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Assessing the effects of a commercial fungicide and an herbicide, alone and in combination, on *Apis mellifera*: Insights from biomarkers and cognitive analysis

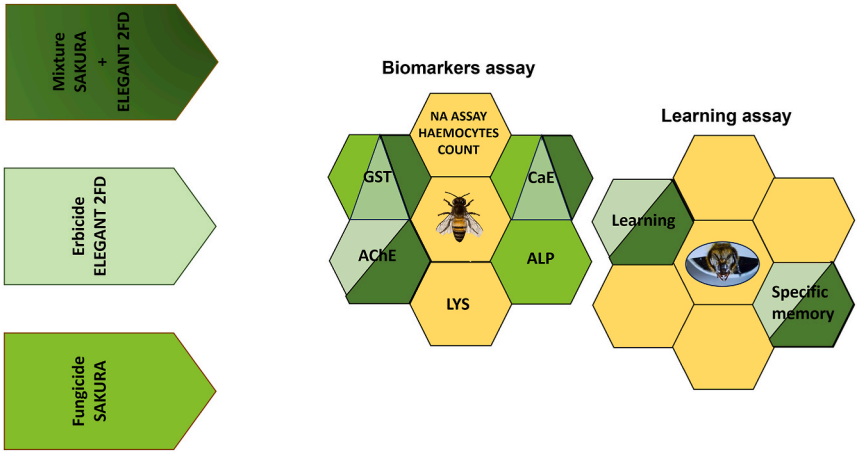
Agata Di Noi [a], Ilaria Caliani [b,*], Antonella D'Agostino [c], Giampiero Cai [a], Marco Romi [a], Tommaso Campani [b], Federico Ferrante [d], David Baracchi [e], Silvia Casini [b]

[a] Department of Life Sciences, University of Siena, Via Mattioli, 4, Siena, 53100, Italy
[b] Department of Physical, Earth and Environmental Sciences, University of Siena, Via Mattioli, 4, Siena, 53100, Italy
[c] Department of Economics and Statistics, University of Siena, Piazza S. Francesco 7, 53100 Siena, Italy
[d] Department of Ecological and Biological Science, Tuscia University, Largo dell'Università s.n.c., 01100 6, Viterbo, Italy
[e] Department of Biology, University of Florence, Via Madonna del Piano, 6, Sesto Fiorentino, 50019, Italy

HIGHLIGHTS

- Effects of commercial fungicide and herbicide, single and in combination, were evaluated.
- Mortality assay, biomarkers and learning and memory tests were performed on honey bees.
- Neurotoxic effects after both mixture treatments could be attributed to the herbicide.
- Herbicide and pesticide mixtures compromise honey bees' behaviour.

GRAPHICAL ABSTRACT



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ABSTRACT

Agrochemicals play a vital role in protecting crops and enhancing agricultural production by reducing threats from pests, pathogens and weeds. The toxicological status of honey bees can be influenced by a number of factors, including pesticides. While extensive research has focused on the lethal and sublethal effects of insecticides on individual bees and colonies, it is important to recognise that fungicides and herbicides can also affect bees' health. Unfortunately, in the field, honey bees are exposed to mixtures of compounds rather than single substances. This study aimed to evaluate the effects of a commercial fungicide and a commercial herbicide, both individually and in combination, on honey bees. Mortality assays, biomarkers and learning and memory

* Corresponding author.
E-mail address: caliani4@unisi.it (I. Caliani).

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Commercial pesticides
Integrated approach

tests were performed, and the results were integrated to assess the toxicological status of honey bees. Neurotoxicity (acetylcholinesterase and carboxylesterase activities), detoxification and metabolic processes (glutathione S-transferase and alkaline phosphatase activities), immune system function (lysozyme activity and haemocytes count) and genotoxicity biomarkers (Nuclear Abnormalities assay) were assessed. The fungicide Sakura® was found to activate detoxification enzymes and affect alkaline phosphatase activity. The herbicide Elegant 2FD and the combination of both pesticides showed neurotoxic effects and induced detoxification processes. Exposure to the herbicide/fungicide mixture impaired learning and memory in honey bees. This study represents a significant advance in understanding the toxicological effects of commonly used commercial pesticides in agriculture and contributes to the development of effective strategies to mitigate their adverse effects on non-target insects.

1. Introduction

Agrochemical products play a crucial role in agricultural systems worldwide. These compounds help safeguard crops against pests and pathogens and eliminate weeds, leading to increased agricultural production (Alexandratos and Bruinsma, 2012; Carvalho, 2017). Insecticides, including organochlorines, organophosphates, carbamates, pyrethroids, neonicotinoids, and biopesticides, are widely used to control insect pests. In addition to insecticides, fungicides, herbicides, nematicides, rodenticides and acaricides, designed to target other organisms, are used. Herbicides, fungicides and insecticides account for more than 95% of the global pesticide market (Delso et al., 2022). Over the years, the use of synthetic and biological herbicides has expanded the most, followed by insecticides and fungicides (Cappa et al., 2022; Carvalho, 2017). Unfortunately, the residues of these agrochemical compounds can spread in the environment, causing significant contamination and harm to terrestrial ecosystems (Cappa et al., 2022; Carvalho, 2017).

Pesticides have a significant impact on various terrestrial organisms, including earthworms, nematodes, and insect pollinators such as honey bees, all of which are essential for the ecosystem services they provide (Cappa et al., 2022; Daam et al., 2011). Honey bees in particular can be affected by a variety of stressors, including pathogens, habitat loss and poor nutrition and the presence of pesticides (Goulson et al., 2015; Potts et al., 2010). Bees can come into contact with these compounds in a variety of ways, including airborne particles like dust and spray droplets, volatile compounds diluted in the air, contaminated surfaces, nesting materials, and food like nectar, pollen and water. Claudianos et al. (2006) hypothesise that honey bees are more vulnerable to pesticides because they possess a limited number of genes, with respect to other insects. Haplodiploid genetic system of honey bees may also contribute to reduced genetic diversity specifically related to xenobiotic detoxifying systems. However, it is worth noting that the low number of detoxification genes in honey bees could be due to their social lifestyle and their organisational, behavioural and physiological adaptations (termed “social immunity”) to prevent the establishment and spread of parasites and pathogens (Baracchi and Tragust, 2017; Cremer et al., 2007; Otti et al., 2014). Numerous studies have focused on the lethal and sublethal effects of insecticides on honey bees at both individual (Al Naggar et al., 2015; Badawy et al., 2015; Qi et al., 2020)) and colony level (Imran et al., 2019; Ingram et al., 2015; Monchanin et al., 2019). In addition, fungicides and herbicides have been shown to affect honey bee’s health status through behavioural, molecular, biochemical and cellular changes (Di Noi et al., 2021); these effects could develop into irreversible physiological alterations and permanent damage to honey bee populations (Caliani et al., 2021a).

In the environment, it is common to find not only individual compounds, but also mixtures of pesticides, which honey bees are exposed to. These mixtures may have similar modes of action or synergistic, antagonistic or additive interactions (Piggott et al., 2015). Various studies have evaluated the effects of mixtures of different insecticides (Christen et al., 2017; Wang et al., 2020, 2021; Yao et al., 2018a, 2018b; Zhu et al., 2017a) and the interactions between insecticides and fungicides (Bjergager et al., 2017; Colin and Belzunces, 1992; Iwasa et al., 2004; Meled et al., 1998; Schmuck et al., 2003; Thompson et al., 2014; Zhu et al., 2017a, 2017b). However, despite the high likelihood of honey bees being exposed to mixture of fungicides and herbicides, there is a significant gap in the assessment of these mixtures.

Under natural conditions, honey bees are exposed to commercial pesticides containing both active ingredients and co-formulants or adjuvants, also known as “inerts”, which are added to enhance the absorption and stability of the active ingredient (Travlos et al., 2017). These co-formulants can be toxic to non-target species (Mesnage and Antoniou, 2018), as also reported in several studies on adjuvants in commercial formulations (Nagy et al., 2020). Up to date, to the best of our knowledge, the study of the effects of commercial formulations used in agriculture on honey bees are extremely limited. Recently, the European Food Safety Authority (EFSA Working GroupDraft Guidance Document) published a guidance document for the risk assessment of plant protection products (PPPs), highlighting the importance of evaluating the effects of mixtures on domestic and wild bees, with a particular attention to synergistic effects (EFSA, Draft).

Biomarkers are sensitive and useful tools to study the sublethal effects of pollutants on the ecotoxicological health status of pollinators. They provide a better understanding of the effects of anthropogenic stressors (Cajaraville et al., 2000; Caliani et al., 2021a, 2021b; Campani et al., 2017; Sanchez and Porcher, 2009; Tlili et al., 2013). In honey bees, biomarkers have been developed to assess the exposure to and effects of various contaminants (Badawy et al., 2015; Caliani et al., 2021a; Carvalho et al., 2013; Roat et al., 2017). Yet, so far, only a limited number of studies have used a multi-biomarker approach to gain a thorough understanding of the extent of chemical stress caused by the exposure of honey bees to pesticide active ingredient, both individually and in combination (Badiou-Bénéteau, 2013; Badiou-Bénéteau et al., 2012; Qi et al., 2020; Yao et al., 2018a). Nevertheless, only Caliani et al. (2021a) have investigated the sublethal effects of commercial PPPs using a biomarker approach. To date, no data are available on the effects of mixtures of commercial pesticides using biomarker assays.

A wide variety of enzymes and of cellular processes are commonly used as biomarkers in many species. For instance, acetylcholinesterase (AChE) is a key enzyme that controls and modulates neural transmission (Badiou et al., 2008) and can be inhibited not only by organophosphorus and carbamate insecticides, but also by other contaminants such as fungicides and metals (Boily et al., 2013; Caliani et al., 2021a; Carvalho et al., 2013; Frasco et al., 2005; Fukuto, 1990), making it a sensitive biomarker of neurotoxicity. Carboxylesterases (CaEs) are hydrolases catalysing the reactions of a wide range of aliphatic/aromatic esters and choline esters, as well as some xenobiotics (Dauterman and Hodgson, 1990). They are involved in phase I of detoxification processes but also have a defensive function, protecting AChE from its inactivation (Jackson et al., 2013; Yan et al., 2009). Glutathione S-transferase (GST) is an enzyme involved in phase II biotransformation processes, in fact it is responsible for detoxifying various contaminants and most likely contributes to cellular protection against oxidative damage (Babczyńska et al., 2006; Barata et al., 2005). The lipophilic compounds are able to induce GST activity, as GST is involved in the conjugation of a wide variety of drugs and metabolites with GSH (Letelier et al., 2010). Alkaline phosphatase (ALP) is a member of a family of enzymes involved

in digestive processes, cell signalling, and the transport of metabolites and antioxidants through the hydrolysis of phosphate groups (Bounias et al., 1996). Although ALP is not involved in detoxification processes, a change in its activity has been observed following exposure of honey bees to pesticides and metals (Bounias et al., 1996; Caliani et al., 2021a), making it a good biomarker of exposure to these compounds (Suresh and Reju, 1993). The immune system of bees includes a humoral component, with enzymes such as lysozyme and phenoloxidase, and a cellular component, characterised by haemocytes. While lysozyme (LYS) acts against bacteria, by degrading the bacterial cell wall, with its synthesis occurring throughout development (Lazarov et al., 2016), the haemocytes (prohemocytes, plasmatocytes, granulocytes, oenocytoids and macrophage-like cells) are involved in phagocytosis, encapsulation, nodulation, and wound repair processes (Marmaras and Lampropoulou, 2009; Strand, 2008)). Among the different haemocytes, plasmatocytes and granulocytes are the most abundant circulating cell types (Giglio et al., 2015). Granulocytes have a relevant role in phagocytosis, being the first cells to come into contact with a foreign body at the onset of nodule formation, while plasmatocytes in encapsulation response (Richardson et al., 2018). When in contact with the foreign body, granulocytes release their granular content, attracting plasmatocytes or helping them to build the capsule (Ribeiro and Brehélin, 2006). The number of circulating haemocytes reflects the ability of organisms to cope with immunogenic challenges (Doums et al., 2002; Kraaijeveld et al., 2001). Exposure to genotoxic compounds may alter the DNA integrity of an organism and Nuclear Abnormalities (NA) assay is a valid tool to assess the presence of genetic damage, counting the number and types of abnormalities of haemocyte nuclei. There are very few studies on this test applied to *Apis mellifera*. Caliani et al. (2021a) observed a significant increase in nuclear abnormalities after treatment with 0.1 g/L and 2.5 g/L CdSO4, and 200 g/L Amistar®Xtra, almost double the control and with a frequency of 50‰.

In the context of above, which emphasises the importance of a better understanding of potential negative effects of commercial pesticide use on bees, we investigated the toxicological effects of a commercial fungicide (Sakura®) and a commercial herbicide (Elegant 2FD), alone and in combination, on honey bees. We performed a mortality assay, and evaluated detoxification and metabolic alterations (GST and ALP activities, respectively), immune system (LYS activity and haemocytes count) and genotoxicity responses (NA assay), neurotoxicity effects (AChE and CaEs activities) and learning and memory abilities (Proboscis Extension Reflex - PER). The biomarkers used in this study were selected to provide a complete overview of the possible effects of these pesticides on *Apis mellifera*, which is not the target of these compounds. For this reason, we chose more general biomarkers, such as GST and CaE, which give an indication of detoxification activities, and more specific ones, such as AChE and NA assay, to observe if fundamental processes of the organisms were damaged. Neurotoxicity and DNA damage have been observed in honey bees following exposure to compounds with similar modes of action to ours, such as cyproconazole (Caliani et al., 2021a). Neurotoxicity was also observed in non-target vertebrates exposed to 2,4 D (United States Environmental Protection Agency), the active ingredient of our herbicide, whereas only the LD50 assay was performed on *Apis mellifera*. The ALP activity was already been used as a biomarker of exposure to pesticide toxicity. We also focused on learning and memory because the study of cognition provides a sensitive approach to investigates toxicological and sublethal effects (Cappa et al., 2022; Desneux et al., 2007; Tosi et al., 2022). As central-place foraging animals, social pollinators rely heavily on learning and memory, which are fundamental requirements to optimise foraging and ensure colony success (Baracchi, 2019; Klein et al., 2017). Consequently, the detection of even minor impairments in these cognitive abilities would signal their vulnerability and place them at significant risk (Klein et al., 2017).

2. Material and methods

2.1. Chemicals

All of the following compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA): monobasic and dibasic sodium phosphate, sodium chloride (NaCl), Triton X-100, protease inhibitor cocktail powder; acetylthiocholine iodide (AcSCh.I), 5,5-dithiol-bis (2, nitrobenzoic acid) (DTNB); 1-chloro-2,4-dinitrobenzene (DNCB), reduced L-glutathione (GSH); Fast Garnet GBC, sodium dodecyl sulphate (SDS), α-naphthyl acetate (α-NA); tris-hydroxy-methyl-aminomethane (Tris), magnesium chloride ($MgCl_2$), *p*-nitrophenyl phosphate (*p*-NPP); *Micrococcus lysodeikticus* solution, egg whites from chicken (HEL); monobasic potassium phosphate and bovine serum albumin (BSA) for enzymatic assays. Also BioRad Protein stain (BioRad, Segrate, Italy) was used for enzymatic assays. For the NA assay, Diff-Quick dye from Bio-optica, Milano, Italy was used. Sakura® was obtained by Nufarm S.A.S. (France) while Elegant 2FD was obtained by Adama Agan L.t.d. (Israel).

2.2. Honey bees

For the biomarker's experiments, honey bee foragers (*Apis mellifera ligustica*) were collected from the hives of a beekeeper located close to a nature reserve area (Pisa, Tuscany, Italy). The bees were collected on the day before the experiment and placed in seven cages (75 cm × 75 cm x 115 cm, Bug-Dorm-2400 Insect Rearing Tent, MegaView Science Co., Ltd., Taiwan), to rest overnight at 25 °C and 60% relative humidity with honey and water ad libitum. A leafless branch of *Prunus spinosa* (Rosaceae) was placed in the cages as a support for the bees.

For the mortality and the behavioural experiments, honey bee foragers were collected when they landed on a feeder containing a 50% sucrose solution (w/w), located about 10 m away from a small apiary (Department of Biology, University of Florence). The bees were immediately transported to the laboratory and randomly assigned to either the experimental or control groups and processed according to the experiment.

2.3. Pesticides

The pesticides used in the experiments were commercial formulations; the fungicide Sakura® and the herbicide Elegant 2FD. These two pesticides are widely used in wheat crops and are often sprayed in overlapping periods during the uprooting stage of the plant. Bees can be accidently exposed to these two chemicals because the pesticides can reach flowers near wheat crops, where pollinators collect pollen and nectar. In this way, foraging bees could transport the pesticides to the hive, exposing the entire colony, from larvae to the queen, to the risk of contamination.

The active ingredients of Sakura® are bromuconazole (15,86%) and tebuconazole (10,17%), while Elegant 2FD consists mainly of 2,4D (42,3 g/100 g of product) and florasulam (0,6 g/100 g of product). The commercial pesticides mentioned above also contain co-formulants, the composition of which is not specified on the labels.

In all experiments, bees were exposed topically to: 1) the fungicide Sakura® at two concentrations, 200 g/L and 400 g/L; 2) the herbicide Elegant 2FD at two concentrations, 225 g/L and 450 g/L; 3) the mixture of the pesticides at two concentrations, Sakura® 200 g/L + Elegant 2FD 225 g/L and Sakura® 400 g/L + Elegant 2FD 450 g/L; 4) acetone 80%, considered as control. All the pesticides were dissolved in 80% acetone. The topical treatment was chosen because these compounds are sprayed on crops and therefore contact is one of the main routes of exposure for honey bees.

The lowest concentrations used were those recommended for field use in cereal crops, while the highest concentrations were twice those recommended for field use, which could represent a potential overexposure of honey bees to these compounds.

2.4. Exposure protocols

2.4.1. Experiment for mortality assay

According to standard methods for toxicology research in *A. mellifera* (Williams et al., 2013), bees were collected and treated topically on the thorax with experimental solutions or the solvent solution as described above. Subsequently, they were housed in groups of 10 individuals in 120 ml Plexiglas cages. Each cage was equipped with a 20 ml syringe providing unrestricted access to a 50% sucrose solution. To facilitate easy access to food while preventing any sugar solution leakage, the syringes were deprived of the cone luer (Carlesso et al., 2020). A total of 490 cages, each containing 10 bees, were used (7 cages per group). Mortality and sucrose consumption were measured daily for ten days by counting dead bees and weighing the syringes, respectively. Daily sucrose consumption was normalized to the number of live bees in each cage on that day (Carlesso et al., 2020). Bees were kept in a dark and humid place (~60%) at 23 ± 2 °C throughout the assay.

2.4.2. Experiment for biomarker analysis

On the day of the experiment, the honey bees were mildly anesthetized with CO_2 and 2 μL of each contaminant solution, was applied to the dorsal thorax of bees from each experimental group using a Burkard hand micro applicator equipped with a one-mL syringe (Bedini et al., 2017). Each group of treated bees (50 each) was put in a separate cage for four days before biomarkers analysis. At the end of the exposure, bees were anesthetized in ice (4 °C) for 30 min before being handled. When asleep, the back of the thorax was incised with a scalpel and the haemolymph was collected with a micropipette and used to perform the haemocytes differential count and Nuclear Abnormalities (NA) assay. The midgut was then removed with tweezers and the head was separated from the rest of the body. The samples, head and midgut, were immediately frozen and stored at -80 °C. The haemolymph was used for the differential haemocytes count and NA assay; the extract from head nervous tissues were used to evaluate esterase activity (AChE and CaE). GST, ALP and lysozyme activities were evaluated on midgut extracts. For the preparation of each extract, tissue samples from 3 specimens were pooled and supernatants were obtained according to methods by Caliani et al. (2021a). All the enzymatic assays were performed according to Caliani et al. (2021a). Acetylcholinesterase (AChE) activity was measured at 412 nm in a medium containing 0.1 M sodium phosphate buffer (pH 7.4), 10 mM DTNB, 41.5 mM acetylthiocoline. The carboxylesterase (CaE) enzyme was quantified at 538 nm adding 100 mM sodium phosphate buffer (pH 7.4) to the head extract and incubating at 25 °C for 5 min. The reaction was started by adding 0.4 mM α-NA as a substrate and stopped by adding 1.5% SDS and 0.4 mg/L Fast Garnet GBC. GST activity was measured at 340 nm in a medium containing 8 mM GSH (reduced glutathione), 8 mM DNCB (2,4-dinitrochlorobenzene) as substrate, and 100 mM sodium phosphate buffer (pH 7.4). ALP was monitored at 405 nm in a medium containing 100 mM $MgCl_2$, 100 m Mp-nitrophenyl phosphate as substrate, and 100 mM Tris-HCl pH 8.5. AChE, CaE, GST and ALP activities were quantified spectrophotometrically with a Cary UV 60 Agilent spectrophotometer. LYS activity was measured using a turbidity test and the absorbance was monitored at 450 nm with a Microplate Reader (Multiskan Skyhigh Thermo Scientific). Protein concentrations were estimated using the method described by Bradford (1976), with bovine serum albumin as the standard.

The NA assay and the differential count of granulocytes and plasmatocytes were performed on the same slide for each sample. 10 μL of two honey bees' haemolymph were placed on a slide previously spread with poly-lysine (2.5 mg/mL). The slides were left to dry and stained with Diff-Quick stain (Bio-Optica).

The NA assay was performed following the procedure according to Pacheco and Santos (1997) with some modifications. Cells were counted using an immersion light microscope (Olympus BX41) and abnormalities were assigned to one of the following categories according to Caliani et al. (2021a): micronuclei, lobed nuclei, segmented nuclei and kidney-shaped nuclei. Apoptotic cells were also counted. The results were expressed as the number of nuclear abnormalities/1000 cells.

Granulocytes and plasmatocytes were counted following Sapcaliu et al. (2009). One thousand blood cells were counted, and the results expressed as the number of cells/1000.

2.4.3. Experiment for learning and memory assay

On the day of the experiment, immediately after collection, the honey bees were cold anesthetized in ice for 5 min, and 2 μL of every contaminant solution was applied on the dorsal thorax of the bees as previously described (see 2.4.2 section). The animals were put in separate cages (50 animals each) and kept in a dark and humid place (~60%) at 23 ± 2 °C for four days. Immediately after exposure, cold-anesthetized bees were individually placed inside 3D-printed tubes with a strip of duct tape placed between their head and thorax (Balzani et al., 2022). A small drop wax was applied behind the head to prevent free movement, except for their antennae and mouthparts. The bees were then fed with 5 μl of 50% sucrose solution to equalize their level of hunger and rested for 2 h in a dark and humid place (~60%) at room temperature (23 ± 2 °C). To test whether fungicides and herbicides affect bees' learning and memory abilities, the bees were subjected to differential olfactory conditioning of the proboscis extension reflex (PER) (Balzani et al., 2022). The bees were trained to discriminate a rewarding odor (CS+) from a non-rewarding odor (CS-) during ten trials (5 CS + trials and 5 CS- trials). Both odors (nonanal and 1-hexanol, Sigma Aldrich, Italy) were used either as CS + or CS- in a counterbalanced design. Odor presentations were spaced by 10 min and were pseudorandomized according to their contingency (i.e., rewarded vs. unrewarded) so that the same stimulus was never presented more than twice consecutively (Balzani et al., 2022). An odor releaser controlled by the microcontroller board Arduino Uno was used to deliver the two odors to the bees. At the beginning of the training, a bee was placed 2 cm in front of the odor releaser, which provided a continuous airflow of clean air. After 10 s of familiarization, a CS (either 1-hexanol or nonanal) was provided for 4 s and continuously carried away through an exhaust system. In the reinforced trials, after 3 s from the odor onset, the bee was stimulated with a 30% sucrose solution (unconditioned stimulus, US) by means of a toothpick on the antennae and proboscis for 4 s. To account for the existing laterality in sucrose responsiveness, only the same antennae (left) was stimulated using a wooden toothpick during each presentation (Baracchi et al., 2018). The bee was left in position with clean airflow for a further 10 s. In the unreinforced trials, no US was provided to the bee after the onset of the CS-. For each bee, PER occurrence during the CS presentation was recorded over the 10 conditioning trials. Bees that showed PER to the first presentation of the CS+ were immediately discarded as learning could not be addressed (Balzani et al., 2022). After conditioning, the bees were kept under the same condition (in the dark, 60% humidity, 23 ± 2 °C) and tested 2 and 24 h later for short-term and mid-long term memory retention respectively. During the test, each bee was presented with both CS+ and CS- without reinforcement with an inter-trial interval of 10 min, and the PER occurrence was recorded for each individual. The order of the presentation of the two CSs was randomized between bees (Balzani et al., 2022). We tested a total of 727 bees: 85 bees as a control group for the fungicide, and 81 and 86 bees with a low and a high dose treatment respectively. For the herbicide, we tested 74 bees as a control group, 73 bees with a low concentration treatment, and 76 bees with a high concentration treatment. Finally, for the combination of both pesticides, we tested 82 bees as a control group, 83 bees with a low concentration treatment, and 87 bees with a high concentration treatment.

2.5. Statistical analysis

We tested for significant differences in each biomarker between control group, the fungicide Sakura®, the herbicide Elegant 2FD and the

mixture samples using Kruskal-Wallis (KW) test (Kruskal and Wallis, 1952). This is a non-parametric test, and it is used when the data does not satisfy the normality property and contains outliers. Furthermore, Dunn's test with a Benjamini–Hochberg stepwise adjustment (Benjamini and Hochberg, 1995) was applied for pairwise multiple-comparison when the null hypothesis of the KW test was rejected. Linear mixed model (LMM) was used for studying the dependence of food consumption on groups (Raudenbush and Bryk, 2002). A mixed effects Cox regression (Allison, 2014; Fox and Weisberg, 2002; Lin and Zelterman, 2002) was used to estimate the effect of groups on mortality rate. The odd ratios, their confidence intervals and p-values were represented using the function "ggforest" (available in the R package "survminer"). Finally, generalized linear mixed models (GLMM) were estimated for studying the response PER occurrence (PER, 1 = response, 0 = no response) over trials on groups controlling for other regressors (Garson, 2013). Precisely, in the GLMM models for acquisition, 'treatment' (control, dose 1, dose 2) and 'CS' (CS+, CS-) as fixed factors, and 'conditioning trial' (1, 2, 3, 4, 5) as a covariate. Interaction between 'trial' and 'CS' was also considered. In all models, individual identity 'ID' was included as a random factor to account for repeated measures. Statistical analyses have been implemented by STATA 17-software (StataCorp, 2021) and R (Core Team, 2021).

3. Results

3.1. Mortality assay

Exposure to pesticides may cause alterations in honey bees sucrose intake and lifespan. To investigate this further, we compared the daily food consumption and mortality rates of exposed and control bees over a 10-day observation period. For all groups of caged bees, the food consumption decreased over the course of the test (LMM, day: $\chi2 = 18.80$, $df = 1$, $p < 0.001$, Supp. Fig. 1S). The treatment with the fungicide or the herbicide or both did not affect the per capita daily food consumption regardless of the concentration used (LMM: treat: $\chi2 = 1.79$, $df = 6$, $p = 0.93$, Suppl. Fig. 1S).

By contrast, the estimated mixed effects Cox regression suggested that mortality rate differed between groups (LR test: $\chi2 = 17.41$, $df = 6$, $p = 0.008$). The detailed results of the mixed effects Cox regression are presented in Table 1.

In the model the reference group is the "control" group. All estimated hazards are statistically significant and greater than 1, except one. Accordingly, the results suggest that five treatments are negatively associated with the length of survival. The mortality of bees treated with the fungicide at both doses is higher than the control group (FUNG 200 g/L: $p = 0.006$; FUNG 400 g/L: $p = 0.001$). Whereas the mortality of bees treated with herbicide at the highest dose (HERB 450 g/L: $p = 0.006$) is significantly higher than the control group, the herbicide at the lower dose did not affect bee survival (HERB 225 g/L: $p = 0.33$). Bees exposed to the combination of the two pesticides died faster than control bees, but the effect was comparable to the one by the fungicide alone (MIX 200 g/L+225 g/L: $p = 0.006$; MIX 400 g/L+450 g/L: $p = 0.003$).

Table 1
Estimated hazard ratios and p-values which are derived from the mixed effects Cox regression. Hazard ratio >1 indicates an increased risk of death for bees belonging to the experimental groups. A Hazard ratio <1, on the other hand, indicates a decreased risk compared to the reference group (i.e., Control bees).

GROUP	Hazard ratio	p-values	
Control (reference group)			
FUNG 200 g/L	1.72	0.006	***
FUNG 400 g/L	1.85	0.001	***
HERB 225 g/L	1.23	0.326	
HERB 450 g/L	1.74	0.006	***
MIX 200 g/L+225 g/L	1.69	0.006	***
MIX 400 g/L+450 g/L	1.74	0.003	***

3.2. Biomarker responses

We assessed whether exposure to Sakura®, Elegant 2FD and the combination of the two, causes neurotoxicity (AChE and CaE), alters bee metabolism (ALP and GST), affects the immune system (LYS and haemocytes count) and induces genotoxicity (NA assay).

Fig. 1 (a and b) shows the results obtained for AChE and CaE activities. AChE activity was found to be statistically different in bees exposed to both doses of the fungicide ($p < 0.01$; Table 1S). In contrast to the fungicide, HERB 450 g/L showed significant AChE inhibition compared to the control and to HERB 225 g/L (Fig. 1a, $p < 0.01$). The MIX highest dose was significantly inhibited compared to the control ($p < 0.05$) and to FUNG 400 g/L ($p < 0.01$), and the MIX lowest dose showed a significant inhibition compared to FUNG 200 g/L ($p < 0.01$).

Groups treated with fungicides only (at both concentrations) and mixture treatment (high concentration) showed lower CaE activity compared to the control group (Fig. 1b) ($p < 0.01$, Table 1S). MIX lowest concentration showed a significant induction with respect to FUNG 200 g/L treatment group ($p < 0.01$) while the MIX highest dose showed a statistically significant inhibition with respect to HERB 450 g/L ($p < 0.05$) and the MIX lowest one ($p < 0.01$) and induction with respect to FUNG 200 g/L ($p < 0.01$).

The enzymatic assay results also showed that GST followed different patterns depending on the exposure to fungicide and herbicide. Indeed, GST activity was inhibited after treatment with FUNG 400 g/L, with a statistically significant difference compared to the control while HERB 450 g/L, and both mixture treatments showed significant induction (Fig. 2a; $p < 0.01$, Table 1S). A statistically significant difference was observed between the two herbicide doses, with HERB 450 g/L showing higher values compared to HERB 225 g/L ($p < 0.05$). Similarly, there was a significant difference between the two fungicide doses ($p < 0.01$), with GST activity decreasing from FUNG 200 g/L to FUNG 400 g/L. Furthermore, GST activity increased significantly from the MIX lowest concentration to the MIX highest one ($p < 0.05$). The two MIX doses showed activities similar to those of the herbicide, and each of them showed a statistically significant difference with respect to the corresponding fungicide dose.

Both fungicide treatments significantly inhibited ALP activity in a dose-dependent manner ($p < 0.01$, Table 1S). The two different mix doses showed values that were similar to the herbicide and control values and statistically different from the corresponding fungicide dose (Fig. 2b; $p < 0.01$).

Lysozyme activity did not show differences among the treatments (Fig. 3a).

Plasmatocytes (Supplementary material, Table 5) showed a statistically significant decrease in FUNG 200 g/L group with respect to the control and MIX at the lowest concentration ($p < 0.05$, Table 1S).

The Nuclear Abnormalities (NA) assay values didn't show differences among the treatments (Fig. 3b).

Table 2 summarize the statistically significant differences found between the control and each treatment. Table 3 report the differences observed in FUNG 200 g/L and HERB 225 g/L compared to MIX 200 g/L + 225 g/L, as well as in FUNG 400 g/L and HERB 450 g/L with respect to MIX 400 g/L + 450 g/L.

3.2.1. Cognition and behaviour

To assess whether exposure to Sakura®, Elegant 2FD and the combination of the two, would cause cognitive deficits (learning and memory) 4 days after treatment, bees were subjected to a differential conditioning procedure in which they had to learn to discriminate between two olfactory stimuli, one rewarding (CS+) and one unrewarding (CS-). Fig. 4 (a, c, e) shows the percentage of PER to the CS+ (solid lines) and CS- (dotted lines) exhibited over the five conditioning trials by bees exposed to the fungicide, the herbicide and a mixture of the two and the corresponding control bees. Different model specifications were compared for estimating the response PER across trials on groups

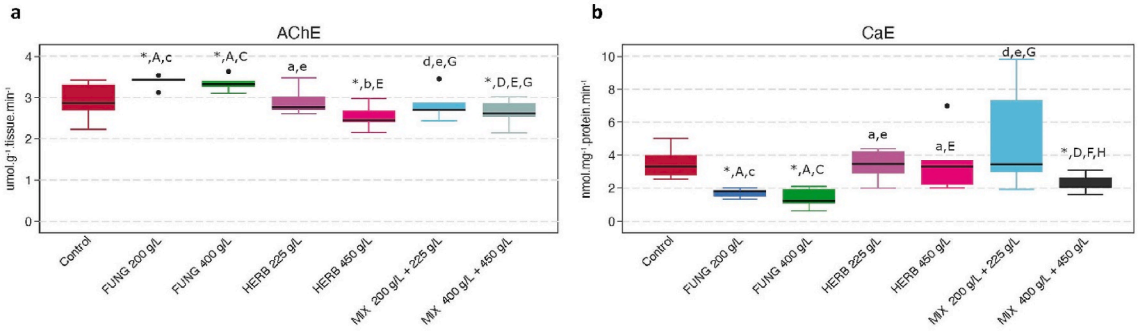

Fig. 1. AChE (a) and CaE (b) activities measured in the head of honey bees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures. Different letters indicate a statistically significant difference ($p < 0.05$). "*" indicates differences with respect to control; "A,B" indicate differences between the fungicide doses; "a,b" indicate differences between the herbicide doses; "c,d" indicate differences between FUNG 200 g/L and MIX 200 g/L+225 g/L; "C,D" indicate differences between FUNG 400 g/L and MIX 400 g/L+450 g/L; "e,f" indicate differences between HERB 225 g/L and MIX 200 g/L+225 g/L; "E,F" indicate differences between HERB 450 g/L and MIX 400 g/L+450 g/L; "G,H" indicate differences between MIX 200 g/L+225 g/L and MIX 400 g/L+450 g/L.

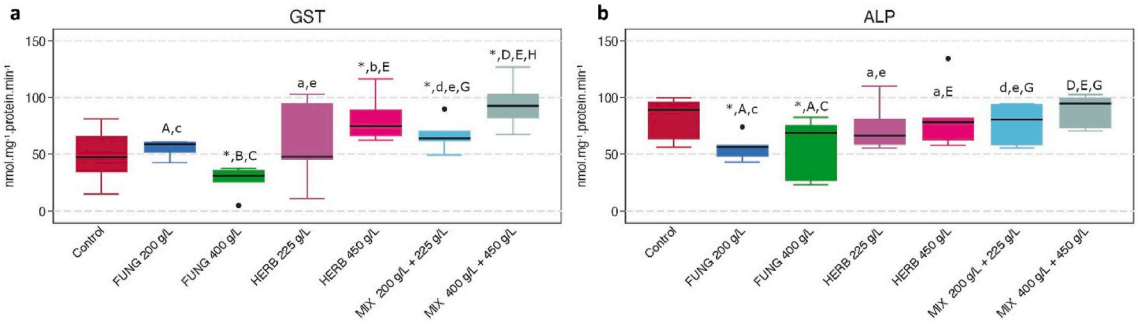

Fig. 2. GST (a) and ALP (b) activity measured in the midgut of honey bees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures. Different letters indicate a statistically significant difference ($p < 0.05$). "*" indicates differences with respect to control; "A,B" indicate differences between the fungicide doses; "a,b" indicate differences between the herbicide doses; "c,d" indicate differences between FUNG 200 g/L and MIX 200 g/L+225 g/L; "C,D" indicate differences between FUNG 400 g/L and MIX 400 g/L+450 g/L; "e,f" indicate differences between HERB 225 g/L and MIX 200 g/L+225 g/L; "E,F" indicate differences between HERB 450 g/L and MIX 400 g/L+450 g/L; "G,H" indicate differences between MIX 200 g/L+225 g/L and MIX 400 g/L+450 g/L.

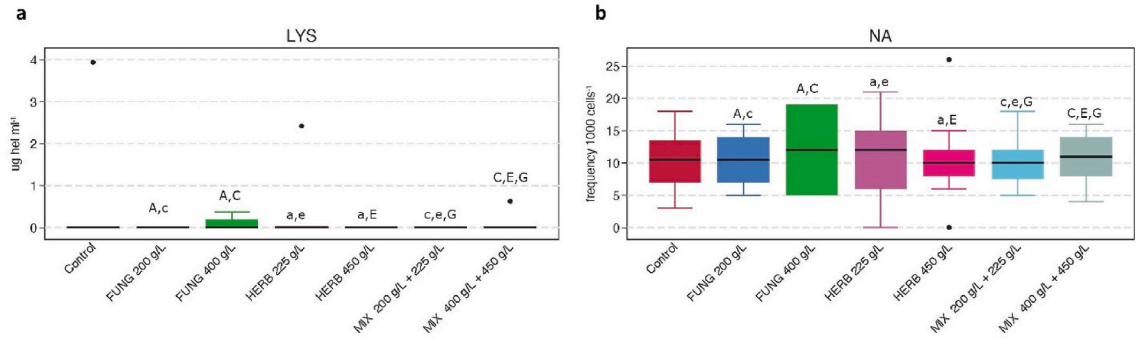

Fig. 3. LYS activity (a) and NA assay (b) measured in the midgut of honey bees (*Apis mellifera*) exposed to the fungicide Sakura® (200 and 400 g/L), the herbicide Elegant 2FD (225 and 450 g/L), and their mixtures. Different letters indicate a statistically significant difference ($p < 0.05$). "*" indicates differences with respect to control; "A,B" indicate differences between the fungicide doses; "a,b" indicate differences between the herbicide doses; "c,d" indicate differences between FUNG 200 g/L and MIX 200 g/L+225 g/L; "C,D" indicate differences between FUNG 400 g/L and MIX 400 g/L+450 g/L; "e,f" indicate differences between HERB 225 g/L and MIX 200 g/L+225 g/L; "E,F" indicate differences between HERB 450 g/L and MIX 400 g/L+450 g/L; "G,H" indicate differences between MIX 200 g/L+225 g/L and MIX 400 g/L+450 g/L.

controlling for other regressors (see Supplementary Tables 2, 3 and 4). Based on the selected model (model N°5), all groups of bees learned to respond differently to CS+ and CS- over the conditioning trials (the interaction effect between CS and trial was statistically significant at $p < 0.001$). However, in all cases we found a significant difference between bees treated with compounds, regardless of concentration and combination, and control bees. Specifically, control bees performed better than bees exposed to the fungicide regardless of its dose (FUNG 200 g/L: $p = 0.02$; FUNG 400 g/L: $p = 0.005$) and better than bees exposed to the highest dose of the herbicide (HERB 225 g/L: $p = 0.14$; HERB 450 g/L: $p = 0.02$). Bees exposed to the mixture of the two pesticides performed worse than control bees (MIX 200 g/L + 225 g/L: $p =$

Table 2
Statistically significant differences in the seven analysed biomarkers in each treatment compared to acetone. Red "+" are for differences where the values are higher than the control ones; green "-" are for values lower compared to the control; no ticks are for the absence of statistically significant differences.

	FUNG 200 g/L	HERB 225 g/L	MIX 200 g/L + 225 g/L	FUNG 400 g/L	HERB 450 g/L	MIX 400 g/L + 450 g/L
AChE	+ (**)			+ (**)	- (**)	- (*)
CaE	- (**)			- (**)		- (**)
GST			+ (*)	- (**)	+ (**)	+ (**)
ALP	- (**)			- (**)		
LYS						
PLASM		- (*)				
NA assay						

Table 3
Statistically significant differences in the seven analysed biomarkers compared to MIX. FUNG 200 g/L and HERB 225 g/L are compared to MIX 200 g/L+ 225 g/L; FUNG 400 g/L and HERB 450 g/L are compared to MIX 400 g/L+ 450 g/L. Red "+" are for differences where the values are higher than the mix ones; green "-" are for values lower compared to the mix; no ticks are for the absence of statistically significant differences.

	FUNG 200 g/L	HERB 225 g/L	FUNG 400 g/L	HERB 450 g/L
AChE	+ (**)		+ (**)	
CaE	- (**)		- (*)	+ (*)
GST	- (*)		- (**)	
ALP	- (*)		- (**)	
LYS				
PLASM		- (*)		
NA assay				

0.02; MIX 400 g/L + 450 g/L: p = 0.03).

Furthermore, control bees and bees exposed to the fungicide differed in the 2-h memory test (specific learners: p = 0.06, Fig. 4b). At 24 h this difference disappeared specific memory: p = 0.88). The herbicide at the high dose impaired memory recall (specific memory: p = 0.003, Fig. 4d). As with the fungicide, this effect disappeared after 24 h (specific memory: p = 0.55). The mixture had a stronger effect especially at 24 h (2h: specific memory: p = 0.0098; 24h: p = 0.0002p = 0.0057, Fig. 4f).

4. Discussions

The overall aim of this study was to investigate the effects of two commercial pesticides, alone and as mixtures, on honey bees, evaluating responses ranging from molecular and physiological changes to behavioural alterations. The selected fungicide Sakura® was shown to affect the metabolism and the detoxification system of *A. mellifera*, mostly at the highest dose. Elegant 2FD at the highest dose caused neurotoxic damage and also activated the detoxification system. The results showed that the herbicide and the mixtures had no effects on the immune system. In addition, the herbicide appeared to play a significant role in the responses observed following the combination treatments.

AChE activity was found to be statistically different in bees exposed to both doses of the fungicide. The result differs from that obtained by Caliani et al. (2021a), who showed that the commercial fungicide Amistar®Xtra, composed mainly of azoxystrobin, a strobilurin, co-formulated with cyproconazole, inhibited AChE activity. On the contrary, no changes in AChE activity were observed after the 10 days of treatment with difenoconazole, a curative and preventive fungicide belonging to the triazoles class (Almasri et al., 2020). The results of our work suggest that the triazoles, unlike the strobilurins, are not able to cause variations in AChE activity. HERB 450 g/L showed significant AChE inhibition compared to the control and to HERB 225 g/L. To the best of our knowledge, the sublethal effects of Elegant 2FD and the active principle 2,4 D have never been investigated in *Apis mellifera* before. Recently, Almasri et al. (2020) exposed *A. mellifera* specimens to the herbicide glyphosate (0.1 and 1 μg/L) for 10 and 20 days, and observed AChE inhibition at the highest dose. The results obtained in our study lead us to hypothesise that the herbicide is capable of causing neurotoxicity. Both mixture treatments showed an inhibition compared to the corresponding fungicide concentrations. The neurotoxic effects after both mix treatments could be attributed to the herbicide, as the fungicide alone showed no changes, while the herbicide was able to inhibit this enzyme activity.

The effects of these two pesticides on AChE activity may partly explain their effects on honeybee cognition. Notably, the only treatment that had no significant effect on AChE activity (HERB 445 g/L) was also the only treatment that had no significant effect on honeybee learning and memory. Although the causal relationship between AChE and learning is not immediately obvious and remains inconclusive based on our experiments, we can understand the potential link between AChE enzyme activity and learning and memory in bees through the role of acetylcholine (ACh), a key neurotransmitter in the bee nervous system. ACh is a primary excitatory neurotransmitter in the insect brain and is closely associated with learning, memory and cognitive processes (Gauthier, 2010). AChE plays a critical role in rapidly terminating the action of AChE at the synaptic cleft, preventing prolonged stimulation of the postsynaptic neuron. An imbalance in AChE activity could lead to excessive ACh degradation, impairing neurotransmission and potentially affecting the bee's ability to acquire and retrieve information. Indeed, for example, agonists of nACh receptors such as nicotine, caffeine and nicotine-derived neonicotinoid pesticides such as imidacloprid, thiamethoxam and clothianidin have been shown to affect learning and memory in bees (Baracchi et al., 2017; Blacquière et al., 2012; Grünewald and Siefert, 2019; Wright et al., 2013).

In particular, our PER conditioning was based on olfactory cues (odors). It's well known that projection neurons from the antennal lobes, responsible for processing olfactory information, connect to Kenyon cells in the mushroom bodies, which are involved in sensory integration and learning in insects (Hourcade et al., 2010; Oleskevich, 1999). These projection neurons release ACh, which binds to ACh receptors in the dendrites of Kenyon cells. Therefore, we hypothesise that altered ACh activity at the level of the antennal lobes or mushroom bodies may contribute to the observed impairment in olfactory appetitive learning and memory in honey bees exposed to the fungicide and herbicide compared to control bees. However, it's important to recognise that there are other plausible hypotheses to consider. In particular, the in-cage mortality data highlight the high toxicity of these compounds, with the exception of the low-dose herbicide, which doesn't cause increased mortality. The bees were, therefore, significantly weakened, suggesting that the observed cognitive changes may be an indirect consequence (secondary effect) of either the general poor condition and/or the increased energy demands associated with detoxification processes. On the other hand, it's worth noting that there were no differences in average daily food intake between the different treatments.

The induction of CaE activity observed in our study is consistent with that obtained by various authors after the exposure of *A. mellifera* samples to different classes of pesticides (Badiou-Bénéteau et al., 2012; Carvalho et al., 2013; Hashimoto et al., 2003; Roat et al., 2017). In particular, the induction observed after treatment with our lowest mixture cannot be compared with any study. The inhibition obtained after both fungicide treatments and mix highest one is in agreement with the results reported by Caliani et al. (2021a) and Almasri et al. (2020), who observed a decrease in CaE activity after exposure to the fungicide Amistar®Xtra (200 g/L) and the difenoconazole (0.1 and 1 μg/L), respectively. The lack of reduction in AChE and concomitant reduction in CaE activity after exposure to the fungicide may indicate that CaE acts

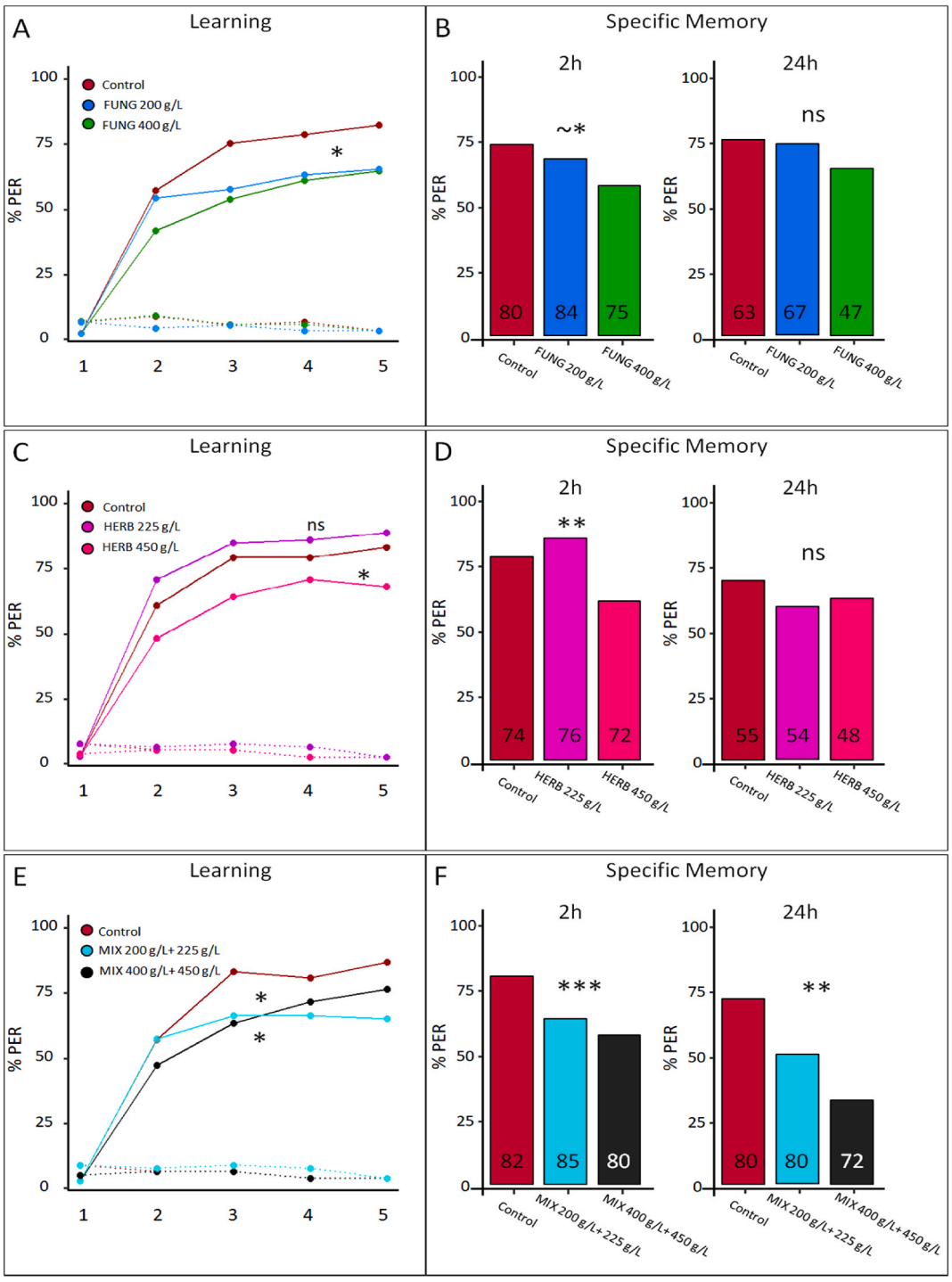

Fig. 4. Effects of the pesticides on associative learning and memory retention in honey bees. Percentage of PER evoked by the five CS+ (rewarded odorant, solid lines) and the five CS- (unrewarded odorant, dotted lines) presentations in control bees and bees exposed to the fungicide (A), the herbicide (C) and a mixture of them (E). Specific 2h and 24h memory recall of control bees and bees exposed to the fungicide (B), the herbicide (D) and a mixture of them (F). Sample sizes of the memory test are indicated inside the bars. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

as detoxifying enzyme rather than to protect AChE. The only author who evaluated sublethal effects in honey bees after exposure to the herbicide glyphosate (0.1 and 1 μg/L) found a decrease in CaE (Almasri et al., 2020), while no changes were found after Elegant 2FD treatments. The observed AChE reduction and no increase in CaE activity after the herbicide and mix treatments could indicate that CaE did not provide a protection to AChE activity, demonstrating that these compounds could affect bees. The highest concentration of each compound caused a decrease in CaE activity compared to those found at the field concentrations, especially the fungicide showed the most evident inhibition at both doses. Carvalho et al. (2013) observed CaE1 inhibition in honey bees at the lowest deltamethrin dose (5.07 ng/bee); similarly, the lowest CaE levels were observed at the highest pesticide doses in our study. These results may indicate that high concentrations of these compounds can modulate this phase I biotransformation enzyme.

The two mix doses showed GST activities similar to the herbicide ones, and each of them showed a difference with respect to the corresponding fungicide dose, as already observed in the AChE results. This

suggests that the herbicide is mainly responsible for the GST increase observed in the mix treatments. GST induction is a well-known process in vertebrate species exposed to lipophilic compounds (Topić Popović et al., 2023), while in pollinator species there are few publications on the modulation of GST activity after the exposure to chemical compounds (Koirala et al., 2022) and especially to pesticides (Araújo et al., 2023; Caliani et al., 2021a; Martins et al., 2022). Caliani et al. (2021a) observed an increase in GST activity in honey bees exposed to a fungicide containing cyproconazole and azoxystrobine. On the contrary, in agreement with our results, Martins et al. (2022) observed a decrease in this activity in *Osmia bicornis* samples exposed to an insecticide, the Confidor® (the main active principle is imidacloprid), and a fungicide, the Folicur® SE (consisting of tebuconazole). The reduced GST activity in bees exposed to fungicides could be related to, or a manifestation of, an adaptation mechanism to a condition of oxidative stress. Indeed, triazole fungicides have been shown to cause oxidative stress in zebrafish specimens (Huang et al., 2022; Zhu et al., 2021). This type of pesticide may indeed be able to exceed the ability of the enzyme to detoxify, resulting in a decrease in the GST levels. On the other hand, our results suggest that the herbicide and consequently the mix treatments are able to induce and activate the detoxification processes.

The decrease in ALP response obtained after Sakura® treatments was also observed by Caliani et al. (2021a) following the treatment of honey bees with the fungicide Amistar®Xtra. Although ALP is not known to be involved in pesticide detoxification (Caliani et al., 2021a), the fungicide Sakura® could affect the ALP activity in honey bees. This could potentially cause irregularities in nutrient transport and absorption at the intestinal level, ultimately weakening the animals. Regarding the mix treatments, the herbicide appears to counteract the effects of the fungicide in response to the combination treatments.

Lysozyme and haemocytes are the fundamental components of the immune system of honey bees. Lysozyme activity in honey bees decreased after the exposure to a fungicide, as did the number of plasmatocytes by Caliani et al. (2021a), suggesting that pesticide exposure may cause an alteration in immune responses. To the best of our knowledge, no other ecotoxicological studies have examined these types of responses to evaluate the effects of pesticide treatments in honey bees. In this work, no changes in lysozyme activity and haemocyte counts were observed, indicating that the investigated compounds did not affect the immune system.

To date, no genotoxic effects on terrestrial organisms have been reported for commercial pesticide formulations. However, Caliani et al. (2021a) observed an increase in NA assay values after exposure of honey bees to the commercial fungicide Amistar®Xtra. The absence of nuclear changes in all treatment groups in of our study suggests that both fungicide and herbicide, alone and in combination, did not cause genotoxic effects in honey bees.

The obtained results permitted to study the ecotoxicological effects of these commercial compounds. In future research, it is crucial to test commercial pesticides and active ingredients separately, in order to gain the full understanding of the potential effects of co-formulants on non-target species. Indeed, “inerts” used to enhance the effects of active principles have biological activity of their own and may be even more toxic to non-target organisms than the active ingredients (Adams et al., 2021; Wagner et al., 2015).

5. Conclusions

The results of this study provide valuable insights into the potential impact of herbicides, fungicides and pesticide mixtures on honey bee health. They also highlighted the effectiveness of using a set of biomarkers to monitor sublethal effects in *Apis mellifera*. Specifically, the study showed that the fungicide Sakura® activated both the detoxification enzymes (CaE and GST) and affected the ALP activity. The herbicide Elegant 2FD was able to cause a neurotoxic effect and to activate detoxification processes. The combination of these compounds shows the same effects of the herbicide treatments for all the biomarkers except for the ALP activity, where the herbicide appears to counteract the effects of the fungicide in the mixed treatments. Our results also showed that exposure to the herbicide and the pesticide mixture can undermine honey bee behaviour, particularly cognitive skills such as learning and memory. These cognitive skills are essential for the foraging flexibility and ecological success of honey bees and other wild pollinators. It is therefore important and urgent to consider the potential adverse effects of these chemicals on the behaviour of pollinators, which play a crucial role in maintaining ecosystem health and biodiversity. Overall, our results reinforce the idea that there is an urgent need for more in-depth investigation and research into the effects of pesticides like fungicides and herbicides, which are widely used in agriculture, on pollinators. We strongly recommend that future studies investigating the effects of pesticides on non-target insects should not only test the active ingredients, but also focus on the investigation of commercial pesticides, including their co-formulants or adjuvants.

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CRediT authorship contribution statement

Agata Di Noi: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Ilaria Caliani:** Conceptualization, Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Antonella D’Agostino:** Formal analysis, Writing – review & editing. **Giampiero Cai:** Writing – review & editing. **Marco Romi:** Writing – review & editing. **Tommaso Campani:** Investigation, Methodology, Writing – review & editing. **Federico Ferrante:** Investigation. **David Baracchi:** Conceptualization, Formal analysis, Methodology, Writing – review & editing. **Silvia Casini:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142307.

References

Adams, E., Gerstle, V., Schmitt, T., Brühl, C.A., 2021. Co-formulants and adjuvants affect the acute aquatic and terrestrial toxicity of a cycloxydim herbicide formulation to European common frogs (Rana temporaria). Sci. Total Environ. 789, 147865 https://doi.org/10.1016/j.scitotenv.2021.147865.

Al Naggar, Y., Wiseman, S., Sun, J., Cutler, G.C., Aboul-Soud, M., Naiem, E., Mona, M., Seif, A., Giesy, J.P., 2015. Effects of environmentally-relevant mixtures of four common organophosphorus insecticides on the honey bee (Apis mellifera L.). J. Insect Physiol. 82, 85–91. https://doi.org/10.1016/j.jinsphys.2015.09.004.

Alexandratos, N., Bruinsma, J., 2012. World agriculture towards 2030/2050: the 2012 revision. ESA Working paper No. 12-03. Rome, Food and Agriculture Organization of the United Nations. https://doi.org/10.22004/ag.econ.288998.

Allison, P.D., 2014. Event History and Survival Analysis, second ed. Sage, Thousand Oaks CA.

Almasri, H., Tavares, D.A., Pioz, M., Sené, D., Tchamitchian, S., Cousin, M., Brunet, J.-L., Belzunces, L.P., 2020. Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter Apis mellifera honey bees. Ecotoxicol. Environ. Saf. 203, 111013 https://doi.org/10.1016/j.ecoenv.2020.111013.

Araújo, R., dos, S., Lopes, M.P., Viana, T.A., Bastos, D.S.S., Machado-Neves, M., Botina, L. L., Martins, G.F., 2023. Bioinsecticide spinosad poses multiple harmful effects on foragers of Apis mellifera. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-023-27143-6.

Babczyńska, A., Wilczek, G., Migula, P., 2006. Effects of dimethoate on spiders from metal pollution gradient. Sci. Total Environ. 370, 352–359. https://doi.org/10.1016/j.scitotenv.2006.06.024.

Badawy, M.E.I., Nasr, H.M., Rabea, E.I., 2015. Toxicity and biochemical changes in the honey bee Apis mellifera exposed to four insecticides under laboratory conditions. Apidologie 46, 177–193. https://doi.org/10.1007/s13592-014-0315-0.

Badiou, A., Meled, M., Belzunces, L.P., 2008. Honeybee Apis mellifera acetylcholinesterase—a biomarker to detect deltamethrin exposure. Ecotoxicol. Environ. Saf. 69, 246–253. https://doi.org/10.1016/j.ecoenv.2006.11.020.

Badiou-Bénéteau, A., 2013. Honeybee biomarkers as promising tools to monitor environmental quality. Environ. Int. 11.

Badiou-Bénéteau, A., Carvalho, S.M., Brunet, J.-L., Carvalho, G.A., Buleté, A., Giroud, B., Belzunces, L.P., 2012. Development of biomarkers of exposure to xenobiotics in the honey bee Apis mellifera: application to the systemic insecticide thiamethoxam. Ecotoxicol. Environ. Saf. 82, 22–31. https://doi.org/10.1016/j.ecoenv.2012.05.005.

Balzani, P., Galeotti, G., Scheggi, S., Masoni, A., Santini, G., Baracchi, D., 2022. Acute and chronic ingestion of polyethylene (PE) microplastics has mild effects on honey bee health and cognition. Environmental Pollution 305, 119318. https://doi.org/10.1016/j.envpol.2022.119318.

Baracchi, D., 2019. Cognitive ecology of pollinators and the main determinants of foraging plasticity. Current Zoology 65, 421–424. https://doi.org/10.1093/cz/zoz036.

Baracchi, D., Marples, A., Jenkins, A.J., Leitch, A.R., Chittka, L., 2017. Nicotine in floral nectar pharmacologically influences bumblebee learning of floral features. Sci. Rep. 7, 1951. https://doi.org/10.1038/s41598-017-01980-1.

Baracchi, D., Rigosi, E., de Brito Sanchez, G., Giurfa, M., 2018. Lateralization of sucrose responsiveness and non-associative learning in honeybees. Front. Psychol. 9, 425. https://doi.org/10.3389/fpsyg.2018.00425.

Baracchi, D., Tragust, S., 2017. Venom as a component of external immune Defense in hymenoptera. In: Malhotra, A. (Ed.), Evolution of Venomous Animals and Their Toxins. Toxinology. Springer, Netherlands, Dordrecht, pp. 213–233. https://doi.org/10.1007/978-94-007-6458-3_3.

Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005. Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae Hydropsyche exocellata from the Llobregat river basin (NE Spain). Aquat. Toxicol. 74, 3–19. https://doi.org/10.1016/j.aquatox.2005.04.002.

Bedini, S., Flamini, G., Cosci, F., Ascrizzi, R., Echeverria, M.C., Guidi, L., Landi, M., Lucchi, A., Conti, B., 2017. Artemisia spp. essential oils against the disease-carrying blowfly Calliphora vomitoria. Parasites Vectors 10, 80. https://doi.org/10.1186/s13071-017-2006-y.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Stat. Soc. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Bjergager, M.-B.A., Dalhoff, K., Kretschmann, A., Nørgaard, K.B., Mayer, P., Cedergreen, N., 2017. Determining lower threshold concentrations for synergistic effects. Aquat. Toxicol. 182, 79–90. https://doi.org/10.1016/j.aquatox.2016.10.020.

Blacquière, T., Smagghe, G., van Gestel, C.A.M., Mommaerts, V., 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology 21, 973–992. https://doi.org/10.1007/s10646-012-0863-x.

Boily, M., Sarrasin, B., DeBlois, C., Aras, P., Chagnon, M., 2013. Acetylcholinesterase in honey bees (Apis mellifera) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments. Environ. Sci. Pollut. Res. 20, 5603–5614. https://doi.org/10.1007/s11356-013-1568-2.

Bounias, M., Kruk, I., Nectoux, M., Popeskovic, D., 1996. Toxicology of cupric salts on honeybees. V. Gluconate and sulfate action on gut alkaline and acid phosphatases. Ecotoxicol. Environ. Saf. 35, 67–76. https://doi.org/10.1006/eesa.1996.0082.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of Protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295–311. https://doi.org/10.1016/S0048-9697(99)00499-4.

Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Ammendola, A., Di Noi, A., Gori, A., Casini, S., 2021a. Multi-biomarker approach and IBR index to evaluate the effects of different contaminants on the ecotoxicological status of Apis mellifera. Ecotoxicol. Environ. Saf. 208, 111486 https://doi.org/10.1016/j.ecoenv.2020.111486.

Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Giovanetti, L., Di Noi, A., Casini, S., 2021b. First application of an Integrated Biological Response index to assess the ecotoxicological status of honeybees from rural and urban areas. Environ. Sci. Pollut. Res. 28, 47418–47428. https://doi.org/10.1007/s11356-021-14037-8.

Campani, T., Caliani, I., Pozzuoli, C., Romi, M., Fossi, M.C., Casini, S., 2017. Assessment of toxicological effects of raw and bioremediated olive mill waste in the earthworm Eisenia fetida: a biomarker approach for sustainable agriculture. Appl. Soil Ecol. 119, 18–25. https://doi.org/10.1016/j.apsoil.2017.05.016.

Cappa, F., Baracchi, D., Cervo, R., 2022. Biopesticides and insect pollinators: detrimental effects, outdated guidelines, and future directions. Sci. Total Environ. 837, 155714 https://doi.org/10.1016/j.scitotenv.2022.155714.

Carlesso, D., Smargiassi, S., Sassoli, L., Cappa, F., Cervo, R., Baracchi, D., 2020. Exposure to a biopesticide interferes with sucrose responsiveness and learning in honey bees. Sci. Rep. 10, 19929 https://doi.org/10.1038/s41598-020-76852-2.

Carvalho, F.P., 2017. Pesticides, environment, and food safety. Food Energy Secur. 6, 48–60. https://doi.org/10.1002/fes3.108.

Carvalho, S.M., Belzunces, L.P., Carvalho, G.A., Brunet, J.-L., Badiou-Beneteau, A., 2013. Enzymatic biomarkers as tools to assess environmental quality: a case study of exposure of the honeybee *Apis mellifera* to insecticides: biomarker responses in honeybees exposed to pesticides. Environ. Toxicol. Chem. 32, 2117–2124. https://doi.org/10.1002/etc.2288.

Christen, V., Bachofer, S., Fent, K., 2017. Binary mixtures of neonicotinoids show different transcriptional changes than single neonicotinoids in honeybees (Apis mellifera). Environmental Pollution 220, 1264–1270. https://doi.org/10.1016/j.envpol.2016.10.105.

Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit BlackwellPublishingLtd of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 22.

Colin, M.-E., Belzunces, L.P., 1992. Evidence of synergy between prochloraz and deltamethrin in *apis mellifera* L.: a convenient biological approach. Pestic. Sci. 36, 115–119. https://doi.org/10.1002/ps.2780360206.

Core Team, 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Cremer, S., Armitage, S.A.O., Schmid-Hempel, P., 2007. Social immunity. Curr. Biol. 17, R693–R702. https://doi.org/10.1016/j.cub.2007.06.008.

Daam, M.A., Leitão, S., Cerejeira, M.J., Paulo Sousa, J., 2011. Comparing the sensitivity of soil invertebrates to pesticides with that of Eisenia fetida. Chemosphere 85, 1040–1047. https://doi.org/10.1016/j.chemosphere.2011.07.032.

Dauterman, W.C., Hodgson, E., 1990. Metabolism of Xenobiotics. Safer Insecticides: Development and Use. Basel, New York, pp. 19–55.

Delso, N.S., Mędrzycki, P., Sgolastra, F., Fourrier, J., Azpiazu, C., Tosi, S., Hatjina, F., 2022. Apitox - the COLOSS task force to investigate the impact of pesticides on bees. Bee World 99, 32–34. https://doi.org/10.1080/0005772X.2021.2016291.

Desneux, N., Decourtye, A., Delpuech, J.-M., 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52, 81–106. https://doi.org/10.1146/annurev.ento.52.110405.091440.

Di Noi, A., Casini, S., Campani, T., Cai, G., Caliani, I., 2021. Review on sublethal effects of environmental contaminants in honey bees (Apis mellifera), knowledge gaps and future perspectives. IJERPH 18, 1863. https://doi.org/10.3390/ijerph18041863.

Doums, C., Moret, Y., Benelli, E., Schmid-Hempel, P., 2002. Senescence of immune defence in Bombus workers. Ecol. Entomol. 27, 138–144. https://doi.org/10.1046/j.1365-2311.2002.00388.x.

EFSA Working Group, Draft Guidance Document. Revised guidance on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees). https://doi.org/10.2903/j.efsa.201Y.xxxx10.2903/j.efsa.201Y.xxxx.

Fox, J., Weisberg, S., 2002. Cox Proportional-Hazards Regression for Survival Data in R.

Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L., 2005. Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10, 360–375. https://doi.org/10.1080/13547500500264660.

Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspect. 87, 245–254.

Garson, G.D., 2013. Fundamentals of hierarchical linear and multilevel modeling. In: Hierarchical Linear Modeling: Guide and Applications. SAGE Publications, Inc., Thousand Oaks California 91320 United States, pp. 3–26. https://doi.org/10.4135/9781483384450.n1, 2455 Teller Road.

Gauthier, M., 2010. State of the art on insect nicotinic acetylcholine receptor function in learning and memory. Insect Nicotinic Acetylcholine Receptors 97–115. https://doi.org/10.1007/978-1-4419-6445-8_9.

Giglio, A., Brandmayr, P., Pasqua, T., Angelone, T., Battistella, S., Giulianini, P.G., 2015. Immune challenges trigger cellular and humoral responses in adults of Pterostichus melas italicus (Coleoptera, Carabidae). Arthropod Struct. Dev. 44, 209–217. https://doi.org/10.1016/j.asd.2015.01.002.

Goulson, D., Nicholls, E., Botias, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347. https://doi.org/10.1126/science.1255957, 1255957–1255957.

Grünewald, B., Siefert, P., 2019. Acetylcholine and its receptors in honeybees: involvement in development and impairments by neonicotinoids. Insects 10, 420. https://doi.org/10.3390/insects10120420.

Hashimoto, J.H., Ruvolo-Takasusuki, M.C.C., de Toledo, V.A.A., 2003. Evaluation of the use of the inhibition esterases activity on Apis mellifera as bioindicators of insecticide thiamethoxam pesticide residues. Sociobiology 42 (3), 693–699.

Hourcade, B., Muenz, T.S., Sandoz, J.-C., Rössler, W., Devaud, J.-M., 2010. Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? J. Neurosci. 30, 6461–6465. https://doi.org/10.1523/JNEUROSCI.0841-10.2010.

Huang, T., Jiang, H., Zhao, Y., He, J., Cheng, H., Martyniuk, C.J., 2022. A comprehensive review of 1,2,4-triazole fungicide toxicity in zebrafish (Danio rerio): a mitochondrial and metabolic perspective. Sci. Total Environ. 809, 151177 https://doi.org/10.1016/j.scitotenv.2021.151177.

Imran, M., Sheikh, U.A.A., Nasir, M., Ghaffar, M.A., Tamkeen, A., Iqbal, M.A., 2019. Do neonicotinoid insecticides impaired olfactory learning behavior in Apis mellifera? International Journal of Industrial Entomology 38, 1–5. https://doi.org/10.7852/IJIE.2019.38.1.1.

Ingram, E.M., Augustin, J., Ellis, M.D., Siegfried, B.D., 2015. Evaluating sub-lethal effects of orchard-applied pyrethroids using video-tracking software to quantify honey bee behaviors. Chemosphere 135, 272–277. https://doi.org/10.1016/j.chemosphere.2015.04.022.

Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, R.M., 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera. Crop Protect. 23, 371–378. https://doi.org/10.1016/j.cropro.2003.08.018.

Jackson, C.J., Liu, J.-W., Carr, P.D., Younus, F., Coppin, C., Meirelles, T., Lethier, M., Pandey, G., Ollis, D.L., Russell, R.J., Weik, M., Oakeshott, J.G., 2013. Structure and function of an insect α-carboxylesterase (α *Esterase* 7) associated with insecticide resistance. Proc. Natl. Acad. Sci. U.S.A. 110, 10177–10182. https://doi.org/10.1073/pnas.1304097110.

Klein, S., Cabirol, A., Devaud, J.-M., Barron, A.B., Lihoreau, M., 2017. Why bees are so vulnerable to environmental stressors. Trends Ecol. Evol. 32, 268–278. https://doi.org/10.1016/j.tree.2016.12.009.

Koirala, B.K.S., Moural, T., Zhu, F., 2022. Functional and structural diversity of insect glutathione S-transferases in xenobiotic adaptation. Int. J. Biol. Sci. 18, 5713–5723. https://doi.org/10.7150/ijbs.77141.

Kraaijeveld, A.R., Limentani, E.C., Godfray, H.C.J., 2001. Basis of the trade–off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. Proc. Roy. Soc. Lond. B 268, 259–261. https://doi.org/10.1098/rspb.2000.1354.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47 (260), 583–621. https://doi.org/10.1080/01621459.1952.10483441.

Lazarov, S., Zhelyazkova, I., Salkova, D., Shumkova, R., Takova, S., 2016. Lysozyme levels in haemolymph of worker bees (Apis mellifera L.) from bee colonies with different degree of expression of hygienic behaviour. AST 8, 201–204. https://doi.org/10.15547/ast.2016.03.037.

Letelier, M.E., Molina-Berríos, A., Cortés-Troncoso, J., Jara-Sandoval, J.A., Müller, A., Aracena-Parks, P., 2010. Comparative effects of superoxide anion and hydrogen peroxide on microsomal and cytosolic glutathione S-transferase activities of rat liver. Biol. Trace Elem. Res. 134, 203–211. https://doi.org/10.1007/s12011-009-8461-3.

Lin, H., Zelterman, D., 2002. Modeling survival data: extending the Cox model. Technometrics 44. https://doi.org/10.1198/tech.2002.s656.

Marmaras, V.J., Lampropoulou, M., 2009. Regulators and signalling in insect haemocyte immunity. Cell. Signal. 21, 186–195. https://doi.org/10.1016/j.cellsig.2008.08.014.

Martins, C.A.H., Caliani, I., D'Agostino, A., Di Noi, A., Casini, S., Parrilli, M., Azpiazu, C., Bosch, J., Sgolastra, F., 2022. Biochemical responses, feeding and survival in the solitary bee Osmia bicornis following exposure to an insecticide and a fungicide alone and in combination. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-022-24061-x.

Meled, M., Thrasyvoulou, A., Belzunces, L.P., 1998. Seasonal variations in susceptibility of *Apis mellifera* to the synergistic action of prochloraz and deltamethrin. Environ. Toxicol. Chem. 17, 2517–2520. https://doi.org/10.1002/etc.5620171220.

Mesnage, R., Antoniou, M.N., 2018. Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Front. Public Health 5, 361. https://doi.org/10.3389/fpubh.2017.00361.

Monchanin, C., Henry, M., Decourtye, A., Dalmon, A., Fortini, D., Bœuf, E., Dubuisson, L., Aupinel, P., Chevallereau, C., Petit, J., Fourrier, J., 2019. Hazard of a neonicotinoid insecticide on the homing flight of the honeybee depends on climatic conditions and Varroa infestation. Chemosphere 224, 360–368. https://doi.org/10.1016/j.chemosphere.2019.02.129.

Nagy, K., Duca, R.C., Lovas, S., Creta, M., Scheepers, P.T.J., Godderis, L., Ádám, B., 2020. Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. Environ. Res. 181, 108926 https://doi.org/10.1016/j.envres.2019.108926.

Oleskevich, S., 1999. Cholinergic synaptic transmission in insect mushroom bodies in vitro. J. Neurophysiol. https://doi.org/10.1152/jn.1999.82.2.1091.

Otti, O., Tragust, S., Feldhaar, H., 2014. Unifying external and internal immune defences. Trends Ecol. Evol. 29, 625–634. https://doi.org/10.1016/j.tree.2014.09.002.

Pacheco, M., Santos, M.A., 1997. Induction of EROD activity and genotoxic effects by polycyclic aromatic hydrocarbons and resin acids on the juvenile eel (Anguilla anguillaL.). Ecotoxicol. Environ. Saf. 38, 252–259. https://doi.org/10.1006/eesa.1997.1585.

Piggott, J.J., Townsend, C.R., Matthaei, C.D., 2015. Reconceptualizing synergism and antagonism among multiple stressors. Ecol. Evol. 5, 1538–1547. https://doi.org/10.1002/ece3.1465.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25, 345–353. https://doi.org/10.1016/j.tree.2010.01.007.

Qi, S., Niu, X., Wang, D. hui, Wang, C., Zhu, L., Xue, X., Zhang, Z., Wu, L., 2020. Flumethrin at sublethal concentrations induces stresses in adult honey bees (Apis mellifera L.). Sci. Total Environ. 700, 134500 https://doi.org/10.1016/j.scitotenv.2019.134500.

Raudenbush, S.W., Bryk, A.S., 2002. Hierarchical linear models: applications and data analysis methods. Advanced Quantitative Techniques in the Social Sciences Series, No. 1), second ed. sage, Thousand Oaks, CA.

Ribeiro, C., Brehélin, M., 2006. Insect haemocytes: what type of cell is that? J. Insect Physiol. 52, 417–429. https://doi.org/10.1016/j.jinsphys.2006.01.005.

Richardson, R.T., Ballinger, M.N., Qian, F., Christman, J.W., Johnson, R.M., 2018. Morphological and functional characterization of honey bee, Apis mellifera, hemocyte cell communities. Apidologie 49, 397–410. https://doi.org/10.1007/s13592-018-0566-2.

Roat, T.C., Carvalho, S.M., Palma, M.S., Malaspina, O., 2017. Biochemical response of the Africanized honeybee exposed to fipronil: enzymatic assessment of honeybees exposed to fipronil. Environ. Toxicol. Chem. 36, 1652–1660. https://doi.org/10.1002/etc.3699.

Sanchez, W., Porcher, J.-M., 2009. Fish biomarkers for environmental monitoring within the water framework directive of the European union. TrAC, Trends Anal. Chem. 28, 150–158. https://doi.org/10.1016/j.trac.2008.10.012.

Sapcaliu, A., Radoi, I., Pavel, C., Tudor, N., Cauia, E., Siceanu, A., Meiu, F., 2009. Research regarding haemocyte profile from Apis mellifera carpatica bee haemolymph originated in the south of Romania. Lucr. Stiintifice Univ. Stiinte Agric. Banat. Timis. Med. Vet 42, 393–397.

Schmuck, R., Stadler, T., Schmidt, H.-W., 2003. Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee (*Apis mellifera* L, Hymenoptera): synergistic effect between fungicide and thiacloprid in honeybee. Pest Manag. Sci. 59, 279–286. https://doi.org/10.1002/ps.626.

StataCorp, 2021. Stata Statistical Software: Release 17. StataCorp LLC, College Station, TX.

Strand, M.R., 2008. The insect cellular immune response. Insect Sci. 15, 1–14. https://doi.org/10.1111/j.1744-7917.2008.00183.x.

Suresh, P.G., Reju, M.K.Mohandas, 1993. Haemolymph phosphatase activity levels in two fresh-water gastropods exposed to copper. Sci. Total Environ. 134, 1265–1277. https://doi.org/10.1016/S0048-9697(05)80132-9.

Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in honeybees (Apis mellifera) of exposure to neonicotinoids and sprayed fungicides in crops. Apidologie 45, 545–553. https://doi.org/10.1007/s13592-014-0273-6.

Tlili, S., Minguez, L., Giamberini, L., Geffard, A., Boussetta, H., Mouneyrac, C., 2013. Assessment of the health status of Donax trunculus from the Gulf of Tunis using integrative biomarker indices. Ecol. Indicat. 32, 285–293. https://doi.org/10.1016/j.ecolind.2013.04.003.

Topić Popović, N., Čižmek, L., Babić, S., Strunjak-Perović, I., Čož-Rakovac, R., 2023. Fish liver damage related to the wastewater treatment plant effluents. Environ. Sci. Pollut. Res. 30, 48739–48768. https://doi.org/10.1007/s11356-023-26187-y.

Tosi, S., Sfeir, C., Carnesecchi, E.Chauzat, 2022. Lethal, sublethal, and combined effects of pesticides on bees: a meta-analysis and new risk assessment tools. Sci. Total Environ. 844, 156857 https://doi.org/10.1016/j.scitotenv.2022.156857.

Travlos, I., Cheimona, N., Bilalis, D., 2017. Glyphosate efficacy of different salt formulations and adjuvant additives on various weeds. Agronomy 7, 60. https://doi.org/10.3390/agronomy7030060.

Wagner, N., Lötters, S., Veith, M., Viertel, B., 2015. Acute toxic effects of the herbicide formulation and the active ingredient used in cycloxydim-tolerant maize cultivation on embryos and larvae of the african clawed frog, Xenopus laevis. Bull. Environ. Contam. Toxicol. 94, 412–418. https://doi.org/10.1007/s00128-015-1474-z.

Wang, Y., Zhu, Y.C., Li, W., 2020. Comparative examination on synergistic toxicities of chlorpyrifos, acephate, or tetraconazole mixed with pyrethroid insecticides to honey bees (Apis mellifera L.). Environ. Sci. Pollut. Res. 27, 6971–6980. https://doi.org/10.1007/s11356-019-07214-3.

Wang, Y., Zhu, Y.-C., Li, W., Yao, J., Reddy, G.V.P., Lv, L., 2021. Binary and ternary toxicological interactions of clothianidin and eight commonly used pesticides on honey bees (Apis mellifera). Ecotoxicol. Environ. Saf. 223, 112563 https://doi.org/10.1016/j.ecoenv.2021.112563.

Williams, G.R., Alaux, C., Costa, C., Csáki, T., Doublet, V., Eisenhardt, D., Fries, I., Kuhn, R., McMahon, D.P., Medrzycki, P., Murray, T.E., Natsopoulou, M.E., Neumann, P., Oliver, R., Paxton, R.J., Pernal, S.F., Shutler, D., Tanner, G., van der Steen, J.J.M., Brodschneider, R., 2013. Standard methods for maintaining adult *Apis mellifera* in cages under *in vitro* laboratory conditions. J. Apicult. Res. 52, 1–36. https://doi.org/10.3896/IBRA.1.52.1.04.

Wright, G.A., Baker, D.D., Palmer, M.J., Stabler, D., Mustard, J.A., Power, E.F., Borland, A.M., Stevenson, P.C., 2013. Caffeine in floral nectar enhances a pollinator's memory of reward. Science 339, 1202–1204. https://doi.org/10.1126/science.1228806.

Yan, S., Cui, F., Qiao, C., 2009. Structure, function and applications of carboxylesterases from insects for insecticide resistance. PPL 16, 1181–1188. https://doi.org/10.2174/092986609789071243.

Yao, J., Zhu, Y.C., Adamczyk, J., 2018a. Responses of honey bees to lethal and sublethal doses of formulated clothianidin alone and mixtures. J. Econ. Entomol. 111, 1517–1525. https://doi.org/10.1093/jee/toy140.

Yao, J., Zhu, Y.C., Adamczyk, J., Luttrell, R., 2018b. Influences of acephate and mixtures with other commonly used pesticides on honey bee (Apis mellifera) survival and detoxification enzyme activities. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 209, 9–17. https://doi.org/10.1016/j.cbpc.2018.03.005.

Zhu, J., Liu, C., Wang, J., Liang, Y., Gong, X., You, L., Ji, C., Wang, S.-L., Wang, C., Chi, X., 2021. Difenoconazole induces cardiovascular toxicity through oxidative stress-mediated apoptosis in early life stages of zebrafish (Danio rerio). Ecotoxicol. Environ. Saf. 216, 112227 https://doi.org/10.1016/j.ecoenv.2021.112227.

Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017a. Feeding toxicity and impact of imidacloprid formulation and mixtures with six representative pesticides at residue concentrations on honey bee physiology (Apis mellifera). PLoS One 12, e0178421. https://doi.org/10.1371/journal.pone.0178421.

Zhu, Y.C., Yao, J., Adamczyk, J., Luttrell, R., 2017b. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (Apis mellifera). PLoS One 12, e0176837. https://doi.org/10.1371/journal.pone.0176837.