Physiological and toxicological responses of honeybees and stingless bees to individual and combined exposure to imidacloprid, glyphosate, and pyraclostrobin

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Cover Letter

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Dear Co-Editors-in-Chief in Journal of Hazardous Materials,

We would like to submit our manuscript entitled "**Physiological and toxicological responses of honeybees and stingless bees to individual and combined exposure to imidacloprid, glyphosate, and pyraclostrobin**" for publication in the *Journal of Hazardous Materials*.

The manuscript addresses the differential toxicity and physiological impacts of imidacloprid, glyphosate, and pyraclostrobin pesticide pollutants (alone or mixed) on *Apis mellifera*, *Melipona scutellaris*, and *Scaptotrigona postica*, important pollinators of the native vegetation and Brazilian agricultural crops. This research is relevant to Ecotoxicology and is expected to contribute to the ongoing discussion in the field of Environmental Toxicology.

The manuscript investigates the chronic effects of commonly used pesticides (glyphosate, pyraclostrobin, and imidacloprid) on the survival and physiological functions of Africanized honeybees and native stingless bees. By examining the combined effects of these pesticides, the study identifies complex interactions and differences in sensitivity between bee species. The research integrates toxicological bioassays and enzyme activity analysis to provide insights into the sublethal impacts of pesticide exposure, emphasizing the importance of considering pesticide mixtures in environmental risk assessments. The findings highlight the need for tailored regulatory measures to protect bee populations, particularly native species.

We have chosen to submit our manuscript to *Journal of Hazardous Materials* because of its well-established reputation for publishing high-quality research in Environmental Toxicology and Biodiversity. Furthermore, we have noted several recent publications in your journal that have explored related themes, such as: "*Biopesticide spinosad induces transcriptional alterations in genes associated with energy production in honey bees (Apis mellifera) at sublethal concentrations*", and "*Environmental exposure to metallic pollution impairs honey bee brain development and cognition*".

The current work represents an original research paper accomplished by the authors that has not been published or is being considered for publication by another journal. We declare that there is no conflict of interest associated with this research. All co-authors have read and approved of this submission.

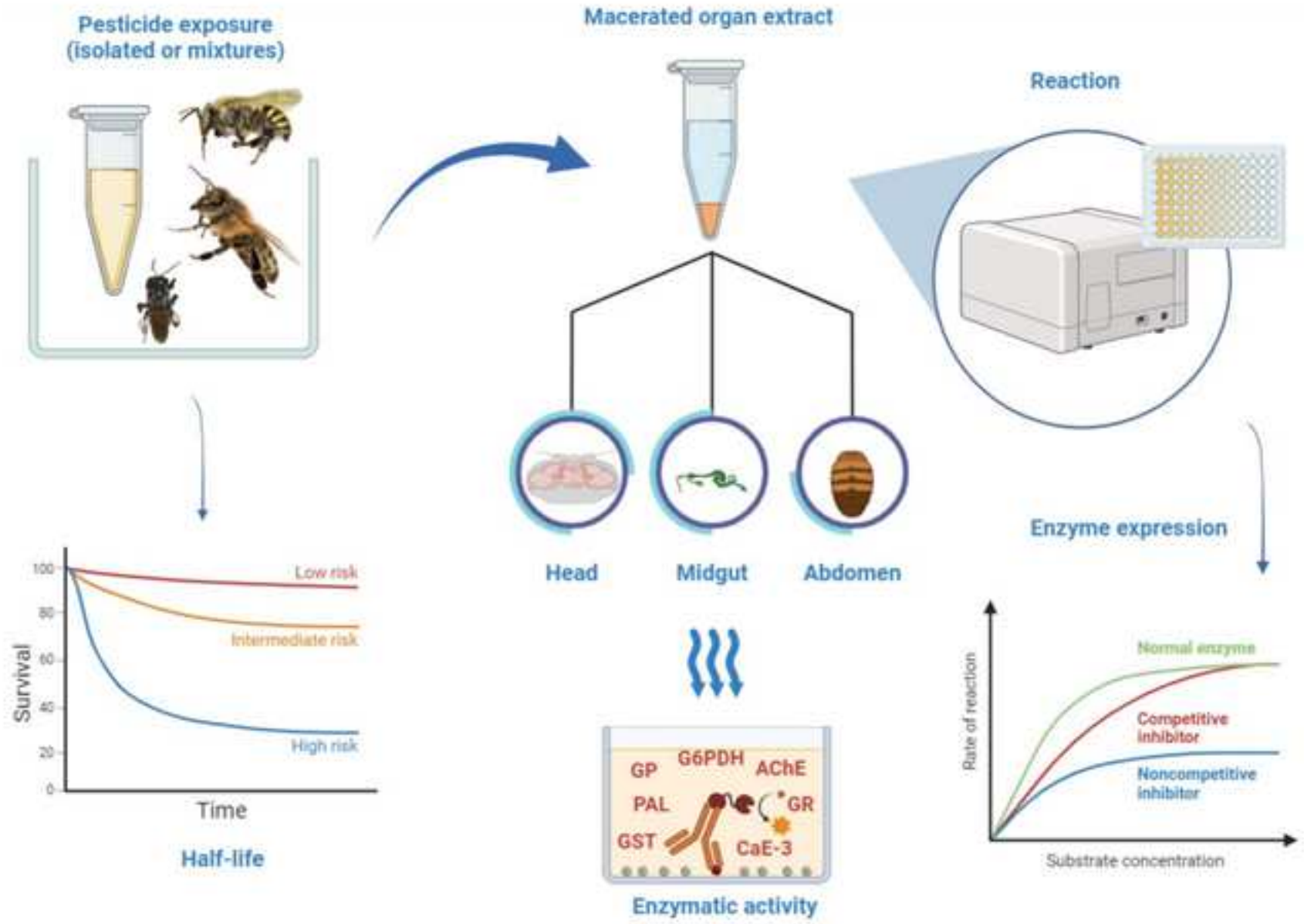
Thank you for considering our manuscript for publication in *Journal of Hazardous Materials*.

Best Regards.

Environmental implication

Imidacloprid, glyphosate, and pyraclostrobin, isolated or mixed, represent significant environmental risks to honeybees and stingless bees. Chronic exposure to these chemicals can compromise survival, physiological functions, and bee health. Consecutively, the critical role in natural and agricultural ecosystems pollinating is potentially reduced.

Graphical Abstract



Highlights

Apis and non-*Apis* bees were exposed to pesticides alone or mixed.

The survival and interaction pesticides were different for bee species.

Biomarkers enzymatic were used for study the physiological statement.

The physiological condition of *Apis* and non-*Apis* can be differently affected.

Physiological and toxicological responses of honeybees and stingless bees to individual and combined exposure to imidacloprid, glyphosate, and pyraclostrobin

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Abbreviations

LT_{50}, lethal time; AChE, acetylcholinesterase; CaE-3, carboxylesterase-3; ALP, alkaline phosphatase; GP, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; G6PDH, glucose-6-phosphate dehydrogenase.

Abstract: Bees play crucial roles in pollination. They face pesticide exposure, adversely affecting their survival and physiological functions. Pesticides like glyphosate, pyraclostrobin, and imidacloprid are commonly used in Brazilian agroecosystems, with residues found in bee food resources. Our study investigates the chronic effects of these pesticides, alone and in mixtures, on the survival and physiological response of *Apis mellifera*, *Melipona scutellaris*, and *Scaptotrigona postica*. We conducted toxicological bioassays exposure of the bees to residual concentrations of imidacloprid (0.7 µg/L), glyphosate (1.8 µg/L), and pyraclostrobin (0.2 µg/L), both individually and combined. We determined the mean lethal time (LT_{50}) and analyzed enzyme activity in exposed bees. Results showed varying sensitivities among bee species to pesticide exposure. Pesticide mixtures generally led to the most significant reductions in bee survival, with interactions showing complex effects. Enzyme activity analysis revealed alterations in important physiological compartments, indicating potential sublethal effects of pesticide exposure. This study highlighted the need for further research on the effects of mixtures of pesticides, especially on native bee species, to better understand their impact on bee populations. In addition, these findings underscore the need for further research to inform regulatory decisions on pesticide management.

Keywords: Pesticides. Ecotoxicology. Bioassays. Native bees. Physiological markers.

1. Introduction

Bees are important pollinators in natural environments and agroecosystems. In Brazil, in addition to the exotic honey bee *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apoidae: Apini), there are also approximately 3000 species of bees, of which 244 are stingless bees (Hymenoptera : Apidae: Meliponini) including *Melipona scutellaris* Latreille, 1811 and *Scaptotrigona postica*, Latreille, 1807 (Pedro, 2014; Michener, 2007). Meliponines are responsible for 90% of the pollination of native plant species in ecosystems such as the Atlantic Forest and contribute to the maintenance of natural systems (Imperatriz-Fonseca et al., 2012). In addition to native plants, stingless bees pollinate crops such as coffee, tomato, strawberry, and beans (Giannini et al., 2015; Wolowski et al., 2019). In Brazil, meliponiculture is a widespread and important economic activity, as it can favor economic development by improving the quality of life of rural families by rearing stingless bees and the sale of colony products (Jaffé et al., 2015).

The potential pollinator decline around the world has been reported, with several causes identified, such as diseases, increase in urban areas, climate change, and intensive use of pesticides (Potts et al., 2010; Goulson et al., 2015; Kok et al., 2017; Ratto et al., 2017). Since the beginning of investigations on the toxicity of pesticides to bees, most of the results have been obtained from studies on the model species *A. mellifera*. However, it is not certain that the data obtained for *A. mellifera* could be applied to non-*Apis* bees (Arena and Sgolastra, 2014; Cham et al., 2019; Dietzsch and Jütte 2020; Rosa-Fontana et al., 2020). The evidence of pesticide toxicity to other, non-*Apis* bee species motivates further studies to better understand the sublethal effects of pesticides on stingless bees (Jacob et al., 2013; Costa, L.M., et al., 2015; Soares et al., 2015; Heard et al., 2017; Miotelo et al., 2021; Jacob et al., 2019a; Domingues et al., 2020a; Assis et al., 2022).

Bees can be exposed to pesticides in a variety of ways, including oral contamination through contaminated pollen, nectar, and honey (Blacquière et al., 2012; Krupke et al., 2012; van der Sluijs et al., 2013; Belsky and Joshi 2020). Although insecticide toxicity to bees has been widely studied,

there is a lack of data regarding the toxicity of herbicides and fungicides (Soares et al., 2015; Belsky and Joshi 2020). Therefore, spraying pesticides on crops can potentially expose bees to harmful chemicals, not only to a particular pesticide but also to mixtures that occur in the colony matrices. Several studies have demonstrated that interactions between stressors can induce deleterious impacts on honey bees, such as lethal synergies (Colin and Belzunces, 1992; Mullin et al., 2010; Chauzat et al., 2011; Almasri et al., 2020; Schuhmann et al., 2022) and adverse physiological effects (Tadei et al., 2019; Almasri et al., 2020; Almasri et al., 2021a; Almasri et al., 2021b; Wang et al., 2021).

Glyphosate, pyraclostrobin, and imidacloprid are among the most commonly pesticides used in agroecosystems for controlling weeds, diseases, and pests, respectively. Because of that, data on contamination in beehive honey reported concentrations of 0.14-0.275 µg/L of imidacloprid (Nguyen et al., 2009; Chauzat et al., 2011; Lambert et al., 2013; López et al., 2016), and 17-342 µg/L of glyphosate (Rubio et al., 2014; Berg et al., 2018; El Agrebi et al., 2020), and a median content of 359.93 µg/L was reported for pyraclostrobin in pollen of beehives (Graham et al., 2022). In this case, the isolated effects of imidacloprid on *A. melifera* (Decourtye et al., 2003; Henry et al., 2012; Almasri et al., 2020; Carneiro et al., 2022) and non-*Apis* bees (Tomé et al., 2012; da Costa, et al., 2015; Soares et al., 2015; Brito et al., 2020; Costa et al., 2020; Miotelo et al., 2021; Siviter et al., 2021; Aguiar et al., 2023) have been extensively reported. However, the exposure to glyphosate caused some little-known sublethal effects in honey bees (Balbuena et al., 2015. Helmer et al., 2015; Luo et al., 2021; Faita et al., 2022; Vázquez et al., 2023), and stingless bees (Seide et al., 2018; da Silva et al., 2022). Likewise, the fungicide pyraclostrobin was considered potentially harmful to bees (Domingues et al., 2020a; Domingues et al., 2020b; Domingues et al., 2023; Xiong et al., 2023).

Exposure to sublethal concentrations of combined mixtures of glyphosate, pyraclostrobin and imidacloprid showed retardation of locomotor activity and cell abnormalities on the fat body of *M. scutellaris* (Farder-Gomes et al., 2024a) and *S. postica* (Farder-Gomes et al., 2024b). However, the

effects of chronic exposure to residual concentrations of those pesticides on survival and physiological response have not yet been explored in honey bees and non-Apis bees.

The exposure to sublethal concentrations of combined mixtures of glyphosate, pyraclostrobin, and imidacloprid showed retardation of locomotor activity and cell abnormalities on the fat body of *M. scutellaris* (Farder-Gomes et al., 2024a) and *S. postica* (Farder-Gomes et al., 2024b). However, the effects of chronic exposure to residual concentrations of those pesticides on survival and physiological response have not been documented for honey bees and non-Apis bees. To understand the effects of pesticides on the physiology of bees, physiological biomarkers are commonly used (Badiou et al., 2008; Badiou-Bénéteau et al., 2012; Carvalho et al., 2013; Kairo et al., 2017; Almasri et al., 2020; Decio et al., 2021). The performance of essential metabolic functions, such as detoxification and defenses against oxidative stress, can be easily assessed for enzymatic expression, such as acetylcholinesterase (AChE), carboxylesterase (CaE-3), glutathione-S-transferase (GST), glutathione peroxidase (GP), glutathione reductase (GR), alkaline phosphatase (ALP), and glucose-6-phosphate (G6PDH) (Berenbaum and Johnson, 2015; Gong and Diao, 2017).

Therefore, here we assess the chronic exposure to field residual concentrations of glyphosate, pyraclostrobin, and imidacloprid (alone and in mixtures) on the *A. mellifera*, *M. scutellaris*, and *S. postica*, and their adverse effects on the survival and physiological biomarker enzymes.

2. Materials and methods

2.1. Reagents

Imidacloprid (98% pure, CAS number 138261-41-3), glyphosate (98% pure, CAS number 1071-83-6), and pyraclostrobin (98% pure, CAS number 175013-18-0) were purchased from Sigma Aldrich analytical standards. Triton X-100, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl), pepstatin A, leupeptin, aprotinin, trypsin, antipain, acetylthiocholine iodide (AcSCh), 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), 1,5-bis(4-

allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51), 4-nitrophenyl acetate (*p*-NPA), ethanol, monopotassium phosphate (KH_2PO_4), disodium ethylene-diaminetetraacetate dihydrate (EDTA), reduced L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), reduced β-nicotinamide adenine dinucleotide 2′-phosphate tetrasodium salt hydrate, L-Glutathione oxidized, tert-butyl hydroperoxide, glutathione reductase, 4-nitrophenyl phosphate bis (tris) salt (*p*-NPP), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), tris base, D-glucose-6-phosphatase disodium salt hydrate (G6P), β -nicotinamide adenine dinucleotide phosphate hydrate (β-NADP+), and acetone were purchased from Sigma Aldrich.

2.2. Residual concentrations

Residual concentrations for glyphosate, pyraclostrobin, and imidacloprid were estimated by BeeREX model for pesticide risk assessment version 1.0 (USEPA 2015), using the recommended dose to control weeds, diseases, and key pests of coffee and cotton crops. The values estimated by BeeREX corresponded to the minimum amounts found in the nectar, reported in the scientific literature (Mullin et al., 2010; Chauzat et al., 2011; Lambert et al., 2013; Rubio et al., 2014; Lopéz et al., 2016; Berg et al., 2018; El Agrebi et al., 2020; Graham et al., 2022). In this case, we obtained a residual concentration of 0.7 µg a.i. L^{-1} for imidacloprid (I), 1.8 a.i. L^{-1} for (H) glyphosate, and a.i. L^{-1} for pyraclostrobin (F). To prepare the residual concentrations, a stock solution containing 100 a.i. L^{-1} was prepared. Subsequently, the stock solution was diluted in sucrose solution (50% sugar + 50% water; w/v), until reaching the estimated final concentration. The glyphosate stock solution was made in water. The stock solution of pyraclostrobin and imidacloprid were made in 4% and 15% acetone respectively, and subsequently diluted in water so that the percentage of acetone in the final solution did not exceed 1% as recommended by the OEDC (1998). The binary and ternary mixture were made from individual stock solutions previously prepared and stored. Therefore, in total, each bee species was exposed to eight distinct groups, as follows: Control sucrose (C); imidacloprid (I); glyphosate

(H); pyraclostrobin (F); imidacloprid + glyphosate (IH), imidacloprid + pyraclostrobin (IF), glyphosate + pyraclostrobin (HF), and imidacloprid + glyphosate + pyraclostrobin (IHF).

2.3. Toxicological bioassays

Adult forager bees of *A. mellifera*, *S. postica* and *M. scutellaris* were collected from four healthy colonies kept in the experimental apiary and meliponary of the Institute of Bioscience, Universidade Estadual Paulista (UNESP), Campus Rio Claro, São Paulo, Brazil. The bees were collected at the entrance of each colony with a cylindrical plastic container (250 mL, 9cm x 7cm), containing small holes on the sides to allow for aeration during the assessment time and containing feeders with sucrose solution (50% sugar and 50% water, wt/wt).

The mean of lethal time (LT_{50}) was determined according to OECD nº 245 guidelines (OECD, 2017), with adjustments. For each group of pesticide (alone or mixed), the bees were continually fed with a contaminated sucrose solution for the last forager died. Mortality was recorded daily. The dead bees were counted and discarded to preserve hygienic conditions of the experimental unit. The experimental design of the bioassays was completely randomized. All bioassays were conducted in a climate-controlled chamber at 32 ± 2 °C for *A. mellifera* and 28 ± 2 °C for *S. postica* and *M. scutellaris* at 75 ± 5% relative humidity (RH), and kept in darkness.

2.4. Determination of pesticide interactions

The evaluation of the type of interaction between pesticides was based on Colin and Belzunces (1992) and Piggott et al. (2015), as validated by Almasri et al. (2020). The interaction rate (IR) was determined by the ratio between the corrected mortality and the predicted mortality. According to IR value, the interactions for the each exposure group were classified in: additive effect (when IR = 1); synergistic effect (when IR > 1); antagonistic effect (when IR < 1 and the mortality from the mixture is lower than the toxicity of the mixture substance alone); sub-additive (when IR < 1 and the mortality from the mixture is higher than the toxicity of the substance alone; or when the effect of the mixture

is between the least toxic substance and the most toxic, the interaction is also considered sub-additive). The effect of the mixture was judged as independent when the mixture induced a similar mortality to each pesticide. The interaction rate was calculated from the LT_{50} of the control group.

2.5. Physiological biomarkers

To evaluate the physiological effects induced by chronic oral exposure to pesticides (isolated or mixed), the expression of seven enzymes involved in important biochemical processes was realized, following as: acetylcholinesterase (AChE), responsible for the neural activity, such as learning and memory processing (Gauthier et al., 1992; Guez et al., 2010); the carboxylesterase-3 (CaE-3), responsible to the cell detoxification (Badiou-Bénéteau et al., 2012); alkaline phosphatase (ALP), involved in the gut metabolic activity and immunity system (Chen et al., 2011; Vlahović et al., 2009); and glutathione peroxidase (GP), glutathione reductase (GR), glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH), involved in defense against the oxidative stress and detoxification (Efferth et al., 2006; Badiou-Bénéteau et al., 2012; Qin et al., 2013; Yan et al., 2013; Berenbaum and Johnson, 2015; Gong and Diao, 2017).

2.6 *Enzymatic assays*

Foraging bees were collected from four different colonies in the apiary and meliponary, according to the protocols described in item 2.3. The bees were kept in plastic cages (250 mL) and exposed continuously to residual concentrations of pesticides (isolated or mixed) and control group with sucrose solution. To assess the physiological effects of pesticides five replicates with three alive bees were recovered from the cages of each group in the mean lethal time determined on the toxicological bioassays (Table 1). The recovered samples were kept at -80C until the extraction of proteins and modulation of the enzymes of interest.

2.6.1 *Protein extraction*

The enzyme expression was assessed in tissues with relevant biological and metabolic activities (Almasri et al., 2021). Therefore, we assessed the AChE, CaE-3, GST, GP, and GR were in the three bee species head; CaE-3, GST, and ALP in the gut of the *A. mellifera* and *M. scutellaris*; and CaE-3, GST, and G6PDH in the abdomen of *A. mellifera* and *M. scutellaris*. Due to the small body size of *S. postica*, CaE-3, GST, ALP, and G6PDH were extracted from the full abdomen.

To obtain the protein extracts, the head was separated from the thorax, and the midgut was pulled out from the abdomen. Subsequently, the heads, abdomens (whit or devoid of midgut), and midguts were placed in microcentrifuge tubes (2 mL) and weighed. For each treatment, five repetitions (n = 5 samples) of pooled tissues from three bees per sample were analyzed, and each sample was assayed in triplicate during the measurement of enzymatic activity. The tissues were homogenized using a buffer consisting of 10 mM sodium chloride, 1% (w/v) Triton X-100, 40 mM sodium phosphate pH 7.4, and protease inhibitors (2 µg/mL pepstatin A, leupeptin, and aprotinin, 0.1 mg/mL soybean trypsin inhibitor, and 25 units/mL antipain) to make a 10% (w/v) extract. Tissue homogenization was carried out just before analysis with the aid of a Loccus® L-Beader 6 tissue lyser through 2 cycles. In each cycle, the samples were homogenized by automatic agitation at 2,500 rpm, 5 times for 10 s each, with intervals of 30 s. The samples were centrifuged at 4 °C for 20 min at 15,000 x gav, and the supernatant was saved and stored on ice util analysis.

2.6.2. Enzyme activity

AChE activity was assayed at 412 nm in a medium containing tissue extract, 1.5 mM DTNB, 0.3 mM AcSCh as substrate, and 100 mM sodium phosphate pH 7.0 (Belzunces et al., 1988). CaE-3 was assayed at 410 nM in a medium containing the tissue extract, 10 µM BW284C51 (acetylcholinesterase inhibitor), 0.1 mM *p*-NPA as the substrate, and 100 mM sodium phosphate pH 7.0 (Badiou-Bénéteau et al., 2012; Renzi et al., 2016). GST activity was determined by measuring the conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. The reaction medium

contained 1 mM EDTA, 2.5 mM CDNB, and 100 mM Na/K phosphate pH 7.4 (Carvalho et al., 2013). GP was assayed using *tert*-butyl hydroperoxide (TBHP) as the substrate. The GSSG generated was reduced by glutathione reductase (GR) in the presence of NADPH to generate GSH and NADP. The conversion of NADPH to NADP+ was followed at 340 nm. The reaction medium contained 1 mM EDTA, 0.2 mM TBHP, 0.85 mM GSSG, 0.16 mM NADPH, 0.25 U/mL GR, and 50 mM Na/K phosphate pH 7.4. GR activity was assayed at 340 nm by the conversion of NADPH to $NADP^+$. The reaction medium contained 1 mM EDTA, 0.85 mM GSSG, 0.16 mM NADPH, and 50 mM Na/K phosphate pH 7.4 (Kairo et al., 2017; Decio et al., 2021). ALP activity was assayed at 410 nm in a medium containing the tissue extract, 20 μM $MgCl_2$, 2 nM *p*-NPP as the substrate, and 100 mM Tris-HCl pH 8.5 (Bounias et al., 1985). G6PDH activity was measured at 340 nm through the formation of NADPH in a medium containing the extract, 1 mM G6P as substrate, 0.5 nM $NADP^+$ as the coenzyme, 10 mM $MgCl_2$, and 100 mM Tris-HCl pH 7.4 (Renzi et al., 2016). Readings were performed in 96-well cell culture plates, using an Infinite® 200 PRO spectrophotometer microplate reader (TECAN, Mannedorf, Switzerland) at 25 °C. Each sample was assayed in triplicate.

2.7. Data analyses

Statistical analyses were performed using the software RStudio 4.1.2 (R Core Team, 2022). The mean of lethal time (LT_{50}) was calculated based on the daily mortality data. To perform the analysis the "survival" (Therneau and Grambsch 2000, Therneau and Lumley 2024) and “survminer” (Kassambara and Kosinski 2021) packages was used. For this, the Kaplan-Meier method (log-rank test) followed by a post hoc test, with correction of fdr ($p \leq 0.05$) was performed.

The enzyme expression data for different exposed groups were analyzed according to normality and homoscedasticity, using the ks. teste, Levene test, and Bartlett test, respectively. The data did not satisfy the normality and variance homogeneity, even after transformations, so Kruskal-Wallis non-parametric Dunn test with Benjamini-Hochberg correction ($p < 0.05$) was performed.

3. Results

3.1 *Toxicological bioassays*

The mean of lethal time (LT_{50}) of each pesticide alone or mixed for the bee species was significantly different (Table 1). For example, the LT_{50} of *A. mellifera* was reduced by all pesticides (isolated or mixed) when compared with the control group ($\chi 2 = 46.16$, df = 7, $p < 0.001$). In this case, the (F) isolated or mixed with (H), and the (I) mixed with (H) were harmful to *A. mellifera*, because the 50% of bees mortality occurred in the shortest time. In contrast, *M. scutellaris* forager bees showed LT_{50} a significative reduction ($\chi 2 = 30.00$, df = 7, $p < 0.001$), when exposed to: (IH) < (IHF) < (HF) < (I) < (IF). While *S. postica* showed LT_{50} reduction ($\chi 2 = 48.74$, df = 7, $p < 0.001$), when the bees were exposed to (F), (IHF), and (H).

3.2 *Pesticide interactions*

Apis mellifera, *M. scutellaris* and *S. postica* showed different response types to pesticide mixture interactions (Table 2). Sub-additive effects were observed in *A. mellifera* forage bees exposure to (IH), (IF), and (IHF), and additive effect on the bees exposed to (HF). The sub-additive effects were also observed in *M. scutellaris* exposure to (IF). However, synergistic effects were observed in *M. scutellaris* exposure to (IH), (HF), and (IHF). For *S. postica*, in addition, sub-additive effects were observed when *S. postica* was exposed to (IH), (IF), and (IHF), while the antagonistic effect occurred in those exposed to (HF).

3.3 *Enzyme expression*

Apis mellifera, *M. scutellaris* and *S. postica* exposed to pesticides (isolated or mixed) showed different response regarding enzymatic expression of the physiological biomarkers (Fig. 1-3). Overall, these findings indicate complex responses of bee enzymatic systems to pesticide exposure, with variations observed across species and enzyme types. For example, the AChE activity increased

in the head of *S. postica* exposure to (IF) ($\chi2 = 16.00$, df = 7, $p = 0.0251$). While GST head activity decreased in *A. mellifera* exposure to (IH), (HF), and (IHF) ($\chi2 = 26.57$, df = 7, $p < 0.001$), and in *M. scutellaris* ($\chi2 = 25.76$, df = 7, $p < 0.001$) exposure to (IH), (IF), (HF), (IHF). When *S. postica* was exposed to (IH), (IF), (HF), and (IHF), the GST head activity decreased. However, the GST head activity increase when *S. postica* was exposure to (F) ($\chi2 = 3.25$, df = 7, $p < 0.001$). In addition, head GP activities decreased when *A. mellifera* ($\chi2 = 33.58$, df = 7, $p < 0.001$) and *M. scutellaris* ($\chi2 = 14.20$, df = 7, $p = 0.004$) were exposure to all the mixtures, while GR activity was reduced in the head of *A. mellifera* exposed to (IH), (IF), (IC), and (IHF) ($\chi2 = 30.88$, df = 7, $p < 0.001$), and increasing activity in the head of *M. scutellaris* exposed to (F) ($\chi2 = 15$, df = 7, $p = 0.030$).

The CaE-3 enzyme activity was expressed only for *S. postica* full abdomen, increasing when the bees were exposed to (I), (IH), (IF), and (HF) ($\chi2 = 31.33$, df = 7, $p < 0.001$). In contrast, there was a reduction in CaE-3 activity when the *S. postica* was exposed to (H) and (IHF). The activity of GST was reduced in the midgut of *A. mellifera* exposure to (IF), (IC), and (IHF) mixtures ($\chi2 = 26.70$, df = 7, $p = 0.003$) while the GST activity on the midgut of *M. scutellaris* decreased after the exposure to (IF) and (HF) ($\chi2 = 17.40$, df = 7, $p = 0.015$). In addition, *S. postica* exposure to (H), (IH), and (IHF) showed a significant reduction in GST activity of the full abdomen ($\chi2 = 26.97$, df = 7, $p < 0.001$). Regarding ALP midgut activity, there was a decrease when *A. mellifera* was exposed to (H), (IH), (IF), (HF), and (IHF) ($\chi2 = 31.80$, df = 7, $p < 0.001$). In contrast, *M. scutellaris* exposed to (IH) and (IHF) showed an increase in ALP enzyme expression ($\chi2 = 18.90$, df = 7, $p = 0.009$). Likewise, the ALP activity increases on the full abdomen of *S. postica* after exposed to (I), (H), (IH), and (IHF) ($\chi2 = 24.98$, df = 7, $p < 0.001$).

Finally, GST abdomen activity was reduced in *A. mellifera* exposed to (I), (H), (IH), (HF), and (IHF) ($\chi2 = 21.94$, df = 7, $p = 0.003$). However, GST and G6PDH abdomen activities were unaltered in *M. scutellaris.* While the G6PDH abdomen activity was reduced in *A. mellifera* exposed to (I), (H), and (IHF) ($\chi2 = 19.90$, df = 7, $p = 0.006$). In addition, the full abdomen of *S. postica*

showed G6PDH activity decrease after exposure to (H), (IHF), and an increase in (I), (IH), and (IF) ($\chi 2 = 30.45$, df = 7, $p < 0.001$).

4. Discussion

4.1 *Toxicological bioassays*

Ecotoxicological bee research has predominantly focused on the honey bee *A. mellifera*, and considering the effects of isolated exposure to pesticides (Rosa-Fontana et al., 2020; Almasri et al., 2020; Dicks et al., 2021), leaving gaps in understanding the impact on native bee populations.

In our study, we exposed three bee species to common pesticides, alone and in mixtures, simulating realistic oral exposures. In this case, we obtained the LT_{50} of bees varied among species and treatments, with *A. mellifera* exhibiting significantly reduced LT_{50} in all treatments, while *S. postica* showed more significant reductions in LT_{50} with (H) and (F) treatments alone and in mixtures. However, *M. scutellaris* experienced reduced LT_{50} primarily with (IH) and (IHF) treatments. These findings suggest varying sensitivities among species to the tested pesticides and mixtures. Sensitivity to pesticides has been compared between *Apis* and 17 species of non-*Apis* bees, including *M. scutellaris* and *S. postica* (Arena and Sgolastra, 2014). Despite this, the complexity of sensitivity cases reported by Arena and Sgolastra (2014) suggests that more studies are needed to better understand the impacts of pesticides on native bee populations. Like the studies of Miotelo et al. (2021), Lourencetti et al. (2023), and Jacob (2019a) that showed higher sensitivity to thiamethoxam on for brasilian native bees *M. scutellaris*, and *S. postica*, and *Tetragonisca angustula* (Latreille, 1811; Hymenoptera: Apidae: Meliponini), respectivally. In addition, we highlight that many factors can influence the sensitivity of bees to pesticides (Johnson 2015), such as food sources, diseases, parasites, weather, and other abiotic and biotic stressors.

Pesticide mixtures generally caused the most significant reduction in LT_{50} for the bee species assessed. In this case, the interactions between specific pesticide combinations, such as (IH) and (HF),

negatively affect *A. mellifera* survival (Almasri et al., 2020). Nevertheless, *M. scutellaris* exhibited superior LT_{50} with (H) treatment, possibly indicating compensatory immune responses. Perhaps, in this case, an increase in immunity genes compensated for the effects of exposure (Castelli et al., 2021).

Research on bee exposure to fungicides and herbicides remains limited compared to insecticides, particularly for native stingless bees. Although fungicide alone did not alter *M. scutellaris* LT_{50}, it led to morphological changes in midgut when combined with other pesticides (Domingues et al., 2020a). Similarly, exposure to fungicides like pyraclostrobin had physiological consequences on *A. mellifera* (Domingues et al., 2020b), impacting gene expression related to vital functions (Zaluski et al., 2020). Previous exposure to other pesticides can weaken the bees and make them more susceptible to the effects of fungicides or to the same herbicides (Almasri et al., 2021a). Regarding *S. postica*, a few published observations address the toxicity of pesticides to this bee species. In our findings, *S. postica* was less negatively affected compared to *A. mellifera* and *M. scutellaris*. However, the LC_{50} found by Jacob et al. (2019b) indicates that *S. postica* is more highly susceptible to acetamiprid, imidacloprid, and triacloprid than *A. mellifera*. In this case, although the results are reliable, complementary studies are needed, especially when dealing with the susceptibility of populations of *S. postica* under the effect of other biotic stressors. The reason is, as mentioned above, that any other stimulus can alter the physiological response of a species.

Ecotoxicology studies of bees using fungicides and herbicides are few compared to bee exposure to insecticides. Data are even scarcer for native stingless bees. Here, although the fungicide did not change the LT_{50} for *M. scutellaris*, these bees showed several changes in midgut morphology when exposed to sublethal concentrations of pyraclostrobin (0.125, 0.025, and 0.005 ng a.i. μL^{-1}), in concentrations higher than those used in this investigation (0.0002 ng a.i μL^{-1}) (Domingues et al., 2020a). Interestingly, although (F) alone did not change the LT_{50} in *M. scutellaris*, the interaction with the other pesticides caused sub-additive effects (IF) and synergistic effects (HF and IHF).

The response to effects of the exposure to fungicides and herbicides remains limited compared to insecticides, particularly for native stingless bees. Although fungicide alone did not alter *M. scutellaris* LT_{50}, it led to morphological changes in midgut (Domingues et al., 2020a; Domingues et al., 2023). Similarly, exposure to fungicides like pyraclostrobin had histopathological consequences on *A. mellifera* (Domingues et al., 2020b), impacting gene expression related to vital functions (Zaluski et al., 2020). Previous exposure to other pesticides can weaken the bees and make them more susceptible to the effects of fungicides or the same herbicides (Almasri et al., 2021a). Regarding *S. postica,* a few published observations address the toxicity of pesticides to this bee species. In our findings, *S. postica* was less negatively affected compared to *A. mellifera* and *M. scutellaris*. However, the LC_{50} found by Jacob et al. (2019b) indicates that *S. postica* is more highly susceptible to acetamiprid, imidacloprid, and triacloprid than *A. mellifera*. In this case, although the results are reliable, complementary studies are needed, especially when dealing with the susceptibility of populations of *S. postica* under the effect of other biotic stressors. The reason is, as mentioned above, that any other stimulus can alter the physiological response.

4.2 *Pesticide interactions*

The interaction between the mixed pesticides produced different responses in each species. *A. mellifera* and *S. postica* showed mostly sub-additive effects, while *M. scutellaris* showed mostly synergistic effects. These species-specific responses underscore the challenge of generalizing results across bee species. Additionally, pesticide interactions can vary depending on concentration and seasonal factors, as shown in studies by Almasri et al. (2020) that endorse the interaction between imidacloprid, glyphosate, and difenoconazole evaluated in winter on honey bees caused different effects according to the concentrations to which the bees were exposed. In this case, the differences in response depend on the concentration as well as the season of the year (Almasri et al., 2020). In addition to the interaction between pesticides, other xenobiotics can accumulate in the body (do Prado

et al., 2020). Moreover, do Prado et al. (2020) concluded that because of this there was an accumulation of the fungicide difenoconazole in *M. scutellaris*.

4.3 *Enzyme expression*

Enzyme biomarkers have been crucial in evaluating bee responses to xenobiotics like pesticides (Carvalho et al., 2013; Kairo et al., 2017; Tavares et al., 2017; Almasri et al., 2020; Almasri et al., 2021a; Decio et al., 2021). Our findings indicate significant alterations in enzyme expression across assessed physiological compartments, particularly with pesticide mixtures. This demonstrated that the mixtures of pesticides can induce disturbances in the detoxification process, in defense against oxidative stress, as well as in metabolism, negatively affecting the physiology of exposed bees. For instance, AChE modulation was evident in *S. postica* exposed to (IF), potentially affecting cognitive functions, behavior, and locomotion activity. Therefore, these factors can contribute to the weakening and subsequent premature death of bees.

The CaE-3 is involved in detoxification, showed activity changes in *S. postica*, correlating with its resilience to LT_{50} alterations. GST activity varied across species and biological compartments, reflecting detoxification capacity and oxidative stress resistance. While GP and GR enzymes exhibited altered activity in *M. scutellaris* exposed to (F), while mixtures reduced their activity in *A. mellifera* and *M. scutellaris*, potentially compromising metabolic processes.

Glutathione-S-transferase plays a vital role in xenobiotic detoxification and oxidative stress resistance (Kairo et al., 2017). The observed alterations in GST activity suggest differential metabolic responses to pesticide exposure, potentially compromising detoxification capacity. Furthermore, GP and GR enzyme activity increased in *M. scutellaris* exposed to treatment (F), whereas *A. mellifera* and *M. scutellaris* exposed to mixtures showed lower activity. This reduction in GP and GR activity could exacerbate oxidative stress, leading to compromised metabolic processes and increased vulnerability to adverse effects. Meanwhile, ALP is crucial in digestion and immunity, showing

varied responses, with reduced activity in *A. mellifera* exposed to mixtures, while *M. scutellaris* and *S. postica* exhibited increased activity in specific treatments. G6PDH activity fluctuated across treatments, indicating antioxidant function and metabolic adaptation.

Despite the complexity of enzyme responses, GST and CaE-3 emerged as sensitive indicators across all analyzed compartments. GST particularly proved effective as a biomarker for *A. mellifera* and stingless bees, suggesting its utility in future studies involving other stingless bee species.

5. Conclusions

In conclusion, our study revealed significant variations in the mean lethal time (LT_{50}) among bee species exposed to different pesticides, either alone or in mixtures. Pesticide interactions yielded diverse responses among the bee species, with *A. mellifera* and *S. postica* mostly exhibiting sub-additive effects, while *M. scutellaris* displayed predominantly synergistic effects. Enzyme expression analysis revealed complex responses to pesticide exposure, with alterations observed across species and enzyme types. Notably, GST and CaE-3 emerged as sensitive biomarkers across all biological compartments assessed, with GST particularly showing promise for future stingless bee studies. These findings underscore the importance of considering species-specific responses and enzyme biomarkers in ecotoxicological assessments, highlighting the need for further research to better understand the impacts of pesticides on native bee populations.

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Declaration of competing interest

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

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Table 1. Mean of lethal time (LT50) in days and confidence interval (CI) of *Apis mellifera*, *Melipona scutellaris* and *Scaptotrigona postica* after oral chronic exposure to sucrose solution treated with imidacloprid, glyphosate, and pyraclostrobin, isolates or mixed.

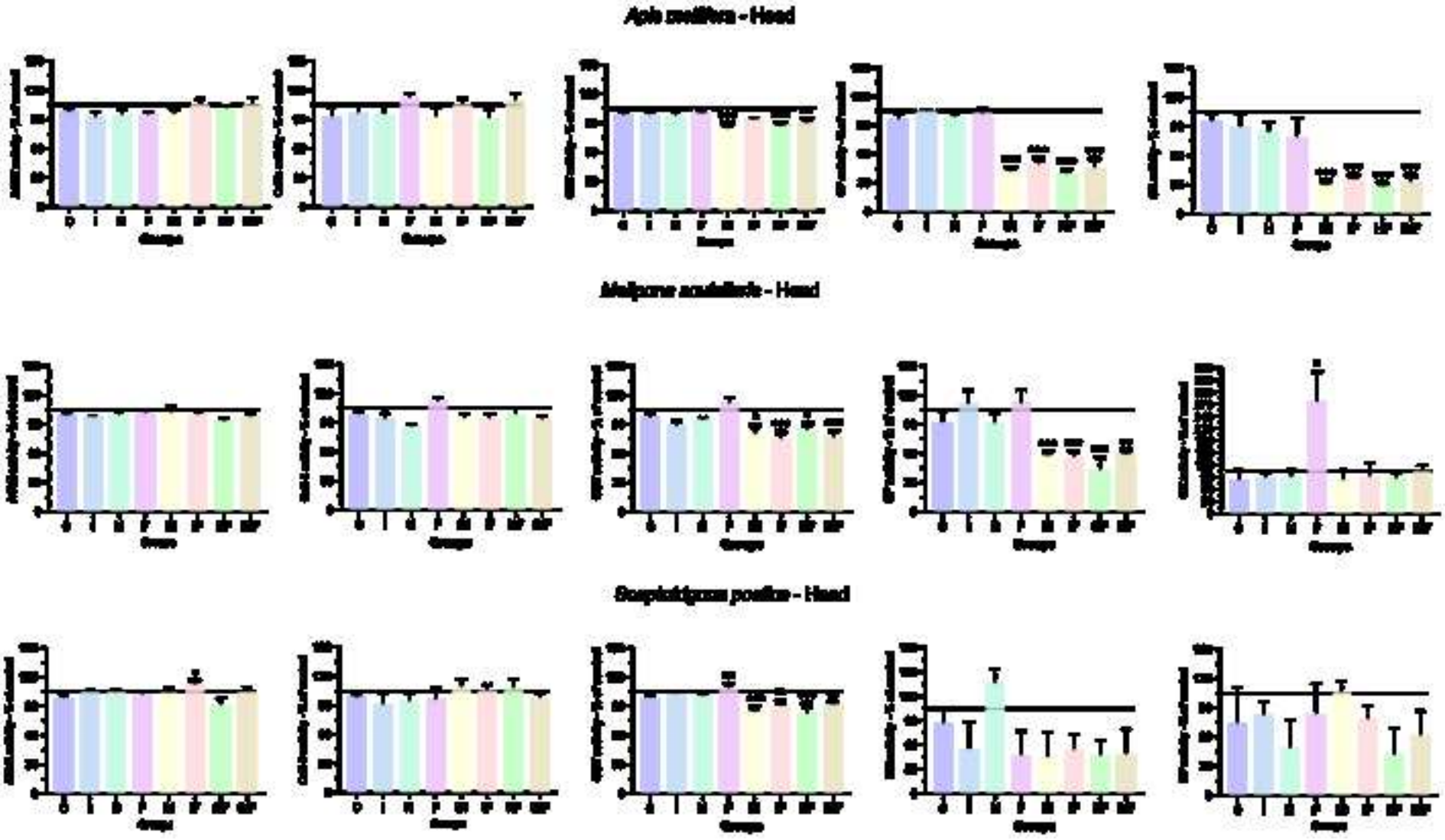
Treatment / Groups	**LT50 (95% CL) [*A. mellifera*]**	**LT50 (95% CL) [*S. postica*]**	**LT50 (95% CL) [*M.scutellaris*]**
Control (C)	**5.57** (5.18 - 5.95) aA	**9.52** (8.20 - 10.8) aC	**15.7** (14.3 - 17.2) aB
Imidacloprid (I)	**4.24** (3.77 - 4.68) bA	**8.95** (8.34 - 9.54) aC	**11.7** (10.7 - 12.6) bB
Glyphosate (H)	**4.38** (3.59 - 5.09) bA	**6.80** (6.04 - 7.51) bcC	**20.6** (18.5 - 23.3) cB
Piraclostrobyn (F)	**2.76** (2.03 - 3.36) cA	**6.12** (5.09 - 7.05) bC	**13.2** (11.7 - 14.8) abB
Imidacloprid + Glyphosate (IH)	**3.00** (2.50 - 3.43) cA	**8.24** (7.60 - 8.84) aB	**8.94** (7.97 - 9.87) dB
Imidacloprid + Piraclostrobyn (IF)	**4.48** (3.87 - 5.04) bA	**10.2** (9.51 - 10.9) aC	**12.6** (11.2 - 14.1) bB
Glyphosate + Piraclostrobyn (HF)	**3.02** (2.70 - 3.31) cA	**7.98** (7.21 - 8.72) acC	**11.5** (10.3 - 12.8) bB
Imidacloprid + Glyphosate + Piraclostrobyn (IHF)	**4.49** (4.00 - 4.96) bA	**6.79** (6.24 - 7.32) bcB	**9.50** (7.01 - 11.8) bdB
p-value	<0.001	<0.001	<0.001
df	7	7	7
χ^2	46.16	30.00	48.74

Lowercase letters by column values and higher case letters by row values in parentheses indicated significant differences. Significant differences between values are represented by different letters by contrasts from log-rank test followed by a post hoc test, with fdr correction ($p \leq 0.05$). Pesticides groups corresponding = Control with sucrose solution (C); imidacloprid (I); glyphosate (H); pyraclostrobin (F); imidacloprid + glyphosate (IH); imidacloprid + pyraclostrobin (IF); glyphosate + pyraclostrobin (HF); and imidacloprid + glyphosate + pyraclostrobin (IHF).

Table 2. Mortality rates in days, corrected mortality, expected mortality, and pesticide interaction rates of pesticides on *Apis mellifera*, *Melipona scutellaris*, and *Scaptotrigona postica* exposed to isolated and mixed pesticides.

Treatment / Group		Mortality rate (%) ± SD	Corrected mortality	Expected mortality	IR	Effect	Statistical significance LT50 ($p < 0.05$)
Apis mellifera	C	55.55±2.88					a
	I	75.55±3.20	20.00				b
	H	57.77±2.30	2.22				b
	F	88.88±4.60	33.33				c
	IH	73.33±2.00	17.17	22.22	0.80	sub-additivity	c
	IF	68.88±0.57	16.66	33.33	0.25	sub-additivity	b
	HF	91.11±1.52	35.55	35.55	1.00	additivity	c
	IHF	68.88±2.08	13.33	55.55	0.24	sub-additivity	b
Melipona scutellaris	C	40.00±1.73					a
	I	63.33±1.52	23.33				b
	H	33.33±3.21	-6.66				c
	F	50±2.00	10				ab
	IH	76.66±1.15	36.66	16.66	2.20	synergistic	d
	IF	56.66±2.08	16.66	33.33	0.50	sub-additivity	b
	HF	70±2.00	30	3.33	9.00	synergistic	b
	IHF	70±4.35	30	26.66	1.12	synergistic	b
Scaptotrigona postica	C	51.11±2.30					
	I	62.22±2.08	22.22				a
	H	80±4	40.00				a
	F	73.33±6.08	33.33				bc
	IH	82.22±2.51	42.22	62.22	0.67	sub-additivity	b
	IF	66.66±3.6	26.66	55.55	0.48	sub-additivity	a
	HF	60±4.58	20.00	73.33	0.27	antagonistic	ac
	IHF	77.77±1.52	37.77	95.55	0.39	sub-additivity	bc

Mortality rates (% ± SD) on the lethal time (LT50) of *Apis mellifera*, *Melipona scutellaris* and *Scaptotrigona postica* after oral chronic exposure to imidacloprid, glyphosate and pyraclostrobin (isolated or mixed); pesticides were exposed at the residual concentrations determined for BeeREX model. Mean values followed by different letters are significantly different by contrasts from a binomial generalized linear model ($p \leq 0.05$). Pesticides groups corresponding = Control with sucrose solution (C); imidacloprid (I); glyphosate (H); pyraclostrobin (F); imidacloprid + glyphosate (IH); imidacloprid + pyraclostrobin (IF); glyphosate + pyraclostrobin (HF); and imidacloprid + glyphosate + pyraclostrobin (IHF). The corrected and expected mortality was calculated from the sum of the values of the corrected mortality of the isolated pesticides. The interaction ratio (IR) was calculated by: Mortality rate/Expected mortality.



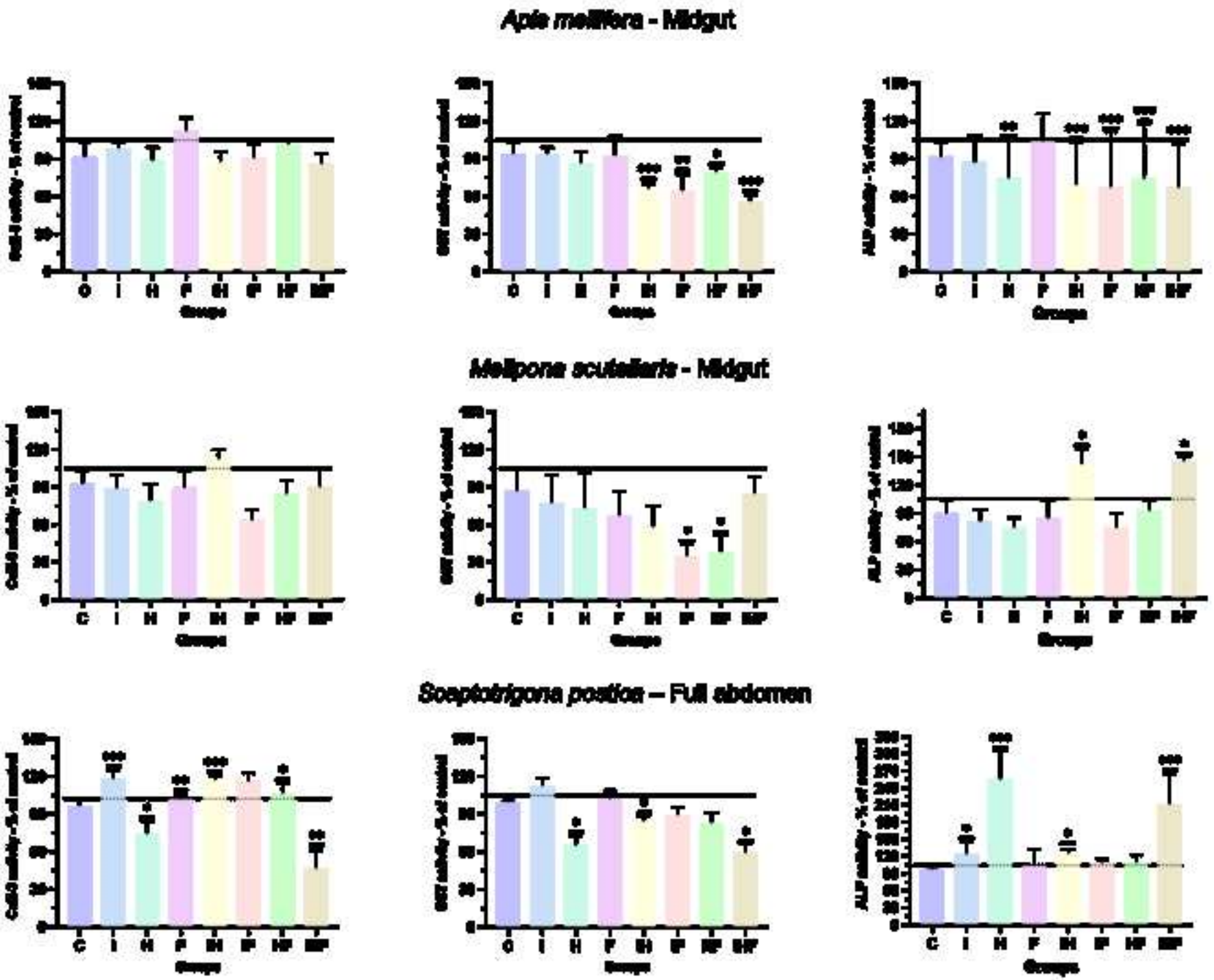
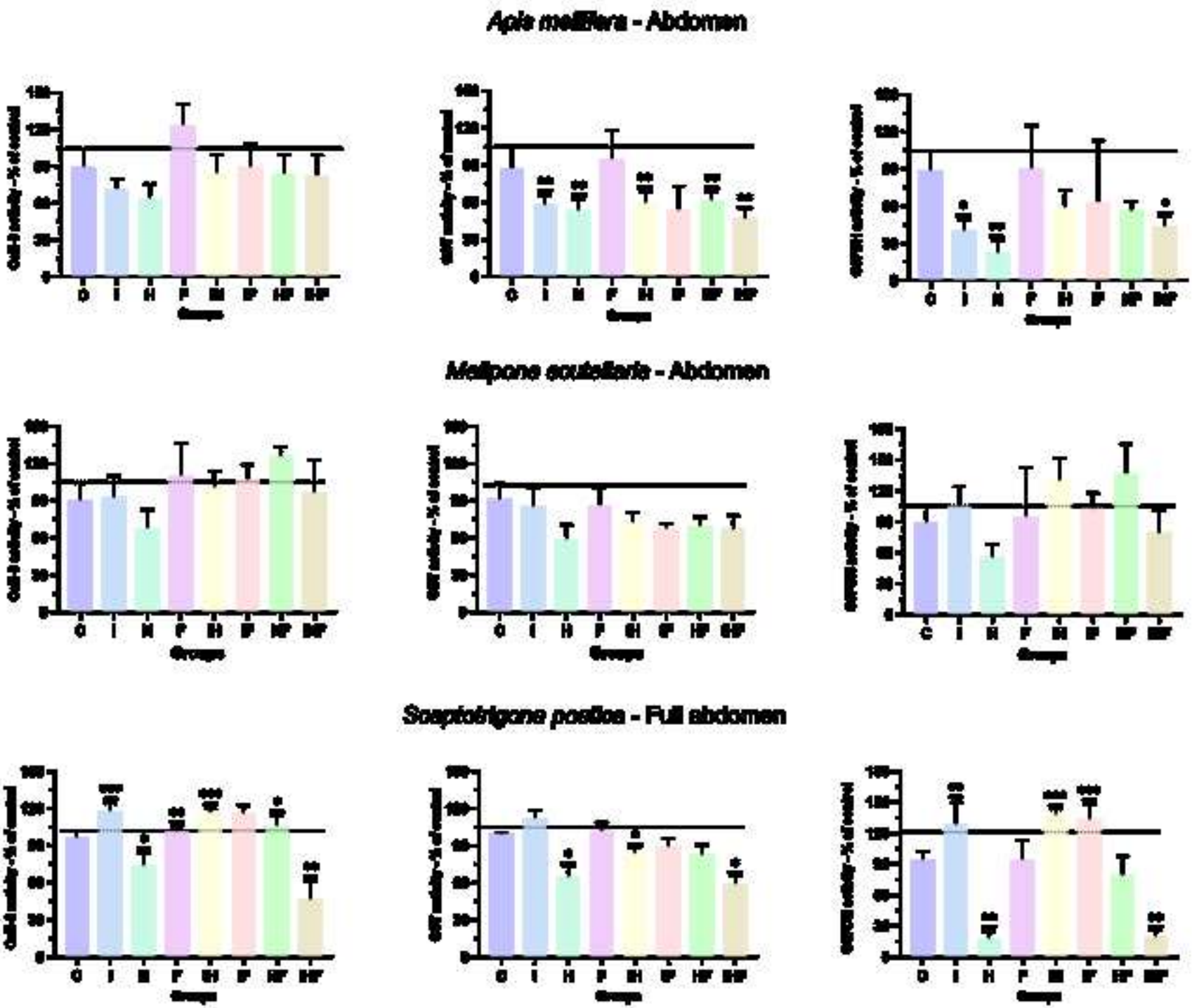


Figure 1. Physiological effects of different pesticides isolated or in mixed on head. Different physiological biomarkers were followed: Acetylcholinesterase (AChE), Carboxylesterase (CaE-3), Glutathione-S-transferase (GST), Glutathione peroxidase (GP) and Glutathione reductase (GR) were extracted from head of *A. mellifera*, *M. scutellaris* and *S. postica*. The bees were exposed to pesticides alone or in mixtures: imidacloprid (I), glyphosate (H), pyraclostrobin (F) imidacloprid + glyphosate (IH), imidacloprid + pyraclostrobin (IF), glyphosate + pyraclostrobin (HF) and imidacloprid + glyphosate + pyraclostrobin (IHF) at concentrations of of 0.7 µg/L (I), 1.8 µg/L (H) and 0.2 µg/L. To make the data comparable and easy to interpret, the values were expressed as percentages of the respective controls. Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.0001$).

Figure 2. Effects of different pesticides alone or in mixtures on the enzymatic activity of Acetylcholinesterase (AChE), Carboxylesterase (CaE-3), Glutathione-S-transferase (GST), Glutathione peroxidase (GP) and Glutathione reductase (GR) extracted from midgut of *A. mellifera*, *M. scutellaris* and from full abdomen of *S. postica*. The bees were exposed to pesticides alone or in mixtures for the time in days referring to the mean lethal time LT_{50} (previously determined) for each group of pesticide exposure, imidacloprid (I), glyphosate (H), pyraclostrobin (F) imidacloprid + glyphosate (IH), imidacloprid + pyraclostrobin (IF), glyphosate + pyraclostrobin (HF) and imidacloprid + glyphosate + pyraclostrobin (IHF) at concentrations of 0.7 µg/L (I), 1.8 µg/L (H) and 0.2 µg/L when isolated or in mixtures. To make the data comparable and easy to interpret, the values were expressed as a percentage from the control group. Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.0001$).

Figure 3. Effects of different pesticides alone or in mixtures on the enzymatic activity of Acetylcholinesterase (AChE), Carboxylesterase (CaE-3), Glutathione-S-transferase (GST), Glutathione peroxidase (GP) and Glutathione reductase (GR) extracted from abdomen of *A. mellifera*, *M. scutellaris* and from full abdomen of *S. postica*. The bees were exposed to pesticides alone or in mixtures for the time in days referring to the mean lethal time LT_{50} (previously determined) for each group of pesticide exposure, imidacloprid (I), glyphosate (H), pyraclostrobin (F) imidacloprid + glyphosate (IH), imidacloprid + pyraclostrobin (IF), glyphosate + pyraclostrobin (HF) and imidacloprid + glyphosate + pyraclostrobin (IHF) at concentrations of 0.7 µg/L (I), 1.8 µg/L (H) and 0.2 µg/L when isolated or in mixtures. To make the data comparable and easy to interpret, the values were expressed as a percentage from the control group. Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.0001$).



Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: