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ORIGINAL ARTICLE

Genetic and molecular oxidative stress responses in honeybees exposed to pesticide residues

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Abstract

Pesticides pose a significant challenge to non-target organisms, particularly honeybees (*Apis mellifera* Linnaeus, 1758). Worker honeybees encounter pesticide residues during foraging in both open fields and hives, and these residues have been identified in bee products. However, the effects of these pesticides on bees at detected concentrations are not well understood. Therefore, this study aimed to assess the potential impacts of pesticide residues, including seven fungicides, six insecticides, and three herbicides, on gene expression and the activities of enzymes related to oxidative stress in worker bees. The following enzymes were included in the study: glucose oxidase, cytochrome P-450, acetylcholinesterase, general esterases, glutathione-S-transferase, butyrylcholinesterase, catalase, and adenosine triphosphatase. The results showed that chlorpyrifos (15.30 ng · ml^{-1}), cypermethrin (10.72 ng · ml^{-1}), and pendimethalin (3.42 ng · ml^{-1}) significantly affected gene expression and the activities of the tested enzymes, while the control group and other pesticides did not. Untreated bees had higher specific enzyme activity and gene expression compared to most of the pesticides tested. Results from fludioxonil (14.45 ng · ml^{-1}) and tolclofos-methyl (18.14 ng · ml^{-1}) closely resembled those of the control group, indicating that these fungicides, at the tested concentrations, may not pose a threat to bee health. This research offers valuable insights into the potential negative effects of pesticide residues on the physiological balance of bees.

Keywords: gene expressions, honeybees, oxidative stress enzymes, pesticide residues

Introduction

Honeybees, *Apis mellifera* Linnaeus, 1758, significantly contribute to human food security due to their vital pollination services. Unfortunately, the decline in pollinators is a concurrent global problem, including the collapse of honeybee colonies (Goulson 2019; Wagner 2020). The collapse of colonies can be attributed to various biotic and abiotic factors, including the extensive use of pesticides (e.g., Liu *et al.* 2016). Pesticides may contaminate colonies through various pathways, such as drifting during application or through worker bees during their foraging activities. Honeybee colonies are exposed to two categories of pesticides: ex-hive (also called out-of-hive) and in-hive (Johnson *et al.* 2010).

The ex-hive pesticides are residues that bees collect from the fields and bring back to their colonies after foraging. In contrast, in-hive pesticides are those deliberately introduced into the hives by beekeepers for colony treatments, such as acaricides for *Varroa* mites

(Vilarem *et al.* 2021). The accumulation of pesticides in bee products, including wax, pollen, and honey, has been frequently reported (Manning 2018; Nassar *et al.* 2024). Insecticides like neonicotinoids have demonstrated considerable harmful impacts on honeybees' development, survival, behavior, and productivity (Ohlinger *et al.* 2022; Zhao *et al.* 2022). Consequently, most regulatory authorities worldwide have restricted their use in greenhouses only to minimize their potential adverse impact on bees. However, irresponsible use of this group of insecticides by some farmers may cause adverse impacts on pollinators and farm animals. Also, fungicides have shown negative effects on honeybee colonies both individually and in combination with other pesticides (Belsky and Joshi 2020; Rondeau and Raine 2022). Similarly, herbicides have been found to negatively impact the behavior and survival of honeybees (Faita *et al.* 2020; Motta *et al.* 2020).

The exposure of bees to abiotic stressors, including pesticides, significantly affects the expression of stress-related genes and oxidative stress enzymes, resulting in a disturbance of the physiological balance of bees. Oxidative stress occurs due to the presence of free radicals in the body, which triggers the activity of antioxidant enzymes such as glutathione-S-transferase (GST), catalase (CAT), and glutathione peroxidase (GPx) (Olgun *et al.* 2020). These and other antioxidant enzymes serve as biomarkers for assessing the physiological status of bees when exposed to pesticide stress (Carvalho *et al.* 2013; Shan *et al.* 2022). However, knowledge regarding the effects of different types of pesticides on essential physiological stress biomarkers is limited. Therefore, the objective of the current study was to investigate the genetic and molecular effects of pesticide residues previously detected in bee products (Nassar *et al.* 2024) on the specific activity and gene expression of stress-related enzymes in treated honeybees.

Materials and Methods

Pesticides

In this study, residues of 7 fungicides, 6 insecticides, and 3 herbicides in bee products (Table 1) were investigated based on their amounts detected by Nassar *et al.* (2024). Certified Reference Materials (CRMs) for each of the tested pesticides were purchased from Agilent Technologies through local distributors.

Honeybees and treatments

Worker honeybees at foraging age were randomly collected at the hive entrance from bee colonies in El-Beheira Governorate (Latitude: 30°53'30" N and Longitude: 30°39'27"E). The bees were then transferred to the laboratory and maintained under conditions of 24°C and at relative humidity (~35%). After a 2-hour period of starvation, the bees were exposed to pesticide residues prepared from the CRM of each tested pesticide in a 50% sucrose feeding solution. In contrast, the control group of bees was fed a pesticide-free sucrose solution. The treatments were conducted in 200 ml transparent plastic cups covered with a white piece of cloth. Feeding was performed through a cotton plug attached to Pasteur pipettes. Each concentration was repeated three times, and each replicate consisted of 20 randomly selected forager bees, totaling 60 bees per treatment group. The assay period lasted 48 h. Bees were exposed to residue concentrations for 4 h, after which the sucrose feeding solution was replaced with pesticide-free sucrose solution. After the 48 h assay period, bees were collected in sterilized 15 ml Falcon tubes and preserved at –80°C for further analyses. Gene expression analysis was then performed on three bees from each replicate in every treatment (amounting to 9 bees × treatment^{-1}) while the rest of the bees were designated for enzymatic activity analysis.

Table 1. Pesticides used in the study are listed in alphabetical order

Name	Type	Concentration [ng · ml^{-1}]
Atrazine	herbicide	6.61
Chlorpyrifos	insecticide	15.30
Cypermethrin	insecticide	10.72
Cyprodinil	fungicide	21.5
Diazinon	insecticide	11.64
Dichlorvos	insecticide	1.60
Fludioxonil	fungicide	14.45
Flusilazole	fungicide	21.29
Metalaxyl	fungicide	34.50
Metribuzin	herbicide	38.9
Penconazole	fungicide	8.48
Pendimethalin	herbicide	3.42
Profenofos	insecticide	48.63
Propiconazole	fungicide	14.49
Tolclofos-methyl	fungicide	18.14
Triazophos	insecticide	29.46

Gene expression analysis

Expression analysis of GST, glucose oxidase (GO), esterase (GE), cytochrome P-450 (Cyt-P450), and acetylcholinesterase (AChE) genes was completed using real-time qPCR after treating bees with detected levels of pesticides in bee products according to the kits manufacturer guidelines.

RNA isolation and cDNA synthesis

Total RNA of each specimen was extracted using a GStract™ RNA Isolation kit II (Maxim Biotech Inc, USA) following the manufacturer's instructions. Reverse transcription (RT) or first-strand cDNA was synthesized from total RNA with oligo (dT) primer, dNTPs, and M-MLV Reverse Transcriptase enzyme (Fermentas, USA).

Real-time PCR preparations

The RT-PCR Reaction (25 µl) contained 12.5 µl of 2X Quanti Tech SYBR® Green RT Mix (Fermentas, USA), 2 µl of 10 pmol · µl^{-1} (1 µl each of forward and reverse primers) (Table 2), 1 µl of template cDNA (50 ng), and 9.5 µl of RNase-free water. The PCR reaction included an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min using a Rotor-Gene 6000 (QIAGEN, New Cairo, Egypt). After the amplification stage, a dissociation stage was added to verify the specificity of the PCR products. The threshold cycle (Ct) values were measured during the exponential phase of amplification using Agilent Mx3000P real-time PCR and Mx-Pro Excel software. Relative quantification of gene expression was calculated by the equations ΔCq = Cq – reference gene, ΔΔCq = Cq – control, and ΔΔCq expression = 2 (−ΔΔCq). The expression levels of the target genes were normalized relative to the actin rRNA gene and relative expression of untreated controls. Real-time PCR products were electrophoretically separated on a 1.1% agarose gel stained with ethidium bromide (10 µg · ml^{-1}) in TBE buffer [Tris base pH 7.6, boric acid (89 mM), and EDTA (2 mM)]. The gel was photographed using gel documentation (Bio-Rad Gel DOCTM EZ Imager).

Biochemical responses

Frozen bees from each replicate were pooled and dissected to separate the head capsule, which was used for the analysis of AChE and adenosine triphosphatase (ATPase), while the thorax and abdomen (hereafter referred to as body tissue) were used in the analysis of butyrylcholinesterase (BuChE) and catalase (CAT).

Acetylcholinesterase (AChE)

About 0.1 g of the combined head capsules of the bees was homogenized with 0.1 M phosphate buffer pH 8.0 (1 : 10 w · v^{-1}) in an ice bath. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was used as an enzyme source. In each test tube, 2.9 ml of 0.1 M phosphate buffer, pH 8.0, 0.1 ml of 10X dilution of DTNB reagent solution [39.5 mg of 5,5'-dithiobis (2-nitrobenzoic acid)], and 15 mg of sodium bicarbonate in 10 ml of 0.1 M phosphate buffer pH 7.0 were mixed with 20 µl of the enzyme. Then, aliquots (20 µl) of 0.075 M acetylthiocholine iodide (ASChI) were added. The optical density of the developed yellow color was recorded after 10 min against the blank at 412 nm using a Spectronic 20D spectrophotometer. The enzyme activity was calculated as µM of substrate hydrolyzed · mg^{-1} × protein · min^{-1}.

Butyrylcholinesterase (BuChE)

The activity of BuChE was determined and calculated as µM of substrate hydrolyzed · mg^{-1} × protein · min^{-1}. An aliquot (0.1 g) of the insect's body tissue was homogenized as described above in the case of AChE, and the resulting supernatant was used as the enzyme source. For each test tube, 2.9 ml of 0.1 M phosphate buffer, pH 8.0, and 0.1 ml of a 10X dilution of the DTNB reagent solution were mixed with 20 µl of the

Table 2. Sequence of primers used in the Real-Time PCR with annealing temperature 60°C

Primers		Primer sequence	Reference*
Acetylcholinesterase (AChE)	F	5′-CCTCCATGCGGTACAGAGTT-3′	1
	R	3′-GTCCCTGAGCCATCTGAGAG-5′	
Esterase (GE)	F	5′-GTTTACGTGCCGGCAGATAG-3′	2
	R	3′-TTCTGAAACCCATCGCAACG-5′	
Glutathione-S-transferase (GST)	F	5′-AAATTCCGTGTGTGGTCGTTTAGATAAC-3′	3
	R	3′-ATGACAGAAAAACAACCGCTTTAC-5′	
Glucose oxidase (GO)	F	5′- AGGATTACCCGAGATCACCTGC-3′	4
	R	5′- GAGGGCGGAAAATCATCAGACC-3′	
Cytochrome P450 (Cyt-P450)	F	5′- ACCGCCAATAAACTCAGAGGAATGT-3′	5
	R	5′- ACTTCTCGCACATTGACAGGTTCTC-3′	
β-actin (reference gene)	F	5′- TGCCAACACTGTCCTTTCTG-3′	--
	R	5′- AGAATTGACCCACCAATCCA-3′	

*1 – Samson-Robert *et al.* (2015); 2 – Bomtorin *et al.* (2014); 3 – Shan *et al.* (2022); 4 – Bucekova *et al.* (2014); 5 – Wu *et al.* (2020)

enzyme. Subsequently, aliquots (20 µl) of 0.075 M butyrylthiocholine iodide (BuSChI) were added. The optical density of the resulting yellow color was recorded after 10 min against the blank at 412 nm using a Spectronic 20D spectrophotometer.

Catalase (CAT)

About 0.5 g of each treatment (insect's body tissue) was homogenized with ice-cold saline solution ($1:10\ w \cdot v^{-1}$). The extract was centrifuged at 12,000 rpm for 10 min at 4°C. The activity of CAT was quantified subsequently by the decline of absorbance at 240 nm, attributed to hydrogen peroxide (H_2O_2) depletion. In the cuvette, 1 ml of 12.5 mM H_2O_2 (substrate), 2 ml of 66.7 mM phosphate buffer, pH 7.0, and an aliquot of the enzyme source were added. The enzyme activity was assessed as $U \cdot mg^{-1} \times$ protein.

Adenosine triphosphatase (ATPase)

A hundred mg of head capsule tissue was homogenized in 20 mM Tris-HCl buffer pH 7.4 ($1: 5\ w \cdot v^{-1}$). The homogenate was centrifuged at 1,000 rpm at 4°C for 10 min. This method was used to quantify the amount of inorganic phosphate (Pi) produced from the hydrolytic reaction of ATP by ATPase. An aliquot (30 µl) of enzyme source was added to a medium containing 20 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, and 5 mM ATP, then the mixture was incubated at 37°C for 5 min with shaking. The reaction was stopped by adding 5% trichloroacetic acid (TCA), followed by the color reagent. The concentration of Pi was measured at 740 nm and the activity of the enzyme was expressed as $\mu M\ Pi \cdot mg^{-1} \times$ protein $\cdot\ min^{-1}$.

Statistical analysis

The expression levels of the tested enzymes were reported as means with their standard deviations (SD). The analysis was conducted using Statistical Analysis System (SAS) version 9.3, with a significance level of $p \leq 0.05$, to identify statistical variations between the means using the Tukey test.

Results

Gene expression analysis

For the used primers, the amplification efficiency was >95% and the R^2 value was > 0.98, indicating a high level of accuracy in amplification. All treated bees exhibited significantly higher expression levels of the GO enzyme ($p < 0.05$) compared to the control group, except for fluzilazole and profenofos (Table 3). Worker bees exposed to cypermethrin displayed the highest expression level of glucose oxidase (4.77 ± 0.18 fold), followed by chlorpyrifos (4.58 ± 0.004 fold), while the control group had the lowest expression level (1.47 ± 0.09 fold). Regarding the Cyt-P450, all bees treated with different pesticides exhibited significantly higher expression levels ($p < 0.05$) compared to the control group (Table 3). Worker bees exposed to chlorpyrifos, cypermethrin, and pendimethalin displayed the highest expression levels of Cyt-P450 with values of 4 ± 0.09, 3.76 ± 0.162, and 3.76 ± 0.016 fold, respectively, while the control group had the lowest expression level of 1.38 ± 0.030 fold. No significant differences ($p > 0.05$) were observed between the groups treated with pendimethalin, metribuzin, triazophos, chlorpyrifos, and cypermethrin.

All treatments exhibited significant differences ($p < 0.05$) compared to the control group, except for cyprodinil in the AChE (Table 3). The highest effects were observed with metribuzin, chlorpyrifos, and cypermethrin (6.63 ± 0.018, 6.71 ± 0.097, and 6.82 ± 0.175 folds, respectively), followed by pendimethalin, atrazine, and profenofos (5.59 ± 0.175, 5.89 ± 0.268, and 5.98 ± 0.176 folds, respectively), as well as triazophos and dichlorvos (4.96 ± 0.268 and 5.00 ± 0.054 folds) without significant differences ($p > 0.05$) within each group of treatments. The bees treated with penconazole, flusilazole, fludioxonil, tolclofos-methyl, cyprodinil, propiconazole, and profenofos did not exhibit significant differences ($p > 0.05$) in general esterases compared to the control group, unlike those treated with the other pesticides (Table 3). Worker bees exposed to pendimethalin and chlorpyrifos displayed the highest expression levels of general esterases, with 3.05 ± 0.100 and 2.92 ± 0.167-folds increases, respectively, without significant differences between them when compared to the control group. No significant differences ($p > 0.05$) were detected between the groups treated with atrazine, dichlorvos, metribuzin, and cypermethrin.

Regarding glutathione-S-transferase, all bees treated with different pesticides exhibited significantly higher expression levels ($p < 0.05$) compared to the control group (Table 3). Worker bees exposed to pendimethalin, cypermethrin, and chlorpyrifos displayed the highest expression levels of GST with values of 4.06 ± 0.018, 4.07 ± 0.175, and 4.33 ± 0.101 folds, respectively, without significant differences among them. The control group had the lowest expression level recorded at 1.50 ± 0.005. Additionally, no significant differences ($p > 0.05$) were detected between the groups treated with diazinon, flusilazole, and atrazine, which also exhibited notable effects on worker bees.

Table 3. Gene expression (fold of expression; Mean ± SD) of glucose oxidase (GO), cytochrome P450 (Cyt-P450), acetylcholinesterase (AChE), general esterases (GE), and glutathione-S-transferase (GST) after treating bees with pesticide residue levels expressed as ΔCt

Pesticides		Gene expression (Mean ± SD)				
		GO	Cyt-P450	AChE	GE	GST
Control		1.47j ± 0.095	1.38i ± 0.030	1.47h ± 0.030	1.25f ± 0.015	1.50i ± 0.005
Herbicides	Atrazine	4.22bc ± 0.154	2.79bc ± 0.118	5.89b ± 0.268	2.14bcd ± 0.007	3.22b ± 0.019
	Pendimethalin	4.42ab ± 0.005	3.76a ± 0.016	5.59b ± 0.175	2.92a ± 0.167	4.06a ± 0.018
	Metribuzin	4.42ab ± 0.092	2.97b ± 0.017	6.63a ± 0.018	2.41bc ± 0.152	2.55def ± 0.184
Insecticides	Profenofos	1.98ij ± 0.444	2.36def ± 0.158	5.98b ± 0.176	1.61ef ± 0.157	1.99gh ± 0.036
	Dichlorvos	3.16ef ± 0.065	2.36def ± 0.037	5.00c ± 0.054	2.16bcd ± 0.362	2.26fg ± 0.176
	Diazinon	3.80cd ± 0.029	2.63cd ± 0.095	4.41d ± 0.008	2.05cd ± 0.175	2.84cd ± 0.102
	Triazophos	4.57ab ± 0.089	2.55cde ± 0.054	4.96c ± 0.268	2.06cd ± 0.104	2.55def ± 0.041
	Chlorpyrifos	4.58ab ± 0.004	4.00a ± 0.094	6.71a ± 0.097	3.05a ± 0.100	4.33a ± 0.101
	Cypermethrin	4.77a ± 0.180	3.76a ± 0.162	6.82a ± 0.175	2.49b ± 0.096	4.07a ± 0.175
Fungicides	Cyprodinil	2.36ghi ± 0.180	1.84gh ± 0.016	1.87h ± 0.010	1.52ef ± 0.075	2.76cde ± 0.058
	Fludioxonil	2.20hi ± 0.063	1.72h ± 0.104	2.37g ± 0.175	1.45ef ± 0.016	1.86h ± 0.113
	Flusilazole	1.91ij ± 0.184	1.78gh ± 0.131	2.87ef ± 0.025	1.44ef ± 0.174	3.01bc ± 0.128
	Metalaxyl	2.59gh ± 0.156	2.36def ± 0.170	3.15e ± 0.175	1.82de ± 0.035	2.55def ± 0.171
	Penconazole	3.46de ± 0.195	1.84gh ± 0.033	3.26e ± 0.176	1.27f ± 0.033	1.99gh ± 0.018
	Propiconazole	2.83fg ± 0.181	2.27ef ± 0.075	3.28e ± 0.018	1.61ef ± 0.019	2.46ef ± 0.081
	Tolclofos-methyl	2.37ghi ± 0.212	2.10fg ± 0.163	2.64fg ± 0.175	1.45ef ± 0.013	1.93h ± 0.141

Significant differences between treatments (different letters in each column) are based on Tukey's test, $p \leq 0.05$

Biochemical responses

Metalaxyl showed the highest activity of AChE enzyme in bees treated with pesticides (Table 4). Only three treatments, pendimethalin, fludioxonil, and metalaxyl, were significantly higher than the control group. Conversely, the control group exhibited significantly higher activity ($p \leq 0.05$) compared to the remaining treatments. The lowest activity was observed with profenofos. No significant differences ($p > 0.05$) were detected between triazophos, chlorpyrifos, cypermethrin, atrazine, penconazole, dichlorvos, metribuzin, and profenofos. The specific activity of BuChE in bees treated with pesticides (Table 4) increased significantly with penconazole and profenofos ($p \leq 0.05$) compared to the control group. All other treatments resulted in significantly lower BuChE activity compared to the control group. No significant differences ($p > 0.05$) were found between atrazine, cypermethrin, diazinon, propiconazole, triazophos, flusilazole, tolclofos-methyl, pendimethalin, metribuzin, cyprodinil, metalaxyl, dichlorvos, fludioxonil, and chlorpyrifos.

Tolclofos-methyl and cyprodinil showed the highest specific activity of CAT in treated bees (Table 4). Only three treatments, dichlorvos, tolcophos, and cyprodinil, were significantly ($p \leq 0.05$) higher than the other treatments. The control group exhibited significantly higher activity than only two treatments, chlorpyrifos and metribuzin. The specific activity of the ATPase of bees treated with pesticides (Table 4) was significantly higher in the control group compared to all other treatments ($p \leq 0.05$). Propiconazole and flusilazole were not significantly different from each other. The lowest activities were observed in dichlorvos and atrazine treatments. Diazinon, penconazole, metalaxyl, propiconazole, and flusilazole pesticides showed increased ATPase activity compared to the other tested pesticides. The pesticide residues studied demonstrated varied specific activities of the enzymes investigated. For instance, metalaxyl exhibited the highest effects on AChE exclusively, without significant effects on the other enzymes. The untreated bees (control group) displayed higher specific activity in all enzymes compared to those treated with most pesticides.

Discussion

The available knowledge supports the adverse effects of pesticides on the development and survival of bees

Table 4. Mean ± SD of specific activity (μM mg^{-1} × protein · min^{-1}) of acetylcholinesterase (AChE) enzyme, (μM mg^{-1} × protein · min^{-1}) of butyrylcholinesterase (BuChE) enzyme, (U · mg^{-1} × protein) of catalase (CAT) enzyme, and (μM Pi · mg^{-1} × protein · min^{-1}) of adenosine triphosphatase (ATPase) enzyme in bees treated with pesticide residue levels

Pesticides		Mean ± SD of specific activity			
		AChE	BuChE	CAT	ATPase
Control		2.13c ± 0.28	0.45b ± 0.04	0.53cdef ± 0.08	5.36a ± 0.33
Herbicides	Atrazine	0.49fgh ± 0.03	0.003c ± 0.0002	0.61cd ± 0.11	0.78g ± 0.04
	Pendimethalin	2.46bc ± 0.15	0.03c ± 0.001	0.65cd ± 0.29	1.16ef ± 0.02
	Metribuzin	0.66efgh ± 0.008	0.03c ± 0.005	0.12g ± 0.03	1.04efg ± 0.12
Insecticides	Profenofos	0.19h ± 0.003	1.08a ± 0.07	0.56cde ± 0.06	1.54cd ± 0.06
	Dichlorvos	0.63fgh ± 0.053	0.04c ± 0.009	1.26b ± 0.25	0.75g ± 0.09
	Diazinon	0.86defg ± 0.05	0.01c ± 0.001	0.41cdefg ± 0.09	1.72c ± 0.15
	Triazophos	0.24h ± 0.02	0.01c ± 0.002	0.20efg ± 0.005	1.26de ± 0.07
	Chlorpyrifos	0.37gh ± 0.05	0.08c ± 0.01	0.04g ± 0.02	1.10efg ± 0.03
	Cypermethrin	0.39gh ± 0.05	0.09c ± 0.0009	0.33cdefg ± 0.06	0.88fg ± 0.07
Fungicides	Cyprodinil	0.96def ± 0.09	0.03c ± 0.002	1.69a ± 0.05	1.08efg ± 0.08
	Fludioxonil	2.79b ± 0.46	0.04c ± 0.01	0.41cdefg ± 0.20	1.25de ± 0.07
	Flusilazole	1.20d ± 0.15	0.02c ± 0.004	0.13c ± 0.009	2.19b ± 0.08
	Metalaxyl	3.85a ± 0.23	0.03c ± 0.009	0.69c ± 0.11	1.79c ± 0.11
	Penconazole	0.49fgh ± 0.02	1.08a ± 0.08	0.62cd ± 0.07	1.75c ± 0.01
	Propiconazole	1.22d ± 0.20	0.01c ± 0.002	0.28defg ± 0.08	1.89bc ± 0.09
	Tolclofos-methyl	1.15de ± 0.01	0.02c ± 0.005	1.37ab ± 0.19	0.93efg ± 0.08

Significant differences between treatments (different letters in each column) are based on Tukey's test, $p \leq 0.05$

(Johnson *et al.* 2010), as well as on the productivity and activities of bee colonies (Tosi and Nieh 2019). Also, pesticides have the potential to alter gene expression and enzymatic activities in the exposed bees (Murawska *et al.* 2021). This study supports such alterations when bees are exposed to fungicides, insecticides, and herbicides. Regarding fungicides, the gene expression analysis revealed the lowest effects mainly for fluzilazole and cyprodinil. The fungicides that exhibited the highest specific activity were mainly fludioxonil and metalaxyl (AChE), penconazole (BuChE), tolclofos-methyl and cyprodinil (CAT), as well as propiconazole and flusilazole (ATPase). Consistent with the current findings, propiconazole has been shown to affect the AChE activity, GST, and Cyt-P450 (midgut) in Asian bees, *Apis cerana* (Han *et al.* 2019).

Regarding the tested insecticides, the gene expression results showed variations among them. Profenofos had the lowest effect on glucose oxidase, while cypermethrin and chlorpyrifos exhibited high effects on glucose oxidase, Cyt-P450, AChE, and GST. Additionally, chlorpyrifos showed elevated effects on general esterases. The gene expression was generally high in most cases for chlorpyrifos and cypermethrin compared to the other insecticides. Consistent with these findings, chlorpyrifos has altered GST activity in the midgut of *A. mellifera* (Yao *et al.* 2018). Moreover, insecticides like acephate inhibited the activities of esterase, GST, and AChE (Yao *et al.* 2018). With regard to the tested herbicides, pendimethalin exhibited the highest expression levels of Cyt-P450, GE, and GST, while metribuzin had the strongest effects on AChE. Most reports on the impact of herbicides on non-target organisms have indicated alterations in survival due to changes in the population of host plants. These reductions are attributed to the removal of plant hosts, pollen, nectar, shelter, nesting, and overwintering sites by the herbicides (Rands and Whitney 2011). This study presents additional potential negative effects of herbicides on oxidative stress-related biomarkers of honeybees.

Regarding biochemical responses, dissimilar enzymatic changes in bee workers have been reported for other pesticides such as deltamethrin, spinosad, and fipronil, affecting carboxylesterases (CE) and alkaline phosphatase (ALP) (Carvalho *et al.* 2013). The chemical composition and toxicity of each pesticide play a significant role in inducing such enzymatic changes in the target organism. In this study, we also observed dissimilar enzymatic activities for each pesticide using spectrophotometric methods. The untreated bees exhibited higher specific enzyme activity compared to most treated bees, indicating the negative effects of the tested pesticides on bee physiology. Among the

tested insecticides, profenofos exhibited the highest BuChE activity, but showed the least activity for the other enzymes. Regarding fungicides, this study revealed that fludioxonil and tolclophos-methyl caused similar effects to those of the control group, indicating that these fungicides may not endanger bee health at the concentrations examined. Additionally, research by Gradish *et al.* (2010) supports the safety of fludioxonil for bees in greenhouse environments. Among the tested herbicides, pendimethalin significantly impacted the specific activity of AChE, while metribuzin had the least effect on the specific activity of CAT. In contrast, atrazine exhibited the lowest specific activity of ATPase. This study supports the use of spectrophotometric methods to screen enzymes as biomarkers for assessing bee exposure to stress.

When comparing all the tested pesticides, the enzymes did not respond to the pesticides in the same manner, as indicated by the results. Chlorpyrifos, cypermethrin, and pendimethalin each caused the highest effects on the target enzymes, suggesting their strong negative impact on the health of worker bees at the tested concentrations. This finding is significant for bee health, particularly given that chlorpyrifos is a commonly detected pesticide in honeybee samples (Villalba *et al.* 2020). Chlorpyrifos also exhibits strong synergistic toxicity with other pesticides in honeybees (Wang *et al.* 2020). Cypermethrin is known to cause health issues in honeybees (Fent *et al.* 2020). Additionally, pendimethalin can induce enzymatic changes in honeybee larvae (He *et al.* 2022). The results highlight the effects of these pesticides on the physiological biomarkers of adult bees, which have not been sufficiently documented before. It has been stated that mixtures of pesticides can have synergistic effects on bees (e.g., Johnson *et al.* 2010). Studying the effects of the tested pesticides as mixtures is therefore recommended for future studies to gain more insight into their potential synergistic effects on honeybees.

Conclusions

Treated worker bees with detectable residue amounts of pesticides found in bee products generally exhibited adverse effects on antioxidant enzymes. Chlorpyrifos, cypermethrin, and pendimethalin exhibited high up-regulation levels of the tested genes compared to both the control group and the other tested pesticides. Moreover, the tested pesticides led to a reduction in the specific activity of certain enzymes compared to the control bees. The exposure of bees to these pesticides induced stress, resulting in observable changes in their enzyme levels. This study provides insights into the physiological balance of bees following exposure to specific pesticides at residue amounts found in bee products. Since these pesticides were individually examined in this study on honeybees, it would be valuable to investigate their potential synergistic effects in future research. Furthermore, future studies are encouraged to explore the potential correlation between these detrimental physiological and genetic impacts caused by pesticides and their influence on the productivity of bee colonies. Routine screening for pesticide residues, particularly for chlorpyrifos, cypermethrin, and pendimethalin, is recommended, especially for colonies experiencing health issues. Implementing this proactive measure could help address the health challenges facing bee colonies and ensure their well-being.

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