Environmental Pollution 229 (2017) 386–393

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Exposure of larvae to thiamethoxam affects the survival and physiology of the honey bee at post-embryonic stages☆

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ARTICLE INFO

Article history:
Received 12 April 2017
Received in revised form
29 May 2017
Accepted 29 May 2017
Available online 12 June 2017

Keywords:
Apis mellifera
Neonicotinoids
Larvae
Pupae
Toxicity
Development

ABSTRACT

Under laboratory conditions, the effects of thiamethoxam were investigated in larvae, pupae and emerging honey bees after exposure at larval stages with different concentrations in the food (0.00001 ng/μL, 0.001 ng/μL and 1.44 ng/μL). Thiamethoxam reduced the survival of larvae and pupae and consequently decreased the percentage of emerging honey bees. Thiamethoxam induced important physiological disturbances. It increased acetylcholinesterase (AChE) activity at all developmental stages and increased glutathione-*S*-transferase (GST) and carboxylesterase *para* (CaE*p*) activities at the pupal stages. For midgut alkaline phosphatase (ALP), no activity was detected in pupae stages, and no effect was observed in larvae and emerging bees. We assume that the effects of thiamethoxam on the survival, emergence and physiology of honey bees may affect the development of the colony. These results showed that attention should be paid to the exposure to pesticides during the developmental stages of the honey bee. This study represents the first investigation of the effects of thiamethoxam on the development of *A. mellifera* following larval exposure.

1. Introduction

The honey bee *Apis mellifera* plays an important role at the economic and environmental levels. It contributes to more than 80% of the total pollination in agriculture and plays an important role in the pollination in ecosystems (Klein et al., 2007; Breeze et al., 2011). Gallai et al. (2009) estimated that the economic value of pollination in global scales is approximately €153 billion per year. However, there is an increasing number of reports on the decline of the bee population worldwide. This decline is characterized by the mass disappearance of bees in the United States (including colony collapse disorder (CCD)) and, in numerous cases, by losses of managed and wild pollinators in Europe and Asia (Stokstad, 2007; Potts et al., 2010). This raises the discussion among beekeepers, researchers, the chemical industry and governmental agencies about the factors involved in this syndrome. Several causes have been identified to explain the decline in the bee population, highlighting beekeeping management, agricultural practices, loss of floral diversity, habitat fragmentation and rarefication, pathogens, parasites and pesticides (Ratnieks and Carreck, 2010; Goulson et al., 2015).

An epidemiological study was performed in 2013 to explain the loss of honey bee colonies in Europe (Chauzat et al., 2013). If we exclude the problems of diseases (e.g., varroosis or nosemosis), the intoxication with pesticides could also, at least partially, explain the colony losses. Among pesticides, neonicotinoid insecticides represent the main family of insecticides used worldwide (Blacquiere

☆ This paper has been recommended for acceptance by Dr. Chen Da.

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http://dx.doi.org/10.1016/j.envpol.2017.05.092
0269-7491/

et al., 2012; Sparks and Nauen, 2014). They act as agonists of the nicotinic acetylcholine receptors of insects (nAChR) (Tomizawa and Casida, 2003). They are characterized by the systemic properties and xylem and phloem transports enable them to be distributed in all plant tissues and to contaminate pollen and nectar, the two main food resources of bees (Rortais et al., 2005). They thus have adverse effects on bees, such as behaviour impairment or morphological and physiological disturbances (Henry et al., 2012; Goulson et al., 2015). Therefore, the effects and the properties of neonicotinoids suggest that they pose the greatest risk to honey bees (Sanchez-Bayo and Goka, 2014).

Within this class of insecticides, thiamethoxam, a second-generation neonicotinoid, can be regarded as an active substance of high concern (Maienfisch et al., 2001). Honey bees can be exposed to thiamethoxam and other neonicotinoids because they usually explore areas in a radius up to 12 km around the hive to collect floral resources, water and resins, increasing the risk of exposure at lethal and sublethal levels (Beekman and Ratnieks, 2000).

In addition to honey bee foragers, larvae could also be exposed to neonicotinoids from residues contained in pollen, nectar, water and wax stored in the hive (Rortais et al., 2005; Desneux et al., 2007; Couvillon et al., 2014; Sanchez-Bayo and Goka, 2014; Johnson, 2015). Thiamethoxam has been found at concentrations ranging from 1 to 100 μg/kg in nectar, pollen and plant secretions (and some other environmental matrices) (Blacquiere et al., 2012; Bonmatin et al., 2014; Kessler et al., 2015) and at a concentration of 0.6 μg/kg in beebread (Giroud et al., 2013).

The post-embryonic period of honey bees may be considered crucial because the exposure to xenobiotics can cause irreversible damage at the cellular, physiological and morphological levels, which can jeopardize the development of the honey bee (Becher et al., 2013; Tavares et al., 2015). At present, the side effects induced by an exposure to neonicotinoids during the post-embryonic period are poorly investigated (Desneux et al., 2007; Blacquiere et al., 2012), although some studies have demonstrated toxicity to bumblebees, which includes an increased mortality, a reduced efficiency of pollen collection and a reduced growth rate (Mommaerts et al., 2010; Whitehorn et al., 2012; Gill et al., 2012; Elston et al., 2013; Laycock et al., 2014).

To study honey bee health, the approach involving the use of biomarkers appears to be particularly pertinent to assess the physiological responses of honey bees after exposure to xenobiotics and to understand the mechanisms involved in the toxicity and the adaptation to environmental changes (Jovanovic-Galovic et al., 2004; Badiou-Beneteau et al., 2012; Boily et al., 2013; Carvalho et al., 2013; Badawy et al., 2015). Some biomarkers are particularly used to assess the physiological effects of environmental stressors. Acetylcholinesterase (AChE, EC 3.1.1.7) is an enzyme that controls the neuronal activity of cholinergic synapses (Badiou et al., 2008). Carboxylesterases (CaE, EC 3.1.1.1) and glutathione-*S*-transferase (GST 2.5.1.1.8) are phase I and phase II enzymes involved in the detoxification and endocrine systems (Yu et al., 1984; Maxwell, 1992; Diao et al., 2006). Alkaline phosphatase (ALP, EC 3.1.3.1) hydrolyses the phosphate group of different substrates and is involved in the absorption of substances, in intestinal integrity and homeostasis, and in the immunity process (Moss, 1992; Millán, 2006; Lallès, 2010).

In this study, we have investigated the effects of thiamethoxam in larvae, pupae and emerging workers following exposure at the larval stage. The study was focused on the success of post-embryonic development and on the physiological disturbances assessed by the modulation of the biomarkers AChE, GST, CaE*p* and ALP.

2. Materials and methods

2.1. Chemicals

Thiamethoxam (98.5% pure) was purchased from Dr. Ehrenstorfer GmbH. Yeast extract, D-glucose, D-fructose, antipain, aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, monosodium phosphate, sodium chloride (NaCl), Triton® X-100, acetylthiocholine iodide (AcSCh.I), 5,5'dithio-bis(2,nitrobenzoic acid) (DTNB), reduced L-glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), 1-chloro-2,4-dinitrobenzene (CDNB), Trizma® base (Tris), hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), *p*-nitrophenyl phosphate (*p*-NPP), 1,5-bis(4-allyldimethylammonium-phenyl)pentan-3-one-dibromide (BW284C51) and *p*-nitrophenyl acetate (*p*-NPA) were obtained from Sigma Aldrich (France). Royal jelly was purchased from Ickowicz Apiculture (Bollène, France).

2.2. Collection of honey bee larvae and maintenance during development

Four colonies of *Apis mellifera* honey bees, previously checked for their health status, were selected from the experimental apiary of the *Abeilles & Environment* Research Unit (INRA, Avignon, France). Each colony had 6 to 7 brood frames and was supervised during the experiments to ensure good condition of the individuals. Larvae rearing was performed according to the method developed by Aupinel et al. (2005, 2007) and adopted by OECD (2013). To obtain larvae of a known age, three days before the experiment, combs containing empty cells were previously equipped with a queen excluder and placed in the hive for egg laying. The fourth day, 1st instar larvae were transferred into plastic queen-starter-cells and placed in an incubator under controlled conditions (34 ± 2 °C and 95 ± 5% relative humidity (RH)). At the 7th day, the RH was changed to 80% for the pupation period. On the 15th day (emergence period), each plate was individually sealed with a thin layer of beeswax so that each cell was individualized. In each cell, orifices were made on the top to enable air exchange. The plates were individually accommodated in pots upright, simulating colony conditions. Temperature and RH were identical to those of the pupation period. To feed the emerging honey bees, candy and distilled water were provided ad libitum.

2.3. Larvae feeding

Larvae were provided with food at the daily intakes recommended for each developmental stage (OECD, 2013). Food was composed of 1 volume of royal jelly and 1 volume of an aqueous solution containing 12% (w/v) glucose, 12% fructose and 2% yeast extract (diet A); 15% glucose, 15% fructose and 3% yeast extract (diet B); or 18% glucose, 18% fructose and 4% yeast extract, plus or minus (control) thiamethoxam (diet C). The daily feeding of larvae (volume per diet and per day) was performed from the 1st day (grafting) to the 6th day, except for the 2nd day, which was considered a period of acclimatization. The diet was: 20 μL of diet A on the 1st day, 20 μL of diet B on the 3rd day, and 30, 40, and 50 μL of diet C on the 4th, 5th and 6th days, respectively.

2.4. Exposure to thiamethoxam

To expose larvae, three concentrations of thiamethoxam were selected: 0.00001, 0.001 and 1.44 ng/μL of the diet. The two lowest concentrations were close to the levels of residues found in nectar, pollen and beebread (Rortais et al., 2005; Desneux et al., 2007; Mullin et al., 2010; Blacquiere et al., 2012; Krupke et al., 2012;

Stoner and Eitzer, 2012; Giroud et al., 2013; Pilling et al., 2013; Bonmatin et al., 2014; Couvillon et al., 2014; Sanchez-Bayo and Goka, 2014; Johnson, 2015; Kessler et al., 2015). The highest concentration was selected according to a previous study (Tavares et al., 2015) and was equivalent to 1/10 of the LC_{50} in Africanized *Apis mellifera* larvae. Thiamethoxam was added directly to the larval food (diet C) from a stock solution prepared in distilled water after purity correction. The control group received only the uncontaminated diet. Acute exposure was performed on the 4th day after grafting (PG) by providing 30 μL of diet C containing thiamethoxam at the appropriate concentrations. The concentrations of the stock solutions of thiamethoxam were checked according to Wiest et al. (2011), and the relative standard deviations (RS) were less than 10%. At the thiamethoxam concentrations used, the doses per larvae were 0.0003, 0.03 and 43.2 ng/larvae. Larval mortality was checked individually by observation under a stereomicroscope at the 5th, 6th and 8th days PG. Pupal mortality was checked at the 11th, 13th and 15th days PG, and the percentage of adult emergence enabled the estimation of the success of the pupae phase, which occurred between the 15th and the 20th day PG. Bees were considered emerging when individuals left the artificial cells. The experiments were conducted from May to June 2014. For the bioassays, experimental replicates of 48 larvae from at least 3 colonies were adopted. For each bioassay, an experimental duplicate or triplicate was made $n = 96$ larvae per group or $n = 144$ larvae per group, and data were analysed.

2.5. Analysis of the physiological markers

The samples collected at the different developmental stages were 5th instar larvae, black-eye pupae with the cuticle slightly tanned (Pdl) and newly emerged workers (emerging bees) (Rembold et al., 1980; Michelette and Soares, 1993; Cruz-Landim, 2009). Different tissues were used for enzymatic analysis. AChE and GST were extracted from the whole body of larvae and the head of pupae and emerging bees. CaE*p* and ALP were extracted from the whole body of larvae, the abdomens of pupae and the midgut of emerging bees. For each enzyme and at each exposure condition and developmental stage, 7 extracts each containing 3 body parts were assayed in triplicate. Bee tissues were homogenized with TissueLyser (Qiagen®) to make a 10% (w/v) tissue extract, for 5 × 10 s at 30 Hz, in the extraction medium composed of 10 mM NaCl, 1% Triton X-100, 40 mM sodium phosphate, pH 7.4, and containing 2 mg/ml antipain, 2 mg/ml leupeptin, 2 mg/ml pepstatin A, 25 units/ml aprotinin and 0.1 mg/ml soybean trypsin inhibitor. After homogenization, samples were centrifuged for 20 min at 15,000 *g*, and the supernatants were recovered for analysis. All procedures were performed at 4 °C. Enzyme kinetics were followed with a multimode microplate reader Infinite F500 (Tecan®) at 25 °C in a final reaction volume of 200 μL. AChE activity was measured at 412 nm in a medium containing 0.3 mM AcSCh.I, 1.5 mM DTNB and 100 mM sodium phosphate pH 7.0. GST activity was measured at 340 nm in a medium containing 2.5 mM GSH, 1 mM EDTA, 1 mM CDNB and 100 mM Na/K phosphate, pH 7.4. CaE*p* activity was measured at 410 nm in a reaction medium containing 0.01 mM BW284C51, an AChE inhibitor, 0.1 mM of *p*-NPA and 100 mM sodium phosphate, pH 7.5. ALP activity was measured at 410 nm in a medium containing 20 mM $MgCl_2$, 2 mM *p*-NPP and 100 mM Tris-HCl, pH 8.5.

2.6. Statistical analyses

All data were analysed using the statistical R Development Core Team (2015). Survival data were analysed using the Cox proportional hazards regression model (survival package), and significant differences were denoted when $P < 0.05$. The influence of thiamethoxam on emergence success was analysed by a chi-square test that makes pairwise comparisons between exposed and control groups, with 1 df and $P < 0.001$. Enzymatic data were analysed by the pairwise Wilcoxon rank sum tests from the package “stats”.

3. Results

3.1. Honey bee survival

The exposure of larvae to thiamethoxam at the concentration of 1.44 ng/μL affected the survival of individuals with higher mortality rates at day 8 (Cox model $P \leq 0.01$, Fig. 1). Pupae exposed to the concentrations of 0.001 and 1.44 ng/μL exhibited a significant decrease of the survival rate (Cox model $P \leq 0.05$, Fig. 2). However, there was no effect on survival in larvae exposed to 0.00001 and 0.001 ng/μL and in pupae exposed to 0.00001 ng/μL thiamethoxam (Figs. 1 and 2). Regarding the adult emergence rate, a significant decrease was observed with exposures at the concentration of 0.001 and 1.44 ng/μL thiamethoxam because of the increased mortality of larvae and pupae (Chi-Square test $P \leq 0.001$, Fig. 3). Furthermore, independent of the experimental group, an increase in mortality was observed during the periods of transition from larvae to pupae, which occurred at day 13, and from pupae to adult, which occurred at day 20 (Fig. 4).

3.2. Physiological effects of thiamethoxam

The physiological effects of thiamethoxam on individuals of different ages (immature stages and adults) were assessed with physiological biomarkers. Thiamethoxam modulated the activity of AChE in larvae, pupae and emerging bees. Larvae exposed at 1.44 ng/μL showed an increase of AChE activity (5.51 ± 1.26 and 7.56 ± 0.40 mAU/min/mg tissue in control and exposed bees, respectively) (Mann Whitney *U* tests, $P \leq 0.05$, Fig. 5). For pupae and emerging bees, a significant increase of AChE activity was observed in all thiamethoxam-exposed groups (Mann-Whitney *U* test, $P \leq 0.05$, Fig. 5). For pupae exposed to thiamethoxam at 0.00001, 0.001 and 1.44 ng/μL, AChE activities were 61.06 ± 11.54, 55.80 ± 10.82 and 60.30 ± 6.99 mAU/min/mg tissue, respectively, and were higher than that of the control (of 40.99 ± 6.93 mAU/min/mg tissue). For emerging bees exposed to thiamethoxam at 0.00001, 0.001 and 1.44 ng/μL, AChE activities were 114.38 ± 11.02; 117.14 ± 9.91 and 117.47 ± 8.77 mAU/min/mg tissue and were higher than that of the control (93.2 8 ± 9.89 mAU/min/mg tissue).

For the enzymes related to the metabolism of xenobiotics, the activities of GST and CaE*p* showed significant increases only in pupae (Mann-Whitney *U* tests, $P < 0.05$, Figs. 6 and 7). The activity of GST was 202.26 ± 6.99; 197.16 ± 3.16 and 194.7 5 ± 2.31 mAU/min/mg tissue in pupae exposed to thiamethoxam at 0.00001, 0.001 and 1.44 ng/μL, respectively, and 188.82 ± 6.27 mAU/min/mg tissue in the control (Fig. 6). For CaE*p*, the activities were 201.88 ± 6.99; 196.78 ± 3.16 and 194.37 ± 2.31 mUA/min/mg tissue in pupae exposed to thiamethoxam at 0.00001, 0.001 and 1.44 ng/μL, respectively, and 188.45 ± 6.27 mAU/min/mg tissue in the control (Fig. 7). No effect of thiamethoxam was observed in larvae and emerging bees (Fig. 8). In the pupal stages, ALP activity was not detected.

4. Discussion

Few studies have evaluated the effects of insecticides, and particularly thiamethoxam, on the developmental phase of honey bees, although the success of this phase is dependent on large changes in tissues, such as the modelling of the brain (Chapman,

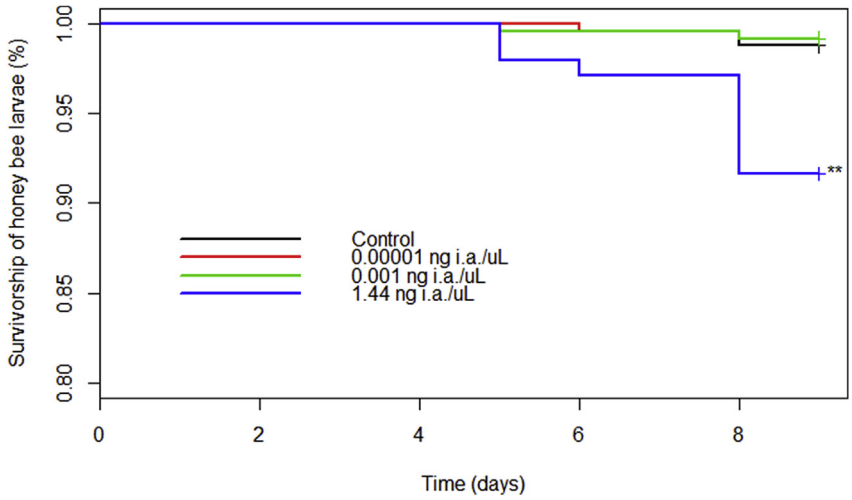

Fig. 1. Effect of thiamethoxam on honey bee larvae survival. Bees were acutely exposed to thiamethoxam at the 4th day PG at the concentrations of 0.00001, 0.001 and 1.44 ng/μL. The data represent the survival rate of larvae following exposure. Asterisks denote significant differences between exposed and control groups analysed by Cox proportional hazards regression model (**$p \leq 0.01$). ($n = 336$ individuals per group from 7 replicates).

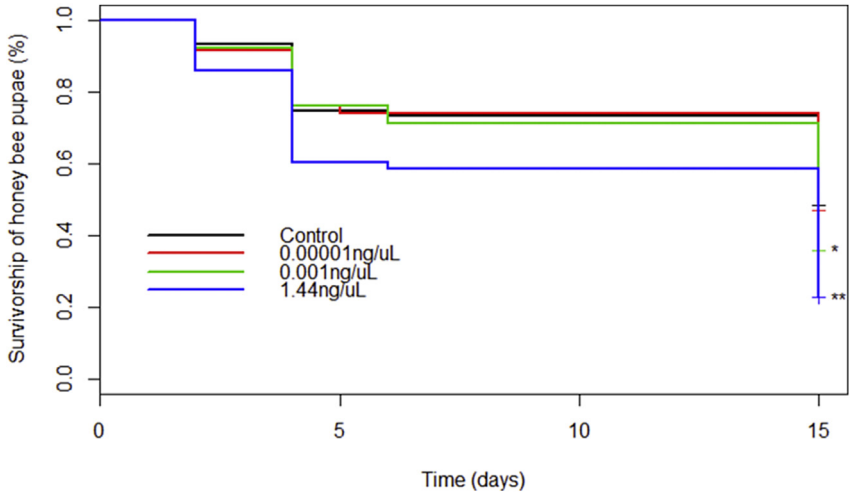

Fig. 2. Effect of thiamethoxam on honey bee pupae survival. Bees were acutely exposed to thiamethoxam at the 4th day PG at the concentrations of 0.00001, 0.001 and 1.44 ng/μL. The data represent the survival rate of pupae following exposure. Asterisks denote significant differences between exposed and control groups analysed by Cox proportional hazards regression model (*$p \leq 0.05$ and **$p \leq 0.01$) ($n = 240$ individuals per group from 5 replicates).

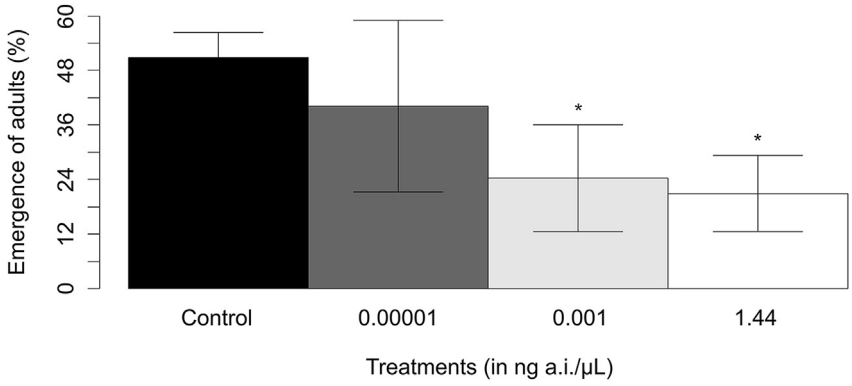

Fig. 3. Effect of thiamethoxam on the emergence success of honey bee adults. Bees were acutely exposed to thiamethoxam at the 4th day PG at the concentrations of 0.00001, 0.001 and 1.44 ng/μL. The percentage of emergence was calculated from the number of pupae in each experimental group: (i) control, $n = 120$; (ii) 0.00001 ng/μL, $n = 107$; (iii) 0.001 ng/μL, $n = 111$ and (iv) 1.44 ng/μL, $n = 105$. The comparisons between exposed groups and controls were performed with the Chi-square test with 1 df (*$p \leq 0.001$) ($n = 144$ individuals per group from 3 replicates). Asterisks denote significant differences between exposed and control groups. Bars represent the mean values ± standard deviations from 3 repetitions.

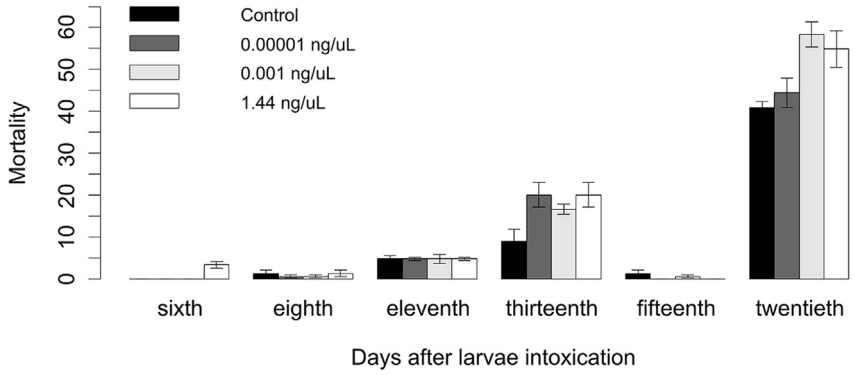

Fig. 4. Effect of thiamethoxam on the mortality of honey bees during development. Bees were acutely exposed to thiamethoxam at the 4th day PG at the concentrations of 0.00001, 0.001 and 1.44 ng/μL (n = 144 individuals per group from 3 replicates). Bars represent the mean mortality values ± standard deviations from 3 repetitions.

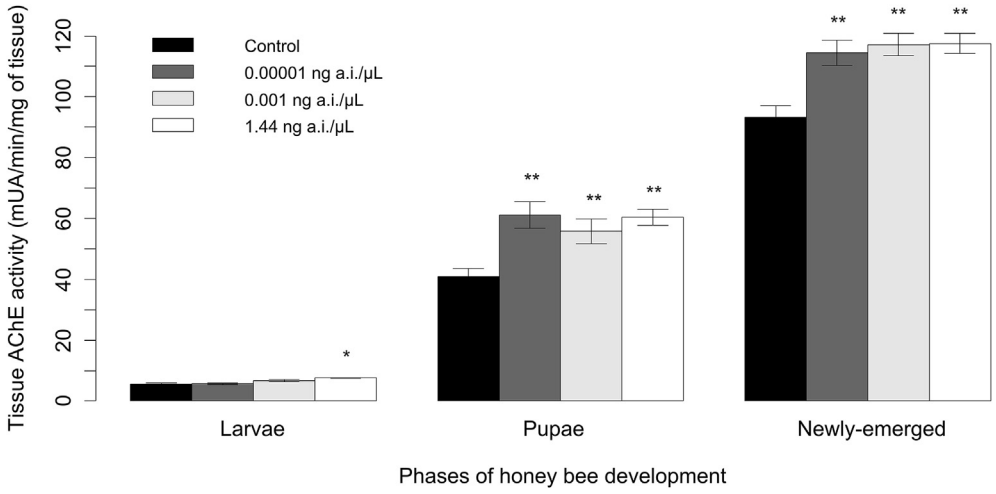

Fig. 5. Effects of thiamethoxam on acetylcholinesterase. Bees were acutely exposed to thiamethoxam at the 4th day PG at the concentrations of 0.00001, 0.001 and 1.44 ng/μL. Acetylcholinesterase (AChE) activity was measured in 5th instar larvae (A), the head of pupae (Pdl) (B) and the head of emerging bees (C). Bars represent the mean values ± SD of 7 repetitions performed in triplicate. Asterisks denote significant differences between exposed groups and their respective control groups analysed by Mann-Whitney U tests ($^{*}p \leq 0.05$, $^{**}p \leq 0.005$).

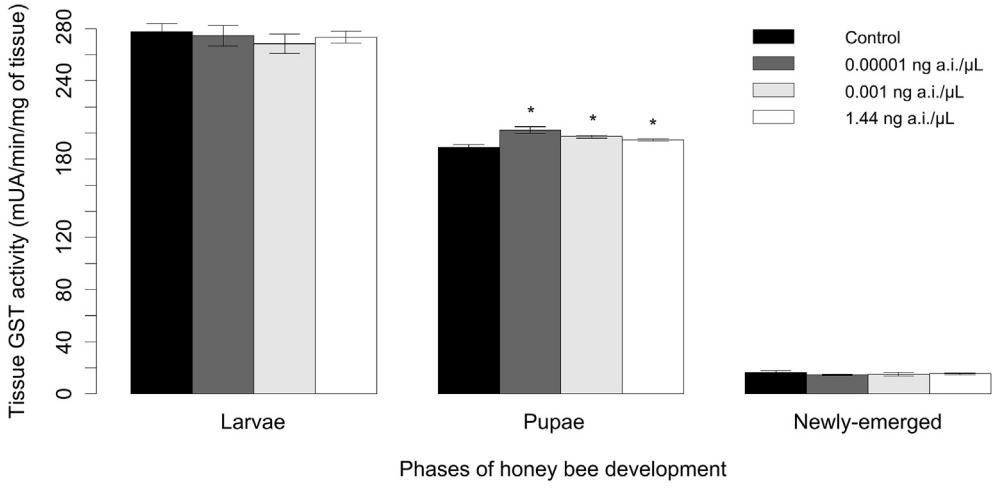

Fig. 6. Effects of thiamethoxam on glutathione-*S*-transferase. Bees were acutely exposed to thiamethoxam at the 4th day PG at the concentrations of 0.00001, 0.001 and 1.44 ng/μL. Glutathione-*S*-transferase (GST) was measured in 5th instar larvae (A), the head of pupae (Pdl) (B) and the head of emerging bees (C). Bars represent the mean values ± SD of 7 repetitions. Asterisks denote significant differences between exposed groups and their respective control groups analysed by Mann-Whitney U tests ($^{*}p \leq 0.05$, $^{**}p \leq 0.005$).

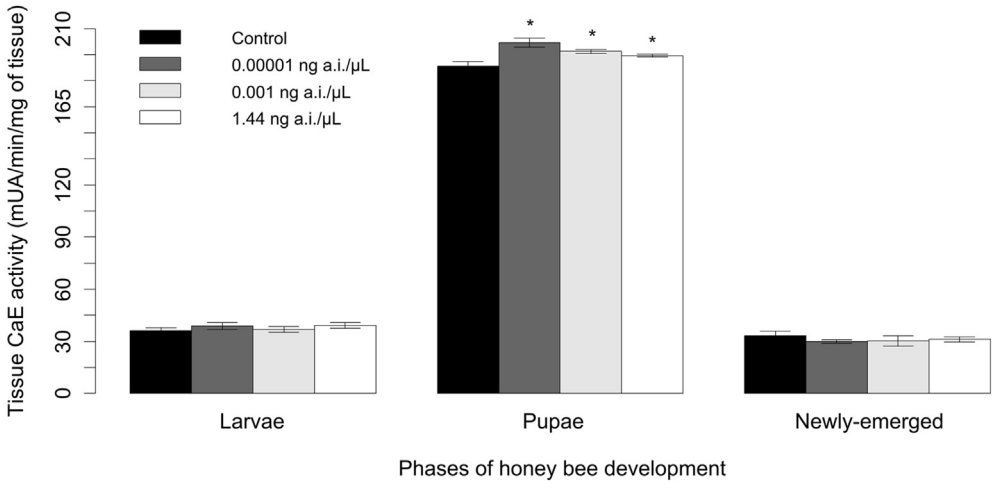

Fig. 7. Effects of thiamethoxam on Carboxylesterase. *Para.* Carboxylesterase *para* (CaE*p*) was measured in 5th instar larvae (A), the head of pupae (Pdl) (B) and the head of emerging bees (C). Bars represent the mean values ± SD of 7 repetitions performed in triplicate. Asterisks denote significant differences between exposed groups and their respective control groups analysed by Mann-Whitney *U* tests ($^{*}p \leq 0.05$, $^{**}p \leq 0.005$).

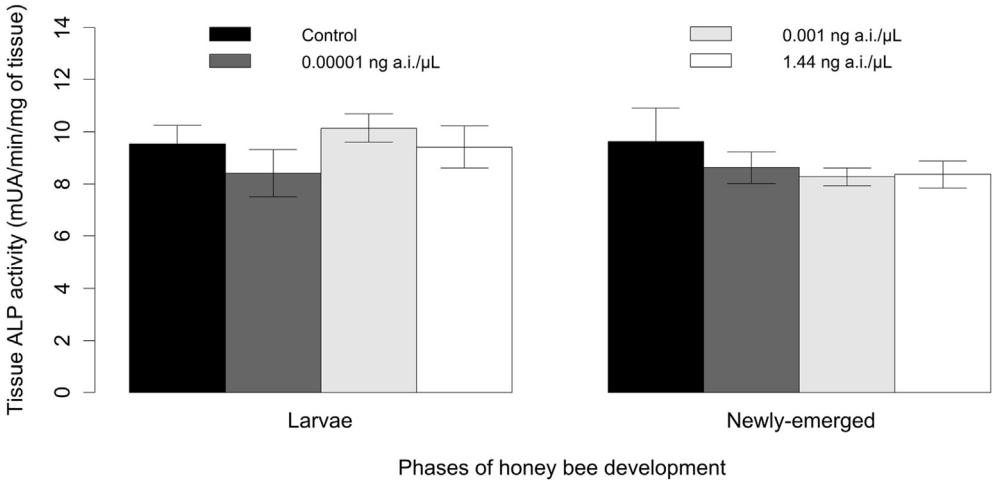

Fig. 8. Effects of thiamethoxam on alkaline phosphatase. Bees were acutely exposed to thiamethoxam at the 4th day PG at the concentration of 0.00001, 0.001 and 1.44 ng/µL. Alkaline phosphatase (ALP) was measured in 5th instar larvae (A), the head of pupae (Pdl) (B) and the head of emerging bees (C). Bars represent the mean values ± SD of 7 repetitions performed in triplicate. Mann-Whitney *U* tests ($^{*}p \leq 0.05$, $^{**}p \leq 0.005$).

1998; Roat and Landim, 2010), and is crucial for the fitness of adults (Desneux et al., 2007; Yang et al., 2012; Cousin et al., 2013). This study represents the first investigation on the effects of thiamethoxam on the development of *A. mellifera* after exposure at larval stages. Our results demonstrate that acute exposures of *A. mellifera* larvae to thiamethoxam reduce the survival of larvae, pupae and the emergence rate of adult honey bees. They also disrupt the physiology by modifying the activity of AChE, at all developmental stages, and GST and CaE*p* at pupal stages.

Regarding larval survival, only the highest thiamethoxam concentration had a lethal effect on larvae. However, it is very important to consider that delayed effects may occur during development or at the adult stage. This can be exemplified by the concentration of 0.001 ng/µL that did not change the survival rate of larvae but decreased the survival of pupae and thus the percentage of emerging bees. Grillone et al. (2017) in evaluating the effects of thiamethoxam on the development of *A. mellifera* found different sublethal late effects including like brownish larvae, duplication of the pupal integument, delay in development, and deformed adults.

In addition to survival, it should be considered that late effects on development can occur and that the functional integrity of adults may be impaired. Thus, exposure to neonicotinoids at doses that did not induce mortality in larvae can impair learning performance in adults (Yang et al., 2012) or can induce the appearance of atypical neurons in the brains of larvae (Tavares et al., 2015).

The effects on survival were observed only from the 8th day. Thus, it is possible to assume that both thiamethoxam and its metabolites are involved in the toxicity. This assumption is in accordance with the results showing that neonicotinoids may accumulate in bee tissues and that metabolites are involved in the toxicity through a metabolic relay (Suchail et al., 2001, 2004; Yang et al., 2012; Zhu et al., 2014). In addition, as honey bee larvae do no defecate until the pupal stage, metabolites may accumulate and thus increase the toxicity of the parent substance (Michelette and Soares, 1993; Cruz-Landim, 2009; Suchail et al., 2001, 2004).

In this study, it was observed that mortality presented peaks that coincided with the periods of transition in the development, larva to pupa (13-d) and pupa to adult (20-d) both in control and exposed individuals. This outcome not only highlights the importance of these periods in the success of honey bee development

(Cruz-Landim, 2009) but also reveals that these periods should be considered at risk for exposures to pesticides. Thus, effects on the survival of pupae and adult emergence might originate from a particular susceptibility during metamorphosis.

After exposure to thiamethoxam, pupae and emerging bees exhibit a significant increase in AChE activity in all exposed groups. AChE is an enzyme that hydrolyses the neurotransmitter acetylcholine (ACh) in cholinergic synapses for the rapid and precise control of nerve transmission (Massoulié et al., 1993; Badiou et al., 2008). Thus, the increase of AChE might be a biological response to compensate the permanent activation of cholinergic neurons due to the strong binding of thiamethoxam to nicotinic acetylcholine receptors (nAChR). However, whatever the mechanisms by which AChE is increased, these results exemplify the interest in AChE as a pertinent biomarker of exposure to neonicotinoids (Boily et al., 2013; Badiou-Beneteau et al., 2013).

CaE*p* and GST are phase I and phase II enzymes involved in the detoxification of xenobiotics (Claudianos et al., 2006; Badawy et al., 2015; Berenbaum and Johnson, 2015; Dussaubat et al., 2016). CaE*p* and GST showed an increase in activity during the pupal stage and in the individuals of all exposed groups. Their activity could have increased as a biological response to detoxify thiamethoxam. However, GST is also involved in the detoxification of reactive oxygen species, and its increase could be the consequence of the induction of oxidative stress by thiamethoxam (Kostaropoulos et al., 2001; Barata et al., 2005; Babczynska et al., 2006). In addition, under normal conditions the amount of GST is higher in pupae than in larvae and adults, as reported by Diao et al. (2006) and according to our results. Since there is a high amount of this enzyme in the pupae phase, its increase following exposure to thiamethoxam should be a rapid physiological protective response. Nielsen et al. (2000) evaluated the effect of flumethrin on *Apis mellifera linguistica* Spinola, and also observed an increase of GST in larvae, pupae and nurse bees, which is more notable in larvae and pupae. This demonstrates the importance of GST in detoxification during the pupal phase and suggests that its activity may be very important in the metabolization of insecticides during this phase.

Concerning carboxylesterases, these enzymes appear very sensitive to the exposure to pesticides and can be easily modulated by neonicotinoids, as shown for CaEα, CaEβ and CaE*p* (Badiou-Beneteau et al., 2012; Dussaubat et al., 2016). They are enzymes that besides being involved in detoxification, also act in the degradation of the juvenile hormone during the development of *A. mellifera*. Since juvenile hormone is associated with development, we can relate the high amount of carboxylesterases in the pupal phase with the decrease of the juvenile hormone that occurs at this stage (Truman and Riddiford, 1999). For larvae and newly-emerged honey bees, there were no differences in the enzymatic activity of GST and CaE*p*. It is important to consider that each stage of life must rely on its specific defence mechanisms. For example, larvae have a large amount of fat body that probably plays an important role in detoxification because this tissue is rich in cytochromes P450. For adults honey bees, the susceptibility of *A. mellifera* to xenobiotics may also be related to the fact that there is a low amount of GST and other detoxification enzymes (Claudianos et al., 2006).

The basic structure of the intestine of larvae and pupae is similar but, during metamorphosis, the intestine is extensively reorganized to fit the body of the adult bees (Cruz-Landim, 2009; Moss, 1992). However, ALP activity was not detected during the pupal stage. This could be due to the fact that this enzyme is involved in the hydrolysis of phosphate groups and in the absorption of substances not present in the food of pupae.

We demonstrated that the exposure of *A. mellifera* larvae to thiamethoxam, at concentrations similar to or lower than those found in field, may affect the survival rate, the emergence success and the physiology of bees at the larval, post-embryonic and adult stages. It is legitimate to think that these effects may jeopardize the survival of *A. mellifera* colonies, alone or in combination with effects induced by other chemical or biological stressors. These results are important for the process of pesticide registration because they emphasize the necessity of assessing the toxicity of plant protection products on developmental stages.

Acknowledgements

The authors would like to thank the São Paulo Research Foundation for providing the scholarship BEPE (grants 2013/21634-8) and the thematic project (grants 2012/50197-2). We thank also G. Kairo and M. Cousin for their technical help.

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