Side-Effects of Thiamethoxam on the Brain and Midgut of the Africanized Honeybee *Apis mellifera* (Hymenopptera: Apidae)

Regiane Alves Oliveira, Thaisa Cristina Roat, Stephan Malfitano Carvalho, Osmar Malaspina

Centro de Estudos de Insetos Sociais, Departamento de Biologia, Instituto de Biociências de Rio Claro, UNESP-Univ. Estadual Paulista, Av. 24A, 1515, Bela Vista, 13.500-900 Rio Claro São Paulo, Brazil

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ABSTRACT: The development of agricultural activities coincides with the increased use of pesticides to control pests, which can also be harmful to nontarget insects such as bees. Thus, the goal of this work was assess the toxic effects of thiamethoxam on newly emerged worker bees of *Apis mellifera* (africanized honeybee—AHB). Initially, we determined that the lethal concentration 50 (LC_{50}) of thiamethoxam was 4.28 ng a.i./μL of diet. To determine the lethal time 50 (LT_{50}), a survival assay was conducted using diets containing sublethal doses of thiamethoxam equal to 1/10 and 1/100 of the $LC_{50.}$ The group of bees exposed to 1/10 of the LC_{50} had a 41.2% reduction of lifespan. When AHB samples were analyzed by morphological technique we found the presence of condensed cells in the mushroom bodies and optical lobes in exposed honeybees. Through Xylidine Ponceau technique, we found cells which stained more intensely in groups exposed to thiamethoxam. The digestive and regenerative cells of the midgut from exposed bees also showed morphological and histochemical alterations, like cytoplasm vacuolization, increased apocrine secretion and increased cell elimination. Thus, intoxication with a sublethal doses of thiamethoxam can cause impairment in the brain and midgut of AHB and contribute to the honeybee lifespan reduction. Environ Toxicol 00: 000–000, 2013.

Keywords: *Apis mellifera*; neonicotinoid; toxicity; morphology; histochemistry; mushroom bodies; optical lobes; digestive cells; regenerative cell

INTRODUCTION

Honeybees *Apis mellifera* L., 1758 (Hymenoptera: Apidae) are insects that provide high value products, such as honey, propolis, royal jelly, beeswax, and venom. Additionally, its represents 85% of all insect pollinators, upon which 90% of fruiting plants are dependent for reproduction (Tautz, 2008). In Brazil, there is predominantly the hybrid originated from the natural crossing between the European species *A. mellifera mellifera* L., 1758 and the African specie *A. mellifera scutellata* Lepeletier, 1836. This honeybee which is called Africanized honeybee (AHB), has well adapted on the environmental conditions of Brazil and is considered as important pollinator of several economic crops (Crane, 2000).

Several cases of decreasing numbers of honeybee colonies have been reported in the northern hemisphere, (Neumann and Carreck, 2010). This phenomenon, termed colony collapse disorder (CCD), has recently occurred in the USA (Stokstad, 2007) and its cause has not fully elucidated. CCD is not only caused by a single factor but also by a set of factors that can occur simultaneously and influence each other

Correspondence to: T. C. Roat; e-mail: thaisaroat@yahoo.com.br

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up. Diseases, parasites, predators and even the insecticides may contribute to a weakening of the colony and cause severe damage (Oldroyd, 2007; vanEngelsdorp et al., 2009).

There have been profound changes to the current model of agriculture that can affect pollinators. For example, we have observed an increase in cultivated areas for monoculture as well as high use of fertilizers and pesticides that can contribute to cause the decline of honeybees.

Among the various pesticides employed in agriculture, special attention is given to those belonging to the neonicotinoid class, particularly thiamethoxam. This insecticide is a second generation neonicotinoid showing low toxicity to mammals with exceptional translaminar and systemic action. Thiamethoxam is used to control aphids, whiteflies, leafhoppers, trips, some beetles and caterpillars. Furthermore, thiamethoxam is becoming one of the most widely used insecticides in various agroecosystems in Brazil due to its successful application through various methods (terrestrial and aerial spraying and soil and seed treatment), its control efficiency and its moderate residual effects (Maienfisch et al., 2001a; Andrei, 2009; Girolami et al., 2009).

AHBs are often found in agroecosystems where thiamethoxam is used, where they are also affected by its toxic effects through either direct (death of the bee) or indirect damage (sublethal effects) (Devillers, 2002; Desneux et al., 2007). Several studies on laboratorial condition confirm that neonicotinoids are harmful to honeybees. For example, Maienfisch et al. (2001b) and Iwasa et al. (2004) found that for *A. mellifera*, thiamethoxam has a toxicity up to 192 times greater than the neonicotinoids acetamiprid and thiacloprid. In another study, Carvalho et al. (2009) found that a thiamethoxam concentration of 37.5 g active ingredient (a.i.)/100 L H_2O (maximum concentration for citrus crops, Brazil), applied through different routes (spraying, ingestion and residue on the crop surface), is extremely toxic to the AHB; on average, it kills 50% of the bees within 214 min. Studies have also suggested that neonicotinoids, such as thiamethoxam, might affect the homing flight of the honeybee (Decourtye and Devillers, 2010), as shown for imidacloprid by Blacquière et al. (2012).

Neonicotinoids are substances that act directly on the insect nervous system through agonistic action on nicotinic acetylcholine receptors (nAChR) (Tomizawa and Casida, 2003; Tan et al., 2007). In the honey bee brain and ganglia, nAChRs are widely distributed and involved in pathways controlling a variety of physiological functions (El Hassani et al., 2008). Thus, it is important to check the toxicity of this insecticide on the nervous system, and more specifically in the brain, of exposed AHBs.

Secondary targets may also be affected by xenobiotics. Thiamethoxam exhibits systemic action in plants, and honeybees may become affected through ingestion of contaminated pollen and nectar. It is therefore important to analyze the cytotoxicity of thiamethoxam in tissues reached via the metabolism of contaminated food containing this compound. For example, recently Badiou-Bénéteau et al. (2012) reported a battery of biomarkers in honeybees that may be modulated after intoxication with thiamethoxam. Furthermore, it is necessary to analyze the midgut, as it is an absorption organ and therefore the source of first contact with an orally administered insecticide. The purpose of this work was to analyze the toxic effect of thiamethoxam on newly emerged honeybees and to examine the cytotoxic effect on the brain and midgut through morphological and histochemical techniques.

MATERIALS AND METHODS

Chemicals

The analytical standard thiamethoxam (92.5% of purity) was obtained from Syngenta Crop Protection (Brazil). Sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4), paraformaldehyde, formaldehyde, glacial acetic acid, picric acid, ethanol, hematoxylin, eosin, xylidine ponceau, periodic-acid, Schiff's reagent, and hydrochloride acid (HCl) were obtain from Sigma Aldrich (Brazil). The historesin embedding kit was purchased from Leica Microsystems (Germany).

Honeybee Collection

To obtain the newly emerged AHB, three frames with sealed broods (near of adult emergence) were collected on a queen-right colony and kept in a controlled climate room (34 ± 2°C, relative humidity (RH) of 80 ± 10% and in darkness). Using this procedure, we obtained specimens of a known age (0 to 24 h). For all experiments, adults were put into disposable cages (11 × 11 × 7 cm^3), fed a sucrose + H_2O solution (1:1), and maintained at 32 ± 2°C with a 70 ± 10% of RH in darkness.

Acute Toxicity Test by Ingestion of Thiamethoxam

A thiamethoxam stock solution (1000 ng a.i./μL) was prepared using acetone as a solvent. More 10 concentrations ranging from 1000 to 0.01 ng a.i./μL were prepared directly in the diet constituted of sucrose + H_2O (1:1, w/v), with a maximum acetone concentration less than 0.1%. A total of 75 newly emerged honeybees from each treatment were divided into three replicates/cages of 25 honeybees, previously starved for 2 h. Each treatment received an average of 10 μL diet/bee packaged in a plastic container at the cage bottom as well as a cotton swab soaked in distilled water (CEB, 2003). In these experiments, two controls were used: control without solvent (C−), providing only food sucrose + H_2O (1:1), and solvent control (C+), which added acetone to the diet

sucrose + H_2O (1:1); they were provided in the same ratios as used in the experimental groups treated with thiamethoxam. At 24 h, the number of dead bees for each treatment was recorded and subjected to dose–response analysis to determine the lethal concentration (LC_{50}).

Honeybee Survival After Exposure to Thiamethoxam

The honeybee toxicity assay using a sublethal dose of thiamethoxam was performed with the LC_{50} dose (see ''Acute toxicity test by ingestion of thiamethoxam'' section). From the stock solution (1000 ng a.i./μL acetone), we prepared diets with thiamethoxam final solutions of 1/10 and 1/100 of LC_{50}. A total of 75 newly emerged honeybees were equally divided into three disposable cages (250 mL). The bees were collectively fed the contaminated syrup, with the total volume adjusted so that each bee could daily consume 10 μL of sucrose solution containing 0.0428 ng/μL or 0.00428 ng/μL thiamethoxam per bee (i.e., 250 μL of enriched diet per cage). Therefore, each bee ingested 0.428 ng/μL thiamethoxam per day (1/10 of LC_{50}) or 0.0428 ng/μL thiamethoxam per day (1/100 of LC_{50}) (CEB, 2003). Every day, the number of dead bees was counted, and the total volume of syrup was adjusted to the number of remaining live bees. Two experimental controls were used in these experiments: (1) control without acetone, where the bees were fed only with sucrose and H_2O (1:1), and (2) control with acetone, in which the sucrose and H_2O (1:1) contained acetone at the same concentration used when bees were fed thiamethoxam (0.01%).

Bioassays of Intoxication and Body Dissection

The specimens used for morphological studies were obtained in an independent intoxication assay with procedures described previously (''Honeybee survival after exposure to thiamethoxam'' section). Honeybees were collected at intervals of one, three, 5 and 8 days after the start of the bioassay. Five samples were taken per treatment/time.

For the morphological and histochemical studies, AHBs were immobilized in low temperatures (−5°C). Brains and midguts were obtained by dissections through a stereomicroscope, scissors, tweezers, and buffer solution of 20 mM Na_2HPO_4/KH_2PO_4, pH 7.4 + 130 mM of NaCl (modified of Dade, 2009). Posteriorly, the organs were immersed in the specific fixing solutions for morphological analysis (4% paraformaldehyde), for proteins (sodium phosphate buffer 100 mM pH 7.4 + 4% paraformaldehyde) or for polysaccharides and chromatin (aqueous Bouin—75% saturated picric acid solution, 25% of formaldehyde, and 5% of glacial acetic acid).

Procedures to Morphological and Histochemical Analysis

Organs were bathed three times with the same buffer solution used for fixation and then subjected to dehydration. Dehydration was carried out using an ascending ethanol series (from 15 to 95%) lasting 2 h for each bath. After this step, brains and midguts were embedded in resin without catalyst for 3 days and then finally embedded in historesin. Sections measuring 5 μm were cut with a Leica Microtome (Germany). Some posterior sections of brain and midgut fixed in paraformaldehyde were stained with hematoxylin-eosin (HE) for morphological analysis (Junqueira and Junqueira, 1983). Other sections fixed with 100 mM sodium phosphate buffer pH 7.4 + 4% of paraformaldehyde were subjected to histochemical staining with Xylidine-Ponceau to detect total proteins (Junqueira and Junqueira, 1983). The material fixed with aqueous Bouin was processed with the PAS reaction (periodic-acid Schiff stain) to detect acidic and neutral polysaccharides (Mcmanus, 1946) and with the Feulgen reaction to analyze the levels of chromatin compaction (Feulgen and Rossenbeck, 1924). Slides were examined by light microscopy (Olympus BX51—Olympus America Inc), and images were obtained with a digital camera (Olympus DP-71). Image acquisition was conducted with DP Controller software.

Statistical Analysis

Analyses were performed using R® software (2012). Preliminary analyze using the Shapiro–Wilk test (p-value = 0.007106), showed that data were not normally distributed, so the data obtained in acute toxicity test were subjected to dose–response curve analysis with package *drc* taking a binomial distribution (Ritz and Streibig, 2005). The LC_{50}, 95% confidence interval and chi-square values were determined. The survival experimental data were analyzed using the package Survival (Therneau and Lumley, 2012) using LogLogistic distribution to determine the LT_{50} value. The similarities between treatments were compared by cluster analysis using the contrasts between templates (Moncharmont et al., 2003).

RESULTS

Acute toxicity of thiamethoxam and honeybee survival

The LC_{50} of thiamethoxam for newly emerged AHBs was 4.28 ng a.i/μL diet (Fig. 1). Using this value, the sublethal concentrations equivalent to 1/10 and 1/100 of LC_{50} (0.428 and 0.0428 ng a.i./μL diet, respectively) were calculated for use in subsequent experiments.

The survival rate of honeybees was also determined using the sublethal concentration values for thiamethoxam

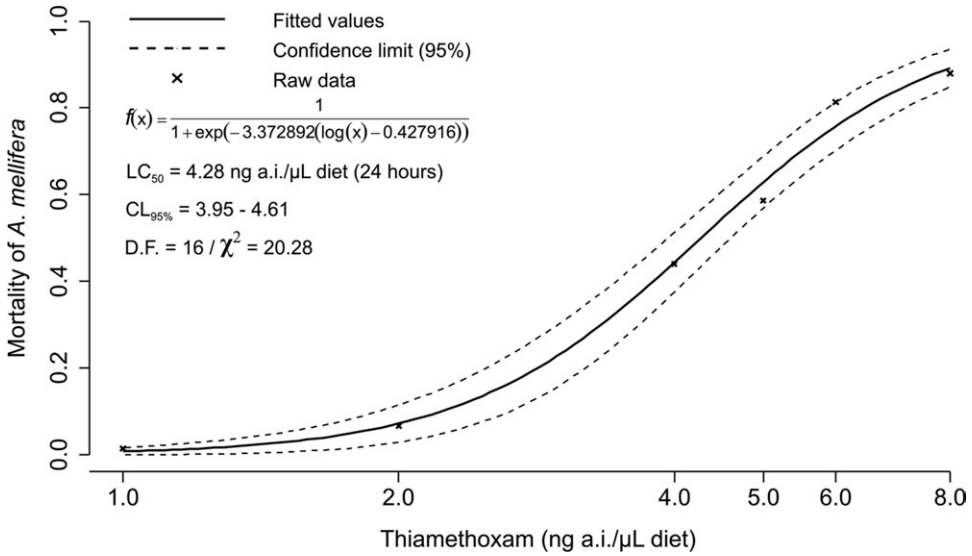

Fig. 1. Mortality of newly emerged workers of Africanized *A. mellifera* according to the ingestion of food contaminated with several doses of thiamethoxam (1–8 ng/μL diet).

(Fig. 2). No significant differences were observed between the groups C−, C+ and LC_{50}/100, all of which resulted in an LT_{50} of 8.04 days. However, the bee group treated with LC_{50}/10 showed a significant decrease in survival (LT_{50} of 5.22 days).

Morphological and Histochemical Assessment of the Brain

The results of the morphological and histochemical analysis in brains of AHBs either exposed to thiamethoxam or not are shown in Figures 3 and 4 and summarized in Table I. Honeybees exposed to thiamethoxam presented morphological and histochemical alterations of the mushroom bodies and optical lobes of their brains; however, alterations of the antennal lobe were not observed (data not shown).

Mushroom Bodies

The Kenyon cells of AHBs exposed to thiamethoxam showed morphological alterations, as observed through HE staining. In the groups of bees exposed to 1/100 or 1/10 of

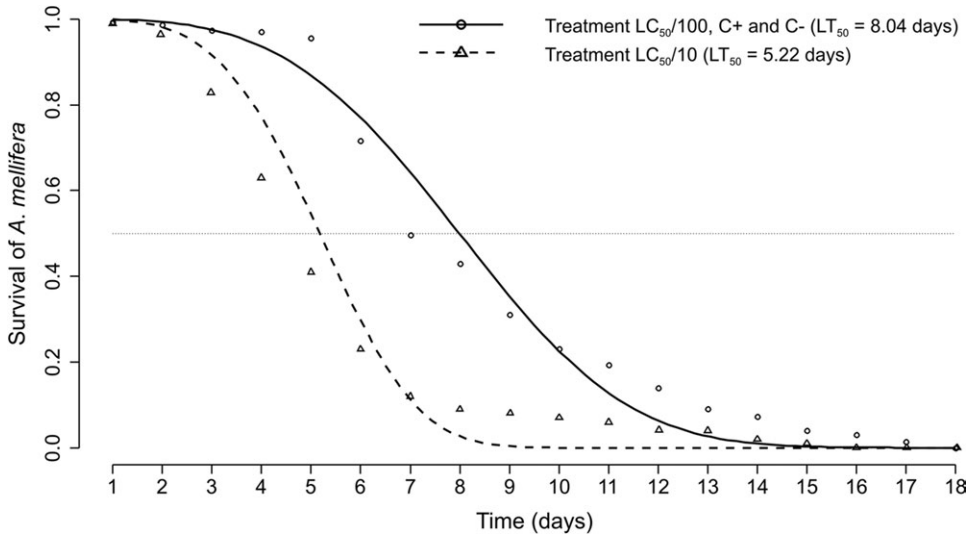

Fig. 2. Survival rate of newly emerged workers of Africanized *A. mellifera* according to the food intake during different treatments: control without solvent (C−); solvent control (C+); 1/10 of LC_{50} of thiamethoxam (0.428 ng i.a./μL diet) and 1/100 of LC_{50} of thiamethoxam (0.0428 ng i.a./μL diet).

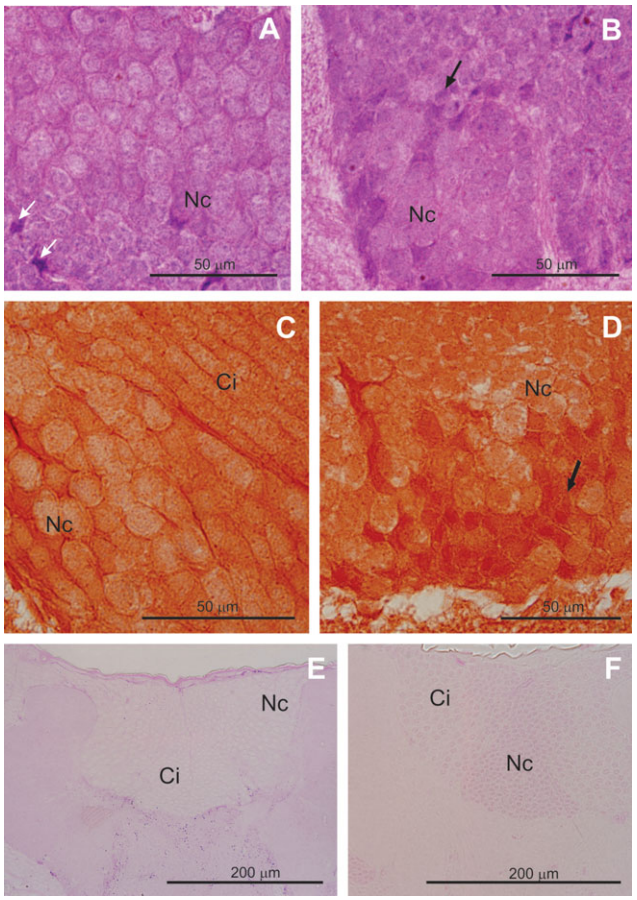

Fig. 3. Images of the mushroom bodies of newly emerged workers of Africanized *A. mellifera*, stained with HE (A and B), subjected to Xylidine-Ponceau staining (C and D), PAS reaction (E), and the Feulgen reaction (F), with or without exposure to thiamethoxam. Note the presence of Glial cells (white arrow). (A) Mushroom body detail from a control honeybee treated without solvent with 1 day, with no observed morphological changes, stained with HE. (B) Detail of the mushroom body of a honeybee exposed to the LC_{50}/100 for 3 days, showing the presence of strongly stained cells (arrow). (C) detail of the mushroom body from a control honeybee without solvent, at 8 days of treatment, showing cells stained uniformly, subject to Xylidine-Ponceau staining. (D) Details of the mushroom body of a honeybee exposed to LC_{50}/10 for 3 days, showing the presence of strongly stained cells (arrow), indicating increased protein synthesis or cell compaction. (E) overview of the mushroom body from a control honeybee without solvent, for 3 days, subjected to PAS reaction. (F) Details of the mushroom body of a honeybee exposed to LC_{50}/100 for 3 days, subjected to the Feulgen reaction. Ci = inner compact Kenyon cells; Nc = non-compact Kenyon cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

LC_{50} on the 3rd and 5th days of the experiments, respectively, the presence of intensely stained cells in the central region of the mushroom bodies' calyx was observed, suggesting the presence of condensed cells [Fig. 3(B) and Table I]. Morphological alterations were not found in AHBs from groups C− and C+ [Fig. 3(A)]. These data show that use of the 1/100 of LC_{50} of thiamethoxam does not present differences in the survival time but that it is cytotoxic for the Kenyon cells of the mushroom bodies.

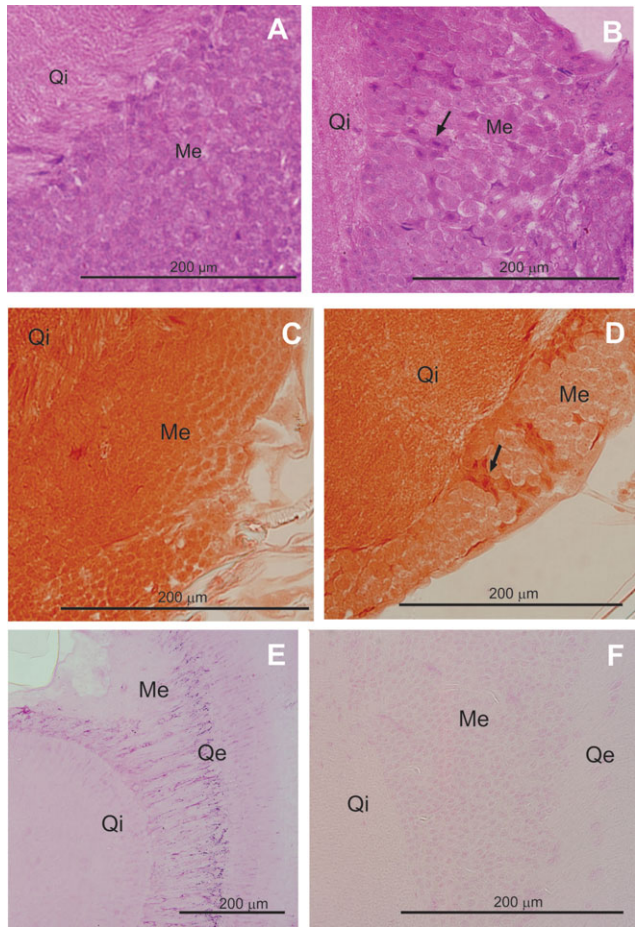

Fig. 4. Images of the optical lobes of newly emerged workers of Africanized *A. mellifera*, stained with HE (A and B), subjected to the Xylidine-Ponceau staining (C and D), the PAS reaction (E), and the Feulgen reaction (F), with or without exposure to thiamethoxam. (A) Details of the optical lobe from a control honeybee after 1 day, with no observable morphological alterations, stained with HE. (B) Details of a honeybee optical lobe exposed to LC_{50}/100 for 3 days. Note condensed cells with more intense staining (arrow). (C) details of an optical lobe from a control honeybee without solvent, after 8 days, showing no alterations, when subjected to the Xylidine-Ponceau staining. (D) details of a honeybee optical lobe exposed to LC_{50}/100 for 1 day, with the presence of cells with intense staining (arrow). (E) Details of a honeybee optical lobe exposed to LC_{50}/10 for 5 days, subject to PAS reaction. (F) Details of a honeybee optical lobe exposed to LC_{50}/100 for 3 days, subjected to the Feulgen reaction. Me = medula; Qe = outer chiasm; Qi = inner chiasm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I. Summary of morphological and histochemical analyses on the brain structures of Africanized honeybees exposed to sublethal doses of thiamethoxam

		Morphological		Histochemical					
				Mushroom Bodies			Optical Lobe		
Treatment		Mushroom Bodies CC	Optical Lobe CC	XYP	PAS	FEU	XYL	PAS	FEU
1 day	C−	−	−	+	+	+	+	+	+
	C+	−	−	+	+	+	+	+	+
	LC_{50}/10	−	−	+++	+	+	++	+	+
	LC_{50}/100	−	+	+	+	+	+++	+	+
3 days	C−	−	−	+	+	+	+	+	+
	C+	−	−	+	+	+	+	+	+
	LC_{50}/10	−	−	+++	+	+	++	+	+
	LC_{50}/100	+	+	+++	+	+	+++	+	+
5 days	C−	−	−	+	+	+	+	+	+
	C+	−	−	+	+	+	+	+	+
	LC_{50}/10	+	−	++	+	+	++	+	+
	LC_{50}/100	+	+	++	+	+	++	+	+
8 days	C−	−	−	+	+	+	+	+	+
	C+	−	−	+	+	+	+	+	+
	LC_{50}/10	+	−	++	+	+	++	+	+
	LC_{50}/100	+	+	+	+	+	++	+	+

(+) Presence of alteration; (++) Alteration moderately present; (+++) Alteration extremely present; (−) alteration absent; (LC_{50}) lethal concentration; (C−) control without acetone; (C+) control with acetone; (CC) condensed cells; (FEU) Fuelgen Reaction; (XYP) Xylidine-Ponceau technique; (PAS) PAS reaction.

Histochemical analyses (Table I) using the Xylidine-Pounceau staining allowed the visualization of Kenyon cells, which stained more intensely in groups exposed to thiamethoxam [Fig. 3(D)] in comparison with control groups (C− and C+) [Fig 3(C)]. For the group exposed to 1/10 of LC_{50}, this alteration was observed in bees as early as the 1st day of exposure, while for the group exposed to 1/100 of LC_{50}, the alteration was observed starting on the 3rd day of exposure. However, on the 5th day of exposure, the cells of bees exposed to either concentration of insecticide showed a decrease in the intensity of staining.

Analysis of mushroom bodies by the PAS reaction indicated the presence of polysaccharide granules, with no observable differences between control groups and those exposed to sublethal doses of thiamethoxam [Fig. 3(E) and Table I]. Similarly, the Fuelgen reaction showed weak cell nucleus staining in all treatments, with no difference between the groups [Fig. 3(F) and Table I].

Optical Lobe

HE staining of the optical lobes showed strongly stained cells, possibly the result of cytoplasmic and nuclear condensation [Fig. 4(B) and Table I], in AHB exposed to 1/100 of LC_{50} since the 1st day of analysis. However, the optical lobe cells of honeybees exposed to 1/10 of LC_{50} did not show morphological alterations [Fig. 4(A)]. This result indicates that the sublethal dose of thiamethoxam LC_{50}/100 is cytotoxic to the optical lobes in addition to the mushroom bodies.

Xylidine-Ponceau staining of optical lobes showed cells with increased staining intensity in honeybees exposed to LC_{50}/10 and in LC_{50}/100 over the entire time course of experimental analysis [Fig. 4(D) and Table I]. This increase in staining may be due to cellular compression and/or increased cellular protein synthesis. No alterations were observed in the control treated cells [Fig. 4(C)].

Using the PAS reaction, polysaccharide granules were observed in all honeybees independent of the treatment [Fig. 4(E) and Table I]. Analysis with the Fuelgen reaction showed a weak staining of the cell nucleus in all groups [Fig. 4(F)], revealing no difference between the controls and groups exposed to thiamethoxam (Table I).

Morphological and Histochemical Assessment on Midgut

Digestive Cells

The morphological analyses of digestive cells in bees exposed to thiamethoxam [Fig. 5(A,B) and Table II] showed cytoplasmic vacuolization, increased apocrine secretion, and increased cell elimination. The presence of a large amount of vacuolization caused by both concentrations of thiamethoxam occurred on the 1st day of analysis, with a tendency to decrease with the course of time. By the

last day of analysis (18th day), there was no evidence of this alteration. The increase of apocrine secretion and cellular elimination was noted on the first day in bees exposed to 1/100 of LC_{50} and by the third day with 1/10 of LC_{50}, with both increasing over the duration of the analysis.

The digestive cells of bees exposed to both concentrations of thiamethoxam presented cytoplasmic regions negative to Xylidine-Ponceau staining since the 1st day of analysis [Fig. 5(C,D) and Table II], indicating cytoplasmic vacuolization. By the 3rd and 5th days, the digestive cells of the exposed groups were similar to those of the control. After 8 days, the cells again showed cytoplasmic regions negative to Xylidine-Ponceau staining. The presence of positive vesicles stained to Xylidine-Ponceau was noted on the 1st day and observed in greater amounts in honeybees exposed to thiamethoxam. These vesicles decreased over time and looked similar to the control on the last (8th) day of analysis. Perichromatin halos were observed in the midgut epithelial cells of honeybees exposed to 1/100 of LC_{50} of thiamethoxam since the 5th day. That finding was not verified in any other treatment.

PAS reaction [Fig. 5(E,F) and Table II] resulted in a negative cytoplasmic staining in all the bee groups on all the analyzed days. PAS reaction positive granules, found mainly in the apical region of the intestinal epithelium and in the lumen of the midgut, were observed in greater amounts in honeybees exposed to either concentration of thiamethoxam when compared with the control groups. The nuclei of digestive cells were weakly stained by the Feulgen reaction, showing no signs of chromatin compaction [Fig. 5(G,H) and Table II].

Regenerative Cells

The regenerative cells of the midgut [Fig. 6(A,B) and Table III] were arranged in nests at the base of the ventricular epithelium. Staining with HE revealed that the cytoplasm was stained, and the nucleus contained uncondensed chromatin with many nucleoli. The regenerative cells of honeybees exposed to 1/10 of LC_{50} showed cytoplasmic vacuolization from the 1st day until the 5th day of the experiments. At a dose of 1/100 of LC_{50}, this vacuolization was only evident from the third day. There was a decrease in the number of regenerative cells in the nests under both treatment

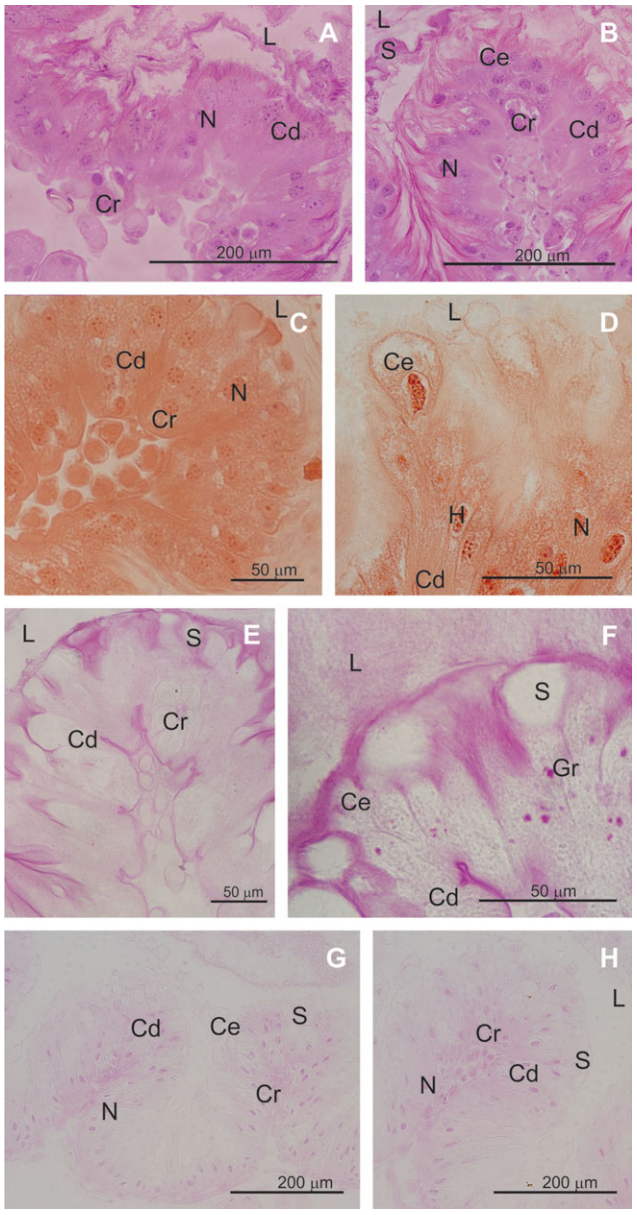

Fig. 5. Images of the midguts from newly emerged workers of Africanized *A. mellifera*, stained with HE (A and B), subjected to Xylidine-Ponceau staining(C and D), the PAS reaction (E and F), and the Feulgen reaction (G and H), with or without exposure to thiamethoxam. (A) Midgut of a honeybee from the control group without solvent, after 1 day, showing the typical morphology of the organ. (B) midgut of a honeybee exposed to LC_{50}/10 for 5 days in which there is an increase of cell elimination (Ce) and the presence of apocrine secretion (S). (C) Midgut of a honeybee from the control group without solvent, after 5 days, subjected to the Xylidine-Ponceau staining, and without changes. (D) Midguts of honeybees exposed to LC_{50}/100 for 5 days showing increased cell elimination (Ce) and the presence of perichromatin halos (H). (E) Midgut of a honeybee from the control group without solvent after 1 day, subject to PAS reaction. (F) Midgut of honeybees exposed to LC_{50}/100 for 8 days. Note positive granules to the PAS reaction (Gr). (G) midgut of a honeybee from the control group without solvent after 1 day, showing a weakly stained nucleus using the Feulgen reaction. (H) Midgut of a honeybee exposed to LC_{50}/100 for 5 days showing, showing nucleus weakly stained by Feulgen reaction. Cd = digestive cell; Cr = regenerative cell; L = lumen; N = nucleus of digestive cell; Ve = vesicle; S = apocrine secretion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II. Summary of morphological and histochemical analyses on the digestive cells of the midgut from Africanized honeybees exposed to sublethal doses of thiamethoxam

					Histochemical						
		Morphological			XYP			PAS			FEU
Treatment		VA	AS	EC	CIT	PV	PH	CIT	PG	PV	CC
1 day	C−	−	−	+	+	−	−	−	+	−	−
	C+	+	−	+	+	−	−	−	+	−	−
	LC_{50}/10	++	−	+	−	++	−	−	++	−	−
	LC_{50}/100	++	+	++	−	++	−	−	+	−	−
3 days	C−	+	−	+	+	+	−	−	+	−	−
	C+	+	+	+	−	+	−	−	+	−	−
	LC_{50}/10	+	+	++	+	++	−	−	+	−	−
	LC_{50}/100	+	+	++	+	++	−	−	++	−	−
5 days	C−	−	+	+	+	+	−	−	−	−	−
	C+	+	+	+	+	+	−	−	−	−	−
	LC_{50}/10	+	+	++	+	++	−	−	+	−	−
	LC_{50}/100	−	++	++	+	+	+	−	+	−	−
8 days	C−	−	−	+	+	+	−	−	+	−	−
	C+	−	−	+	−	+	−	−	−	−	−
	LC_{50}/10	−	++	+++	−	+	−	−	++	−	−
	LC_{50}/100	−	++	+++	−	+	+	−	++	−	−

(+) Presence of alteration; (++) Alteration moderately present; (+++) Alteration extremely present; (−) alteration absent; (LC_{50}) lethal concentration; (C−) control without acetone; (C+) control with acetone; (FEU) Fuelgen Reaction; (XYP) Xylidine-Ponceau technique; (PAS) PAS reaction.; (VA) vacuolization; (AS) apocrine secretion; (EC) eliminated cells; (CIT) cytoplasm; (CC) compacted chromatin; (PH) perichromatin halo; (PG) presence of granules; (PV) presence of vesicles.

conditions on the 1st day for the 1/10 of LC_{50} and on the 3rd day for 1/10 of LC_{50}.

Table III displays the results obtained from histochemical analysis. The regenerative cells of honeybees exposed to thiamethoxam showed negative cytoplasmic regions to Xylidine-Ponceau staining [Fig. 6(D)] for both doses indicating intense cytoplasmic vacuolization. The control groups did not show this alteration [Fig. 6(C)]. PAS positive granules were not observed in the regenerative cells, and all groups studied showed the same characteristics [Fig. 6E,F)]. The nuclei of the regenerative cells stained weakly by the Feulgen reaction [Fig. 6(G,H)] in all treatments.

DISCUSSION

The acute oral toxicity of thiamethoxam (LC_{50} of 4.28 ng i.a./μL diet) shows that this insecticide is highly toxic to newly emerged AHB. According Decourtye and Devillers (2010) report a LC_{50} of thiamethoxam of 5 ng a.i./bee. The high toxicity of neonicotinoids when administered orally was due a low penetration of pesticide through the insect tegument. For example, Badiou-Bénéteau et al. (2012) report a LD_{50} for foragers *A. mellifera mellifera* of 51.16 ng a.i./bee, which was 11.95 fold less toxic than the LC_{50} value found here. Another factor which contributes to the sensitivity of honeybee to thiamethoxam was assessed by Smirle and Winston (1988). Following this work, these researchers found that newly emerged honeybees are more susceptible to environmental pollutants due to the biochemical mechanisms of adaptation and compensation. The main hypothesis for this difference is that the enzymes glutathione S-transferase (GTS) and mixed-function oxidase, which are largely responsible for the metabolism of neonicotinoids in animals (Casida, 2011), have lower activity in newly emerged honeybees. Moreover, Iwasa et al. (2004) compared the toxicity of various neonicotinoid class insecticides and found that those with nitro-substituted compounds, such as imidacloprid, clothianidin, and thiamethoxam, are the most toxic to honeybee species from the northern hemisphere. Regarding the LC_{50} value obtained, it was less than those found in literature.

The survival of AHB is a function of the dose of thiamethoxam given. Although no differences were found among the treatments C−, C+ and 1/100 of LC_{50} (LT_{50} of 8.04 days), the group of bees that was exposed to 1/10 of LC_{50} had a reduction of lifespan of 41.2% (LT_{50} of 5.22 days). In this way is necessary to point out that even sublethal doses can be harmful to honey bee survival in comparison with the high doses. For example, Carvalho et al. (2009) showed that regardless of the mode of exposure, thiamethoxam (37.5 ng a.i./μL) is extremely toxic to AHB, with an average of LT_{50} of 3.57 h. Likewise, Kakamand et al. (2008) observed mortality over of 90% when honeybee were intoxicated orally with thiamethoxam at 0.125 ng a.i./μL.

In this study, both sublethal doses tested caused morphological and histochemical alterations in the brain structures of AHBs. Morphologically, the concentration equivalent to 1/100 of LC_{50} required less time to induce alterations of the mushroom bodies and optical lobes compared with the higher dose (1/10 of LC_{50}). These alterations were characterized by condensed cells observed on the 1st day of exposure to the insecticide, which may be indicative of cell death (Bowen et al, 1998).

An increase in the intensity of Xylidine-Ponceau staining is seen in the cells of treated mushroom bodies, which are important centers of learning and memory (Daly et al, 1998), and in optical lobes, which are responsible for directly processing vision (Ribi et al., 2008). In both structures, the cellular staining was intense at the beginning of the treatment and decreased over time. Xylidine-Ponceau staining detects proteins in tissue, and an increase in staining intensity may indicate an increase in protein synthesis by cells. An increase in protein staining may be due to the expression of heat shock protein (HSP), which is a mechanism of cell protection against an exogenous agent, as observed by Silva-Zacarin et al. (2006), who noted an increase in HSP expression in the salivary glands of *A. mellifera* larvae treated with acaricide. The HSPs are a select group of proteins that are expressed not only after exposure to heat but also when cells are exposed to various types of stress. In these situations, the ability of HSPs to protect cells against adverse effects is an extension of their normal function as chaperones, acting to conserve the functional structures of cellular proteins (Meyer, 1999). However, if exposure to the stressor is extended, as occurred in the present work, the HSPs are overcome, and the cell starts the cell death process, as was observed in the neurons of the mushroom bodies and optical lobes of the AHB.

The PAS reaction indicates the presence of polysaccharides (glucose or glycogen), which are the main energy source to perform neuron processes (Chapman, 2003). However, no difference between the groups was found, demonstrating that the energy demands are similar for honeybees regardless of whether they are exposed to thiamethoxam.

This study demonstrates that the neonicotinoid thiamethoxam directly affects the mushroom bodies and optical

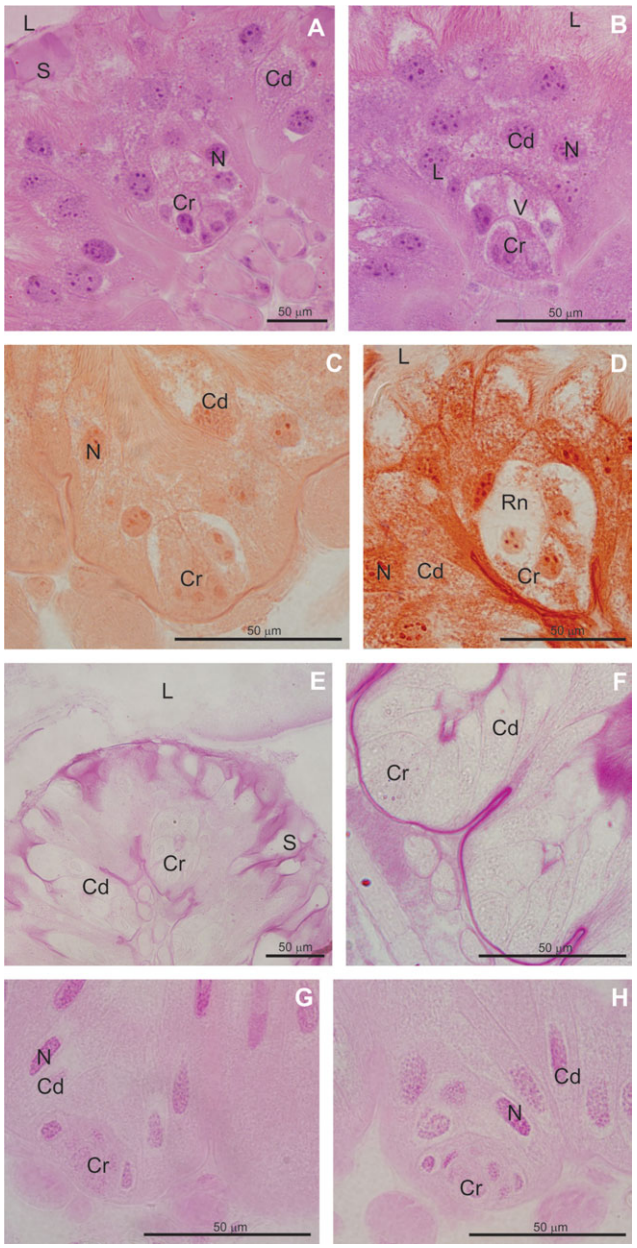

Fig. 6. Images of the nests of regenerative cells in the midguts of newly emerged workers of Africanized *A. mellifera* stained with HE (A and B), subjected to the Xylidine-Ponceau staining (C and D), the PAS reaction (E and F), and the Feulgen reaction (G and H), with or without exposure to thiamethoxam. (A) Nest of regenerative cells with a typical morphology from group without solvent after 5 days. (B) Regenerative cells with cytoplasmic vacuolization (V) from group exposed to LC_{50}/10 for 5 days. (C) Weakly stained cytoplasm of regenerative cells subjected to the Ponceau Xylidine staining from group without solvent after 8 days. (D) Nest of regenerative cells with negative cytoplasmic regions (Rn) to Xylidine-Ponceau staining from group exposed to LC_{50}/10 for 1 day. Note the decrease in the number of regenerative cells composing this nest. (E) Regenerative cells without staining, subject to PAS from group without solvent after 1 day. (F) Nest of regenerative cells without staining, of the honeybee group subjected to LC_{50}/10 for 5 days subject to PAS. (G) Nest of regenerative cells with weak nuclear staining by the Feulgen reaction from group without solvent after 3 days. (H) Nests of regenerative cells from the group subjected to LC_{50}/10 for 3 days (Feulgen reaction), showing staining similar to the control group. Cd = digestive cell; Cr = regenerative cell; L = lumen; N = cell nucleus; S = apocrine secretion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE III. Summary of morphological and histochemical analyses on the regenerative cells from Africanized honeybees exposed to sublethal doses of thiamethoxam

				Histochemical			
		Morphological		XYL	PAS		FEU
Treatment		VA	DC	CIT	CIT	CC	PG
1 day	C−	−	−	+	−	−	−
	C+	−	−	+	−	−	−
	LC_{50}/10	+	+	−	−	−	−
	LC_{50}/100	−	−	−	−	−	−
3 days	C−	−	−	+	−	−	−
	C+	−	−	+	−	−	−
	LC_{50}/10	−	+	−	−	−	−
	LC_{50}/100	+	+	−	−	−	−
5 days	C−	−	−	+	−	−	−
	C+	−	−	+	−	−	−
	LC_{50}/10	+	−	−	−	−	−
	LC_{50}/100	−	−	+	−	−	−
8 days	C−	−	−	+	−	−	−
	C+	−	−	+	−	−	−
	LC_{50}/10	−	−	−	−	−	−
	LC_{50}/100	−	−	−	−	−	−

(+) Presence of alteration; (++) Alteration moderately present; (+++) Alteration extremely present; (−) alteration absent; (LC_{50}) lethal concentration; (C−) control without acetone; (C+) control with acetone; (FEU) Fuelgen reaction; (XYP) Xylidine-Ponceau technique; (PAS) PAS reaction; (VA) vacuolization; (DC) decrease in cell number; (CIT) cytoplasm; (CC) compacted chromatin; (PG) presence of granules.

lobes, even when at sublethal doses. Because these structures are associated with the sensory integration of the whole brain and in the processing of vision, there is strong evidence that this insecticide could aggravate the effects of disorientation in honeybees, as reported in the case of CCD (vanEngelsdorp et al., 2009). To illustrate the real hazard of honeybee toward the neonicotinoids, Girolami et al. (2009) found that germinated plants from corn seeds-coated with thiamethoxam, produces exudates from xylem with maximum concentration of 100 ppm, which correspond to 233-fold more that our high dose tested and that was extremely toxic to AHB.

In addition to changes in the brain of the AHB, this study also demonstrates that thiamethoxam is cytotoxic in the midgut. With regard to cell morphology, cytoplasmic vacuolization, an increase of apocrine secretion and cell elimination were found in both groups of AHBs exposed to thiamethoxam. Histochemical analysis with the Xylidine-Ponceau staining allowed for the observation of negative cytoplasmic regions, high elimination of reaction positive vesicles and the presence of perichromatin halos. The PAS reaction allowed for the visualization of reaction positive granules in both exposed groups, which were described as crystals by Cruz-Landim (2009). These crystals, present in the midgut of bees, are important structures for internal osmotic regulation and to prevent poisoning. Thus, the increased presence of granules in treated groups must be due to cellular mechanisms of defense against the presence of a xenobiotic agent. Staining with the Fuelgen reaction showed an absence of chromatin compaction in digestive cells. These data suggest that cells affected by the insecticide are eliminated to the lumen of the ventricle before finishing the cell death process (Cruz-Landim, 2009).

The digestive cells of exposed bees showed the presence of vacuoles and cytoplasmic regions of negative Xylidine-Ponceau staining. These characteristics were noted during the 1st day of exposure; then, they disappeared for a period, and they reappeared with continued exposure. These data may indicate that the epithelium suffers damage from immediate exposure to thiamethoxam (the 1st day of exposure), which is then followed by a recovery attempt (indicated by the absence of signs, that is, presence of vacuoles and cytoplasmic regions of negative Xylidine-Ponceau staining). In *A. mellifera*, there are three families of enzymes that play a role in the detoxification process: GTSs, cytochrome P450s (P450s) and carboxliesterases (SCCs) (Claudianos et al., 2006). These superfamilies are involved in insecticide metabolism (Feyereisen, 2005; Oakeshott et al., 2005; Ranson and Hemingway, 2005). These enzymes could be attempting to recover the midgut epithelium during insecticide exposure; however, in cases of continuous exposure, the midgut epithelium was unable to fully recover, and the damage reappeared. Some studies have also shown changes caused in the midgut of honeybees that were exposed to xenobiotics agents. Cruz et al. (2010) analyzed *A. mellifera* larvae treated with fipronil and boric acid and found that these compounds increase cellular elimination and cytoplasmic vacuolization.

Regenerative cells are of fundamental importance for epithelium restructuring and are found in cell nests at the base of the midgut epithelium (Cruz-Landim, 2009). The morphological analyses performed showed that thiamethoxam caused cytoplasmic vacuolization and a reduction in the number of regenerative cells present in these nests. Histochemical analysis with the Xylidine-Ponceau stainingshowed negative cytoplasmic staining in the exposed groups, suggesting that exposure to the insecticide may decrease protein synthesis in these cells. This result may be detrimental because these cells are undifferentiated. Thus, these results suggest that thiamethoxam decreases the renewal capacity of the midgut epithelium.

This work showed that thiamethoxam causes morphological and histochemical alterations in the brain and midgut of the AHB. Intoxication with thiamethoxam may cause physiological and behavioral changes at the individual level and throughout the colony. Such changes may lead to a reduction in life expectancy (Carvalho et al., 2009), impairment of the ability to fly (Vandame et al., 1995) and impairment of learning (El Hassani et al., 2008), which may cause the disruption,

decline and disappearance of honeybees (Neumann and Carreck, 2010). Furthermore, the present results show that intoxication with sublethal doses (1/100 of LC_{50}) of thiamethoxam does not affect the survival of bees but that it can be harmful as high doses that cause the immediate death of the insect.

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