among gene expressions of the antioxidant system demonstrated variability between different PAMPs (Table 2). Considering all treatments and exposures, the general correlation matrix showed significant positive correlation between *Mnsod* and *CuZnsod* expressions, between *duox* and *AmPPO*, and a negative correlation between *AmPPO* and *CuZnsod*. Interestingly, the negative correlation between *AmPPO* and *CuZnsod* was not observed in any PAMP exposure groups but on a total scale. We note that the correlation is significant but weak (<0.25) in the mentioned case which could explain the limited observation.

Regarding the control group with pesticide exposures without any PAMP interaction, the only significant correlation was observed between *AmPPO* and *duox* systems. The relationship between *AmPPO* and *duox* is maintained in all cases of PAMP exposure coupled with imidacloprid and amitraz including zymosan, lipopolysaccharide, and peptidoglycan interaction. The correlations among gene expression were similar between the control group and the ZYM interaction. With LPS, correlations that are more significant were obtained in addition to *duox* and *AmPPO*. The mitochondrial SOD gene transcript, *Mnsod*, had a significant positive correlation with *CuZnsod*, *duox*, and *AmPPO*. This indicated that the presence of LPS establishes a relationship between the production of hydrogen peroxide production in by mitochondrial and cytosolic SOD activity and other processes with the mitochondria as the key modulator. This could also explain the diminished effect of imidacloprid and amitraz on the antioxidant/oxidant system gene expressions in honeybees as LPS can counter the gene expression-altering effect of

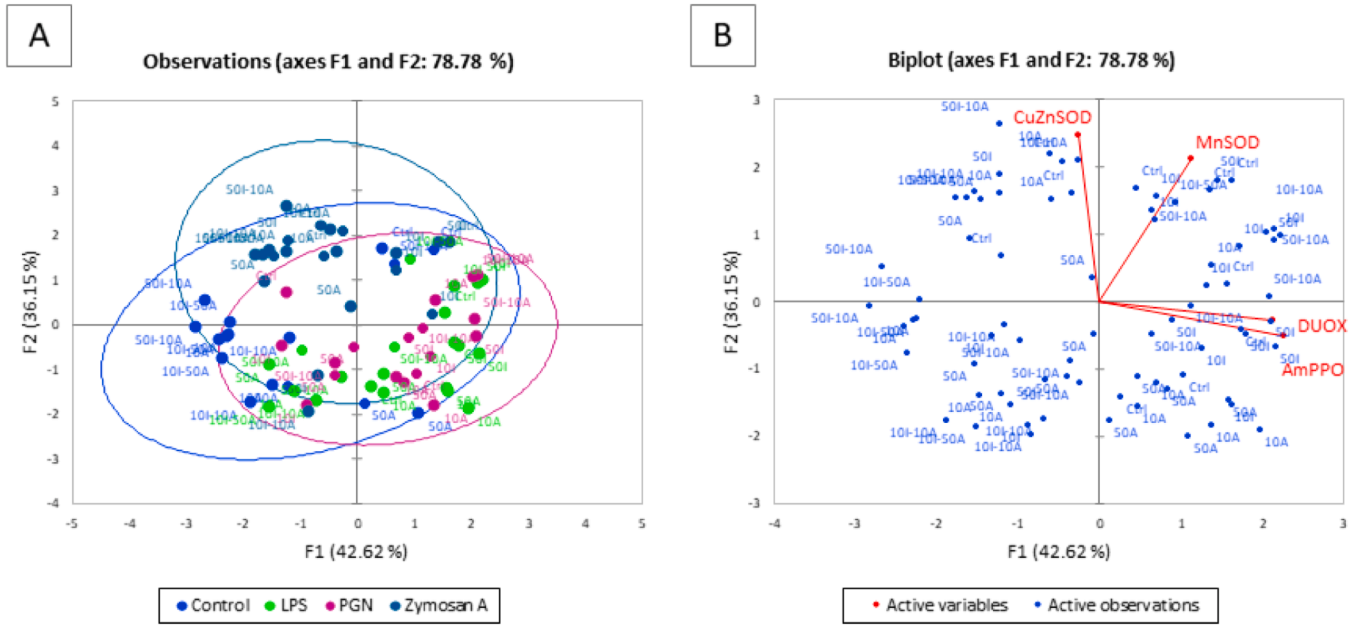

Fig. 6. Principal component analysis (PCA) of honeybee antioxidant genes expressions. Graph A eclipses represent conditions within groups of different immune stimulators (Control, Lipopolysaccharide (LPS), peptidoglycan (PGN), and Zymosan A). Graph B is a biplot separated into four quadrants containing dots representing pesticide treatments as active observations and vectors represent the gene expression of CuZnSOD, MnSOD, DUOX, and AmPPO as active variables. Vectors in the same quadrant are positively correlated while vectors in different quadrants are negatively correlated. Significance of correlations is illustrated by the length of vectors ($n = 3$, $p < 0.05$).

pesticides.

The peptidoglycan (PGN) exposure group showed a positive correlation between *Mnsod* and *CuZnsod* in addition to a positive correlation between *duox* and *AmPPO*. However, unlike the LPS exposure group, there was no correlation between *Mnsod* and *duox* or *AmPPO*. The strength and number of correlations are in increasing order as follows: Control (1 correlation) < ZYM (1 correlation) < PGN (2 correlations) < LPS (3 correlations). Most correlations are present when honeybee hemocytes are exposed to LPS, indicating the effect of LPS on establishing a connection between different elements of the antioxidant system.

Fig. 6 represents the principal component analysis (PCA) for all treatments. Axes F1 includes 42.62 % of the variation, while F2 36.15 % of the variation. The groups of PAMPs/immune stimulators represented by eclipses (Fig. 6A) do not show separation between pesticide treatments, inferring that the effect of PAMPS is limited to the set of pesticide treatments, including imidacloprid (10I and 50I), amitraz (10 A and 50 A), and their combinations (10I+10 A, 10I+50 A, 50I+10 A). The biplot (Fig. 6B) demonstrated vectors of the gene expressions of the studied antioxidant genes including CuZnSOD, MnSOD, DUOX, and AmPPO. The vectors of DUOX and AmPPO show a significant strong positive correlation (acute angle between vectors in the same quadrant) explained in Table 2 with AmPPO strongly contributing to the variation in axis F1. AmPPO also shows a weak but significant positive correlation with MnSOD and a weak but significant negative correlation with CuZnSOD. The fact that groups show no real separation but still show correlation means that the pesticide treatments as single exposures and combinations affect the analysis as they do not represent the usual single-pesticide increasing concentrations in exposure data treatments.

4. Conclusion

Various PAMPs modulate distinct aspects of the antioxidant system in honeybee hemocytes. However, there is always a correlation between the DUOX and phenoloxidase systems. Thus, melanization as an immune response can be affected by altering the regular functioning of DUOX despite the type of microbial interaction but considering pesticide exposure. Imidacloprid and amitraz affect the expression of SOD but the mitochondrial variant is more affected. PAMPs ameliorate the expression of both *CuZnsod* and *Mnsod* noting that PGN has the weakest effect in ameliorating *Mnsod.* In addition, no change between pesticide treatments and the control with hemocytes was challenged by LPS. The effect of PAMPs on honeybee health is crucial to understand how components in honeybee interaction with infections. PAMPs are already being testes as potential beneficiary supplements to increase diseases resistance (Valizadeh et al., 2021).

This study emphasizes the critical need to incorporate microbial interactions into risk assessment frameworks for pesticide regulation. It highlights the necessity of revising pesticide application strategies to reduce unintended harm to beneficial species, such as honeybees. Future research should focus on the effects of pesticide exposure on honeybee colonies and explore the potential of immune stimulators to alleviate these impacts. Moreover, studies should delve into the mechanisms of action and interactions of various risk factors at multiple levels, including biomolecules, cellular compartments, mitochondria, and pesticide target molecules. The findings, when compared to other studies on oxidative responses, suggest that to fully understand the effects of multiple exposures, a broad range of parameters must be considered. This includes the compartmentalization of reactive oxygen species and the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione-S-transferase. The complexity of these interactions must be accounted for in evaluations, moving beyond a focus on single exposures to gain a more accurate understanding of their combined effects in real-world environments. Additionally, comparative studies involving wild pollinators and insects from diverse orders are crucial for developing a comprehensive and ecologically relevant perspective.

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CRediT authorship contribution statement

Laval-Gilly Philippe: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Falla-Angel Jairo:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Bonnefoy Antoine:** Writing – review & editing, Validation, Resources, Methodology. **Wagner Lea:** Writing – review & editing, Validation, Methodology. **Sukkar Dani:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that this study was conducted in the absence of any conflict of interest.

Data availability

Data will be made available on request.

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