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ENZYMATIC BIOMARKERS AS TOOLS TO ASSESS ENVIRONMENTAL QUALITY: A CASE STUDY OF EXPOSURE OF THE HONEYBEE *APIS MELLIFERA* TO INSECTICIDES

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Abstract: The present study was intended to evaluate the responses of enzymes in the honeybee *Apis mellifera* after exposure to deltamethrin, fipronil, and spinosad and their use as biomarkers. After determination of the median lethal doses (LD50), honeybees were exposed at doses of 5.07 ng/bee and 2.53 ng/bee for deltamethrin, 0.58 ng/bee and 0.29 ng/bee for fipronil, and 4.71 ng/bee and 2.36 ng/bee for spinosad (equivalent to 1/10th [LD50/10] and 1/20th [LD50/20] of the LD50, respectively). The responses of acetylcholinesterase (AChE), carboxylesterases (CaEs-1–3), glutathione-*S*-transferase (GST), catalase (CAT), and alkaline phosphatase (ALP) were assessed. The results showed that deltamethrin, fipronil, and spinosad modulated these biomarkers differentially. For the enzyme involved in the defense against oxidative stress, fipronil and spinosad induced CAT activity. For the remaining enzymes, 3 response profiles were identified. First, exposure to deltamethrin induced slight effects and modulated only CaE-1 and CaE-2, with opposite effects. Second, spinosad exhibited an induction profile for most of the biomarkers, except AChE. Third, fipronil did not modulate AChE, CaE-2, or GST, increased CAT and CaE-1, and decreased ALP. Thus, this set of honeybee biomarkers appears to be a promising tool to evaluate environmental and honeybee health, and it could generate fingerprints to characterize exposures to pesticides. *Environ Toxicol Chem* 2013;32:2117–2124. © 2013 SETAC

Keywords: Honeybee Insecticide Biomarker Sublethal effect

INTRODUCTION

The honeybee *Apis mellifera* L., 1758 (Hymenoptera: Apidae), has great agronomic, environmental, and economic importance. It pollinates several plants and thus contributes to increased crop production and quality [1] and to plant biodiversity [2]. Advances in agricultural practices, intense use of pesticides, monocultures, and even interactions with pathogens responsible for honeybee diseases are important factors in vanishing honeybee populations and the corresponding losses in food production [3,4]. While foraging, honeybees can explore as far as 1.5 km to 3.0 km from the hive to collect resources such as nectar, pollen, resins, and water, any of which may be in contact with different pollutants such as pesticides [5].

Monitoring environmental health requires not only analytical but also biological tools such as bioindicators. Among bioindicators, *A. mellifera* may be regarded as a relevant candidate to gauge the environmental impacts of pesticides. It can be used as a bioindicator either directly, by its abundance and its physiological integrity as analyzed using biomarkers, or through the presence of residues in hive products and in honeybees [6]. Different biomarkers have been developed in the honeybee, and some may potentially be used for assessing environmental quality [7–9]. However, compared with other biological species (vertebrates or invertebrates), few studies have used the honeybee as a bioindicator [9,10]. Toxicological studies focused on honeybee enzyme markers were initiated by Metcalf and March [11]. Later, Gilbert and Wilkinson [12] and Yu et al. [13] showed that carboxylesterases (CaEs), glutathione-*S*-transferase (GST), DDT-dehydrochlorinase, and microsomal oxidases can be modulated by pesticides. Two decades were necessary for the use of honeybee acetylcholinesterase (AChE) as a biomarker to assess the impact of organophosphates and carbamates [14–16] and thereafter the use of other biomarker responses after exposure to pesticides [17–19].

Among the different insecticides used in fields, the pyrethroid deltamethrin, the phenylpyrazole fipronil, and the spinosyn spinosad are notable. Their modes of action are characterized, respectively, as blocking voltage-dependent sodium channels and thereby causing rapid membrane depolarization of neurons [20], antagonizing γ-aminobutyric acid receptors (GABAR) [21], and acting as agonists of nicotinic acetylcholine receptors and antagonists of GABAR [22]. These insecticides exhibit high toxicity to the honeybee, with acute median lethal dose (LD50) values ranging from 4 ng/bee to 67 ng/bee [23–26], and can induce adverse physiological and behavioral effects [27]. Thus, because these insecticides belong to the insecticide classes that are most often used in agriculture [28], they can pose problems to beneficial insects [29] and may contribute to honeybee decline [30].

In a previous study, we developed a set of 7 biomarkers and validated them after exposure to thiamethoxam (neonicotinoid) [9]. These biomarkers reveal the integrity of the nervous system (AChE and CaEs), antioxidative defenses (catalase [CAT]), and metabolism (GST and alkaline phosphatase [ALP]). The purpose of the present study was to evaluate the response of this biomarker set to insecticides from 3 other families: deltamethrin (pyrethroid), fipronil (phenylpyrazole), and spinosad (spinosyn). We observed 3 biomarker response profiles that yield valuable information about the insecticides' modes of action. The present study confirms previous results showing that

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biomarker responses may be potentially used to evaluate the presence of environmental insecticides. We propose that the biomarker profiles could represent useful fingerprints that characterize exposure to pesticides.

MATERIALS AND METHODS

Chemicals

The insecticides deltamethrin and fipronil were purchased from Sigma-Aldrich. Spinosad was obtained from Cluzeau Info Labo. Antipain, aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, monobasic and dibasic sodium phosphate, sodium chloride (NaCl), Triton X-100, acetylthiocholine iodide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), α- and β-naphthyl acetate (α-NA or β-NA), *p*-nitrophenyl acetate (*p*-NPA), 1,5-bis (4-allyldimethylammonium-phenyl)pentan-3-one-dibromide (BW284C51), fast garnet GBC, sodium dodecyl sulfate (SDS), hydrogen peroxide (H_2O_2), monobasic potassium phosphate, ethylenediaminetetraacetic acid (EDTA), 1-chloro-2,4-dinitrobenzene (CDNB), reduced L-glutathione (GSH), acetonitrile, acetone, tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), *p*-nitrophenyl phosphate (*p*-NPP), and bovine serum albumin were all obtained from Sigma-Aldrich.

Honeybees

Honeybees (*A. mellifera*) were reared at the experimental apiary of the Institut National de la Recherche Agronomique (INRA), UR 406 Abeilles & Environnement, Avignon, France. The presence of a queen was verified, and the health status of the honeybees was continuously and carefully monitored. Workers were collected from the honey super compartment of the beehive, put in cages (10.5 cm × 7.5 cm × 11.5 cm), fed ad libitum with candy paste and water, and kept at 25 ± 2 °C and 60 ± 10% relative humidity.

Acute toxicity assay

The acute contact toxicity of insecticides to honeybees was assessed by determining the dose–mortality relationship according to the European and Mediterranean Plant Protection Organization guideline 170 [31] but with a higher requirement for control mortality (≤5%). The honeybees were gathered on the day before the experiment, placed in plastic cages (10.5 cm × 7.5 cm × 11.5 cm) in groups of 30 individuals, and allowed to rest overnight at 25 ± 2 °C and 60% relative humidity with candy and water ad libitum. On the day of the experiment, honeybee health and mortality were checked, and dead individuals were replaced. Thereafter, between 8:00 AM and 10:00 AM, the honeybees were mildly anesthetized with CO_2, and 1 μL of insecticide solution containing the appropriate dose was applied to the dorsal thorax by means of a Hamilton microsyringe coupled with an automatic dispenser. Two controls were included in the study: 1 group of honeybees treated with acetone only and 1 group without treatment. Mortality was recorded 24 h and 48 h after treatment and corrected with control mortality.

Exposure to insecticides

Immediately after determining the LD50 at 48 h, a new experiment was conducted to expose honeybees to insecticides at 1/20th of LD50 (LD50/20) and 1/10th of LD50 (LD50/10). After 48 h, the surviving honeybees were removed, and tissues were sampled as described in *Tissue extracts* and immediately frozen at −80 °C until biomarker analysis. The insecticide concentrations in the treatment solutions were verified by gas chromatographic–tandem mass spectrometric chemical analysis, as described by Wiest et al. [32]. For the 25-mg/L mother solutions of insecticides prepared from the 25-g/L stock solution, the actual concentrations were 25.66 ± 0.39 mg/L for deltamethrin and 25.34 ± 1.93 mg/L for fipronil (mean ± standard deviation, $n = 3$). No verification was performed for spinosad, but solutions were prepared as carefully as those for deltamethrin and fipronil.

Tissue extracts

To prevent any animal suffering, all tissues were removed from anesthetized individuals. After anesthesia, honeybee heads were obtained by cutting from the body with a scalpel. Then, midguts were obtained by pulling the stingers from the honeybees. Tissues were placed in microfuge tubes and weighed, and tissue extracts were obtained by adding a phosphate buffer (10 mM sodium chloride and 40 mM sodium phosphate [pH 7.4] containing 2 μg/mL antipain, leupeptin, and pepstatin A, 25 units/mL aprotinin, and 0.1 mg/mL soybean trypsin inhibitor as protease inhibitors) to obtain a 10% (w/v) extract. For head extracts, the phosphate buffer contained 1% (w/v) Triton X-100. Tissues were homogenized using a high-speed homogenizer, the Tissue Lyser II (Qiagen), for 3 periods of 30 s at 30-s intervals. Tissue extracts were centrifuged for 20 min at 13 000 *g*, and the supernatants were stored at −80 °C for analysis. All extraction procedures were conducted at 4 °C.

Enzyme assays

Enzyme assays were performed in a Variant Cary 1E UV-Vis spectrophotometer at 25 °C in a final reaction volume of 1 mL. The activity of each sample was determined in triplicate. For each exposure condition, 3 samples (5 heads or 5 midguts per sample, same individual for each head–midgut couple) from each of 3 cages ($n = 9$) were used to determine the enzyme activity. Protein concentrations were quantified according to Bradford [33] using bovine serum albumin as a standard. For AChE, CaEs, CAT, and ALP, 1 unit of enzyme activity was defined as the quantity of enzyme that, under the assay conditions, hydrolyzed 1 mmol of substrate per minute. For GST, 1 unit of activity corresponded to the quantity of enzyme conjugating 1 mmol of GSH per minute. Results were expressed in terms of specific and tissue activities corresponding to the activity defined above, normalized to the quantity of proteins or tissue, respectively.

The activity of AChE was assessed with 0.3 mM acetylcholine iodide, 1.5 mM DTNB, and 100 mM sodium phosphate buffer at pH 7.0, following the method of Ellman et al. [34] modified by Belzunces et al. [35]. Three CaEs were monitored—CaE-1, CaE-2, and CaE-3—and classified according to their substrate specificity corresponding to hydrolysis of α-NA, β-NA and *p*-NPA, respectively [36]. The crude tissue extract was incubated in a medium containing 1×10^{-4} M BW284C51 as an AChE inhibitor and 100 mM sodium phosphate, pH 7.4, for 20 min at 25 ± 1 °C in the dark. After incubation, the appropriate substrate (α-NA, β-NA or *p*-NPA) was added to a final concentration of 0.4 mM. For CaE-1 and CaE-2, the enzyme reaction was performed for 3 min and stopped with 1.5% SDS and 0.4 mg/mL fast garnet GBC. The reaction products were measured at 568 nm for α-NA and 515 nm for β-NA. For CaE-3, the reaction was monitored continuously at 410 nm. Alkaline phosphatase was monitored continuously at 410 nm in a medium containing 20 mM $MgCl_2$, 2 mM *p*-NPP as the substrate, and 100 mM Tris-HCl buffer at pH 8.5 [37].

Table 1. Acute toxicity (median lethal dose [LD50]) of deltamethrin, fipronil, and spinosad to *Apis mellifera*

Insecticide	Purity (%)	LD50 (ng/bee at 48 h)	95% CI	χ^2	*df*	LD50/20[a]	LD50/10[a]
Deltamethrin	99.8	50.65	43.33–57.97	21.88	15	2.53	5.07
Fipronil	98.6	5.83	5.05–6.60	25.98	21	0.29	0.58
Spinosad	99.0	47.11	44.22–49.99	24.74	18	2.35	4.71

[a]Doses determined from the LD50 value.
CI = confidence interval; *df* = degrees of freedom; LD50/20 = 1/20th of median lethal dose; LD50/10 = 1/10th of median lethal dose.

Glutathione-*S*-transferase was measured at 340 nm in a medium containing 1 mM EDTA, 2.5 mM GSH, 1 mM CDNB as the substrate, and 100 mM sodium phosphate at pH 7.4 [38]. Catalase was measured according to the procedure described by Beers and Sizer [39] in a medium containing 10 mM H_2O_2 and 100 mM phosphate at pH 7.0. The reaction was monitored by the decrease in absorbance at 240 nm due to the consumption of H_2O_2.

Data analyses

Data were analyzed using *R* statistical software [40]. Mortality data from the dose–response assays were analyzed using the drc package [41], and the respective LD50 and 95% confidence interval (95% CI) were calculated. For enzyme assays, data were subjected to one-way analysis of variance, and the means were compared by contrast analysis ($p \leq 0.05$), forming clusters with similar behavior.

RESULTS

Acute toxicity

The acute toxicity of deltamethrin, fipronil, and spinosad was assessed, revealing that among the tested insecticides, fipronil was the most toxic to *A. mellifera*, with an LD50 of 5.83 ng/bee (95% CI 5.05–6.60 ng/bee). The other insecticides, deltamethrin and spinosad, showed similar LD50 values of 50.65 ng/bee (95% CI 43.33–57.97 ng/bee) and 47.11 ng/bee (95% CI 44.22–49.99 ng/bee), respectively (Table 1). From the LD50 values of each insecticide, the sublethal doses LD50/20 and LD50/10 were determined and used in the exposure assays (Table 1).

Biomarker responses to insecticides

The enzyme biomarker responses in honeybees treated with LD50/20 and LD50/10 of each insecticide were analyzed 48 h after exposure. During the experiment, no honeybee mortality was observed, confirming that the doses were sublethal. A knockdown effect was observed for deltamethrin, but honeybees recovered between 1 h and 2 h after exposure to either dose. After exposure of honeybees to deltamethrin, fipronil, and spinosad, the activities of neural (AChE and CaE) and metabolic (ALP, CAT, and GST) biomarkers were assessed.

Neural biomarkers have been demonstrated to be the major target sites of insecticides and are highly modulated by exposure to insecticides. A decrease of AChE activity after exposure to spinosad was observed irrespective of dose when compared to the control (14.26 ± 1.29 μmol/min/g of tissue for control, and 12.93 ± 2.65 μmol/min/g and 12.03 ± 3.05 μmol/min/g of tissue for LD50/20 and LD50/10, respectively) (Figure 1A). Tissue AChE activities were unchanged after exposure to deltamethrin and fipronil. For CaE activities, different patterns were found for the 3 isoforms (Figures 2A, 3A, and 4A). After exposure to deltamethrin, CaE-2 increased irrespective of dose (16.78 ± 2.68 μmol/min/g of tissue for control, and 21.14 ± 4.58 μmol/min/g and 26.67 ± 5.04 μmol/min/g of tissue for LD50/20 and LD50/10, respectively). In contrast, CaE-1 tissue activity decreased (–16.81%) only at the highest dose (10.47 ± 1.58 μmol/min/g of tissue for the control and 8.71 ± 1.56 μmol/min/g of tissue at LD50/10). No significant modulation was observed for CaE-3. After exposure to fipronil, CaE-1 increased slightly irrespective of dose (10.04 ± 2.29 μmol/min/g of tissue for the control, and 11.02 ± 1.60 μmol/min/g and 11.38 ± 2.41 μmol/min/g of tissue at LD50/20 and LD50/10, respectively), whereas CaE-3 showed a decrease (–11.27%) only at the lowest dose (14.55 ± 2.10 μmol/min/g of tissue for the control and 12.91 ± 2.77 μmol/min/g of tissue at LD50/20). No significant modulation was observed for CaE-2. Exposure to spinosad increased the activity of all the CaE isoforms except for CaE-2 at LD50/10. Carboxylesterase-1 activity increased to 11.33 ± 1.35 μmol/min/g (+12.85%) and 12.08 ± 1.61 μmol/min/g (+20.32%) of tissue at LD50/20 and LD50/10, respectively (10.04 ± 2.29 μmol/min/g of tissue for the control), whereas CaE-2 increased (+10.96%) only at LD50/20 and reached 23.18 ± 3.20 μmol/min/g of tissue (20.89 ± 5.13 μmol/min/g of tissue for the control). Specific and tissue activities of AChE and the CaEs exhibited similar patterns (Figures 1–4).

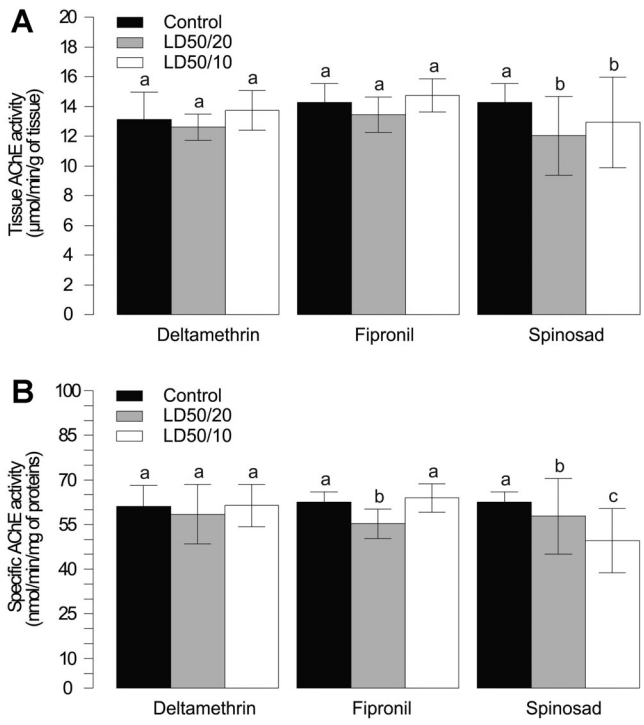

Figure 1. Modulation of acetylcholinesterase (AChE) activity after sublethal exposure to deltamethrin, fipronil, and spinosad. Results are expressed as tissue (**A**) and specific (**B**) activities. Honeybees were subjected to acute contact exposure at different doses: 0 (control), LD50/20 (1/20th of median lethal dose), and LD50/10 (1/10th of median lethal dose), which correspond to 2.53 ng/bee and 5.07 ng/bee for deltamethrin, 0.29 ng/bee and 0.58 ng/bee for fipronil, and 2.36 ng/bee and 4.71 ng/bee for spinosad, respectively. Data correspond to mean ± standard deviation of 9 repetitions performed in triplicate. For a same insecticide, values with different letters significantly differed by cluster analysis ($p \leq 0.05$).

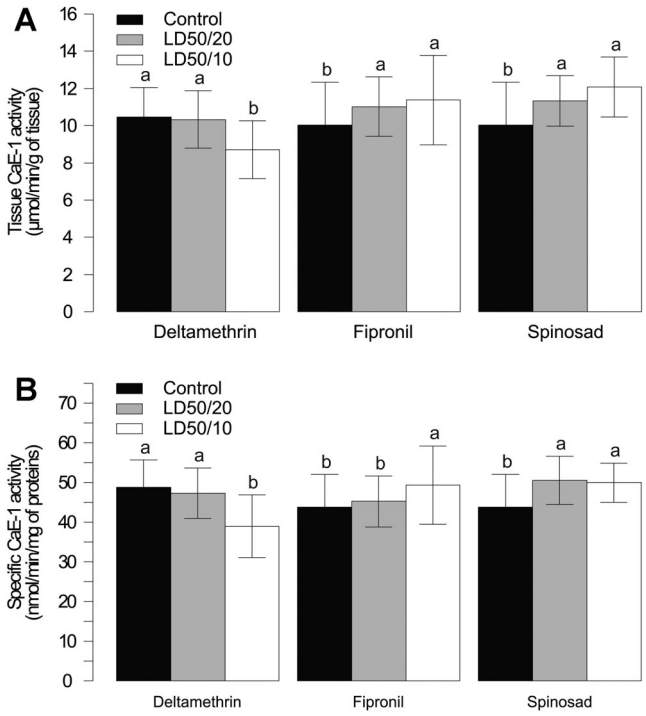

Figure 2. Modulation of carboxylesterase-1 (CaE-1) activity after sublethal exposure to deltamethrin, fipronil, and spinosad. Results are expressed as tissue (**A**) and specific (**B**) activities. Honeybees were subjected to acute contact exposure at different doses: 0 (control), LD50/20 (1/20th of median lethal dose), and LD50/10 (1/10th of median lethal dose), which correspond to 2.53 ng/bee and 5.07 ng/bee for deltamethrin, 0.29 ng/bee and 0.58 ng/bee for fipronil, and 2.36 ng/bee and 4.71 ng/bee for spinosad, respectively. Data correspond to mean ± standard deviation of 9 repetitions performed in triplicate. For a same insecticide, values with different letters significantly differed by cluster analysis ($p \leq 0.05$).

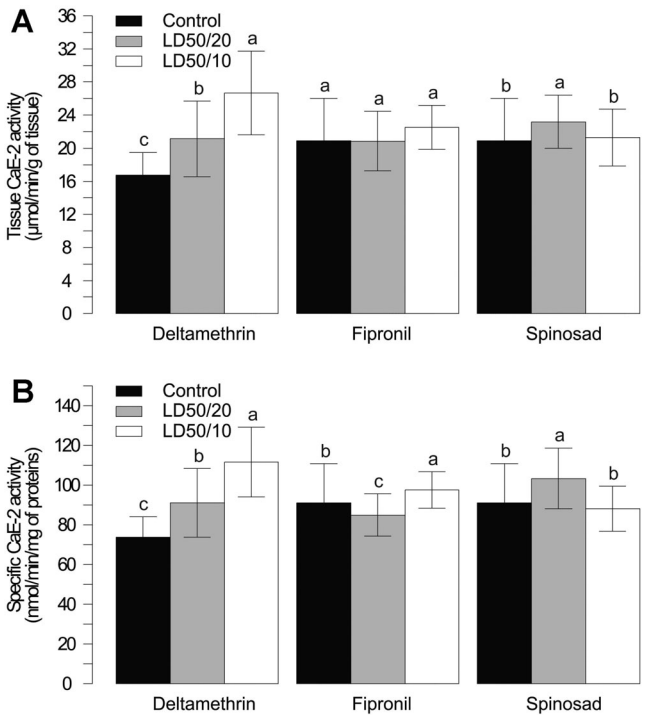

Figure 3. Modulation of carboxylesterase-2 (CaE-2) activity after sublethal exposure to deltamethrin, fipronil, and spinosad. Results are expressed as tissue (**A**) and specific (**B**) activities. Honeybees were subjected to acute contact exposure at different doses: 0 (control), LD50/20 (1/20th of median lethal dose), and LD50/10 (1/10th of median lethal dose), which correspond to 2.53 ng/bee and 5.07 ng/bee for deltamethrin, 0.29 ng/bee and 0.58 ng/bee for fipronil, and 2.36 ng/bee and 4.71 ng/bee for spinosad, respectively. Data correspond to mean ± standard deviation of 9 repetitions performed in triplicate. For a same insecticide, values with different letters significantly differed by cluster analysis ($p \leq 0.05$).

Enzymes that protect against xenobiotics are recognized as sensitive biomarkers, especially those involved in oxidative stress and detoxification processes. Therefore, the activities of ALP, CAT, and GST in the honeybee midgut were assessed. Compared with the control, a strong decrease of ALP (–17.69%) was observed irrespective of fipronil dose (0.65 ± 0.13 µmol/min/g, 0.52 ± 0.17 µmol/min/g, and 0.55 ± 0.10 µmol/min/g of tissue for the control, LD50/20, and LD50/10, respectively), whereas spinosad induced an increase (+9.23%) only at LD50/10 (0.71 ± 0.14 µmol/min/g of tissue; Figure 5A). There were no variations in ALP tissue activity after honeybee exposure to deltamethrin (Figure 5A). Moreover, all doses of fipronil (+12.28%) and spinosad (+20.36%) increased CAT tissue activity, with values of 3.81 ± 0.36 mmol/min/g, 3.69 ± 0.44 mmol/min/g, and 3.34 ± 0.54 mmol/min/g of tissue for fipronil and 4.08 ± 0.44 mmol/min/g, 3.96 ± 0.56 mmol/min/g, and 3.36 ± 0.49 mmol/min/g of tissue for spinosad (LD50/20, LD50/10, and control, respectively; Figure 6A). Deltamethrin had no apparent effect on CAT tissue activity (Figure 6A). Only spinosad induced an increase (+10.72%) in GST tissue activity over the control, with values of 42.08 ± 8.52 µmol/min/g, 46.17 ± 8.09 µmol/min/g, and 47.01 ± 6.47 µmol/min/g of tissue for the control, LD50/20, and LD50/10, respectively (Figure 7A). Deltamethrin had no apparent effect on GST tissue activity (Figure 7A).

In bees exposed to fipronil and spinosad, similar patterns were observed between tissue and specific activities of ALP, CAT, and GST. In bees exposed to deltamethrin, however, the specific activities of these enzymes were increased irrespective of the dose. For control and exposure to LD50/20 and LD50/10, values were, respectively, 4.05 ± 0.50 nmol/min/mg, 4.77 ± 0.71 nmol/min/mg, and 4.97 ± 0.77 nmol/min/mg of proteins for ALP; 14.09 ± 3.29 µmol/min/g, 16.68 ± 5.78 µmol/min/g, and 16.74 ± 4.52 µmol/min/mg of proteins for CAT; and 308.90 ± 20.13 nmol/min/mg, 336.93 ± 22.94 nmol/min/mg, and 347.22 ± 16.37 nmol/min/mg of proteins for GST.

DISCUSSION

In the development of biomarkers, laboratory experiments represent a crucial step in evaluating and validating the power of these molecular tools. A preliminary and important step is determining the sensitivity and the modulation potential of biomarkers by analyzing the dose–response relationships of insecticides on biomarkers. The specific response of *A. mellifera* toward pesticides can vary according to different biotic and abiotic factors such as physiological state, the genetic background of the colony, and the season. Thus, it is important to first conduct a toxicity study on the test colony to assess the sensitivity level of the individuals [42–45]. The results obtained in the acute toxicity test show that all the tested insecticides are toxic to the honeybee and fipronil was the most toxic insecticide with the lowest observed LD50 (5.83 ng/bee). In the present study, the estimated LD50 of fipronil is similar to the values reported by Tingle et al. [26] and Decourtye et al. [23], who found values of 4 ng/bee to 6 ng/bee. Deltamethrin and spinosad induced similar acute toxicity, with LD50 values of 50.65 ng/bee and 47.11 ng/bee, respectively. Atkins et al. [46] and Decourtye et al. [23] reported contact LD50 values for deltamethrin of 62 ng/bee to 67 ng/bee, slightly higher than

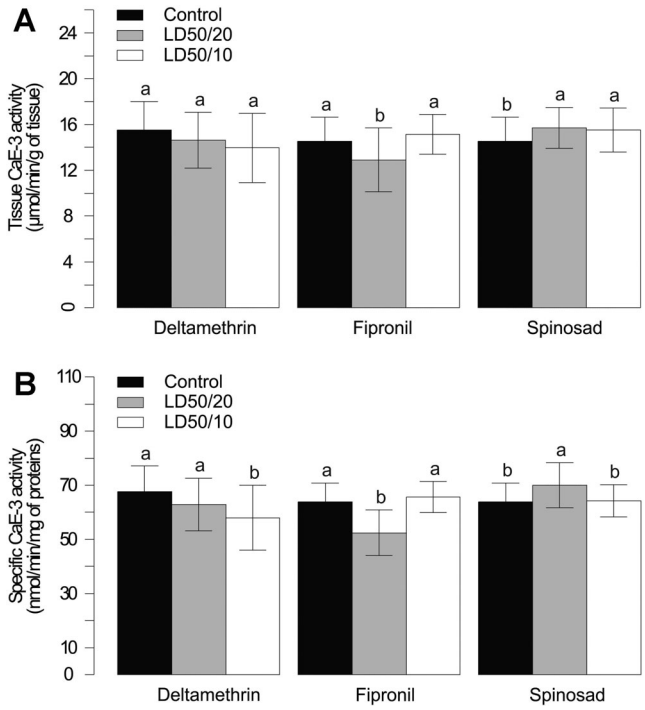

Figure 4. Modulation of carboxylesterase-3 (CaE-3) activity after sublethal exposure to deltamethrin, fipronil, and spinosad. Results are expressed as tissue (**A**) and specific (**B**) activities. Honeybees were subjected to acute contact exposure at different doses: 0 (control), LD50/20 (1/20th of median lethal dose), and LD50/10 (1/10th of median lethal dose), which correspond to 2.53 ng/bee and 5.07 ng/bee for deltamethrin, 0.29 ng/bee and 0.58 ng/bee for fipronil, and 2.36 ng/bee and 4.71 ng/bee for spinosad, respectively. Data correspond to mean ± standard deviation of 9 repetitions performed in triplicate. For a same insecticide, values with different letters significantly differed by cluster analysis ($p \leq 0.05$).

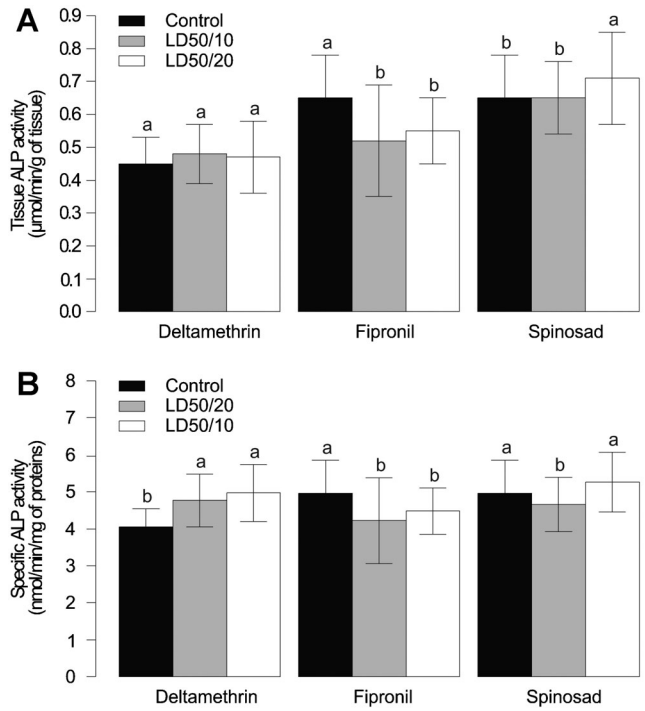

Figure 5. Modulation of alkaline phosphatase (ALP) activity after sublethal exposure to deltamethrin, fipronil, and spinosad. Results are expressed as tissue (**A**) and specific (**B**) activities. Honeybees were subjected to acute contact exposure at different doses: 0 (control), LD50/20 (1/20th of median lethal dose), and LD50/10 (1/10th of median lethal dose), which correspond to 2.53 ng/bee and 5.07 ng/bee for deltamethrin, 0.29 ng/bee and 0.58 ng/bee for fipronil, and 2.36 ng/bee and 4.71 ng/bee for spinosad, respectively. Data correspond to mean ± standard deviation of 9 repetitions performed in triplicate. For a same insecticide, values with different letters significantly differed by cluster analysis ($p \leq 0.05$).

those observed in the present study. For spinosad, the observed LD50 is in accordance with Mayes et al. [22] and Miles [25], who found respective values of 25 ng/bee and 78 ng/bee.

In the present study, the pesticides used elicit different modulations of the biomarkers considered, even at sublethal doses such as LD50/20 and LD50/10. Although no mortality could be observed, the modulation of biomarkers could be regarded as a witness of physiological changes induced by the different insecticides. Thus, it is legitimate to think that an absence of mortality does not always imply a functional integrity of individuals. This is particularly true if we consider that these insecticides can induce various sublethal effects such as impairment of homing flight or associative learning induced by deltamethrin [23,47], increased metabolism of mushroom bodies by fipronil [48], reduced learning performances by fipronil and deltamethrin [23,49], increased sensitivity to *Nosema* by fipronil [50], or alteration of foraging activity and brood development by spinosad [22,51].

Different biomarker responses were obtained after exposure to deltamethrin, fipronil, and spinosad. In contrast to Badiou et al. [17], no AChE response was observed after exposure to deltamethrin. This difference could be partially explained by the dose, which was 5 times higher than the highest LD50/10 dose used in the present study, and that might trigger different cellular responses. For spinosad, we found that this insecticide is able to reduce AChE activity irrespective of the dose. Our results are supported by those of Rabea et al. [19], showing that oral exposure to spinosad elicits a 48% decrease in AChE activity. Similar results have been observed in rats exposed to low doses of spinosad for 2 wk [52]. As AChE is not the target of spinosad, our results highlight the complex effects of this insecticide in the honeybee, which involve an agonist effect on nicotinic acetylcholine receptors [22,53,54].

Independent modulation patterns were found for the 3 CaE isoforms in honeybees exposed to the 3 insecticides. These differences can be explained by the existence of 6 CaE isoforms in *A. mellifera* [55], thus making it necessary to investigate several of them. According to Badiou-Bénéteau et al. [9], each honeybee isoform of CaE has an important biomarker role because CaEs can exhibit potent responses after exposure to sublethal pesticide doses. Some studies report CaE modulation after *A. mellifera* exposure to insecticides such as malathion and methyl-parathion [13,14], dichlorvos [56], and thiamethoxam [9,18], thus highlighting the potential of these enzymes as biomarkers.

As observed previously for thiamethoxam [9], fipronil and spinosad elicit an increase (+16.32% on average) in the CAT activity of the honeybee. Catalase is involved in the detoxification of reactive oxygen species by hydrolyzing H_2O_2 [57]. This enzyme is very active in the honeybee and can be modulated by environmental pollutants [58,59]. Thus, increased CAT activity could be partially explained by the ability of fipronil and thiamethoxam to induce oxidative stress in plants and vertebrates [60–62].

A significant decrease in ALP activity was induced by exposure to fipronil (–17.69%), and the lowest dose of spinosad induced an increase of 9.23%. This contrasts with the absence of any effect observed with deltamethrin. Alkaline phosphatase represents a family of enzymes responsible for hydrolysis in digestive processes, for cell signaling, and for the transport of metabolites [63]. Although it is not involved in pesticide

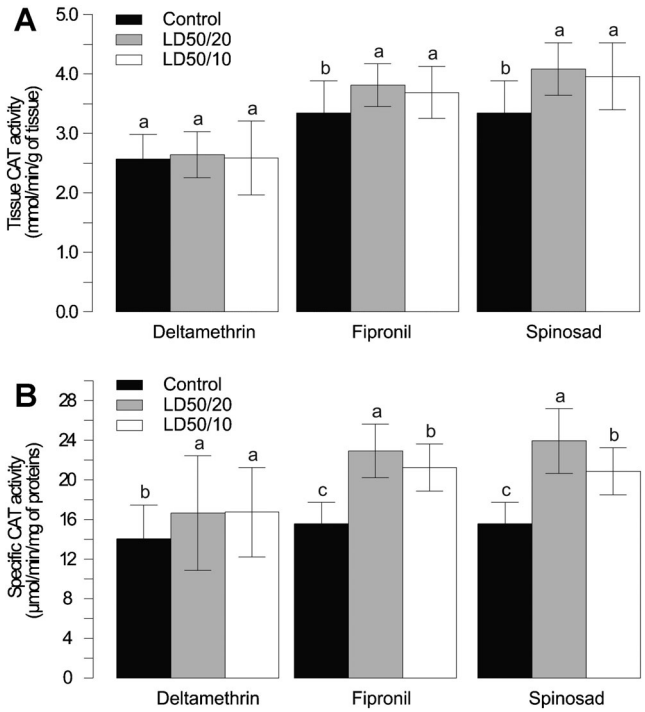

Figure 6. Modulation of catalase (CAT) activity after sublethal exposure to deltamethrin, fipronil, and spinosad. Results are expressed as tissue (**A**) and specific (**B**) activities. Honeybees were subjected to acute contact exposure at different doses: 0 (control), LD50/20 (1/20th of median lethal dose), and LD50/10 (1/10th of median lethal dose), which correspond to 2.53 ng/bee and 5.07 ng/bee for deltamethrin, 0.29 ng/bee and 0.58 ng/bee for fipronil, and 2.36 ng/bee and 4.71 ng/bee for spinosad, respectively. Data correspond to mean ± standard deviation of 9 repetitions performed in triplicate. For a same insecticide, values with different letters significantly differed by cluster analysis ($p \leq 0.05$).

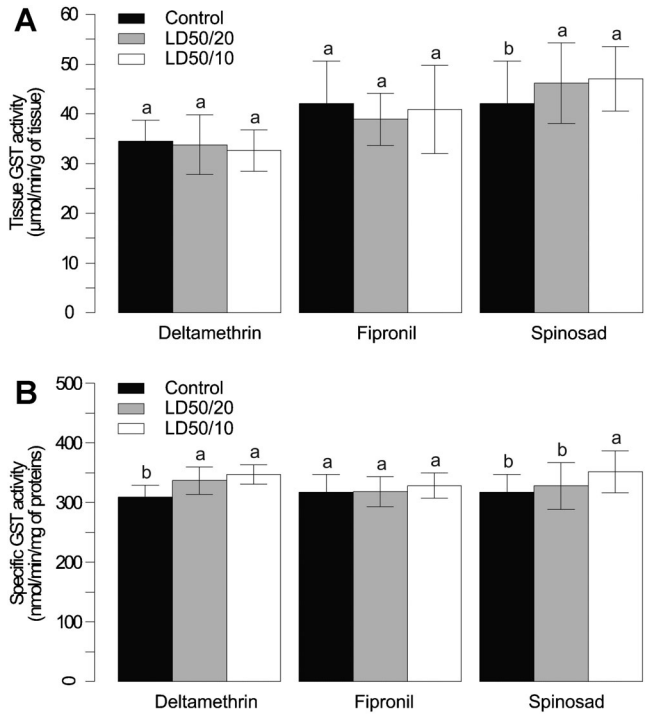

Figure 7. Modulation of glutathione-*S*-transferase (GST) activity after sublethal exposure to deltamethrin, fipronil, and spinosad. Results are expressed as tissue (**A**) and specific (**B**) activities. Honeybees were subjected to acute contact exposure at different doses: 0 (control), LD50/20 (1/20th of median lethal dose), and LD50/10 (1/10th of median lethal dose), which correspond to 2.53 ng/bee and 5.07 ng/bee for deltamethrin, 0.29 ng/bee and 0.58 ng/bee for fipronil, and 2.36 ng/bee and 4.71 ng/bee for spinosad, respectively. Data correspond to mean ± standard deviation of 9 repetitions performed in triplicate. For a same insecticide, values with different letters significantly differed by cluster analysis ($p \leq 0.05$).

detoxification, ALP may be modulated in the honeybee by insecticides [63], fungicides, or acaricides [37], which prompted its use as a biomarker of exposure to pesticides [64]. Its potential as a biomarker has been exploited in the silver catfish (Siluriformes: Heptapteridae), in which ALP activity is increased after exposure to cypermethrin [65].

In honeybees exposed to pesticides, only spinosad modulated the tissue GST level. This could be explained by the fact that an important part of spinosad metabolism involves conjugation to glutathione [66]. Conversely, the absence of an effect with deltamethrin or fipronil could be due to their metabolism being largely dependent on cytochrome P450 [67,68]. Nevertheless, some studies show that the specific activity of GST may be modulated by pesticides. For example, Yu et al. [13], Nielsen et al. [69], and Papadopoulos et al. [70] observed increased GST–specific activity in honeybees poisoned with permethrin, deltamethrin, and flumethrin. The difference observed between tissue and specific activities is explained by the fact that these insecticides do not affect GST activity but do decrease protein content, at least in the studied tissue. This is consistent with our observations in the present study (Figure 7). Thus, it is important to express enzymatic activity as tissue activity, which is the only metric that reflects the actual variation of enzyme activity in a tissue. Specific activity reflects only the proportion of a given protein in comparison with the total protein content [9,17].

CONCLUSIONS

A biomarker is an observable or measurable response at the molecular, biochemical, cellular, physiological, or behavioral level that is modulated by the presence of xenobiotics [71]. In a previous study, we developed a set of enzyme biomarkers in *A. mellifera* validated after exposure to thiamethoxam. We show in the present study that this biomarker set also has the potential to be modulated by other insecticides that are widely used in plant protection. The present study also shows that in vivo studies represent a necessary step to validate biomarkers that are developed for assessing honeybee health and environmental quality. Thus, biomarkers could be used as ancillary tools for monitoring programs on environmental health [9]. Among biological models, the honeybee *A. mellifera* is a particularly relevant bioindicator because of its ecological, social, and economic importance [1,72]. Furthermore, the use of neural and metabolic enzymes enables the investigation of different physiological functions likely to be altered by pesticides.

Our results show that deltamethrin, fipronil, and spinosad are toxic to *A. mellifera*. When used at sublethal doses, they can modulate different enzymes that are responsible for regulating important physiological processes, and that may in turn affect the health and survival of honeybees as well as their cognitive capacities [23,27,48,49] and immune response to pathogens [9,50]. Because these enzymes exhibit distinct modulation patterns after exposure to different pesticides or to different doses of a single pesticide, they represent powerful biomarkers when used as a set. The choice of biomarkers is a crucial step in assessing individual and environmental health. Our results highlight the applicability of these biomarkers and validate the honeybee as a particularly important bioindicator of the presence of insecticides.

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