

# Toxic Effect and Biochemical Study of Chlorfluazuron, Oxymatrine, and Spinosad on Honey Bees (*Apis mellifera*)

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**Abstract** Under laboratory conditions, the comparative effects of two insect growth regulators, chlorfluazuron and oxymatrine, and spinosad as a biopesticide were examined on honey bee workers (*Apis mellifera* L.). Separate groups of bees were left for 24 h to feed on 50% sucrose solution containing different concentrations of the tested insecticides, and the lethal concentration that caused 50% mortality ( $LC_{50}$ ) was estimated. The inhibitory effects on acetylcholinesterase (AChE) and adenosine triphosphatase (ATPase) activities as biochemical indicators were determined in vivo after 24 h in head, thorax, and abdomen of surviving bees obtained after treatments with a view to explore the possible mode of action of these compounds. Results indicated that exposure to spinosad showed toxicity to honey bees with  $LC_{50}$  value of  $7.34 \text{ mg L}^{-1}$ , followed by oxymatrine ( $LC_{50} = 10.68 \text{ mg L}^{-1}$ ), while chlorfluazuron was the least acutely toxic of the tested compounds ( $LC_{50} = 2,526 \text{ mg L}^{-1}$ ). Oxymatrine and spinosad at the same tested concentrations (2.5, 5, 10, and  $20 \text{ mg L}^{-1}$ ) significantly inhibited AChE activity in different organs of honey bee workers, and high inhibition percentage was obtained with the enzyme isolated from the thorax. However, chlorfluazuron at 400, 1,000, 2,000, and  $4,000 \text{ mg L}^{-1}$  caused high inhibition of AChE activity isolated from the head (39.65% and 44.22% at 2,000 and  $4,000 \text{ mg L}^{-1}$ , respectively). In addition, the toxic effects of the tested

compounds on activity of ATPase indicated that spinosad caused the highest inhibitory effect in different organs compared with oxymatrine at the same concentrations, and high inhibition was found with ATPase isolated from the head. The results also indicated that oxymatrine was the least active compound for inhibition of AChE and ATPase.

Among insects, honey bees (*Apis mellifera* L.) are of particular interest because they come into contact with various pollutants during their foraging activity and are considered an environmental indicator of high sensitivity (Wallwork-Barber et al. 1982; Smith and Wilcox 1990). They have increasingly been employed to monitor environmental pollution by heavy metals in territorial and urban surveys (Crane 1984) and as an ideal managed system to determine the impact of pesticide exposure in rural areas (Atkins et al. 1981; Mayer et al. 1987; Celli et al. 1991; Porrini et al. 1996) as well as radionuclides (Tonelli et al. 1990). They are also economically and environmentally important in their contributions as pollinators of crops and wild flowers (Celli and Maccagnani 2003).

Honey bees, as domesticated pollinators, may be constantly exposed to pesticides whenever their colonies are sited in agricultural areas (Stevenson et al. 1978; Haynes 1988; Stone et al. 1997; Weick and Thorn 2002). High exposure levels can kill foragers, but sublethal exposures may also adversely affect colony function (Smirle et al. 1984; Currie 1999). It is crucial to quantify the sublethal effects that various pesticides are having on these bees because such effects may strongly influence individual bee behavior (Haynes 1988; Vandame et al. 1995; Stone et al. 1997) and thus the functioning of bee colonies (Anderson and Atkins 1968). Because the effectiveness of bee colonies for pollination and honey gathering depends on the

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coordination of a suite of worker behaviors involved in the collection and allocation of nectar and pollen (Seeley 1995), any chemical exposure that compromises workers' abilities to carry out these tasks could impact colony performance.

Therefore, the study of pesticide effects on honey bees is important because of the need to control a wide variety of agricultural pests with pesticides without hurting bees that inadvertently come into contact with pesticides (Atkins 1992; Copping and Menn 2000; Porrini et al. 2002, 2003; Ghini et al. 2004; Decourtye et al. 2005; Desneux et al. 2007). Schmidt (1996) reported that imidacloprid at the field dose was highly toxic to honey bees (oral  $LD_{50} = 0.0037 \mu\text{g}/\text{bee}$ , topical  $LD_{50} = 0.081 \mu\text{g}/\text{bee}$ ), but at very low doses does not cause death of honey bees. Bees treated with imidacloprid were less active and their communicative capacity seemed to be impaired (Medrzycki et al. 2003), and this compound can induce behavioral changes, such as foraging activity decrease (Decourtye et al. 2004). Iwasa et al. (2004) added that imidacloprid, clothianidin, and thiamethoxam showed high acute toxicity to honey bee workers with  $LD_{50}$  of 17.9, 21.8, and 29.9 ng/bee, respectively, while dinotefuran and nitenpyram were slightly less toxic with  $LD_{50}$  of 75.0 and 138 ng/bee, respectively. Moreover, Mayes et al. (2003) reported that spinosad showed highly toxic effect to honey bees, bumble bees, alfalfa leafcutter bees, and alkali bees in acute oral and contact toxicity studies. However, dried residues were not harmful to adult honey bees or larvae in laboratory studies, or to adults, brood or foraging rates in field studies.

In this context, insect growth regulators (IGRs) have been developed due to their high activity and selectivity against insects with inherently low toxicity to nontarget wildlife. Due to their mode of action, a subtle effect of these insecticides is likely to pose a greater hazard to larval stages than to adult insects (Darvas and Polgar 1998; Schneider et al. 2003). However, there are few studies on the effects of these types of compounds on the development and overwintering ability of honey bee colonies or on their ability to produce viable drones and queens (Tasei 2001; Thompson et al. 2005; Mommaerts et al. 2006). Abramson et al. (2004) found that exposure to tebufenozide and diflubenzuron as IGRs does not influence learning of harnessed honey bee foragers, whereas an inspection of the learning curves revealed a subtle effect of insecticides on learned behavior.

Bees may act as an indicator of environmental contamination through (1) possible reduced pollination activity related to exposure, (2) the presence of residues in hive matrices (honey, pollen, wax), (3) incidents with mortalities consecutive to exposure to lethal rates of pollutants, and (4) inhibition of certain enzymes that may, when related to relevant effects at the colony level, constitute

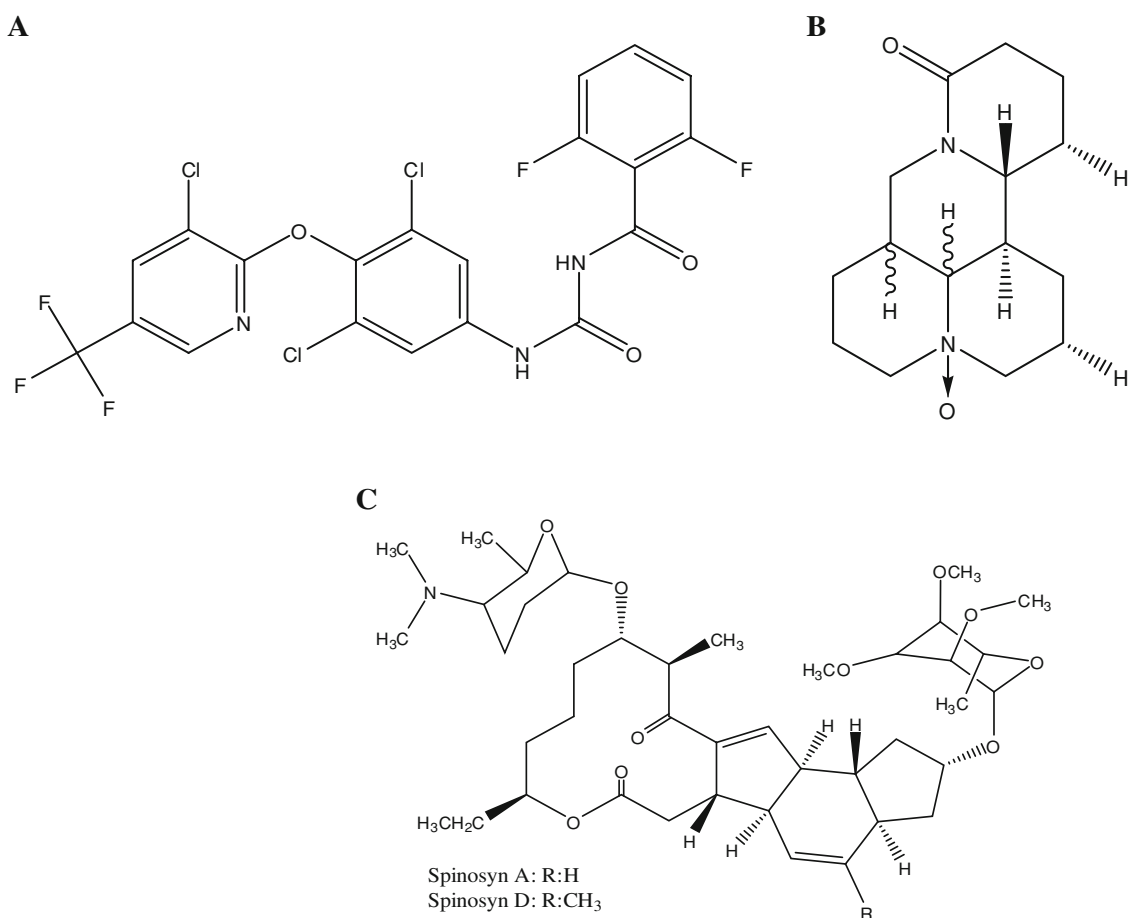
reliable biomarkers (Porrini et al. 2002). Thus honey bees may be considered as a particularly pertinent model for the development of biomarkers to assess environmental contamination (Bendahou et al. 1999; Hyne and Maher 2003). Biomarkers are based on physiological, biochemical, anatomical, and behavioral parameters, the perturbation of which persists after the exposure to the contamination (NRC 1987; Hyne and Maher 2003). The use of biomarkers in environmental pollution assessment enables monitoring of stress responses ranging from the biomolecular/biochemical to the population and community levels (Adams et al. 1989). However, the correlations of population- and community-level changes with variations of selected biomarkers in field-collected organisms are poorly documented. Some biomarkers can be proposed to evaluate the degree of exposure of individuals in order to predict population-level consequences (Lagadic et al. 1994). However, the effects of pesticides exposure on honey bees are difficult to evaluate because there is often a long latent period between exposure and the expression of an adverse effect. Exposure to neurotoxicants results in behavioral changes which could be assessed through the measurement of biomarkers related to nervous system activity (MacKenzie and Winston 1989). Cholinergic effects of some pesticides have sometimes been associated with behavioral changes in invertebrates, but the direct correlation between individual enzyme activity and population- or community-level responses remains to be established.

Therefore, the aim of the present research is to investigate the respective acute toxicity of chlorfluazuron (a benzoyl urea derivative), oxymatrine (a new quinolizidine alkaloid), and spinosad (a microbial biopesticide) on honey bee (*Apis mellifera* L.) workers. These insecticides are known to adversely affect honey bees through mortality and inhibition of physiological enzymes. Thus the enzymes acetylcholinesterase (AChE) and adenosine triphosphatase (ATPase) as well-known biochemical indicators were determined in vivo in bees surviving exposure to these insecticides.

## Materials and Methods

### Chemicals and Test Products

The pesticides tested (Fig. 1) were chlorfluazuron (Capris 5% EC, 1-[3,5-dichloro-4-(3-chloro-5-trifluoromethyl-2-pyridyloxy)phenyl]-3-(2,6-difluorobenzoyl) urea, supplied by Help Co., New Domiat City, Egypt), Oxymatrine (KingBo 0.6% SL, matrine *N*-oxide ammothamnine,  $C_{15}H_{24}N_2O_2$ , a natural plant extract from quinolizidine alkaloid; produced from the roots of *Sophora flavescens*, supplied by EGD Co., Giza Governorate, Egypt), and



**Fig. 1** Chemical structures of chlorfluazuron (a), oxymatrine (b), and spinosad (c)

Spinosad (Tracer 24% SC, Dow AgroSciences, Madrid, Spain). Acetylthiocholine iodide (ATChI), adenosine triphosphate (ATP), bovine serum albumin (BSA), 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB), trichloroacetic acid (TCA), Folin-Ciocalteu phenol reagent, and Tris-HCl were purchased from Sigma-Aldrich Chemical Co., USA.

### Honey Bees

All experiments were conducted in the laboratory using colonies of honey bees *Apis mellifera* L. (Hymenoptera: Apidae). Adult workers were obtained from El-Sabahia Research Station, Agriculture Research Center, Ministry of Agriculture, Alexandria, Egypt. This strain was derived from crosses between Carniolan and Egyptian strains. Foraging bees were rather at the end of life activity of workers (Michener 1974; Winston 1987; Picardnizou et al. 1995) and an extensive literature confirms that foragers are the higher than 20 days bees in typical colonies (reviewed by Michener 1974; Winston 1987). The hives at the time bees were collected were free of obvious diseases that might be observed during routine colony maintenance and

bee collections. No hive treatments to control diseases were conducted prior to our study. Hives were exposed to smoke twice for 30–60 s prior to collection. Honey bees on frames containing honey and pollen were always collected from the top super, and the bees were shaken from the frames into a plastic container. The opening of the container was covered with a solid plastic lid and the bees transported to the laboratory. The bees were maintained at approximately  $25 \pm 2^\circ\text{C}$  during transportation to the laboratory. Immediately upon arrival from the field, the bees were kept in experimental cages ( $10 \times 7 \times 12 \text{ cm}^3$ ) in groups of 50 at  $25 \pm 2^\circ\text{C}$  and  $65 \pm 5\%$  relative humidity and fed 50% (w/v) sucrose solution.

### Acute Toxicity Assay

The acute toxicity of chlorfluazuron, oxymatrine, and spinosad was evaluated on honey bees (*A. mellifera* L.) workers by oral administration through spiked syrup under laboratory conditions. Preliminary screening tests were performed at the application rate in the field (20, 3, and 4.8 g a.i./100 L water for one feddan (0.42 hectare) of

chlorfluazuron, oxymatrine, and spinosad, respectively). Stock solutions of insecticides were prepared in 50% (w/v) sucrose. Prior to treatment with insecticide, bees (20/cup) were anesthetized by exposure to carbon dioxide gas for no longer than 3 min, and each group of each concentration was composed of three plastic cups covered with a nylon mesh containing 60 honey bees. The amount of solutions was applied on cotton bed attached to the upper surface of the cover and bees were left to feed for 24 h. Bees fed with 50% sucrose solution were used as a control. Experiments were carried out by incubating bees at  $25 \pm 2^\circ\text{C}$ ,  $65 \pm 5\%$  relative humidity, and 12:12 (L:D) photoperiod. Bees were considered dead if they were unable to walk or fly. Mortality percentages were recorded after 24 h of treatment. Oxymatrine and spinosad at the previous concentrations (30 and  $48 \text{ mg L}^{-1}$ ) caused 100 % mortality; therefore, lower concentrations (2.5, 5, 10, and  $20 \text{ mg L}^{-1}$ ) were tested to calculate the  $\text{LC}_{50}$  according to Finney (1971). However, chlorfluazuron did not induce any mortality at the application rate in the field ( $200 \text{ mg L}^{-1}$ ); therefore, higher concentrations of 400, 1,000, 2,000, and  $4,000 \text{ mg L}^{-1}$  were then tested to determine the  $\text{LC}_{50}$  value.

#### Total Protein Assay

The Lowry et al. (1951) method was used to determine the protein content in the head, thorax, and abdomen of bees surviving the treatments. Protein extract (100  $\mu\text{L}$ ) was added to 2 mL alkaline copper reagent [48 of 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide + 1 mL 1% (w/v) sodium-potassium tartrate + 1 mL 0.5% (w/v) copper sulfate] and immediately mixed. After 10 min, 0.2 mL Folin-Ciocalteu phenol reagent was added and the samples were thoroughly mixed, then the absorbance of the developed blue color was measured at 600 nm using a Unico 1200 spectrophotometer. The protein content of the sample was determined by comparing to the standard curve of BSA.

#### Acetylcholinesterase (AChE) Activity Assay

After 24 h of feeding on the tested insecticides, we assessed AChE activity (in vivo) in head, thorax, and abdomen of adult honey bees using a procedure of Ellman et al. (1961). The head, thorax, and abdomen were cut from surviving bees obtained after treatments and homogenized in 0.1 M phosphate buffer (pH 7.0). The homogenates were then centrifuged at 5,000 rpm for 20 min at  $0^\circ\text{C}$ . The supernatants were used as enzyme source for assay of AChE activity. Enzyme (150  $\mu\text{L}$ ), 100  $\mu\text{L}$  DTNB (0.01 M), and 30  $\mu\text{L}$  ATChI (0.075 M) were added to 2.8 mL 0.1 M phosphate buffer (pH 8.0). The mixture was incubated at  $37^\circ\text{C}$  for 15 min. The absorbance was measured at 412 nm using Unico 1200 spectrophotometer. All of the treatments were

done in triplicate. The specific activity of AChE was expressed as nmoles of acetylthiocholine iodide hydrolyzed/mg protein/min. Inhibition percentages of the activities against control were considered in the enzymatic assay.

#### Adenosine Triphosphatase (ATPase) Activity Assay

After 24 h of feeding on the tested insecticides, the head, thorax, and abdomen of bees surviving the treatment were cut and homogenized in Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 5,000 rpm for 10 min at  $4^\circ\text{C}$ . The supernatant was then centrifuged at 17,000 rpm for 30 min at  $4^\circ\text{C}$ . The pellets were resuspended in the same buffer. This suspension was used as enzyme source for the assay of ATPase activity. The enzyme activity was determined colorimetrically according to the method of Koch (1969). Enzyme suspension was added to the reaction mixture that contained 100 mM  $\text{Na}^+$ , 20 mM  $\text{K}^+$ , 5 mM  $\text{Mg}^{2+}$ , and 5 mM ATP and the volume was adjusted to 850  $\mu\text{L}$  with Tris-HCl buffer (pH 7.4). This mixture was incubated at  $37^\circ\text{C}$  for 15 min and then stopped with 150  $\mu\text{L}$  TCA. Four milliliters of fresh color reagent (5 g ferrous sulfate in 10 mL ammonium molybdate solution prepared in 10 N sulfuric acid) was added and absorbance was measured at 740 nm by using a Unico 1200 spectrophotometer. The enzyme activity was represented as micromoles inorganic phosphorus (Pi/mg protein/h). Inhibition percentages of the activities compared with control were considered in the enzymatic assay.

#### Statistical Analysis

Statistical analysis was performed using the SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The log dose–response curves allowed determination of the  $\text{LC}_{50}$  values for the insect bioassay according to probit analysis (Finney 1971). The 95% confidence limits for the range of  $\text{LC}_{50}$  were determined by least-square regression analysis of the relative growth rate (percentage of control) against the logarithm of the compound concentration. The data for AChE and ATPase activities were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Student–Newman–Keuls (SNK) test and differences at  $P \leq 0.05$  were considered significant.

## Results

#### Acute Toxicity Assay of Chlorfluazuron, Oxymatrine, and Spinosad on Honey Bees (*A. mellifera* L.)

An oral test with spiked syrup was conducted to determine the lethal concentration of chlorfluazuron, oxymatrine, and

spinosad to honey bees (*A. mellifera* L.). On the basis of LC<sub>50</sub> values, the results (Table 1) indicated that spinosad and oxymatrine have a toxic action to honey bees with LC<sub>50</sub> of 7.34 and 10.68 mg L<sup>-1</sup>, respectively. However, a lower toxicity was obtained with chlorfluazuron, with LC<sub>50</sub> of 2,526 mg L<sup>-1</sup>.

#### Inhibitory Effect of Chlorfluazuron, Oxymatrine, and Spinosad on Acetylcholinesterase (AChE) Activity

The in vivo inhibitory effect of chlorfluazuron, oxymatrine, and spinosad on AChE activity isolated from different parts (head, thorax, and abdomen) of surviving adult honey bees (*A. mellifera* L.) was examined and the results are presented in Tables 2, 3, and 4. Data are expressed as percentage inhibition and specific activity (nmoles of acetylthiocholine iodide hydrolyzed/mg protein/min). All treatments induced a decrease in AChE activity compared with the control. Chlorfluazuron was tested at 400, 1,000, 2,000, and 4,000 mg L<sup>-1</sup> and the data (summarized in Table 2) show that chlorfluazuron has inhibitory effect on AChE activity in all parts of adult honey bees. However, there are significant differences between the specific activity of AChE in head, thorax, and abdomen, and a high specific activity was found in the head (57.49, 30.73, and 10.22 nmoles ATChI hydrolyzed/mg protein/min in head, thorax, and abdomen in untreated bees, respectively). Chlorfluazuron at concentrations from 400 to 4,000 mg L<sup>-1</sup> significantly decreases the activity of bees head AChE from 52.40 to 32.07 nmoles ATChI hydrolyzed/mg protein/min compared with the control (57.49), while in bee thorax, the activity reduced significantly from 25.33 to 20.67

compared with the control (30.73). The lowest levels of the AChE activity were found in the bee abdomen, where the activity declined from 9.62 to 7.12 compared with the control (10.22). A significant inhibition was also observed with AChE in bee head followed by thorax and abdomen, and concentrations of 2,000 and 4,000 mg L<sup>-1</sup> were the most significantly inhibitors (39.65% and 44.22%, respectively, of AChE in bee head).

The inhibitory effect of oxymatrine on AChE activity is presented in Table 3. The data indicate that all of the concentrations tested (2.5, 5, 10, and 20 mg L<sup>-1</sup>) significantly decreased the specific activity of this enzyme compared with the control. The specific activity decreased in the head from 51.39 to 38.73 compared with the control (55.90). AChE activity from the thorax was reduced from 23.59 to 13.77 compared with the control (30.72). However, the lowest levels of the AChE activity was found in the abdomen, where activity declined from 9.59 to 7.68 compared with the control (10.25). The high inhibition of the enzyme activity was recorded in bee thorax, and the concentrations of 5, 10, and 20 mg L<sup>-1</sup> were significantly the highest (42.14%, 49.71%, and 55.57%, respectively).

Results in Table 4 show that spinosad had potency to inhibit AChE activity after 24 h. Concentrations of 2.5, 5, 10, and 20 mg L<sup>-1</sup> significantly reduced the specific activity from 54.02 to 34.86 in bee head and from 27.23 to 15.96 in bee thorax compared with controls (56.44 and 30.80, respectively). However, the specific activity of AChE was reduced from 9.52 to 6.12 in the abdomen compared with the controls (10.31), while there was no significant difference at concentrations of 5, 10, and 20 mg L<sup>-1</sup>. High inhibition of AChE activity was observed in the

**Table 1** Acute toxicity of chlorfluazuron, oxymatrine, and spinosad against honey bees (*A. mellifera* L.)

Compound	Concentration (mg L <sup>-1</sup> )	Mortality (%)	LC <sub>50</sub> <sup>a</sup> (mg L <sup>-1</sup> )	Slope ± SE <sup>b</sup>	Intercept ± SE <sup>c</sup>	(χ <sup>2</sup> ) <sup>d</sup>
Chlorfluazuron	400	6.67	2526 (2166–3040)	2.23 ± 0.22	-7.58 ± 0.76	1.62
	1,000	16.67				
	2,000	33.33				
	4,000	73.33				
Oxymatrine	2.5	10.00	10.68 (9.14–12.81)	2.04 ± 0.22	-2.10 ± 0.21	1.14
	5.0	26.67				
	10.0	43.33				
	20.0	73.33				
Spinosad	2.5	13.33	7.34 (6.56–8.22)	3.02 ± 0.25	-2.61 ± 0.22	1.74
	5.0	23.33				
	10.0	63.33				
	20.0	96.67				

<sup>a</sup> Lethal concentration causing 50% mortality after 24 h with 95% confidence limits

<sup>b</sup> Slope ± standard error of the concentration–mortality regression line

<sup>c</sup> Intercept ± standard error of the regression line

<sup>d</sup> Chi square

**Table 2** In vivo inhibition of acetylcholinesterase (AChE) activity in honey bees (*A. mellifera* L.) by chlorfluazuron

Concentration (mg L <sup>-1</sup> )	nmoles ATChI hydrolyzed/mg protein/min ± SE	Inhibition (%) ± SE
<b>Head</b>		
0.0	57.49 <sup>a</sup> ± 0.97	0.00 <sup>e</sup> ± 0.00
400	52.40 <sup>b</sup> ± 0.73	8.88 <sup>d</sup> ± 1.26
1,000	47.84 <sup>c</sup> ± 1.62	16.80 <sup>c</sup> ± 2.80
2,000	34.70 <sup>d</sup> ± 1.47	39.65 <sup>a</sup> ± 2.55
4,000	32.07 <sup>de</sup> ± 0.47	44.22 <sup>a</sup> ± 0.81
<b>Thorax</b>		
0.0	30.73 <sup>e</sup> ± 2.26	0.00 <sup>e</sup> ± 0.00
400	25.33 <sup>f</sup> ± 1.01	17.48 <sup>c</sup> ± 3.27
1,000	24.57 <sup>f</sup> ± 0.54	19.97 <sup>c</sup> ± 1.75
2,000	24.38 <sup>f</sup> ± 2.03	20.60 <sup>c</sup> ± 6.59
4,000	20.67 <sup>g</sup> ± 0.52	32.68 <sup>b</sup> ± 1.67
<b>Abdomen</b>		
0.0	10.22 <sup>h</sup> ± 0.79	0.00 <sup>e</sup> ± 0.00
400	9.62 <sup>h</sup> ± 0.40	5.68 <sup>de</sup> ± 3.91
1,000	8.69 <sup>h</sup> ± 0.52	14.77 <sup>c</sup> ± 5.10
2,000	8.34 <sup>h</sup> ± 0.68	18.22 <sup>c</sup> ± 6.60
4,000	7.12 <sup>h</sup> ± 0.36	30.24 <sup>b</sup> ± 3.49

Data are averages ± SE of three replicates. Values within a column bearing the same superscript letters are not significantly different ( $P \leq 0.05$ ) according to Student–Newman–Keuls (SNK) test

bee thorax (11.59–48.19%), followed by the abdomen (7.58–40.63%) and the head (4.22–38.19%).

#### Inhibitory Effect of Chlorfluazuron, Oxymatrine, and Spinosad on Adenosine Triphosphatase (ATPase) Activity

The in vivo effect of the two insect growth regulators (chlorfluazuron and oxymatrine) and a biopesticide, spinosad, on the activity of ATPase of the surviving bees obtained after treatments with different concentrations was examined. Results are reported in Tables 5, 6, and 7. High values of the enzyme activity were found in head of untreated bees, followed by thorax and then abdomen (3.64, 1.86, and 1.57  $\mu$ moles Pi/mg protein/h, respectively).

As shown in Table 5, chlorfluazuron at 400, 1,000, 2,000, and 4,000 mg L<sup>-1</sup> decreased significantly the specific activity of ATPase in bee head from 2.36 to 0.64 compared with the control (3.64). The specific activity of the enzyme was also reduced from 1.36 to 0.81 in bee thorax compared with control (1.86). However, ATPase activity was significantly reduced from 1.52 to 0.77 in bee abdomen compared with control (1.57). On the other hand chlorfluazuron caused significant inhibitions of the enzyme activity at tested concentrations, and high significant inhibitions were found with concentration of 2,000 and

**Table 3** In vivo inhibition of acetylcholinesterase (AChE) activity in honey bees (*A. mellifera* L.) by oxymatrine

Concentration (mg L <sup>-1</sup> )	nmoles ATChI hydrolyzed/mg protein/min ± SE	Inhibition (%) ± SE
<b>Head</b>		
0.0	55.90 <sup>a</sup> ± 0.22	0.00 <sup>h</sup> ± 0.00
2.5	51.39 <sup>b</sup> ± 0.09	8.24 <sup>g</sup> ± 0.16
5.0	48.55 <sup>c</sup> ± 0.50	13.30 <sup>f</sup> ± 0.88
10.0	46.88 <sup>c</sup> ± 0.49	16.29 <sup>f</sup> ± 0.87
20.0	38.73 <sup>d</sup> ± 1.70	30.83 <sup>d</sup> ± 3.02
<b>Thorax</b>		
0.0	30.72 <sup>e</sup> ± 0.46	0.00 <sup>h</sup> ± 0.00
2.5	23.59 <sup>f</sup> ± 1.87	23.91 <sup>e</sup> ± 5.99
5.0	17.94 <sup>g</sup> ± 0.31	42.14 <sup>c</sup> ± 0.99
10.0	15.59 <sup>h</sup> ± 1.11	49.71 <sup>b</sup> ± 3.56
20.0	13.77 <sup>h</sup> ± 0.46	55.57 <sup>a</sup> ± 1.49
<b>Abdomen</b>		
0.0	10.25 <sup>i</sup> ± 0.19	0.00 <sup>h</sup> ± 0.00
2.5	9.59 <sup>i</sup> ± 0.18	6.78 <sup>h</sup> ± 1.79
5.0	8.62 <sup>i</sup> ± 0.12	16.20 <sup>f</sup> ± 1.12
10.0	7.95 <sup>i</sup> ± 0.04	22.73 <sup>e</sup> ± 0.36
20.0	7.68 <sup>i</sup> ± 0.04	25.35 <sup>e</sup> ± 0.39

Data are averages ± SE of three replicates. Values within a column bearing the same superscript letters are not significantly different ( $P \leq 0.05$ ) according to Student–Newman–Keuls (SNK) test

4,000 mg L<sup>-1</sup> in bee head (67.82% and 82.28%, respectively).

Table 6 presents the inhibitory effect of oxymatrine on ATPase activity in honey bees at concentrations of 2.5, 5, 10, and 20 mg L<sup>-1</sup>. Significant differences between the activity of bee ATPase in the head, thorax, and abdomen of surviving bees were recorded. The enzyme specific activity was significantly reduced from 3.55 to 2.84 compared with the control (3.64  $\mu$ moles Pi/mg protein/h) in the bee head. The ATPase activity of bee thorax significantly decreased from 1.67 to 1.24 compared with the control (1.86), and in bee abdomen from 1.55 to 1.20 compared with the control (1.57). It was clear that the maximum inhibition percentage was observed significantly in bee thorax at a concentration of 20 mg L<sup>-1</sup> (33.31%).

The data in Table 7 illustrate the inhibitory effect of spinosad on the ATPase activity of the head, thorax, and abdomen obtained from surviving bees after treatment at 2.5, 5, 10, and 20 mg L<sup>-1</sup>. The results indicated that this compound significantly exhibited high reduction in the specific activity of the head ATPase (2.12 to 0.48) compared with the control (3.64  $\mu$ moles Pi/mg protein/h). Thorax ATPase activity was significantly declined from 1.17 to 0.77 compared with the control (1.86). However, a lower enzyme activity was found in the abdomen of the untreated bees (1.57), and the tested compound decreased

**Table 4** In vivo inhibition of acetylcholinesterase (AChE) activity in honey bees (*A. mellifera* L.) by spinosad

Concentration (mg L <sup>-1</sup> )	nmoles ATChI hydrolyzed/mg protein/min ± SE	Inhibition (%) ± SE
<b>Head</b>		
0.0	56.44 <sup>a</sup> ± 1.74	0.00 <sup>h</sup> ± 0.00
2.5	54.02 <sup>b</sup> ± 0.54	4.22 <sup>gh</sup> ± 0.95
5.0	48.23 <sup>c</sup> ± 1.22	14.49 <sup>f</sup> ± 2.16
10.0	40.31 <sup>d</sup> ± 1.20	28.53 <sup>e</sup> ± 2.13
20.0	34.86 <sup>e</sup> ± 1.50	38.19 <sup>bc</sup> ± 2.64
<b>Thorax</b>		
0.0	30.80 <sup>f</sup> ± 0.45	0.00 <sup>h</sup> ± 0.00
2.5	27.23 <sup>g</sup> ± 0.76	11.59 <sup>f</sup> ± 2.44
5.0	20.49 <sup>h</sup> ± 0.05	33.46 <sup>cd</sup> ± 0.16
10.0	19.39 <sup>h</sup> ± 0.55	37.05 <sup>bcd</sup> ± 1.78
20.0	15.96 <sup>i</sup> ± 0.23	48.19 <sup>a</sup> ± 0.74
<b>Abdomen</b>		
0.0	10.31 <sup>j</sup> ± 0.11	0.00 <sup>h</sup> ± 0.00
2.5	9.52 <sup>j</sup> ± 0.12	7.58 <sup>g</sup> ± 1.16
5.0	6.95 <sup>k</sup> ± 0.64	32.50 <sup>d</sup> ± 6.19
10.0	6.48 <sup>k</sup> ± 0.09	37.13 <sup>bcd</sup> ± 0.91
20.0	6.12 <sup>k</sup> ± 0.16	40.63 <sup>b</sup> ± 1.56

Data are averages ± SE of three replicates. Values within a column bearing the same superscript letters are not significantly different ( $P \leq 0.05$ ) according to Student–Newman–Keuls (SNK) test

the enzyme activity to 0.48 μmoles Pi/mg protein/h at 20 mg L<sup>-1</sup>. It was clear that spinosad significantly inhibited the ATPase in different sources at all concentrations tested, and concentrations of 10 and 20 mg L<sup>-1</sup> were significantly the highest in inhibition of the enzyme activity in bee head (77.93% and 86.78%, respectively).

## Discussion

Insect growth regulators (IGRs) and biopesticides are often considered as of lower impact on many beneficial organisms compared with other insecticides. They have attracted considerable attention recently for their inclusion in Integrated Pest Management (IPM) programs, but effects are highly variable depending on the species and studied developmental stage (Darvas and Polgar 1998; Schneider et al. 2003). The present study was carried out in order to compare the toxicity of chlorfluazuron and oxymatrine (IGRs) and spinosad as a biopesticide on honey bee (*A. mellifera* L.) workers. Laboratory toxicity assay of oxymatrine and spinosad clearly indicated toxic effect on honey bees with LC<sub>50</sub> of 10.68 and 7.34 mg L<sup>-1</sup> compared with the application rate in the field (30 and 48 mg L<sup>-1</sup>, respectively). However, chlorfluazuron was the least acutely toxic of the tested compounds (LC<sub>50</sub> = 2,526 mg L<sup>-1</sup>).

**Table 5** In vivo inhibition of adenosine triphosphatase (ATPase) activity in honey bees (*A. mellifera* L.) by chlorfluazuron

Concentration (mg L <sup>-1</sup> )	μmoles Pi/mg protein/h ± SE	Inhibition (%) ± SE
<b>Head</b>		
0.0	3.64 <sup>a</sup> ± 0.09	0.00 <sup>j</sup> ± 0.00
400	2.36 <sup>b</sup> ± 0.04	35.04 <sup>g</sup> ± 1.20
1,000	2.05 <sup>c</sup> ± 0.06	43.76 <sup>e</sup> ± 1.56
2,000	1.17 <sup>g</sup> ± 0.01	67.82 <sup>b</sup> ± 0.17
4,000	0.64 <sup>i</sup> ± 0.08	82.28 <sup>a</sup> ± 2.10
<b>Thorax</b>		
0.0	1.86 <sup>d</sup> ± 0.02	0.00 <sup>j</sup> ± 0.00
400	1.36 <sup>f</sup> ± 0.02	26.93 <sup>h</sup> ± 3.48
1,000	1.12 <sup>g</sup> ± 0.02	39.78 <sup>f</sup> ± 1.19
2,000	1.03 <sup>g</sup> ± 0.03	44.62 <sup>e</sup> ± 1.50
4,000	0.81 <sup>h</sup> ± 0.05	56.35 <sup>c</sup> ± 2.55
<b>Abdomen</b>		
0.0	1.57 <sup>e</sup> ± 0.05	0.00 <sup>j</sup> ± 0.00
400	1.52 <sup>e</sup> ± 0.01	3.11 <sup>j</sup> ± 0.88
1,000	1.39 <sup>f</sup> ± 0.04	11.84 <sup>i</sup> ± 2.34
2,000	0.85 <sup>h</sup> ± 0.04	46.10 <sup>e</sup> ± 2.76
4,000	0.77 <sup>h</sup> ± 0.01	51.09 <sup>d</sup> ± 0.95

Data are averages ± SE of three replicates. Values within a column bearing the same superscript letters are not significantly different ( $P \leq 0.05$ ) according to Student–Newman–Keuls (SNK) test

The study of pesticide effects on the honey bee is important because of the need to control a wide variety of agricultural pests with insecticides (Atkins 1992) without hurting bees that inadvertently come into contact with pesticides when foraging (Haynes 1988; Vandame et al. 1995; Stone et al. 1997; Decourtye et al. 2004, 2005). High exposure levels can kill foragers, but sublethal exposures may also adversely affect colony function (Smirle et al. 1984; Currie 1999). From the wide range of studies conducted much has been learned about the potential effects of pesticides to honey bees (Atkins et al. 1981; Mayer and Lunden 1986; Mayer et al. 1987; Celli et al. 1991; Porrini et al. 1996; Decourtye et al. 2004, 2005). In addition, insecticide actions on the biochemistry of honey bees have been the subject of many studies (M'diaye and Bounias 1993; Bendahou et al. 1999; Badiou et al. 2008). Concerning the biochemical mode of action of insecticides, interaction of several active molecules with neurosecretory cells and receptors of regulatory hormones may be involved (M'diaye and Bounias 1993).

Spinosad is a novel insect control agent derived by fermentation of the actinomycete bacterium, *Saccharopolyspora spinosa*. The active ingredient is composed of two variants, spinosyn A and spinosyn D (Thompson et al. 1997). Spinosad controls many caterpillar pests in some fruit

**Table 6** In vivo inhibition of adenosine triphosphatase (ATPase) activity in honey bees (*A. mellifera* L.) by oxymatrine

Concentration (mg L <sup>-1</sup> )	μmoles Pi/mg protein/h ± SE	Inhibition (%) ± SE
Head		
0.0	3.64 <sup>a</sup> ± 0.09	0.00 <sup>f</sup> ± 0.00
2.5	3.55 <sup>a</sup> ± 0.03	2.33 <sup>f</sup> ± 0.85
5.0	3.39 <sup>b</sup> ± 0.03	6.63 <sup>e</sup> ± 0.93
10.0	3.09 <sup>c</sup> ± 0.04	15.15 <sup>c</sup> ± 1.21
20.0	2.84 <sup>d</sup> ± 0.04	21.82 <sup>b</sup> ± 1.19
Thorax		
0.0	1.86 <sup>e</sup> ± 0.02	0.00 <sup>f</sup> ± 0.00
2.5	1.67 <sup>f</sup> ± 0.05	10.25 <sup>d</sup> ± 2.59
5.0	1.55 <sup>fg</sup> ± 0.01	16.40 <sup>c</sup> ± 0.55
10.0	1.44 <sup>g</sup> ± 0.01	22.68 <sup>b</sup> ± 0.42
20.0	1.24 <sup>h</sup> ± 0.06	33.31 <sup>a</sup> ± 3.13
Abdomen		
0.0	1.57 <sup>fg</sup> ± 0.05	0.00 <sup>f</sup> ± 0.00
2.5	1.55 <sup>fg</sup> ± 0.04	1.27 <sup>f</sup> ± 2.70
5.0	1.52 <sup>g</sup> ± 0.01	3.41 <sup>f</sup> ± 0.61
10.0	1.45 <sup>g</sup> ± 0.01	7.60 <sup>e</sup> ± 0.92
20.0	1.20 <sup>h</sup> ± 0.02	23.38 <sup>b</sup> ± 1.22

Data are averages ± SE of three replicates. Values within a column bearing the same superscript letters are not significantly different ( $P \leq 0.05$ ) according to Student–Newman–Keuls (SNK) test

and vegetables, thrips in tomatoes, peppers, and ornamental cultivation, and dipterous leaf miners in vegetables and ornamentals. The effects of spinosad to honey bees have been investigated (Miles 2003). Our results indicate that exposure to spinosad for 24 h resulted in high mortality of honey bees *A. mellifera* L. at concentrations of 2.5–20 mg L<sup>-1</sup> (LC<sub>50</sub> = 7.34 mg L<sup>-1</sup>). This result can be supported by the Environmental Protection Agency (EPA), i.e., that the topical acute activity of spinosad against honey bees is less than 1 μg/bee, which places spinosad in the highly toxic category to bees. However, once residues have dried completely, toxicity of foraging bees is considered negligible (Mayer et al. 2001). Miles (2003) confirmed that spinosad was highly toxic to worker honey bees under laboratory conditions, whereas in field studies dry residues of spinosad were safe to foraging worker honey bees, with no adverse effects seen on mortality, foraging behavior, brood or queen. Therefore, recommendations for spinosad application include allowing drying time before bee exposure.

The results in the present study also indicates that chlorfluazuron at the application rate for one feddan (20 g a.i./100 L water, 200 mg L<sup>-1</sup>) showed no harmful effect to honey bees (LC<sub>50</sub> = 2,526 mg L<sup>-1</sup>), indicating that this compound can be used in the presence of bees with minimum injury. This finding is in agreement with the results obtained by Heller et al. (1992) and Abramson et al.

**Table 7** In vivo inhibition of adenosine triphosphatase (ATPase) activity in honey bees (*A. mellifera* L.) by spinosad

Concentration (mg L <sup>-1</sup> )	μmoles Pi/mg protein/h ± SE	Inhibition (%) ± SE
Head		
0.0	3.64 <sup>a</sup> ± 0.09	0.00 <sup>g</sup> ± 0.00
2.5	2.12 <sup>b</sup> ± 0.07	41.68 <sup>f</sup> ± 1.82
5.0	1.86 <sup>c</sup> ± 0.11	48.9 <sup>e</sup> ± 2.90
10.0	0.80 <sup>gh</sup> ± 0.07	77.93 <sup>b</sup> ± 1.99
20.0	0.48 <sup>j</sup> ± 0.03	86.78 <sup>a</sup> ± 0.93
Thorax		
0.0	1.86 <sup>c</sup> ± 0.01	0.00 <sup>g</sup> ± 0.59
2.5	1.17 <sup>e</sup> ± 0.04	36.9 <sup>f</sup> ± 3.82
5.0	1.11 <sup>e</sup> ± 0.04	40.06 <sup>f</sup> ± 1.95
10.0	0.93 <sup>fg</sup> ± 0.04	50.20 <sup>e</sup> ± 2.18
20.0	0.77 <sup>ghi</sup> ± 0.05	58.52 <sup>d</sup> ± 2.82
Abdomen		
0.0	1.57 <sup>d</sup> ± 0.02	0.00 <sup>g</sup> ± 3.22
2.5	1.03 <sup>ef</sup> ± 0.03	34.78 <sup>f</sup> ± 1.73
5.0	0.64 <sup>hij</sup> ± 0.02	59.30 <sup>d</sup> ± 1.26
10.0	0.61 <sup>ij</sup> ± 0.01	61.25 <sup>d</sup> ± 0.66
20.0	0.48 <sup>j</sup> ± 0.02	69.46 <sup>c</sup> ± 1.24

Data are averages ± SE of three replicates. Values within a column bearing the same superscript letters are not significantly different ( $P \leq 0.05$ ) according to Student–Newman–Keuls (SNK) test

(2004). They reported that there were no negative effects of the IGRs tebufenozide and diflubenzuron on honey bees. Exposure to these insecticides did not kill or disrupt foraging behavior and had no effect on larval development of honey bees (Heller et al. 1992). They also noted that colonies were not affected after aerial application of 350 g diflubenzuron/ha. Liu et al. (2000) reported that IGRs are considered to be environmentally safer alternatives to broad-spectrum pesticides because of their quick degradation, low toxicity to humans, and low doses of use. Our results indicate however that oxymatrine in the present study showed a significant risk to honey bees (LC<sub>50</sub> = 10.68 mg L<sup>-1</sup>).

In ecotoxicology, enzymes such as AChE and ATPase are particularly useful as they represent the site of action of some insecticides and the degree of inhibition is related to toxic effects (Grue et al. 1991; Badiou et al. 2008). Ideally, the action would be specific to undesirable target organisms, but many nontarget species such as honey bees are affected (Murphy 1986). The study of such enzymes as biomarkers in the honey bee *A. mellifera* is interesting for two reasons. First, the honey bee is an important pollinating agent, sensitive to environmental contaminants (Wallwork-Barber et al. 1982; Smith and Wilcox 1990). The safe-guarding of these beneficial pollinators is very important since mortality of bees will have considerable



impact on honey production and crop pollination (Free 1993). Moreover, its sensitivity to environmental contaminants and its implication in pollination makes the bee a pertinent bioindicator for which it is important to develop accurate biomarkers.

The present study investigates the possibility of using AChE and ATPase activities as biomarkers of exposure to such insecticides in the honey bee *A. mellifera* (Bendahou et al. 1999; Hyne and Maher 2003). The usefulness of measuring such biochemical parameters is in determining the probable cause of lethal and sublethal effects under field conditions or monitoring for the combined adverse effects of long-term exposures to mixtures of toxicants (Giesy and Graney 1989). In fact, biochemical measures are useful in monitoring for effects before they reach the population or community level.

AChE is an important enzyme responsible for rapid hydrolysis of acetylcholine at the cholinergic synapses, thus allowing precise control and modulation of neural transmission. It is largely distributed in the bee brain (Belzunces et al. 1988; Huang and Knowles 1990). This fact was confirmed in our study, where high levels of AChE were found in bee brain (57.49 nmoles ATChI hydrolyzed/mg protein/min) compared with in the thorax and abdomen (30.73 and 10.22, respectively). Recently, evidence has emerged that reduction of AChE activity is not due exclusively to organophosphates and carbamates, but that other classes of environmental contaminants such as complex mixtures of pollutants, detergents, and metals are also involved in AChE reduction (Payne et al. 1996; Bendahou et al. 1999; Frasco et al. 2005; Guilhermino et al. 1998).

ATPase is a group of enzymes that play an important role in intracellular functions and that are considered to be a sensitive indicator of toxicity (Yadwad et al. 1990). They hydrolyze adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate (Pi). In this process, the energy released becomes available for cation transport. These enzymes, especially sodium, potassium-activated ATPase ( $\text{Na}^+$ ,  $\text{K}^+$ -ATPase), play a central role in whole-body osmoregulation, in that they provide energy for the active transport of  $\text{Na}^+$  and  $\text{K}^+$  across the cell membrane. Its activity is inhibited by ouabain; ATPase not inhibited by ouabain is referred to collectively as residual ATPase. Detection of ATPase inhibition could prove to be an important index for tolerable levels of a large group of environmental contaminants (Ozcan Oruc et al. 2002).

In the present study, the statistical tests performed on the data sets for chlorfluazuron, oxymatrine, and spinosad showed significant differences in AChE and ATPase activities attributable to treatments compared with the controls (Tables 2–7). The result showed that chlorfluazuron caused a low toxic effect against the honey bee

*A. mellifera* with a  $\text{LC}_{50}$  of 2,526  $\text{mg L}^{-1}$  and a moderate inhibition of AChE at a high acute treatment (4,000  $\text{mg L}^{-1}$ ). However, at the same concentration, it was highly effective in inhibition of ATPase activity, where the observed inhibition was higher than 50% in the current experiment. On the other hand, oxymatrine and spinosad caused a highly toxic effect against *A. mellifera* ( $\text{LC}_{50} = 10.68$  and  $7.34 \text{ mg L}^{-1}$ , respectively) compared with chlorfluazuron and they had approximately the same efficiency in inhibition of AChE at the tested concentrations (2.5, 5, 10, and 20  $\text{mg L}^{-1}$ ). In addition, spinosad at these concentrations caused the highest inhibition of the ATPase, ranging from 34% to 86% in the current experiment.

Spinosad had potency to inhibit AChE and ATPase activities after 24 h of feeding, indicating that such enzymes could be used as biomarkers of neurotoxicity and exposure to these insecticides in honey bees. This result is in agreement with the study that reported that the mode of action of spinosad is characterized by excitation of the insect nervous system, leading to involuntary muscle contractions, prostration with tremors, and paralysis (Milles and Dutton 2000). These effects are consistent with the activation of nicotinic acetylcholine receptors by a mechanism that is clearly different and unique among known insect control products. Spinosad also has effects on GABA receptor function that may contribute further to its insecticide activity (Salgado 1998; Salgado et al. 1998). Moreover, spinosad is primarily a stomach poison with some contact activity and is particularly active against Lepidoptera and Diptera. This study demonstrates that chlorfluazuron, oxymatrine, and spinosad had inhibitory effects on AChE and ATPase and shows that these enzymes could be used as biomarkers of neurotoxicity and exposure to these insecticides in honey bees.

## Conclusions

The current study compared the toxicity of chlorfluazuron, oxymatrine, and spinosad on honey bee (*A. mellifera* L.) workers. AChE and ATPase activities were quantified to investigate the possibility of using such enzymes as biomarkers of exposure to these insecticides in the honey bees. The results indicated that spinosad was the most harmful of the three substances to honey bees with  $\text{LC}_{50}$  value of 7.34  $\text{mg L}^{-1}$ , followed by oxymatrine ( $\text{LC}_{50} = 10.68 \text{ mg L}^{-1}$ ), whereas chlorfluazuron was the least acutely toxic ( $\text{LC}_{50} = 2,526 \text{ mg L}^{-1}$ ) compared with the rate of application in the field (48, 30, and 200  $\text{mg L}^{-1}$ , respectively). We therefore believe that bees exposed in a field to these doses of compounds could, for example, have impaired behavior with reduced success in returning to hives, thus

depriving the colony of foragers and harming the entire colony. It is therefore suggested that these insecticides must be used only with greatest care as they may impact on honey bees. Further studies are needed to investigate the duration of behavioral effects of these compounds on bees, in relation to biomarker response, particularly at sublethal doses.

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