Arch Environ Contam Toxicol (2013) 64:456–466
DOI 10.1007/s00244-012-9849-1

Effects of Sublethal Dose of Fipronil on Neuron Metabolic Activity of Africanized Honeybees

Thaisa Cristina Roat · Stephan M. Carvalho · Roberta C. F. Nocelli · Elaine C. M. Silva-Zacarin · Mario Sérgio Palma · Osmar Malaspina

Received: 25 July 2012 / Accepted: 14 November 2012 / Published online: 9 December 2012

Abstract Fipronil is a neurotoxic insecticide that inhibits the gamma-aminobutyric acid receptor and can affect gustative perception, olfactory learning, and motor activity of the honeybee *Apis mellifera*. This study determined the lethal dose (LD_{50}) and the lethal concentration (LC_{50}) for Africanized honeybee and evaluated the toxicity of a sublethal dose of fipronil on neuron metabolic activity by way of histochemical analysis using cytochrome oxidase detection in brains from worker bees of different ages. In addition, the present study investigated the recovery mechanism by discontinuing the oral exposure to fipronil. The results showed that mushroom bodies of aged Africanized honeybees are affected by fipronil, which causes changes in metabolism by increasing the respiratory activity of mitochondria. In antennal lobes, the sublethal dose of fipronil did not cause an increase in metabolic activity. The recovery experiments showed that discontinued exposure to a diet contaminated with fipronil did not lead to recovery of neural activity. Our results show that even at very low concentrations, fipronil is harmful to honeybees and can induce several types of injuries to honeybee physiology.

T. C. Roat (✉) · S. M. Carvalho · M. S. Palma · O. Malaspina
Departamento de Biologia, Centro de Estudos de Insetos Sociais, Campus de Rio Claro, UNESP-Univ. Estadual Paulista, Avenida 24-A, n.1515, Bela Vista, Rio Claro, São Paulo 13506-900, Brazil
e-mail: thaisaroat@yahoo.com.br

R. C. F. Nocelli
Centro de Ciências Agrárias, Campus de Araras, UFSCar-Universidade Federal de São Carlos, Rodovia Anhanguera, Km 174, Araras, São Paulo 13600-970, Brazil

E. C. M. Silva-Zacarin
Laboratório de Biologia Estrutural e Funcional, Campus de Sorocaba, UFSCar-Universidade Federal de São Carlos, Rodovia João Leme dos Santos, Km 110, Sorocaba, São Paulo 18052-780, Brazil

Pollinators are crucial for the pollination of agricultural crops and natural areas around the world (Klein et al. 2007). Honeybees are considered excellent pollinating insects in agroecosystems because they visit many flowers on the same day (Kremen et al. 2007). Moreover, the bee *Apis mellifera* Linnaeus 1758 (Hymenoptera: Apidae) is interesting from an economic perspective because it provides products of great value, such as honey, propolis, royal jelly, wax, and apitoxin (bee venom).

During the last decades, agriculture has changed to meet the increasing demand to produce food. There has been expansion of cultivated areas in monoculture and increases use of pesticides. This is evidenced by the annual consumption of >300,000 tons of insecticides formulated in Brazil. The amount of active ingredient consumed annually is approximately 130,000 tons, representing an increase of 700 % during the last 40 years, whereas agricultural area increased only 78 % (Spadotto et al. 2004). The abusive use of pesticides is subjecting pollinator insects to stress as evidenced by a constant decrease in the density of honeybees around agricultural fields in many parts of the world, thus causing economic losses (Neumann and Carreck 2010).

Among the insecticides, fipronil, which belongs the phenylpyrazoles group and has the molecular formula $C_{12}H_4Cl_2F_6N_4OS$, stands out as harmful. In Brazil, it is used to treat seeds and soil against subterranean pests and is sprayed mainly to prevent the attack of leaf cutter ants and to protect wood against termites. Fipronil acts on the nervous system of insects by attaching to gamma-aminobutyric acid (GABA) receptors. These receptors control the flow of chloride ions through ion channel cell membranes

and can inhibit the central nervous system (CNS). Binding of fipronil to GABA receptors induces hyperpolarization (Narahashi et al. 2007). GABA receptors are located in several neuropiles of the honeybee brain (Schäfer and Bicker 1986) that are relevant to the modulation of learning, memory, and sensory perception (El Hassani et al. 2005; Aliouane et al. 2009; Bernadou et al. 2009). Moreover, it has been shown that the insecticides fipronil and neonicotinoids may be involved in colony collapse disorder in the United States (Stokstad 2012).

It is clear that fipronil affects metabolism in the brain; however, it is unknown how low doses of this compound affect neural activity. The metabolic activity of the brain can be studied using the histochemical technique for detection of cytochrome oxidase (CO), a terminal enzyme of the electron transport chain in the mitochondrial respiration process. This technique is based on the principle that neural activity induces metabolism in neurons, and this can increase cellular respiration and, consequently, enzymatic activity of the mitochondria. Alterations in CO activity in the CNS occur concomitantly with memory deficits in the bee (Decourtye et al. 2002). Moreover, the histochemical detection of CO activity in invertebrates is a valuable tool with which to identify the cerebral structures involved in memory processes (Agin et al. 2001; Déglise et al. 2003). For example, Decourtye et al. (2002) verified an increase in CO activity from the calyces of mushroom bodies after *A. mellifera* worker bees were exposed to imidacloprid.

Due to the importance of the use of fipronil in Brazilian crops, the goal of this study was to investigate the acute toxicity (topical and oral) of fipronil and to evaluate the effects of a sublethal dose of this phenylpirazole insecticide on neuron metabolic activity in intoxicated newly emerged and aged honeybees through histochemical technique for the detection of CO enzyme. In addition, using the same parameters for analysis, we studied the capacity for recovery of neuron metabolic activity after exposure to fipronil.

Material and Methods

Chemicals

The pesticide fipronil (95 %) was obtained from Dalian Raiser Pesticides (China), sucrose from Amresco (Brazil), and HistoResin from Leica (Brazil). Sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), paraformaldehyde, cytochrome C, and 3,3'-diaminobenzidine were purchased from Sigma-Aldrich (Brazil).

Honeybees

Honeybees were obtained from queen-right colonies of Africanized *A. mellifera* at the experimental apiary of the Departamento de Biologia, Universidade Estadual Paulista, campus Rio Claro/SP, Brazil. To obtain newly emerged worker bees, three sealed brood combs from three different colonies were put in a BOD incubator at a temperature of 34 ± 2 °C and relative humidity of 70 ± 10 %, and adult bees were collected after emergence. We collected 240 newly emerged honeybees for bioassays, and another 500 bees were marked on the thorax with ink and returned to the colony for later capture when they reached 20 days old (aged honeybees).

Acute Toxicity Assay: Topical Application

The lethal dose 50 (LD_{50}) of fipronil by way of topical application on newly emerged honeybees was determined by dose–response assay. Several concentrations of fipronil (0.5–5.0 ng a.i./μL) were prepared in acetone, and 1 μL of one of the solutions was applied to the thorax of one bee; each concentration group comprised 75 honeybees. The control group received only 1 μL acetone. Immediately afterward, each group of 75 bees was divided into 3 cages with 25 honeybees each and kept at 32 ± 2 °C and 60 ± 10 % relative humidity and fed candy paste ad libitum. Twenty-four hours after intoxication, the number of dead honeybees per treatment was recorded and the data subjected to statistical analysis.

Acute Toxicity Assay: Ingestion

The lethal concentration 50 (LC_{50}) of fipronil by way of ingestion to newly emerged honeybees was determined as previously described (see Acute Toxicity Assay: Topical Application) but with some modifications. Stock solution of fipronil was made in 100 % acetone and then diluted in a sucrose and H_2O mixture (1:1) with a final range of fipronil from 5 to 0.05 ng fipronil/μL. The final solution contained ≤1 % acetone. Groups of 75 honeybees/treatment (25/cage) were kept in experimental cages and subjected to a starvation period of 2 h. Afterward, the honeybees were collectively fed a fipronil-contaminated syrup, and the total volume was adjusted to the number of honeybees so that each bee could consume 10 μL of diet containing different concentrations of fipronil. The number of dead honeybees was recorded and data subjected to statistical analysis 24 h later. The control treatment received the same volume of diet without fipronil.

Table 1 *A. mellifera* age, treatment, and day of sample collection for neuron metabolic activity assays

Age	Type of exposure	Date of sample collection
Newly emerged	Control without acetone (C-3D) Control with acetone (CA-3D) 0.01 ng fipronil/bee/day (F-3D)	Day 3 after initial exposure
Aged	Control without acetone (C-3D) Control with acetone (CA-3D) 0.01 ng fipronil/bee/day (F-3D)	
Newly emerged	Control without acetone (C-5D) Control with acetone (CA-5D) 0.01 ng fipronil/bee/day (F-5D)	Day 5 after initial exposure
Aged	Control without acetone (C-5D) Control with acetone (CA-3D) 0.01 ng fipronil/bee/day (F-5D)	
Newly emerged	Control without acetone (C-8D) Control with acetone (CA-8D) 0.01 ng fipronil/bee/day (F-5D)	Day 8 after initial exposure

Table 2 *A. mellifera* age, treatment, and day of sample collection for neural activity assays including recovery time

Age	Type of exposure	Date of sample collection
Newly emerged Aged (Rec-5D)	Exchange on day 3 of fipronil diet (0.01 ng/bee/day) for control diet without acetone	Day 5 after initial exposure
Newly emerged (Rec-8D)		Day 8 after initial exposure
Newly emerged (Rec-8D2)	Exchange on day 5 of fipronil diet (0.01 ng/bee/day) for control diet without acetone	Day 8 after initial exposure

Honeybee Intoxication Assay

Honeybee intoxication assay with a sublethal dose of fipronil was performed using the LD_{50} value for newly emerged honeybees (see Acute Toxicity Assay: Topical Application) as a reference value and not the LC_{50} value, but these values were similar. To intoxicate the honeybees, a stock solution of fipronil (1,000 ng a.i./μL) was prepared in acetone; from this solution, several dilutions were prepared using a sucrose and H_2O mixture (1:1) as solvent, obtaining a solution with 0.001 ng fipronil/μL diet.

A group of 60 newly emerged and aged honeybees was equally divided into three disposable cages. The honeybees were collectively fed with a fipronil-contaminated syrup, and the total volume was adjusted to the number of honeybees so that each honeybee could consume daily 10 μL sucrose solution containing 0.001 ng a.i./μL diet (i.e., 200 μL diet enriched with fipronil/cage). Therefore, each bee ingested 0.01 ng/μL fipronil/d ($^1/_{100}$ of LD_{50}). Every day the number of dead honeybees was counted and the total volume of syrup adjusted to the number of live honeybees. Two experimental controls were used in these experiments: (1) control without acetone in which honeybees were fed only with sucrose and H_2O (1:1) mixture; and (2) control with acetone in which in the sucrose and H_2O (1:1) mixture contained the solvent acetone at the same concentration used when the honeybees were fed fipronil (0.0001 % of acetone). The food was provided on plastic on the bottom of the experimental cages and covered with plastic mesh. Water was furnished ad libitum by way of impregnated cotton. The cages were kept under climate control of 32 ± 2 °C and 60 ± 10 % relative humidity.

Samples of newly emerged and aged honeybees were collected to assess neuron metabolic activity alterations induced by fipronil at 3, 5, or 8 days after the beginning of exposure by way of continuous supply of contaminated food (Table 1). The recovery effect of honeybees against fipronil was also studied. In the first experiment, the diet containing fipronil was furnished until day 3 and then substituted for diet without insecticide. Honeybees were collected on days 5 or 8. In the other experiment,

honeybees were fed with contaminated diet until day 5 and then fed diet without fipronil, and samples were collected on day 8 (Table 2). The schedules of these assays are listed in Tables 1 and 2.

For histochemical analysis, eight live honeybees were randomly collected from each cage. In this experiment, aged honeybees were used only at days 3 and 5 after exposure but not after day 8 due to the difficulty in obtaining sufficient samples of bees of this age under experimental conditions.

Histochemical Analysis of Neuron Metabolic Activity

This technique was performed according the method described by Wong-Riley (1979) and Armengaud et al. (2000) with some modifications. For each experimental group, the honeybee samples were dissected in buffered saline solution (20 mM Na_2HPO_4/KH_2PO_4 [pH 7.4] + 130 mM NaCl), and brains were fixed in 4 % + 100 mM sodium phosphate buffer [pH 7.4] for 90 min. After this period, the material was immersed for 12 h in 100 mM sodium phosphate buffer [pH 7.4]. Then material was incubated in CO solution (100 mM phosphate buffer [pH 7.4] containing 0.02 % cytochrome C, 0.06 % diaminobenzidine, and 4.5 % sucrose) for 30 min. Afterward, the samples were dehydrated in increasing concentrations of ethanol (70, 80, 90, and 95 %) for 30 min each, incubated in embedding resin for 3 days, and then embedded in historesin. Finally, the material was cut into 7 µm–thick sections and analyzed using a photomicroscope BX 51 (Olympus America).

Densitometry analysis (expressed as grey level) was performed in 29–38 sections from eight honeybees each from the control and treated groups. We employed a magnification of 20× using the photomicroscope. Quantification was performed using computer-aided densitometry of CO histochemistry staining intensity employing Image Pro Plus image analysis software (Media Cybernetics, v.6.0) . The densitometry analysis was performed for mushroom bodies and antennal lobes. In addition, some sections of the brain were stained with hematoxylin and eosin (HE) to serve as a general view of the structures analyzed.

Statistical Analysis

Data from the acute toxicity test (oral and topical application) were subjected to a dose response–type statistical analysis using a log-logistic model from the package "drc" (Ritz and Streibig 2005) compiled with the statistical software R (2012). With the best-fitted model, the values of LD_{50} or LC_{50}, as well as the 5 % confidence limits and the chi-square values, were calculated.

For densitometry analysis, the grey levels of the different brain structures were compared among treatments using one-way analysis of variance ($p \leq 0.05$). When the F value was significant, Fisher's least significant difference test was used to grade the different treatment groups ($p \leq 0.05$).

Results

Acute Toxicity Assay

Acute toxicity assay showed that LD_{50} and LC_{50} values from newly emerged honeybees are very similar. For topical application, the LD_{50} value was 1.06 ng fipronil/bee ($CL_{95\ \%}$ = 0.97 to 1.15; degrees of freedom [df] = 13, χ^2 = 18,386) (Fig. 1); for oral administration, the LC_{50} was 1.27 ($CL_{95\ \%}$ = 1.13 to 1.41; df = 16, χ^2 = 18,474) (Fig. 2). Due to the similar values between the LD_{50} and the LC_{50}, we adopted the first value (LD_{50}) as a reference dose, and then we calculated a sublethal dose of 0.01 ng fipronil/bee ($^1/_{100}$ of the LD_{50}).

Analysis of Neuron Metabolic Activity

Positive marking of histochemical analysis for CO was observed as small dots in the cell because the CO is a mitochondrial enzyme. Figure 3 shows in detail the weakly marked

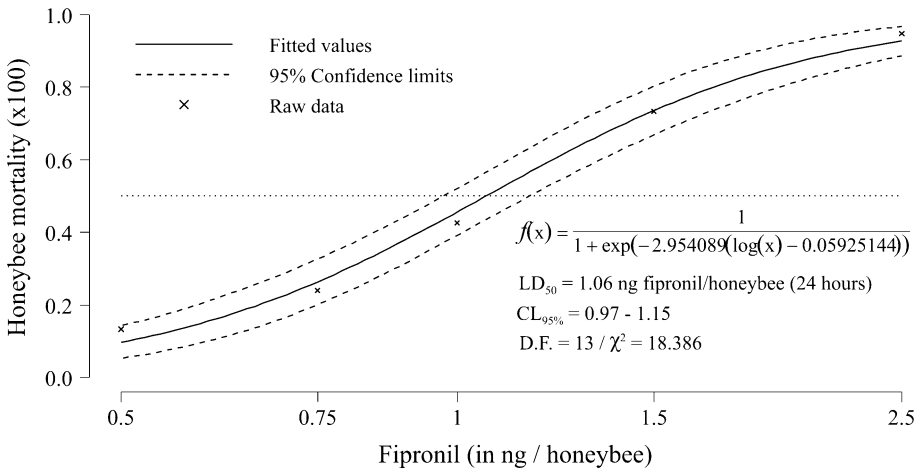

Fig. 1 Acute toxicity assay by contact in which newly emerged *A. mellifera* were exposed to different doses of fipronil to calculate the LD_{50}

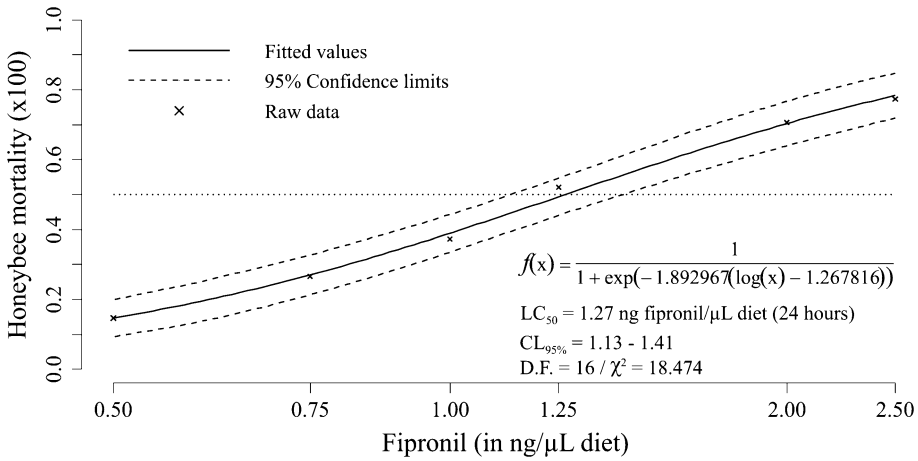

Fig. 2 Acute toxicity assay by oral administration in which newly emerged *A. mellifera* were exposed to different doses of fipronil to calculate the LC_{50}

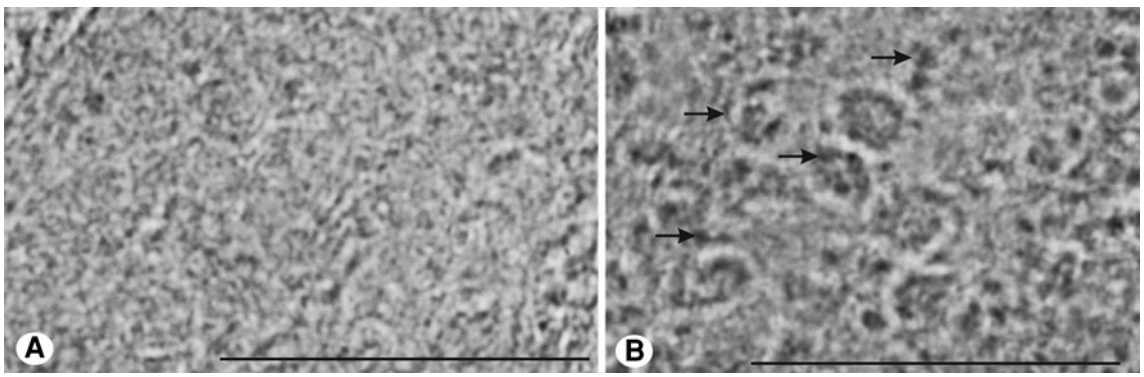

Fig. 3 Photomicrography of Kenyon cells of *A. mellifera* treated for 3 days with fipronil and subjected to a histochemical technique for CO detection. **a** Cells weakly marked reflecting the basal activity of the enzyme in newly emerged honeybees. **b** Cells positively marked for CO histochemistry in aged honeybees. Scale = 100 µm

Kenyon cells (i.e., basal activity of the enzyme) (Fig. 3a) and positive marking (Fig. 3b) for CO histochemistry.

In order not to mask the results obtained by CO marking, we did not perform any counter-staining; because of this, the cerebral structures appeared very clear. Therefore, we used a histological section stained with HE to show the brain structures analyzed and the precise location of the markings obtained. The histological section shown in Fig. 4a gives an overall view of the brain of *A. mellifera* and shows the location of mushroom bodies and antennal lobes, i.e., the cerebral structures analyzed in this study. In Fig. 4b, it is possible to observe the Kenyon cell subtypes that compose the mushroom bodies: the inner compact, noncompact, and outer compact cells. This figure also shows the mushroom body calyx, which contains axons and dentrits of neurons (neuropil area). The other brain structures analyzed in this study, i.e., the antennal lobes formed by glomeruli, are shown in Fig. 4c.

The analysis of CO histochemistry in mushroom bodies of newly emerged bees showed Kenyon cells weakly marked by the reaction of CO activity in the mushroom bodies, depicting the basal activity of this enzyme, in all groups studied. Therefore, for all of the groups (control with acetone, control without acetone, continuous exposure to fipronil, and recovery treatment), we did not detect increased CO activity in Kenyon cells. This shows that the neural activity of bees at this age is not affected by the presence of the insecticide.

After exposure of aged honeybees to fipronil for 3 days, brains of bees in the control groups showed Kenyon cells weakly marked by the reaction of CO activity. However, for the group exposed to fipronil (Fig. 4d), Kenyon cells located in the interior of the calyces of the mushroom bodies were strongly stained, evidencing alterations in neural activity. Figure 4d shows that marked cells are seen mainly in the central region of the mushroom bodies. Considering their morphology and localization, these cells must belong to the Kenyon cell class because they are subtypes of the inner compact cells. On day 5, bees in the control groups showed weakly marked Kenyon cells. However, in the group exposed to fipronil and the group subjected to recovery treatment, there was increased detection of CO activity in cells. It is possible to observe cells that are strongly stained by CO reaction, and regarding localization and morphology, that are part of the subtype of inner compact Kenyon cells.

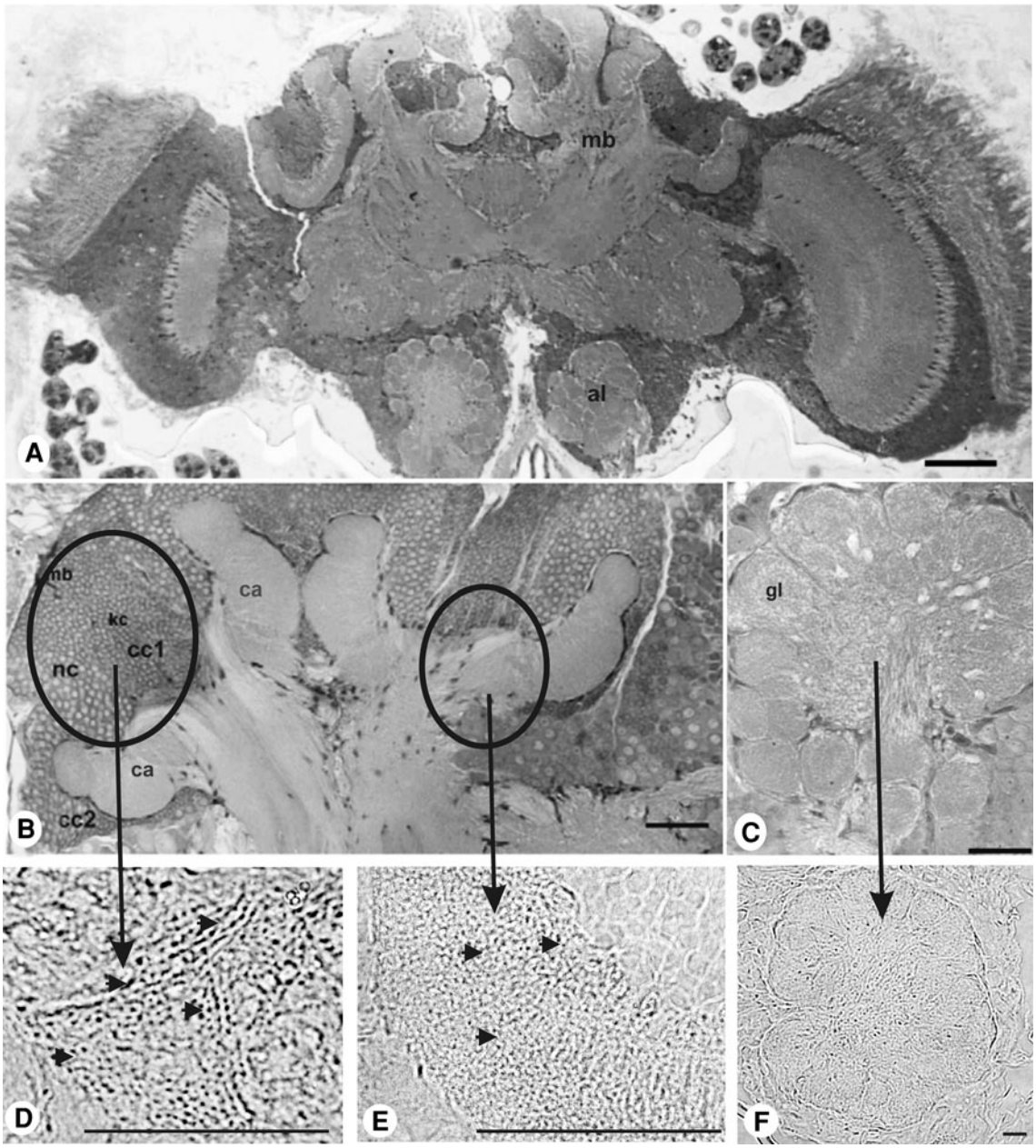

Fig. 4 Photomicrography of a brain of *A. mellifera* stained with HE (**a**–**C**) and subjected to a histochemical technique for CO detection after exposure to fipronil (**d**–**f**). **a** General view showing the localization of the structures analyzed: mushroom bodies (mb) and antennal lobes (al). **b** Detail of the mushroom bodies showing that Kenyon cells (kc) can be divided into three subtypes: inner compact (cc1; highlighted *circles*), noncompact (nc), and outer compact (cc2). Note the calyx (ca; a neuropil region also highlighted with a *circle*). **c** Detail of the antennal lobes showing the glomeruli (gl). **d** Aged honeybees treated for 3 days with fipronil. Note that positively marked cells (*arrowheads*) are present in the central region of mushroom bodies. **e** Detail of mushroom bodies of aged honeybees from the recovery treatment in which exposure to fipronil was ended on day 3 and samples collected on day 5 showing positive markings in neuropil regions (*arrowheads*). **f** Antennal lobes of aged honeybees treated for 5 days with fipronil. Note weakly stained neurons, probably reflecting basal expression of the enzyme. Scale = 100 μm (D = 200 μm)

Mushroom bodies neuropil areas (Fig. 4f) of newly emerged and aged honeybees were positively marked for CO histochemistry.

In all bees evaluated in these experiments, the antennal lobes of newly emerged and aged honeybees subjected to the detection of CO presented weakly stained neurons, probably reflecting the basal expression of this enzyme. Thus, even with continuous exposure to fipronil and after the recovery period, there were no alterations in neural activity of the antennal lobes. We observed similar patterns

of CO activity, without increase, in both newly emerged and aged honeybees (Fig. 4e).

Densitometry Analysis of CO Histochemistry

The results obtained for densitometry analysis confirm the results described for histochemical analysis. Newly emerged honeybees from the control groups, the fipronil-treated groups (Fig. 5), and the recovery-treatment groups (Fig. 6) showed Kenyon cells and neuropil region with basal activity of CO; there were no significant differences among the groups.

CO densitometry analysis of mushroom bodies of aged honeybees revealed significantly increased densitometry in mushroom bodies of honeybees treated with fipronil for 3 and 5 days (Fig. 5) as well as bees subjected to the recovery treatment (Fig. 6). The results show increased CO activity in these groups as evidenced by histochemical analysis.

A summary of densitometry analysis on antennal lobes of newly emerged and aged honeybees treated with fipronil, as well as the groups subjected to the recovery treatment, are shown in Figs. 7 and 8, respectively. The results did not show significant differences of CO activity in antennal lobes among individuals and treatments.

Discussion

The purpose of this study was to assess the toxic effect of fipronil against honeybees as well as the side effect of a sublethal dose fipronil on neuron metabolic activity. Initially, our data obtained for LD_{50} and LC_{50} values of fipronil of newly emerged Africanized honeybees showed that this insecticide is harmful independently of exposure route, presenting toxicity values that are very similar ($LD_{50} = 1.06$ and $LC_{50} = 1.27$ ng fipronil/bee, respectively). However, when compared with the data in the literature, differences can be found. For example, Mayer and Lunden (1999), assessing the toxicity of the same insecticide by way of topical application, showed that fipronil had a LD_{50} value of 13 ng/*A. mellifera* (European subspecies). For this same subspecies, the LD_{50} values can range between 4 (Tingle et al. 2003) and 6.2 ng/bee (Decourtye et al. 2002), showing that fipronil is indeed harmful to bees. Intermediary values were obtained for *Nomia melanderi* Cockerell 1906 (Hymenoptera: Halictidae) ($LD_{50} =$ 1130 ng/bee) and *Megachile rotundata* Fabricius 1787 (Hymenoptera: Megachilidae) ($LD_{50} = 4$ ng/bee).

Various factors may be used to explain these differences. One source of variation is related to the subspecies of honeybee used in the experiments. In Brazil, the Africanized honeybee originated from a cross between the European species *A. mellifera mellifera* L. 1758 and the African species *A. mellifera scutellata* Lepeletier 1836. In contrast, all studies developed around the world are performed using European subspecies, such as black and Italian honeybees, thus highlighting the importance of toxicity studies using Africanized honeybee, which show a particular behavior in toxicity assessments (Danka et al. 1986).

The effects of fipronil in honeybees have mainly been studied in terms of biological, physiological, and behavioral aspects. Using these methods, several studies have shown the effects of insecticides on proboscis extension response (Decourtye et al. 2005, El Hassani et al. 2005, Bernadou et al. 2009), orientation in complex mazes (Decourtye et al. 2008), survival (Aliouane et al. 2009),

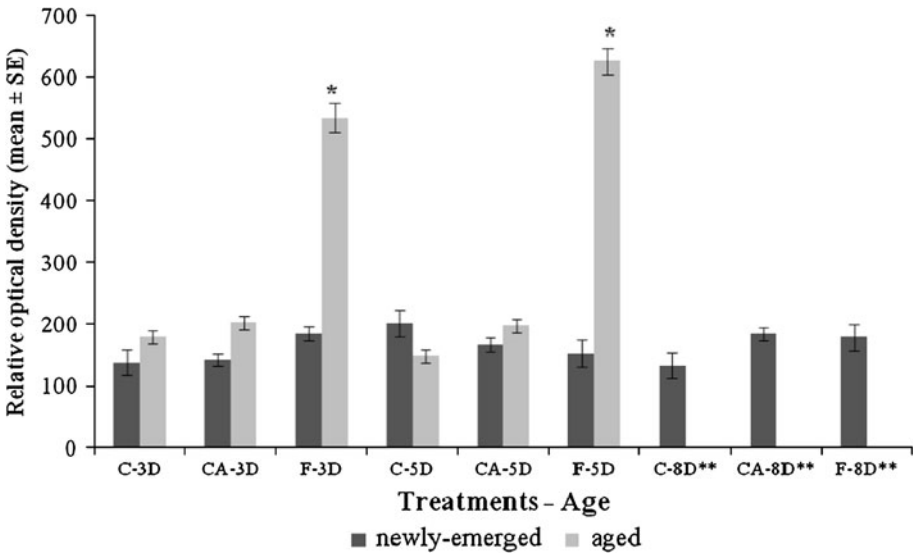

Fig. 5 Effect of fipronil on CO histochemistry in Kenyon cells of mushroom bodies of newly emerged and aged honeybees exposed or not to fipronil. Optical density is expressed as *grey* level. Each value represents the mean ± SE of 29–38 sections from eight bees each from the control and treated groups. *Differ statistically by Fisher's test ($p = 2e^{-16}$). **Not performed for aged honeybees

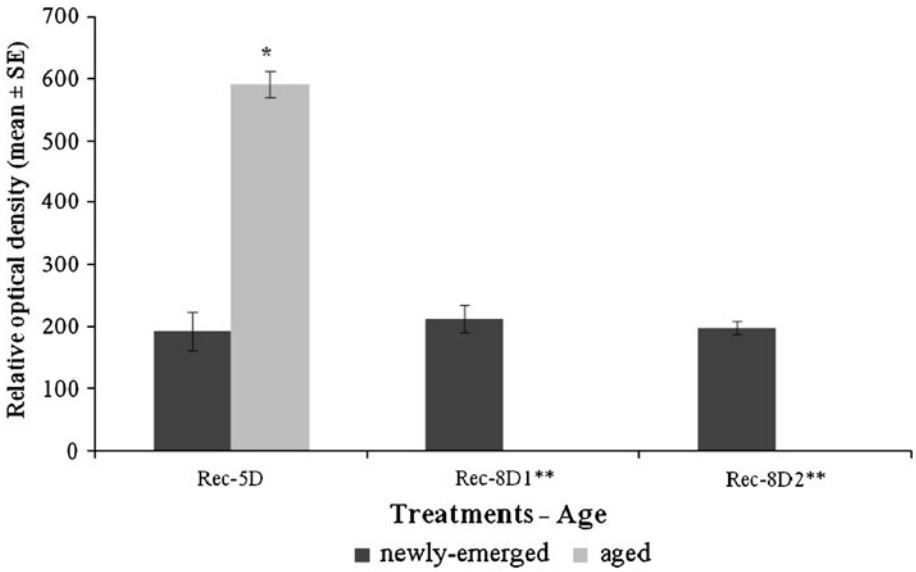

Fig. 6 Effect of fipronil on CO histochemistry in Kenyon cells of mushroom bodies of newly emerged and aged honeybees subject to the recovery treatment. Optical density is expressed as *grey* level. Each value represents the mean ± SE of 29–38 sections from eight bees each from the control and treated groups. *Differ statistically by Fisher's test ($p = 2e^{-16}$). **Not performed for aged honeybees

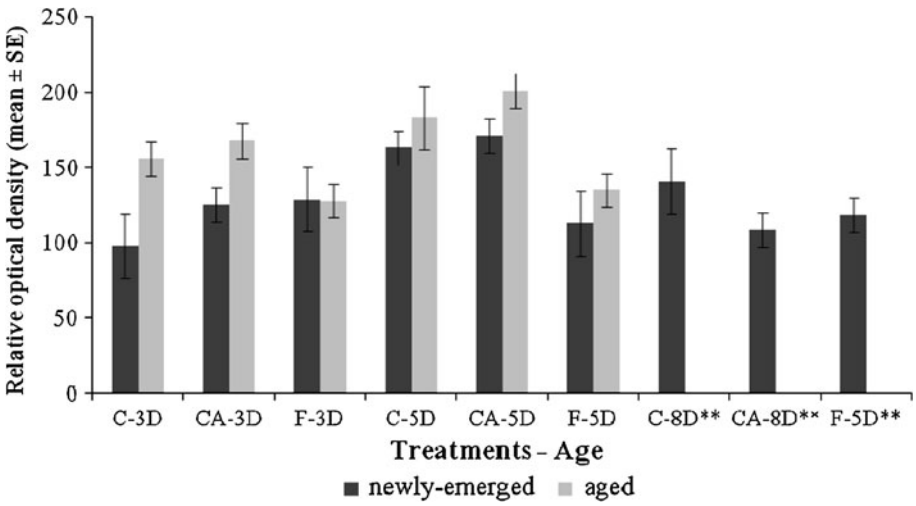

Fig. 7 Effect of fipronil on CO histochemistry in antennal lobes of newly emerged and aged honeybees exposed or not to fipronil. Optical density is expressed as *grey* level. Each value represents the mean ± SE of 29–38 sections from eight bees each from the control and treated groups. **Not performed for aged honeybees

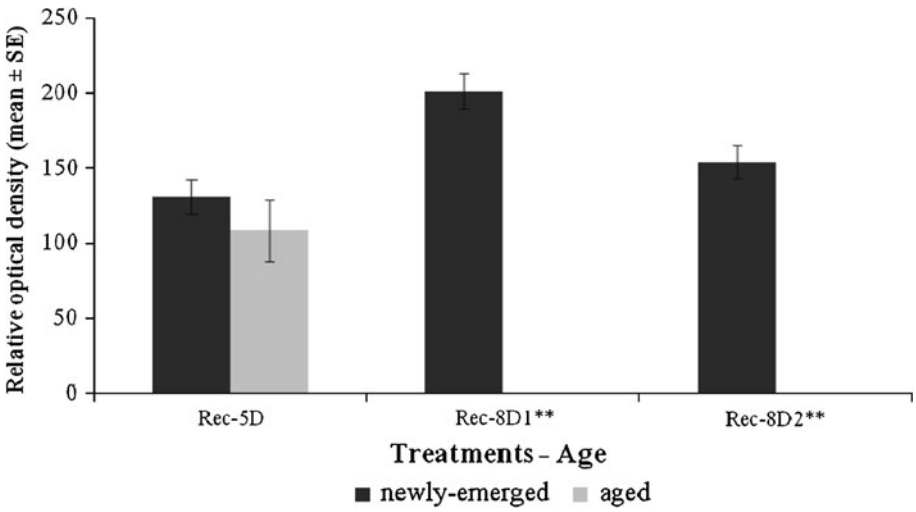

Fig. 8 Effect of fipronil on CO histochemistry in antennal lobes of newly emerged and aged honeybees subject to the recovery treatment. Optical density is expressed as *grey* level. Each value represents the mean ± SE of 29–38 sections from eight bees each from the control and treated groups. **Not performed for aged honeybees

and the use of enzymes as biomarkers (Badiou-Bénéteau et al. 2012). Histological studies focusing on the toxicity of compounds to bees are more rare. Cruz et al. (2010) showed that this phenylpirazole insecticide can affect midgut from honeybee larvae, thus inducing cellular alterations in both cytoplasm and nucleus of digestive cells and accelerating the appearance of degenerative features in midgut epithelium, which are usually present only from the beginning of the pupal phase.

The present study analyzed the effects of a sublethal dose of fipronil on neuron metabolic activity of *A. mellifera* using histochemical technique for the detection of CO activity. The use of CO-activity detection is a valid marker of neuronal metabolic activity because this enzyme is part of complex IV of the mitochondrial respiratory chain that acts in the terminal phase of the electron transport chain in the mitochondrial respiration process to generate the energy required for neural activity. This enzyme catalyzes the last reaction of oxidative metabolism, the main means of energy production in the brain. The distribution of CO activity can be visualized in tissue sections and is an indicator of tissue metabolic activity (Hevner and Wong-Riley 1989).

In histological sections, *A. mellifera* brain presents well-defined areas characterized by neuropil regions that contain dendrites and axons of neurons and cell body regions. Because CO is a marker of mitochondrial activity, it is expected to find positive marking for this enzyme in neuropil and cell body regions. In our study, positive marking for CO was subtle: It was seen as small dots in the cell. This was due the extremely small size of *A. mellifera* neurons. According to Giurfa (2003), the brain of honeybee contains approximately 960,000 neurons and is 1 mm^3 in size.

The results obtained here using CO-activity detection showed that in mushroom bodies, Kenyon cells are weakly marked and neuropil regions positively marked. However, aged honeybees showed increased neural activity in response to exposure to a sublethal dose of fipronil. Thus, this group of honeybees presented increased metabolism of Kenyon cells after 3 days of exposure, and this increase persisted until day 5 of exposure. Similar results were also found by Decourtye et al. (2002) for imidacloprid: Histochemical detection of CO showed increased activity of this enzyme in mushroom bodies of *A. mellifera*. Armengaud et al. (2000) evaluated short-term effects of cholinergic binding by insecticides on the metabolism of different cerebral structures, focusing their investigation on antennal lobe regions and mushroom bodies. These investigators verified that imidacloprid activated cellular metabolism in mushroom bodies and, albeit less intensely, in antennal lobes. Similar results were obtained in this work, which showed that a sublethal dose of fipronil administered to either newly emerged or aged honeybees did not cause increased metabolic activity of antennal lobes.

Interestingly, the exposure of honeybees to a sublethal dose of fipronil mainly altered the metabolism of specific subtypes of Kenyon cells present in mushroom bodies. These structures of the brain contain Kenyon cells, or interneurons, that can be divided in three groups according to their localization and size of their cellular bodies: (1) inner compact cells with interneurons (small cellular bodies found in the center of the calyx); (2) noncompact cells with interneurons in larger cellular bodies that fill the rest of the calyx; and (3) outer compact cells with interneurons and small cellular bodies located in the exterior of the calyx (Zars et al. 2000; Kiya et al. 2007). In honeybees exposed to fipronil, CO-activity detection occurred preferentially in cells belonging to subtypes of inner compact cells.

The existence of subpopulations with distinct characteristics shows that Kenyon cells do not form a homogenous group of neurons. According to Farris et al. (1999) and Strausfeld et al. (2000), the subtypes differ with respect to dendrites, morphology of the axons, content of neuropeptides, and other aspects of gene expression. Several studies of immunolocalization have showed that there is variation in protein expression of these cells. For example, in situ hybridization showed that the gene for a putative inositol 1,4,5-trisphosphate receptor was expressed strongly in large-type Kenyon cells and weakly in small-type Kenyon cells of mushroom bodies (Kamikouchi et al 1998) and that cAMP-dependent protein kinase type II is more strongly expressed in noncompact Kenyon cells (Müller 1997). In contrast, a protein that is a component of royal jelly is expressed by compact cells (Kucharski et al. 1998). Other patterns of gene expression differ between the cells, e.g., due to the activity performed by worker bees (Sawata et al. 1998). Kiya et al. (2007), using a novel immediate early gene, *kakusei*, as a marker of neural activity, showed that compact and noncompact Kenyon cells have increased activity in brains of dancer and forager honeybees; however, only compact Kenyon cells have greater activity in brains of reorienting worker bees, which memorize their hive location during reorienting flights. These findings demonstrate that the preferential activity of small-type (or compact) Kenyon cells is associated with foraging behavior, suggesting its involvement in information integration during foraging flight, which is an essential basis for dance communication. This can be the cause of the increased of neural activity in response to exposure to a sublethal dose of fipronil only in aged bees but not in newly emerged bees.

Vidau et al. (2011) showed that fipronil induces rapid adenosine triphosphate depletion with concomitant activation of anaerobic glycolysis in human neuronal cells.

This cellular response is characteristic of mitochondrial injury associated with a defect of the respiration process. As observed in this study, the increased CO activity induced by fipronil in aged honeybees can be due an increase of cellular respiration as reflected by increased CO enzymatic activity of the mitochondria just before the activation of anaerobic glycolysis. Considering these facts, it can be inferred that fipronil, being toxic and acting on GABA receptors, interferes with the metabolism of Kenyon cells, primarily the compact cell subtype, to modulate their physiology and lead to alterations in their responses.

In addition to investigating the effects of sublethal doses of fipronil on neural activity, the present study performed recovery experiments showing that discontinued exposure to a diet containing a sublethal dose of fipronil did not lead to recovery of neural activity in aged honeybees. Although Smirle and Winston (1988) reported that forager bees present more active enzymes linked to detoxification mechanisms compared with newly emerged bees, the results of the present study show that the neuron metabolic activity in brain of 20-day-old worker bees exposed to a sublethal dose of fipronil is greater than that in newly emerged bees exposed to this same dose of fipronil. In addition, this increased CO activity in older bees did not decrease in the recovery treatment, indicating the persistent effect of fipronil in mushroom bodies. These results demonstrate that exposure to this phenylpyrazole insecticide seriously affects neural activity of bees and might compromise their activities in the colony.

In conclusion, independent of exposure route (topical or ingestion), we verified that fipronil is toxic to Africanized honeybees. The hazard of fipronil's effect on neural activity was shown when honeybees were exposed to a sublethal dose ($^1/_{100}$ of LD_{50}) of fipronil, indicating that even at very low concentrations, this insecticide is harmful to honeybees. Therefore, intoxication by this insecticide can induce several types of injuries to honeybee physiology (e.g., disruption of visual and olfactory capability), thus leading to abnormal behavior and possibly death. Thus, it is suitable to create and suggest mitigatory policies to protect honeybees in the agricultural environment and to minimize the impact of pesticides.

Acknowledgments The authors thank the Fundação de Amparo a Pesquisa do Estado de São Paulo/FAPESP for financial support (Grant No. 2008/05018-7) as well as the Conselho Nacional de Desenvolvimento Científico e Tecnológico/CNPq.

References

Agin V, Chichery R, Chichery M (2001) Effects of learning on cytochrome oxidase activity in cuttlefish brain. NeuroReport 12(1):113–116

Aliouane Y, El Hassani AK, Gary V, Armengaud C, Lambin M, Gauthier M (2009) Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. Environ Toxicol Chem 28(1):113–122

Armengaud C, Causse N, Aït-Oubah J, Ginolhac A, Gauthier M (2000) Functional cytochrome oxidase histochemistry in the honeybee brain. Brain Res 859(2):390–393

Badiou-Bénéteau A, Carvalho SM, Brune J-L, Carvalho GA, Buleté A, Giroud B et al (2012) Development of biomarkers of exposure to xenobiotics in the honeybee *Apis mellifera*: application to the systemic insecticide thiamethoxam. Ecotoxicol Environ Saf 82:22–31

Bernadou A, Démares F, Couret-Fauvel T, Sandoz JC, Gauthier M (2009) Effect of fipronil on side specific antennal tactile learning in the honeybee. J Insect Physiol 55:1099–1106

Cruz AS, Silva-Zacarin ECM, Bueno OC, Malaspina O (2010) Morphological alterations induced by boric acid and fipronil in the midgut of worker honeybee (*Apis mellifera*) larvae. Cell Biol Toxicol 26:165–176

Danka RG, Rinderer TE, Hellmich RL II, Collins AM (1986) Comparative toxicities of four topically applied insecticides to Africanized and European honeybees (Hymenoptera: Apidae). J Econ Entomol 79:18–21

Decourtye A, Armengaud C, Renou M, Devillers J, Cluzeau S, Gauthier M et al (2002) Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). Pestic Biochem Physiol 78:83–92

Decourtye A, Devillers J, Genecque E, Le Menach K, Budzinski H, Cluzeau S et al (2005) Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. Arch Environ Contam Toxicol 48:242–250

Decourtye A, Lefort S, Devillers J, Gauthier M, Aupinel P, Tisseur M (2008) Sublethal effects of fipronil on the ability of honeybee (*Apis mellifera*) to orientate in a complex maze. In: Oomen PA, Thompson HM (eds) Hazard of pesticides to bees. 10th International Symposium of the ICP-BR Bee Protection Group. Julius-Kühn-Archiv, Bucharest, pp 75–83

Déglise P, Dacher M, Dion E, Gauthier M, Armengaud C (2003) Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee. Behav Neurosci 117: 540–547

El Hassani AK, Dacher M, Gauthier M, Armengaud C (2005) Effects of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*). Pharmacol Biochem Behav 82:30–39

Farris SM, Robinson GE, Davis RL, Fahrbach SE (1999) Larval and pupal development of the mushroom bodies in the honeybee, *Apis mellifera*. J Comp Neurol 4(14):97–113

Giurfa M (2003) The amazing mini-brain: lessons from a honeybee. Bee World 84:5–18

Hevner RF, Wong-Riley MTT (1989) Brain cytochrome oxidase: purification, antibody production, and immunohistochemical/histochemical correlations in the CNS. J Neurosci 9(11):3884–3898

Kamikouchi A, Takeuchi H, Sawata M, Ohashi K, Natori S, Kubo T (1998) Preferential expression of the gene for a putative inositol 1,4,5-triphosphate receptor homologue in the mushroom bodies of the brain of the worker honeybee *Apis mellifera* L. Biochem Biophys Res Commun 242:181–186

Kiya T, Kunieda T, Kubo T (2007) Increased neural activity of a mushroom body neuron subtype in the brains of forager honeybees. PLoS ONE 4:370–371

Klein AM, Vaissière BE, Cane JH, Dewenter IS, Cunningham SA, Kremen C et al (2007) Importance of crop pollinators in changing landscape for world crops. Proc R Soc Lond B Biol Sci 274:303–313

Kremen C, Williams NM, Aizen MA, Gemmill-Herren B, Lebuhn G, Minckley R et al (2007) Pollination and other ecosystem services

produced by mobile organism: a conceptual framework for the effects of land-use change. Ecol Lett 10:299–314

Kucharski R, Hayward D, Ball EE, Maleska R (1998) A royal jelly protein is expressed in a subset of Kenyon cells in the mushroom bodies of the honeybee brain. Naturwissenschaften 85:343–346

Mayer DF, Lunden JD (1999) Field and laboratory tests of the effects of fipronil on adult female of *Apis mellifera*, *Megachile rotundata* and *Nomia melanderi*. J Apic Res 38:191–197

Müller U (1997) Neuronal cAMP-dependent protein kinase type II is concentrated in mushroom bodies of *Drosophila melanogaster* and the honeybee *Apis mellifera*. J Neurobiol 33:3–44

Narahashi T, Zhao X, Ikeda T, Nagata K, Yeh JZ (2007) Differential actions of insecticides on target sites: basis for selective toxicity. Hum Exp Toxicol 26(4):361–366

Neumann P, Carreck NL (2010) Honey bee colony losses. J Apic Res 49(1):1–6

R Development Core Team (2012) R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing 2012. Available at: http://www.R-project.org. Accessed 20 Jan 2012

Ritz C, Streibig JC (2005) Bioassay analysis using R. J Stat Softw 12(5):1–22

Sawata M, Takeuchi H, Kamichouchi A, Ohashi K, Natori S, Kubo T (1998) Identification and characterization of a gene expressed specifically in the small type Kenyon cells of the brain of the worker honeybee (*Apis mellifera* L.). Proceedings of the XIII International Congress of the IUSSI, Adelaide, Australia, p 418

Schäfer S, Bicker G (1986) Distribution of GABA-like imunoreactivity in the brain of the honeybee. J Comp Neurol 246:287–300

Smirle MJ, Winston ML (1988) Detoxifying enzyme activity in worker honeybees: an adaptation for foraging in contaminated ecosystems. Can J Zool 66(9):1938–1942

Spadotto CA, Gomes MAF, Luchini LC, Andréa MM (2004) Monitoramento de risco ambiental de agrotóxicos: princípios e recomendações. Embrapa, São Paulo

Stokstad E (2012) Field research on bees raises concern about low-dose pesticides. Science 335:1555

Strausfeld NJ, Homburg U, Kloppenberg P (2000) Parallel organization in honeybee mushroom bodies by peptidergic Kenyon cells. J Comp Neurol 424(1):179–195

Tingle CC, Rother JA, Dewhust CF, Lauer S, King WJ (2003) Fipronil: environmental fate, ecotoxicology, and human health concerns. Rev Environ Contam Toxicol 176:1–66

Vidau C, González-Polo RA, Niso-Santano M, Gomez-Sanchez R, Bravo-San Pedro JM, Pizarro-Estrella E et al (2011) Fipronil is a powerful uncoupler of oxidative phosphorylation that triggers apoptosis in human neuronal cell line SHSY5Y. Neurotoxicology 32(6):935–943

Wong-Riley M (1979) Changes in the visual system of monocularly sutured or enucleated cats demonstrable with cytochrome oxidase histochemistry. Brain Res 171:11–28

Zars T, Fischer M, Schultz R, Heisemberg M (2000) Localization of the short-term memory in *Drosophila*. Science 228:672–675