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Multi-biomarker approach and IBR index to evaluate the effects of different contaminants on the ecotoxicological status of *Apis mellifera*

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ABSTRACT

The honeybee, *Apis mellifera* L. (Hymenoptera: Apidae), a keystone pollinator of wild plant species and agricultural crops, is disappearing globally due to parasites and diseases, habitat loss, genetic constraints, beekeeper management issues and to the widespread use of pesticides. Besides insecticides, widely studied in this species, honeybees are also exposed to herbicides and fungicides and heavy metals whose lethal and sublethal effects need to be investigated. In this context, our study aimed to evaluate the effects of fungicides and of heavy metals on honeybees and to develop and apply a multi-biomarker approach that include an Integrated Biological Index (IBRv2) to assess the toxicological status of this species. Biomarkers of neurotoxicity (AChE and CaE), metabolic alteration (ALP, and GST) and immune system (LYS, granulocytes) were measured, following honeybees' exposure to cadmium or to a crop fungicide, using the genotoxic compound EMS as positive control. A biomarker of genotoxicity (NA assay) was developed and applied for the first time in honeybees. At the doses tested, all the contaminants showed sublethal toxicity to the bees, highlighting in particular genotoxic effects. The data collected were analyzed by an IBRv2 index, which integrated the seven biomarkers used in this study. IBRv2 index increased with increasing cadmium or fungicide concentrations. The IBRv2 represents a simple tool for a general description of honeybees ecotoxicological health status. Results highlight the need for more in-depth investigations on the effects of fungicides on non-target organisms, such as honeybees, using sensitive methods for the determination of sublethal effects. This study contributes to the development of a multi-biomarker approach to be used for a more accurate ecotoxicological environmental monitoring of these animals.

1. Introduction

The honeybee, *Apis mellifera* L. (Hymenoptera Apidae) is probably the most studied insect species. The strong interest of researchers in this species is due not only to its fascinating lifestyle and complex communication systems (Nieh and Roubik, 1995; Nieh, 1998), but also to the importance of the ecosystem services it provides as keystone pollinator of wild plant species and agricultural crops, and to the economic value of hive products (honey, royal jelly, bee wax, bee venom, pollen, and propolis) (Bogdanov et al., 2008; Kurek-Górecka et al., 2020; Simone-Finstrom and Spivak, 2010; Thorp, 2000).

Unfortunately, honeybees are globally endangered due to parasites and diseases, habitat loss, genetic constraints, beekeeper management issues, and overall to the inconsiderate and widespread use of pesticides (Neumann and Carreck, 2010; vanEngelsdorp and Meixner, 2010; Williams et al., 2010).

Pesticides, widely used in modern agriculture, have been recognized as a major stressor affecting honeybee colonies (Desneux et al., 2007; Tosi et al., 2018). The susceptibility of bees to agrochemical pesticides, in particular insecticides, has been the subject of many studies. Sublethal doses of insecticides such as organophosphates and carbamates (Williamson and Wright, 2013), pyrethroids (Chalvet-Monfray et al., 1996) and neonicotinoids (Imran et al., 2019; Ma et al., 2019; Wright et al., 2015) can affect the honeybees essential activities and lead to poor individual performance and population dynamics disorders of the colony (Colin et al., 2004). In 2013, the EU Commission restricted the use of

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plant protection products and treated seeds that contain three neonicotinoids (imidacloprid, clothianidin and thiamethoxam), after a risk assessment performed by the European Food Safety Authority (EFSA), to protect honeybees health.

Insecticides, however, are not the only chemical threat to honeybees, in fact herbicides and fungicides are also significantly present in rural environment being used more widely than insecticides; a recent study by Prado et al. (2019) reported that half of pesticides found in pollen pellet samples from apiaries of three French regions were fungicides. Besides pesticides, bees are also continuously exposed to the heavy metals present in urban and rural environments (Perugini et al., 2011). Heavy metals can reach the hive directly as particles retained on bees body or indirectly through water, nectar, and pollen contaminating the hive and hive products such as honeydew and propolis (Johnson, 2015; Perugini et al., 2011). Fungicides and heavy metals have been up to now, mostly investigated regarding their presence in bees and in products related to honey production (Bargańska et al., 2016; Conti and Botrè, 2001), their acute toxicity (mortality, adult malformations) (Porrini et al., 2002; Devillers, 2002; Ladurner et al., 2005) and for the potential to induce behavioral and ecological alterations in honeybees colonies (Cullen et al., 2019). Despite the growing interest for the potential harm of these compounds, sublethal effects (such as alterations at the molecular, biochemical and cellular level) have been only marginally investigated (Cullen et al., 2019). Such effects, nonetheless, might evolve into irreversible alterations of the physiology and lead to permanent damage of the honeybee populations.

Sublethal effects of contaminants can be investigated by the use of biomarkers, that are applied worldwide as sensitive tools for assessing organisms' ecotoxicological health status in different taxa, thereby contributing to a better understanding of the anthropogenic impact (Cajaraville et al., 2000; Campani et al., 2017; Sanchez and Porcher, 2009; Tlili et al., 2013), but up to now poorly applied to insects monitoring. Recently, researches on honeybees have focused on the development of some biomarkers (Badiou et al., 2008; Badiou-Bénéteau et al., 2012) to assess exposure to and effects of contaminants on honeybees (e.g inhibition of esterases to evaluate neurotoxic effects) (Badawy et al., 2015; Carvalho et al., 2013; Roat et al., 2017), however several responses, such as genotoxicity, immune system alteration, etc. remain unexplored and need a strong research effort.

Moreover, in addition to the development and the validation of a wider set of biomarkers, there is also a lack of adequate methodologies for the integrated processing of biological responses, such as integrated indices and expert systems finalized to assess the ecotoxicological health status and the risk to which honeybees populations are subjected.

In this context the present study aimed to evaluate the effects of fungicides and heavy metals on honeybees and to develop and apply a multi-biomarker approach that include an Integrated Biological Index (IBRv2) to assess the toxicological status of this species. To do that, we exposed honeybee workers to the fungicide Amistar® Xtra, and of Cd and we assessed their effects using a set of biomarkers which cover a wide range of biological responses: acetylcholinesterase (AChE) and carboxylesterase (CaEs) as biomarkers of neurotoxicity, glutathione-*S*-transferase (GST) and alkaline phosphatase (ALP) as biomarkers of phase II and metabolic alteration, lysozyme (LYS) and hemocytes count as markers of immune system. One of the main focus was to develop and apply the Nuclear Abnormalities (NA) assay that represents an important genotoxicity biomarker, never investigated in this species up to now. The multidimensional perspective provided by the use of a large set of biomarkers was used to develop, for the first time in honeybees, a biomarker response index (IBRv2), proposed by Sanchez et al. (2013), which represents a synthetic indicator to evaluate the susceptibility to different contaminants and the general ecotoxicological status of honeybees.

2. Material and methods

2.1. Chemicals

Monobasic and dibasic sodium phosphate, sodium chloride (NaCl), Tritons X-100, protease inhibitor cocktail powder; acetylthiocholine iodide (AcSCh.I), 5,5-dithio-bis(2, nitrobenzoic acid) (DTNB); 1-chloro-2,4-dinitrobenzene (CDNB), reduced L-glutathione (GSH); Fast Garnet GBC, sodium dodecyl sulfate (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, bovine serum albumin (BSA), ethyl methane-sulfonate (EMS) and cadmium sulfate ($CdSO_4$) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). BioRad Protein dye was obtained from BioRad (Segrate, Italy); Diff-Quick dye from Bio-optica (Milano, Italia); Amistar® Xtra was obtained by Syngenta (Basel, Switzerland).

2.2. Honeybees

Honeybee foragers were collected from hives of a beekeeper located close to a nature reserve area (Pisa, Tuscany, Italy). The bees were gathered on the day before the experiment and placed in six cages (75 cm × 75 cm × 115 cm, Bug-Dorm-2400 Insect Rearing Tent, MegaView Science Co., Ltd., Taiwan) 50 individuals each, to rest overnight at 25 °C and 60% relative humidity with honey and water ad libitum. As a support for the bees, the cages contained a leafless branch of *Prunus spinosa* (Rosaceae).

2.3. Exposure conditions

The compounds used for the experiment were: the fungicide Amistar® Xtra at two concentrations (100 g/L, 200 μg per bee; 200 g/L, 400 μg per bee), and $CdSO_4$ at two concentrations (0.1 g/L, 0.2 μg per bee and 2.5 g/L, 5 μg per bee), using acetone (80% in water) as vehicle. EMS (12.4 g/L, 24.8 μg per bee), a well-known genotoxic compound, was used as positive control. The commercial Amistar® Xtra is mainly composed by azoxystrobin in co-formulation with cyproconazole (80 g/L); azoxystrobin is a strobilurin, cyproconazole is a triazole and both are among the most frequently applied fungicides worldwide (Bartlett et al., 2002; Han et al., 2016). Regarding the treatments with the Amistar® Xtra we used recommended field usage concentrations for cereal crops; cadmium concentrations used in our study were below the LD_{50} (6–30 μg per bee) reported by Nikolić et al. (2016).

On the day of the experiment, the honeybees were mildly anesthetized with CO_2, and 2 μL of the different contaminant solution, were applied on the dorsal thorax of the bees by a Burkard hand micro applicator equipped with 1-mL syringe (Bedini et al., 2017). Control bees' group was treated with 2 μL of acetone. Each group of treated bees was put in a separate cage for 5 days, and the mortality recorded every day.

2.4. Collection of tissue samples

Biomarker analysis were conducted on three biological materials: hemolymph, head and gut. 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 hemolymph was collected with a micropipette, then the midgut was removed with tweezers and the head was separated by the rest of the body. The samples were immediately frozen and stored at − 80 °C. Heads and midguts were used for the analysis of the enzymatic biomarkers whereas hemolymph was utilized for the differential count of the hemocytes and NA assay.

2.5. Enzyme assays

Enzyme assays were performed on different biological tissues of the same honeybee. Extract from heads nervous tissues were used to evaluate esterase activity (AChE and CaE) whereas GST, ALP and lysozyme activity were evaluated on midgut extracts. For the preparation of each extract, 5 specimens were pooled, either in the case of heads and guts (Badiou-Bénéteau et al., 2013; Belzunces et al., 1988). Tissue samples were weighted, and extraction medium was added in a volume corresponding to 10% (wt/vol) of the tissue. The buffer contained 40 mM Na phosphate buffer (pH 7.4), a mixture of protease inhibitors enzymes and 1% Triton X-100. The samples were homogenized by a Tissue Lyser homogenizer (Qiagen) for three periods of 30 s at 30-s intervals. Subsequently the homogenates were centrifuged at 4 °C for 20 min at 13,000 and 15,000*g* for heads, and guts samples, respectively. The resulting supernatants were frozen at − 80 °C and used for the analysis.

2.5.1. AChE

The AChE activity was assayed in the head extracts according to Ellman et al. (1961), modified. The reaction mixture was prepared in 3 mL cuvette and contained 2.78 mL sodium phosphate buffer (0.1 M; pH 7.4), 0.1 mL DTNB (10 mM), 0.02 mL acetylthiocholine (41.5 mM) and 5 μL head extract. The activity was monitored continuously for 5 min at 410 nm (25 °C) and expressed in $\mu mol^{-1} g$ tissue^{-1}min.

2.5.2. CaE

CaE activity in the head extracts was measured according to Gomori (1953), modified. A mixture containing 2.820 mL sodium phosphate buffer (100 mM pH 7.4) and 0.1 mL head extract was prepared and incubated at 25 °C for 5 min. The reaction was started by adding 80 μL α-NA (0.4 mM) as a substrate. After 3 min, the reaction was stopped adding 1.5% SDS and 0.4 mg/L Fast Garnet GBC. The products of the reaction were quantified spectrophotometrically (Agilent CARY UV60) at 538 nm (25 °C) and the enzyme activity was expressed as nmol α-NA min^{-1} mg^{-1} protein ($\varepsilon = 23.59 \times 10^3$ mM^{-1} cm^{-1}).

2.5.3. GST

GST activity was measured in the midguts, following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) as described by Habig et al. (1974) with some modification. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.4), 8 mM GSH, 8 mM CDNB and 30 μL extract. Absorbance was recorded with a spectrophotometer (Agilent CARY UV60) at 340 nm (25 °C) and expressed as nmol CDNB conjugate formed min^{-1} mg^{-1} protein ($\varepsilon = 9.6 \times 10^3$ mM^{-1} cm^{-1} ^{1}).

2.5.4. ALP

ALP activity was assayed in the midguts following the formation of p-nitrophenol, a product of the hydrolysis of the substrate (PNPP) due to the enzyme's activity, according to Bounias et al. (1996) modified. The reaction mixture consisted of 100 mM Tris-HCl buffer (pH 8.5), 100 mM $MgCl_2$, 100 mM p-NPP as the substrate and 25 μL guts extract. The reaction was monitored continuously for 5 min at 405 nm (25 °C) at the spectrophotometer (Agilent CARY UV60) and the activity was expressed as nmol p-nNPP min^{-1} mg^{-1} protein ($\varepsilon = 18.81 \times 10^3$ mM^{-1} cm^{-1-1} cm^{-1}).

2.5.5. Lysozyme

Lysozyme activity was measured in the midgut using a turbidity test according to Keller et al., 2006, modified. Two solutions were prepared: a stock solution 1 mg/mL of lysozyme from chicken egg whites (HEL, Sigma, St. Louis, MO) in phosphate buffer (pH 5.9) and a *Micrococcus lysodeikticus* (Sigma, St. Louis, MO) solution 0.3 mg/mL. The HEL was diluted in phosphate buffer to obtain a calibration curve with 0, 0.3, 0.6, 1.25, 2.5, 5, 10 and 20 μg/mL. Aliquots of 25 μL for each concentration were added to a 96-well plate in triplicate and 75 μL of each gut extract was added in quadruplicate to the same plate. The *M. lysodeikticus* solution was quickly added to each standard well (175 μL) and to three sample wells (125 μL). The blank was obtained by adding 125 μL of buffer to the fourth well sample, without *M. lysodeikticus*. The absorbance was monitored at 450 nm with a Microplate Reader (Model 550, Bio Rad) and the lysozyme activity expressed as HEL concentration (μg/mL) by linear regression of the standard curve.

2.5.6. Protein concentrations

The protein concentrations were measured according with the method of Bradford (1976) by BioRad Protein Assay (BioRad), using bovine serum albumin (BSA) as standard.

2.6. NA assay and differential counts of granulocytes and plasmatocytes

The NA assay and the differential counts of granulocytes and plasmatocytes were assayed on the same slide for each sample. Ten μL of two honeybees' hemolymph were placed on two slides 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 carried out following the procedure according to Pacheco and Santos (1997) with some modification. The cells were counted using an immersion light microscope (Olympus BX41) and according to Pacheco and Santos (1997), abnormalities were attributed to one of the following categories: micronuclei, lobed nuclei, segmented nuclei and kidney shaped nuclei. The apoptotic cells were also counted. The results were expressed as the number of nuclear abnormality/1000 cells.

Granulocyte and plasmatocytes count were made following Șapcaliu et al. (2009). A thousand blood cells were counted, and the result was expressed as the number of cells/1000.

2.7. Statistical analysis

Data were analyzed by Kruskal–Wallis (KW) nonparametric test (Kruskal and Wallis, 1952; Hollander et al., 2014). Accordingly, for each biomarker we tested the null hypothesis that the median was the same across control and treatment groups in the population. When the difference among medians were found significant, we performed multiple pairwise comparison tests using the Holm–Sidák adjustment (Holm, 1979). In addition, Spearman's correlation coefficient was used for assessing significant association between each couple of biomarkers and its intensity. Finally, for each treatment group, the Integrated Biological Response (IBRv2) index (Sanchez et al., 2013) was computed to summarize the effect of all biomarkers as follows:

First, the following log transformation was applied to reduce the variance,

$$Y_{kg} = \log[f_0](\frac{\overline{X}_{Kg}}{\overline{X}_{K0}}) =$$

Second, we take the standardized value of Y_{kg} by computing

$$z_{kg} = (Y_{kg} - \overline{Y}_k) \Big/ s_k$$

where $Y_{kg} = \sum_{g=1}^{G} Y_{kg}$ and $s_k = \sum_{g=1}^{G} (Y_{kg} - \overline{Y}_k)$.

Third, we compute the biomarker deviation index as

$$I_{kg} = z_{kg} - z_{k0}$$

where z_{k0}refers to the standardized value of the control group. Finally, the IBRv2 index was computed using the method proposed by Beliaeff and Burgeot (2002), namely as

$$IBRv2 = \sum_{k=1}^{K} I_{kg}$$

where $\overline{X}_{Kg}$is the average value of the k-th (k = 1…K) biomarker in a

particular treatment group g (g = 1... G), and $\overline{X}_{K0}$ is its average value in the control group.

As a general stress index IBRv2 provides a simple interpretation of the level of contamination in a particular group of analysis: the higher its value, the higher the contamination is. The underlying multidimensional data structure was represented by spider graphs and the corresponding IBRv2 values were reported above each of them. The spokes of each spider graph display the values assumed by each biomarker deviation index I_{Kg}. In each spider graph, the area up to zero reflects biomarker induction, and the area down to 0 indicates a biomarker inhibition. All statistical analyses were performed using STATA 14 software (StataCorp., 2015).

3. Results

Biomarkers of neurotoxicity (AChE and CaE), metabolic (ALP, and GST), immune system (LYS) and genotoxicity (NA assay) were assessed, after honeybee's exposure to cadmium, Amistar® Xtra and EMS.

No mortality of the honeybees was observed during the experiment, confirming that the contaminant doses were sublethal.

Data presented in Fig. 1A shows that AChE activity was inhibited in all treatment groups compared to controls, with statistically significant differences for Cd 0.1 g/L, (26% inhibition), Cd 2.5 g/L (36% inhibition), and Amistar® Xtra 200 g/L (26% inhibition). Results highlight a dose dependent inhibition for Cadmium and Amistar® Xtra with a statistical difference between the two doses of each compound.

CaE activity (Fig. 1B) was significantly induced in Amistar® Xtra 100 g/L group with respect to control and to Amistar® Xtra 200 g/L. An increase in CaE activity was also found in Cd 2.5 and EMS groups.

All treatment groups had an increase in GST activity (Fig. 2A), that was significant for Amistar® Xtra at 200 g/L and EMS. The GST values increased with increasing Amistar® Xtra concentrations.

Alkaline phosphatase (ALP) activity was inhibited in all treatments groups when compared to the control (Fig. 2B); Cadmium treatments showed the most evident inhibition with statistical difference respect to control, as well as EMS.

Lysozyme activity was inhibited by all treatments in comparison with control (Fig. 3A), inhibition was particularly evident with both Cd and EMS treatments, whereas Amistar® Xtra slightly inhibit lysozyme activity at both the concentrations. Statistically significant differences were found for Cd 2.5, Amistar® Xtra 200 g/L and EMS compared to the control.

Plasmatocytes showed slight decreases after treatments with the highest doses of Cd and Amistar® Xtra. A statistically significant difference was observed between EMS and control.

There was a general increase of total nuclear abnormalities after all treatments (Fig. 3B). EMS treatment shows the highest number of abnormalities with statistical difference compared to control. Statistically significant differences were also found between Cd 0.1 g/L, Amistar® Xtra 200 g/L and control and between the two Cd doses ($p < 0.05$). The most frequent abnormalities observed were the lobed hemocytes, with a statistical difference between EMS and control and between the two Cd treatments ($p < 0.05$). Regarding segmented and binucleated hemocytes, statistically significant differences between the two Cd treatments were found ($p < 0.05$), while regarding micronuclei, statistically significant differences were observed between the two Amistar® Xtra treatments and in EMS treatment compared to control. The highest apoptosis mean value was found in EMS treatment, with a statistical difference compared to control ($p < 0.05$); moreover, a statistical difference was found between the two Amistar® Xtra treatments ($p < 0.05$).

In Table 1 Spearman correlation coefficients between couple of biomarkers are displayed. As expected, some of these correlations are significantly different from zero. Nevertheless, the intensity of such correlations is not high. Therefore, there is not redundancy in the data and each biomarker contributes with own information to the stress level.

The results of the Integrated Biological Response (IBRv2) in each treatment group are shown in Fig. 4. EMS treatment showed the highest IBRv2 value (13.50), where GST, NA and CaE values were the most discriminant factors for this treatment. Cadmium doses 100 g/L and 200 g/L showed almost the same IBRv2 value (9.68 and 10.85, respectively). In both cadmium treatments, variations in the activity of GST, AChE and CaE and NA frequencies were the most relevant responses that explain the IBRv2 indicator. The Amistar® Xtra highest dose showed an IBRv2 value of 8.19, due to AChE and GST activity changes and to NA frequencies. The Amistar® Xtra lower dose presented the lowest IBRv2 value (5.79) once it was observed minor variations in analyzed biomarkers.

4. Discussion

The exposure of honeybees to environmental pollution raises questions about the risk of colony decline (including pollination services and contamination of beehive products), crop production, food security, and environmental health. We used a multi-biomarker approach to evaluate the effects of different contaminants on the ecotoxicological status of *Apis mellifera*.

AChE is an important enzyme that hydrolyzes acetylcholine at the cholinergic synapses, allowing precise control and modulation of the neural transmission (Badiou et al., 2008) that can be inhibited by some insecticides and other contaminants, being a sensitive biomarker of neurotoxicity. In honeybees, acetylcholinesterases are localized in the head and, in particular, in eye and ocelli (Kral, 1980; Kral and Schneider, 1981). In our work, AChE assay showed no neurotoxic effect of EMS. This result was expected because the compound is known for its genotoxic effects only. Several authors observed that the AChE activity decrease is not due only to organophosphates and carbamates, but also other classes of environmental contaminants such as complex mixtures of pollutants, metals and detergents that can cause AChE inhibition (Diamantino et al., 2003; Frasco et al., 2005; Guilhermino et al., 1998;

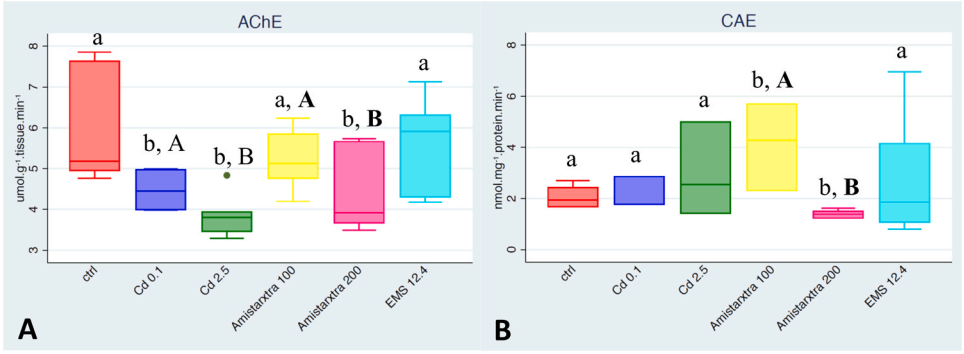

Fig. 1. Activity of AChE (A) and CaE (B) measured in the head of honeybees (*Apis mellifera*) exposed to Amistar® Xtra (100, 200 g/L), Cadmium (0.1, 2.5 g/L) and EMS (12.4 g/L). Different normal lowercase letters (e.g., a, b) indicate significant differences with respect to control ($p < 0.05$); different uppercase letters (e.g., A, B) indicate significant differences between cadmium treatments and different bold uppercase letters (e.g. **A**, **B**) letters indicate significant differences between Amistar® Xtra treatments ($p < 0.05$).

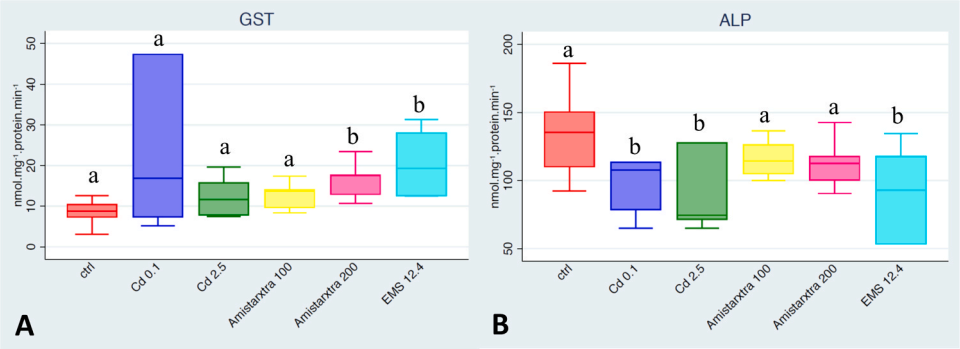

Fig. 2. Activity of GST (A) and ALP (B) measured in the gut of honeybees (*Apis mellifera*) exposed to Amistar® Xtra (100, 200 g/L), Cadmium (0.1, 2.5 g/L) and EMS (12.4 g/L). Different normal lowercase letters (e.g., a, b) indicate significant differences with respect to control ($p < 0.05$).

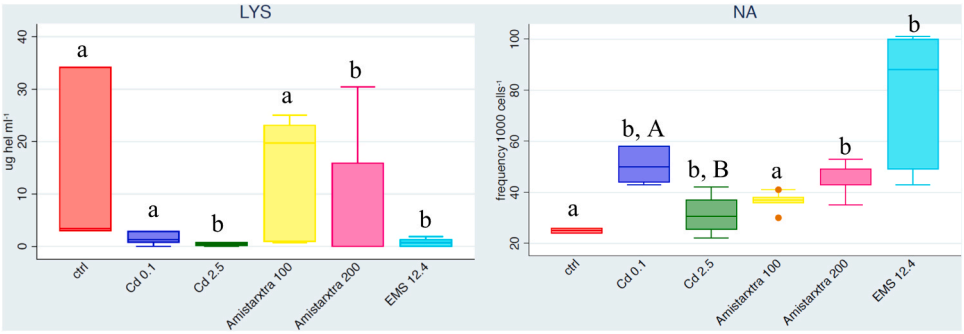

Fig. 3. Activity of LYS (A) and NA (B) measured in the gut and the hemolymph of honeybees (*Apis mellifera*) exposed to Amistar® Xtra (100, 200 g/L), Cadmium (0.1, 2.5 g/L) and EMS (12.4 g/L). Different normal lowercase letters (e.g., a, b) indicate significant differences with respect to control ($p < 0.05$) and different uppercase letters (e.g., A, B) indicate significant differences between cadmium treatments ($p < 0.05$).

Table 1
Spearman correlation index between biomarkers of the honeybees after 5 days exposure to Amistar® Xtra (100, 200 g/L), Cadmium (0.1, 2.5 g/L) and EMS (12.4 g/L). Statistically significant correlations are indicated with * ($p < 0.05$) and ** ($p < 0.01$).

	PLASM	NA	AChE	GST	ALP	LYS
NA	−0.2665 *					
AChE	0.0625	0.0889				
GST	−0.3484 **	0.1664	−0.2600 *			
ALP	0	−0.0515	0.5146 **	−0.1636		
LYS	0.4193 *	−0.1274	0.2384	0.0519	0.2658 *	
CaE	−0.1026	0.1822	0.2469 *	0.3197	−0.2260	−0.1343

Payne et al., 1996). Our results confirm the neurotoxic activity of Cd in honeybees. The AChE inhibition observed in our study after Cd treatment, is in accordance with other authors that demonstrated that five metal ions (nickel, copper, zinc, cadmium and mercury) decrease the AChE activity (Frasco et al., 2005) also in honeybees sampled in a metals polluted site (Badiou-Bénéteau et al., 2013). Our study also highlights an AChE inhibition by Amistar® Xtra. To the best of our knowledge, to date, no study is available on the neurotoxic effects of fungicides on honeybees. Our study demonstrates that Amistar® Xtra has an evident effect on AChE and indicates that AChE could constitute a biomarker for honeybees' exposure to fungicides.

CaEs are hydrolases that catalyze the reactions of a wide range of aliphatic/aromatic esters and choline esters, as well as some xenobiotics (Dauterman, 1985). They play a double role, both as phase I detoxifying enzymes and in the defense, mechanism protecting AChE from the inactivation caused by organophosphates and carbamates. As for CaE, its activity was induced by almost all treatments, except for the highest dose of Amistar® Xtra. Various authors (Badiou-Bénéteau et al., 2012; Carvalho et al., 2013; Hashimoto et al., 2003; Roat et al., 2017) observed a CaE induction after honeybees' exposure to several pesticides, such as spinosad, fipronil and thiamethoxam. Although the CaE decrease observed with the Amistar® Xtra 200 g/L dose cannot be compared with previous studies, due to the lack of studies evaluating the CaE activity following fungicides exposure, a similar result has been previously observed by Carvalho et al. (2013) after honeybees exposure to the insecticide deltamethrin. Our results suggest that CaE activity can be used as a biomarker of honeybees' exposure to cadmium and fungicides.

GST is an enzyme involved in phase II biotransformation processes, being responsible for detoxification of several contaminants and appears to contribute to cellular protection against oxidative damage (Babczyńska et al., 2006; Barata et al., 2005). The observed increase of GST activities strongly suggests the induction of oxidative stress by Amistar® Xtra, cadmium and EMS. It has been demonstrated that metals are able to increase GST activity (Badiou-Bénéteau et al., 2013; Yu et al., 2012). Being EMS a well-known genotoxic compound, it was predictable to observe a GST induction due to EMS exposure, because of the GST role in detoxification processes. It has been also demonstrated that GST activity is modulated by insecticides, which can cause both an increase (Carvalho et al., 2013) or a decrease of its activity (Badiou-Bénéteau et al., 2012; Lupi et al., 2020; Yao et al., 2018) . To the best of our knowledge,

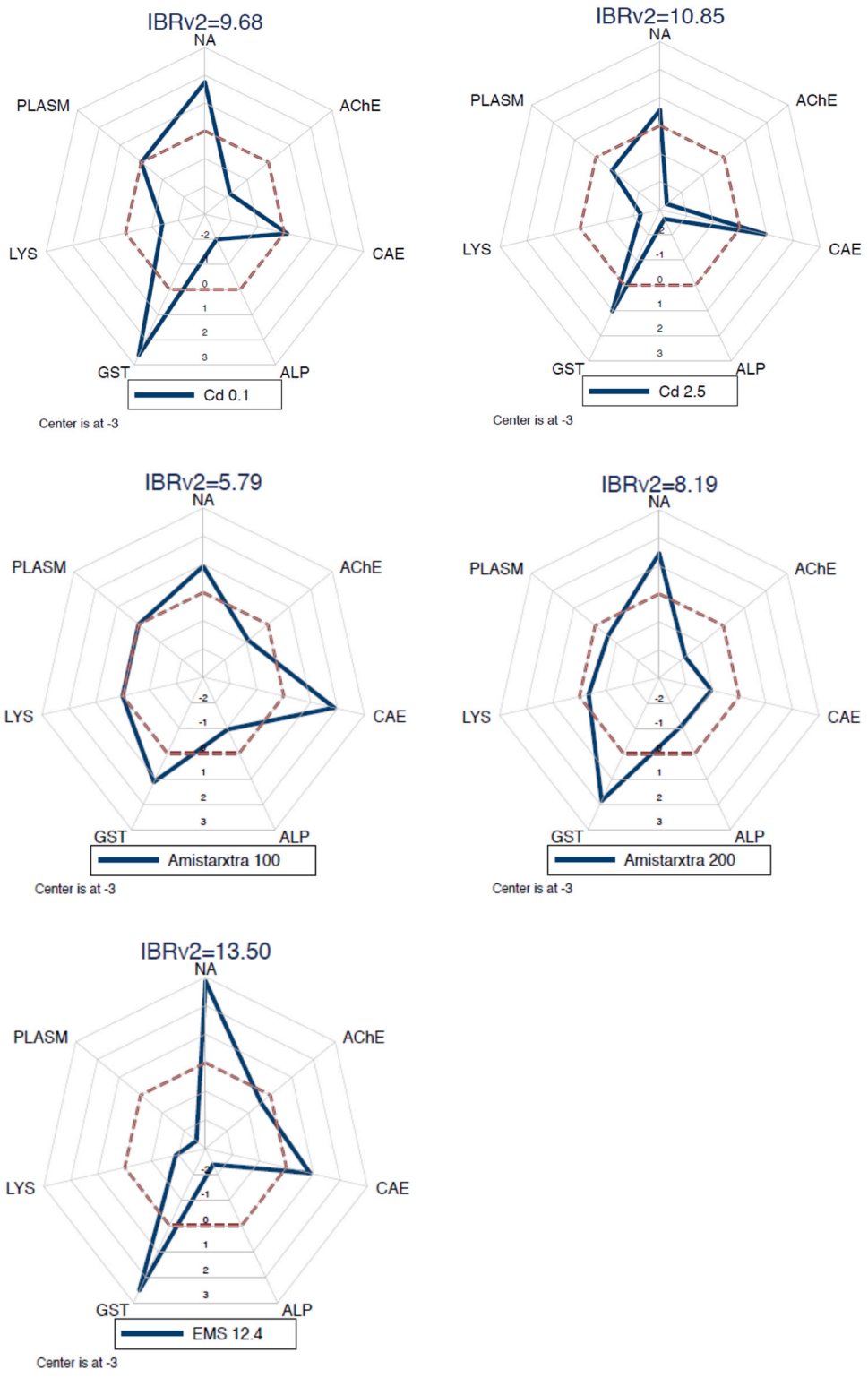

Fig. 4. Spider graphs of Integrated biological response (IBRv2) for each treatment.

there are no studies that investigated the effect of fungicides on GST activity in honeybees. However, Han et al. (2014) observed a dose-dependent increase in the GST activity in the earthworm *Eisenia fetida* (Savigny, 1826) exposed to the fungicide azoxystrobin. Johansen et al. (2007) also observed that exposure to 10 mg/L of fenpropimorph (a demethylation inhibitor fungicide) in the artificial diet of *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae) increased the GST activity. Overall, CaE and GST are both enzymes involved in detoxification processes, in phase I and II, respectively. Both these enzymes can be induced by various chemical compounds because of their role in the detoxification of endogenous and exogenous substances (Barata et al., 2005; Stone et al., 2002). Our results suggest that CaE and GST are involved in the detoxification process; in particular, they seem to modulate cadmium and fungicides detoxification processes in honeybees.

ALP is part of a family of enzymes involved in digestive processes,

cell signaling, and transport of metabolites and antioxidants through the hydrolysis of phosphate groups (Bounias et al., 1985). In the present study, Amistar® Xtra treatments caused a general ALP activity to decrease in tissues. Our results are supported by those of Carvalho et al. (2013), showing that dorsal exposure of honeybees to fipronil elicits a 20% decrease in ALP activity. On the other hand, Badiou-Bénéteau et al. (2012) reported a significant increase in phosphatase activity after thiamethoxam treatment. Although it is known that ALP is not involved in pesticide detoxification, fungicides could affect the ALP activity in honeybees. In our study Cd treatments caused a strong decrease in honeybees ALP activity. Although Bounias et al. (1996) observed an increase in phosphatase activity after copper treatment in honeybees, Vlahović et al. (2009) observed a significant reduction of ALP activity after the acute cadmium exposure. Phosphatase inhibition could be explained by the formation of insoluble clusters, taking into account the strong affinity of cadmium for the sulphydryl groups of proteins (Braeckman et al., 1999; Van Straalenand and Donker, 1994) and/or by the substitution of metal cofactors in the enzyme's active site. For these reasons, ALP can be used as a biomarker of honeybees' exposure to pesticides (Suresh et al., 1993) and metals.

In bees, immune system is made up of hemocytes and proteins present in the hemolymph. The lysozyme enzyme is an important factor of honeybees' immune responses; it is able to degrade the bacterial cell wall and its synthesis is carried out in all development phases (Lazarov et al., 2016). To the best of our knowledge, the data obtained in this study are not comparable with other works about bees. However, studies performed on other taxa show that heavy metals and chemical compounds, such as cadmium and insecticides, can modulate the bees' lysozyme activity (Mdaini et al., 2019; Wu et al., 2007). In our study, the treatment groups with the highest inhibition of lysozyme activity also showed the highest values of granulocytes. This could confirm that cadmium and Amistar® Xtra, at higher doses, are able to affect the immune system of honeybees.

Hemocytes (prohemocytes, plasmatocytes, granulocytes, oenocytoids and macrophage-like cells) have several important immunological functions in phagocytosis, encapsulation, nodulation, and wound repair (Amdam et al., 2005). Among the five different hemocytes, plasmatocytes and granulocytes are the most abundant circulating cell types (Giglio et al., 2015) and plasmatocytes exhibited phagocytic activity following the latex bead immune challenge. Consequently, the loss of immune cells in foragers constitutes a dramatic decline in their immune efficiency (Bedick et al., 2001). Our results showed slight variations after treatments with the highest doses. To the best of our knowledge, no ecotoxicological studies on honeybees examined this kind of response. The only work that investigated the number of functional hemocytes in honeybees was published by Amdam et al. (2005) were a decrease in hemocytes numbers after a change in the insects' diet was observed. Since the number of circulating hemocytes reflects the organism's capacity to cope with immunogenic challenges (Kraaijeveld et al., 2001; Doums et al., 2002), our results permit to hypothesize a good status of the honeybees immune system.

Maintaining DNA integrity is an aspect of great importance to all organisms and the exposure to genotoxic compounds can alter this integrity. For that, Nuclear Abnormalities (NA) assay is a valid instrument to evaluate the presence of genetic damages. In this study, we observed a general increase of nuclear abnormalities after all treatments, with EMS showing the highest values. Moreover, EMS seems to cause the highest number of apoptotic cells. This could be explained by the fact that cells go toward apoptosis and do not replicate to preserve DNA integrity (Brunetti et al., 1988; Das and Nanda, 1986; Guilherme et al., 2008). In the present study, for the first time, a NA assay was developed and validated in *A. mellifera.* This was the main reason for the choice of the treatment with EMS, a direct genotoxic compound, that, providing a clear response, allowed the test validation. Overall, the results showed that Cd and Amistar® Xtra have a genotoxic effect on honeybees, validating the use of NA assay as a biomarker of exposure and effect for these contaminants. We must underline that while genotoxic potential of cadmium was already known (Matić et al., 2016) results for the fungicide are totally new and extremely interesting.

A biomarker response index can quantify the combined biological effects measured by a battery of biomarker and it is able to ensure a more complete diagnosis (Lupi et al., 2020). The index has been widely used to analyze the effects of environmental pollutants on various biological responses to determine the impact of environmental pollutants in organisms (Cao et al., 2019; Liu et al., 2016; Matić et al., 2020). In recent years several authors used the index for the risk assessment of aquatic ecosystems in plants (Li et al., 2020; Wang et al., 2015) and animals such as clams (Arrighetti et al., 2019; Beliaeff and Burgeot, 2002; Damiens et al., 2007; Leiniö and Lehtonen, 2005; Raftopoulou and Dimitriadis, 2010) and, fish (Broeg and Lehtonen, 2006; Oliveira et al., 2009; Sanchez et al., 2013; Vieira et al., 2014). To date, however, no studies that used the IBRv2 index to evaluate honeybee's toxicological health status are available.

In this study, we used the IBRv2 index to integrate the responses of the seven selected biomarkers (AChE, CAE, GST, ALP, LYS, plasmatocytes count and NA assay) to evaluate the toxicity of Amistar® Xtra, cadmium, and EMS in *Apis mellifera*. The investigated biomarkers exhibited a response that was induced or inhibited according to the different treatment groups. The spatial arrangement of these biomarkers in the star plot allowed visualizing more clearly which biomarkers were the most sensitive in this kind of evaluation (Vieira et al., 2014). Among the contaminants tested, EMS showed the highest IBRv2 values. These results were most likely expected, due to the known toxic effects of EMS, which was used as a positive control for the experiment. Regarding both fungicide and cadmium exposure, the higher was the concentration, the greater was the IBRv2 value. Generally, 200 g/L is the Amistar® Xtra dose used to defend cereal crops and sunflowers, and highest IBR value were observed to this concentration, highlighting a modification in ecotoxicological health status of honeybees at the environmental dose of this fungicide. Among the tested biomarkers, NA assay was one of the most affected by the treatment, underlining the genotoxic potential of this compounds, never investigated before in non-target organisms and, in particular, in honeybees.

Environmental monitoring programs reported a cadmium concentration ranging from 0.02 to 0.1 mg/kg in bees (Perugini et al., 2011; Ruschioni et al., 2013; van der Steen et al., 2012), and from 20 to 60 μg/kg in pollen (Conti and Botrè, 2001; Formicki et al., 2013). In our study, IBRv2 index increased with increasing cadmium concentrations. Similar results were obtained by Matić et al. (2020), who observed IBR index increased with increasing cadmium concentrations in specimens of *Lymantria dispar* exposed to two cadmium doses (50 ug and 100 ug Cd/g dry food). Our results demonstrated that IBRv2 index reflect cadmium toxicological potential showing the honeybees high sensitivity to the metal and a dose-response effect.

5. Conclusions

The results obtained in this work highlighted the effectiveness of the applied biomarkers battery. The IBR approach provides a simple tool for a general description of honeybees ecotoxicological health status, combining the different biomarker responses. The results relating to Amistar® Xtra reinforce the idea that it is necessary to carry out more in-depth investigations on fungicides widely used in agriculture, which must be tested on non-target organisms and with sensitive methods for the determination of sublethal effects. These effects can in fact evolve into irreversible alterations and bring permanent damage to natural populations that are integral part of ecosystems, such as honeybees. This study contributes to the development of a multi-biomarker approach, able to investigate the different toxicological responses in honeybees, which represents a fundamental tool for a more accurate environmental monitoring of these animals and the potential danger deriving from anthropic contamination.

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. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Ilaria Caliani: Writing - original draft, Writing - review & editing, Conceptualization, Methodology, Investigation. **Tommaso Campani:** Writing - original draft, Writing - review & editing, Conceptualization, Methodology, Formal analysis. **Barbara Conti:** Writing - original draft, Resources. **Francesca Cosci:** Resources. **Stefano Bedini:** Writing - review & editing. **Antonella D'Agostino:** Formal analysis. **Anna Ammendola:** Investigation. **Agata Di Noi:** Writing - original draft. **Alessandro Gori:** Investigation, Writing - original draft. **Silvia Casini:** Supervision, Conceptualization, Writing - review & editing.

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