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Exposure to thiamethoxam during the larval phase affects synapsin levels in the brain of the honey bee

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ABSTRACT

Thiamethoxam (TMX) is a neurotoxic insecticide widely used for insect pest control. TMX and other neonicotinoids are reported to be potential causes of honey bee decline. Due to its systematic action, TMX may be recovered in pollen, bee bread, nectar, and honey, which make bees likely to be exposed to contaminated diet. In this study, we used immunolabeling to demonstrate that sublethal concentrations of TMX decrease the protein levels of synapsin in the mushroom bodies (MBs) and the antennal lobes (ALs) of pupae and newly emerged worker bees that were exposed through the food to TMX during the larval phase. A decrease in the synapsin level was observed in the MBs of pupae previously exposed to 0.001 and 1.44 ng/μL and in newly emerged bees previously exposed to 1.44 ng/μL and no changes were observed in the optical lobes (OLs). In the ALs, the decrease was observed in pupae and newly emerged bees exposed to 1.44 ng/μL. Because the MBs and ALs are brain structures involved in stimuli reception, learning, and memory consolidation and because synapsin is important for the regulation of neurotransmitter release, we hypothesize that exposure to sublethal concentrations of TMX during the larval stage may cause neurophysiological disorders in honey bees.

1. Introduction

Thiamethoxam (TMX) belongs to the family of neonicotinoid insecticides and is widely used in various agricultural crops against different types of insect pests. This insecticide acts as an agonist on nicotinic acetylcholine receptors (nAChRs) present on post-synaptic membranes in the nervous system of insects. Thus, it mimics the natural neurotransmitter acetylcholine by strongly binding and causing insect death by hyper-excitation followed by paralysis within a few minutes (Maienfisch et al., 2001; Matsuda et al., 2001; Tomizawa and Casida, 2003).

Currently, in addition to other factors, such as infectious agents and loss of habitats, there is a correlation between the increasing loss of honey bee colonies and exposure to pesticides including the neonicotinoid TMX (Goulson et al., 2015). Since the introduction of neonicotinoids into the market, beekeepers began reporting disappearance events of honey bees, disorientation, failure in returning to the colonies and massive loss of colonies (Stokstad, 2007).

Early disappearance of honey bees were reported in the United States and were frequently associated with a "Colony Collapse Disorder" (CCD) (Stokstad, 2007; vanEngelsdorp et al., 2009). Since then, there was a growing concern about the loss of honey bees mainly due to the fact that this insect is not only a pollinator of natural ecosystems and agricultural crops but also a prominent producer of high-value products such as honey, wax and propolis (Potts et al., 2010).

Residues of TMX may be recovered in pollen, nectar, wax and other floral resources. In pollen and nectar, TMX levels range from 2.4 to 53 ng/kg (Krupke et al., 2012; Mullin et al., 2010; Pilling et al., 2013; Stoner and Eitzer, 2012). In addition to exposure during foraging, honey bees may be exposed to TMX during larval development. The contaminated nectar and pollen are the main sources of exposure for the larvae. After being stored inside the hive, these sources are processed into a brood food by nurse bees that can be contaminated by TMX. Some studies demonstrate amounts of TMX in bee bread is between 1.44 and 4.43 ng/kg (Lawrence et al., 2016; Pohorecka et al., 2012; Rumkee et al., 2017).

Although there is there is a real risk of exposure of the larvae, most of the studies evaluating honey bee exposure to TMX were carried out

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in newly emerged or forager honey bees. Rare studies have evaluated the consequences of exposure to TMX in larvae or during post-embryonic development (Desneux et al., 2007; Yang et al., 2012) although they may be exposed to high levels of contamination by clustering of pesticide in the comb Rumkee, (et al. (2017).

The nervous system is essential for their behavior and survival of the bees. Several studies demonstrate the effects of TMX on the nervous system neurophysiology of honey bees including cellular alterations (Friol et al., 2017), alterations in neural activity of acetylcholinesterase (Tavares et al., 2017) and alterations in memory ability (Papach et al., 2017; Peng and Yang, 2016; Wright et al., 2015).

Synapsins are phosphoproteins involved in the transmission of the nervous signal. They are part of a system involved in the communication between the nerve cells, the neurons. Synapsins occur in two forms, synapsin I and synapsin II, bound to synaptic vesicles. They are very important elements for the neural transmission of information because they are involved in the neurotransmitter release (Hilfiker et al., 1999; Humeau et al., 2011). Thus, the study of distribution and abundance of synapsins proteins by immunostaining could help assess the impacts of pesticides on brain structure, particularly because few studies have explored that (Fahrbach and Van Nest, 2016).

To evaluate the influence of TMX on the synapsis levels during the development of honey bees, we exposed the larvae to sublethal concentrations of the insecticide and performed the immunolabeling of synapsin in the brain of pupae and newly emerged. Our evaluations included the mushroom bodies (MBs), the antennal lobes (ALs) and optic lobes (OLs), three important regions of the brain that could relate the possible changes in synapsin levels to the consequences based on the functions of each structure.

2. Materials and methods

2.1. Honey bee colonies

Three colonies of africanized *Apis mellifera* were obtained from the experimental apiary of the Instituto de Biociências da UNESP, Campus Rio Claro. Before the experiments, colonies were monitored for the presence of an egg-laying queen, larvae, pupae, honey and pollen. Three days before the start of the bioassay empty frames were placed inside the hives and the queen was isolated in an excluder cage for oviposition. The experiments were conducted in January and February 2015.

2.2. Larval feeding and exposure to thiamethoxam

For the larvae rearing under laboratory conditions, the OECD (2013) and OECD (2016) methods were adopted. First-instar larvae (L1) were collected from 3 different colonies (12 larvae per colony x 3 colonies = 36 larvae per group), attributed to 3 repetition groups and individually placed to crystal polystyrene grafting cells arranged in 48-well plates containing the larval feed previously prepared. The larvae were fed from day 1 (d-1) to d-6, except on d-2 (a period considered as larval acclimatization in laboratory conditions). The control group received an uncontaminated diet. The experimental groups received the same diet from d-1 and at d-4 the insecticide TMX was incorporated into the diet. For this study, three concentrations of TMX were selected. Concentrations of 0.00001 ng/μL and 0.001 ng/μL (which correspond to 0.001 μg/kg and 1 μg/kg) were selected based on the amounts present in pollen, nectar and bee bread (see Table 1). The highest concentration of 1.44 ng/μL in food (which correspond to 144 μg/kg) was based on the LC_{50} previously determined per Tavares et al. (2015) and corresponds to $LC_{50/10.}$ The larvae were exposed on d-4–30 μL of contaminated diet resulting in the nominal doses of 0.0003, 0.03 and 43.2 ng/larva. TMX was purchased from Cluzeau Info-Labo (99,6% purity) and added directly into the larval diet concentrated from a working solutions at concentration that were three times higher than those of the final concentration in food. To obtain the working solutions, a stock solution of TMX was prepared in water. Cascade dilutions were performed to obtain the working solutions that were added to the larval diet prepares to 2/3 to obtain the nominal TMX concentrations in food. Larval mortality's was observed at d-5, d-6 and d-7 with the aid of a stereomicroscope and the dead larvae were removed. During the larval period, honey bee development was monitored according to the proposed method (OECD, 2013, 2016) until the emergence of honey bees.

Table 1
Concentration of thiamethoxam found in the field. The data on thiamethoxam concentration in pollen, nectar and bee bread were gathered from literature.

Thiamethoxam (μg/kg)			
Polen	**Nectar**	**Bee bread**	**Reference**
53.3	–	–	Mullin et al. (2010)
12	11	–	Stoner and Eitzer (2012)
7.3	–	–	Krupke et al. (2012)
7	2.4	1	Pilling et al. (2013)
–	–	1.44	Lawrence et al. (2016)
–	–	4.3	Pohorecka et al. (2017)

2.3. Neuroanatomy and immunocytochemistry

For the immunocytochemistry analyses, 3 pupae and newly emerged honey bees were collected from each experimental group and we adopted the procedures of Groh et al., (2004) with some adaptations (time of incubation of the antibodies and dilution of the secondary antibody). The pupae were collected on d-15 and had brown eyes and non-pigmented body (Pb stage) (Michelette et al., 1993), and newly emerged honey bees were collected on d-20. The brains of pupae and newly emerged bees were dissected at room temperature in phosphate-buffered saline pH 7.4 (PBS), immediately fixed in 4% formaldehyde in 0.1 M PBS at 4 °C for 2 h and washed 3 times in PBS for 10 min. The brains were embedded in 5% low-melting-point agarose (Agarose II™, 0815-25G Amresco) and sectioned in the frontal plane (100 μm) with a vibrating microtome (Leica TCS-SP5II). To label synapsin, the sections were preincubated for 1 h in PBS containing 2% (v/v) normal goat serum (NGS) and 0.2% (w/v) Triton X-100 at room temperature. The sections were rinsed twice with PBS and incubated for 5 days at 4 °C with the mouse monoclonal antibody SYNORF1 directed against *Drosophila* synaptic vesicle-associated protein synapsin 1 diluted 50-fold in PBS with 1% normal goat serum (1:50). The SYNORF1 antibody was obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa. After incubation, the sections were rinsed three times with PBS and incubated for 1 h with a secondary antibody, goat antimouse antibody conjugated to Cy5 (Molecular Probes, Eugene, OR) diluted 100-fold in PBS with 1% normal goat serum (1:100). Sections were rinsed three times with PBS and incubated for 10 min with DAPI (4′,6′-diamino-2-phenylindole diluted 1:500) for immunofluorescent labeling of the nuclei and, finally, sections were washed 3 times with PBS and mounted using Prolong® Gold Antifade as a mounting medium.

2.4. Laser scanning confocal microscopy

The preparations were visualized with a laser scanning confocal microscope (Leica TCS SP5-II; Leica Microsystems) equipped with an argon laser and an HFT filter using a 633-nm laser wavelength for Cy5 excitation and a 405-nm laser wavelength for DAPI excitation. Optical images of the brain sections were obtained at a depth of 15 μm and range of 1 μm at 40 × magnifications for the MBs and ALs, and at 20 × magnification for the OLs. For the quantification of the synapsin, the fluorescence was converted in grayscale intensity and measured using

the software Leica Application Suite AF 2.6.0. (LAS-AF). Six measurement areas (50 × 50 μm) were used for the MBs: two on the lip (LI), two on the collar (CO) and two on the basal ring (BR). For the ALs, 7 measurement areas (58 × 52 μm) were used around the entire length of the structures, and for the OLs, 5 measurement areas (156 × 156 μm) were used in the extension of the region of the medulla (ME). For each group, three pupae or newly emerged bees were used, and the measurements were made from three sections of each individual brain structure totalizing 54 measurements for MBs, 63 for ALs and 45 for OLs.

2.5. Statistical analyses

For mean values of emitted fluorescence intensity obtained from MBs, ALs and OLs, statistical tests were performed using the SigmaPlot 11.0 program (Systat), and one-way ANOVA was used to compare the means of the groups. Normality was previously verified using the Shapiro-Wilk test. To identify the significant differences between the control group and the exposed groups, the Holm-Sidak test was used with a significance level set at $P < 0.05$. The results are present as the mean ± SD.

3. Results

In the present study we investigated in the laboratorial conditions the effects of sublethal concentrations of TMX in the brain of pupae and newly emerged workers of *A. mellifera* that were exposed in the larval stage. For this, we performed the immunostaining of the protein synapsin and evaluated three regions of the brain. From the images obtained, it was possible to observe synapsin labeling in the MBs of pupae and newly emerged bees throughout the lip (LI), collar (CO) and basal ring (BR) structures and an intense labeling in the pedunculus (P) (Fig. 1A–D). In the AL structures, the immunoreactivity was evident and located in the glomeruli (GL) (Fig. 1E–H). In the OLs, synapsin was visualized throughout the structure, including the chiasma (CH) and the (ME) which was intensely stained (Fig. 1I–L).

The mean values of the synapse immunolabeling intensity showed that the exposure of *A. mellifera* larvae to sublethal concentrations of TMX decreased the levels of synapsin in the brain of pupae and newly emerged bees (Fig. 2). The differences observed between the means of control group (87.980 ± 28.079) and the exposed groups were in the MBs of pupae that were exposed to TMX concentrations of 0.001 (58.113 ± 20.945) and 1.44 ng/μL (57.938 ± 19.739) (one-way ANOVAs: $P = 0.029$ and $P = 0.042$, respectively) and in the MBs of newly emerged bees that were exposed to 1.44 ng/μL (58.086 ± 18.352) ($P = 0.005$, one-way ANOVA) (Fig. 2A–B). The ALs was also particularly affected. Differences were observed in both pupae (59.643 ± 23.130) and newly emerged (58.254 ± 18.369) honey bees compared to controls pupae (100.120 ± 30.778) and control newly emerged (116.859 ± 29.677) when they were previously exposed to 1.44 ng/μL of TMX (one-way ANOVAs: $P = 0.015$ and $P = 0.048$, respectively) (Fig. 2C–D). In the OLs, no effect of the exposure to TMX to synapsin immunolabeling was observed ($P > 0.05$, one-way ANOVA) (Fig. 2E–F).

4. Discussion

The sublethal effects of neonicotinoids during the post-embryonic development of the honey bee remain poorly explored, although there is an intrinsic relationship between integrity of development and colony viability and survival (Blacquière et al., 2012). This study is the first that demonstrates the neurotoxicity of sublethal concentrations of TMX in pupae and newly emerged africanized *A. mellifera* after exposure during the larval phase, as shown by reduced synapsin expression in the MBs and ALs.

The three brain structures investigated in this study, MBs, ALs, and OLs are known for their neurophysiological importance. The MBs are involved in learning and memory formation and consolidation, the ALs underlies the abilities of recognition and olfactory learning, and finally the OLs are responsible for capturing visual stimuli (Menzel, 2012). Although the MBs and ALs structure have their own neural specificity, the can work jointly, especially in the memory of olfactory stimuli (Sandoz, 2011).

In the present study, larvae exposed to TMX exhibit a decrease in the level of the synapsin protein in the MBs of pupae at 0.001 and 1.44 ng/μL, in the MBs of newly emerged bees at 1.44 ng/μL, and in the ALs of pupae and newly emerged bees at the concentration of 1.44 ng/μL.

Synapsins are known as a family of phosphoproteins localized in presynaptic boutons. These proteins regulate the availability of synaptic vesicles for exocytosis of neurotransmitters and their release in the synaptic cleft, and thereby participate to the synaptic transmission of the nerve signal (Hilfiker et al., 1999; Klagges et al., 1996). The fine tuning of synapse formation, plasticity, and remodeling depends on the effectiveness of the synapsins (Cesca et al., 2010; Humeau et al., 2011).

The anti-synapsin antibody used in the current study identifies the synapsin associated with synaptic vesicles (Groh et al., 2014; Klagges et al., 1996). It has been shown that TMX causes a decrease in synapsin. We do not know what physiological events led to this reduction. It is possible the decrease in synapsin as part of an organism response in the attempt to stabilize or balance the levels of neurotransmitters released, as a consequence to the continuous stimulation caused by thiamethoxam, which may have resulted, for example, in a reduction in the number of synaptic vesicles and consequently a reduction of synapsin levels.

Damage caused by lack of synapsin can be exemplified by studies with the *Drosophila* fly. Flies that had the synapsin gene eliminated had no changes in brain morphology or behavior. However, when behavioral responses were analyzed, there were differences between mutant and wild flies, including learning and memory defects (Godenschwege et al., 2004). Analyses from mutants *Drosophila* larvae reveal that without the synapse, flies can learn and remember, but there is no memory formation (Kleber et al., 2015). These works suggest the involvement of the synapse in plasticity including the performance on olfactory association. We can predict that decreases in synapsin as a consequence of the exposure of honey bee larvae to TMX, may compromise neurophysiology and consequently reflect on the behavior of honey bee.

Recently, Peng and Yang (2016) have associated a decrease in the density of micro-glomeruli (MG) which are synaptic complexes, of the MBs with a possible abnormal olfactory and visual functions in honey bees previously exposed to sublethal concentrations of IMD during the larval phase. As the synapsin can be found inside the MG (Frambach et al., 2004), it is possible that the decrease in the level of synapsin observed here is a consequence of the decrease in the density of MG. The relationship between microglomerular density and stable olfactory memory formation was studied by Hourcade et al. (2010). This study showed that there are robust alterations in the synaptic architecture in the calyx region of the MBs as a response to a specific odor.

Here, interestingly, no effects of TMX on the OLs were observed. It is necessary to correlate the effects in the MBs with those in the OLs. In the MBs, the CO and a part of the BR receives inputs of visual information. Thus, we can assume that the observed changes in these MB regions can substantially affect the processing of visual input.

TMX can contaminate the nectar and pollen that will serve as the basis for larvae feeding. Pollen and nectar are processed and will be transformed into bee bread by the addition of honey bee secretions and lactic acid fermentation (Vásquez and Olofsson, 2009; Anderson et al., 2014). Few studies show the amounts of TMX present in bee bread, and to date it is unknown what the mechanisms of chemical biotransformation. The concentrations of TMX in bee bread range from 1 to 4.3 μg/kg (Pilling et al., 2013; Lawrence et al., 2016; Pohorecka

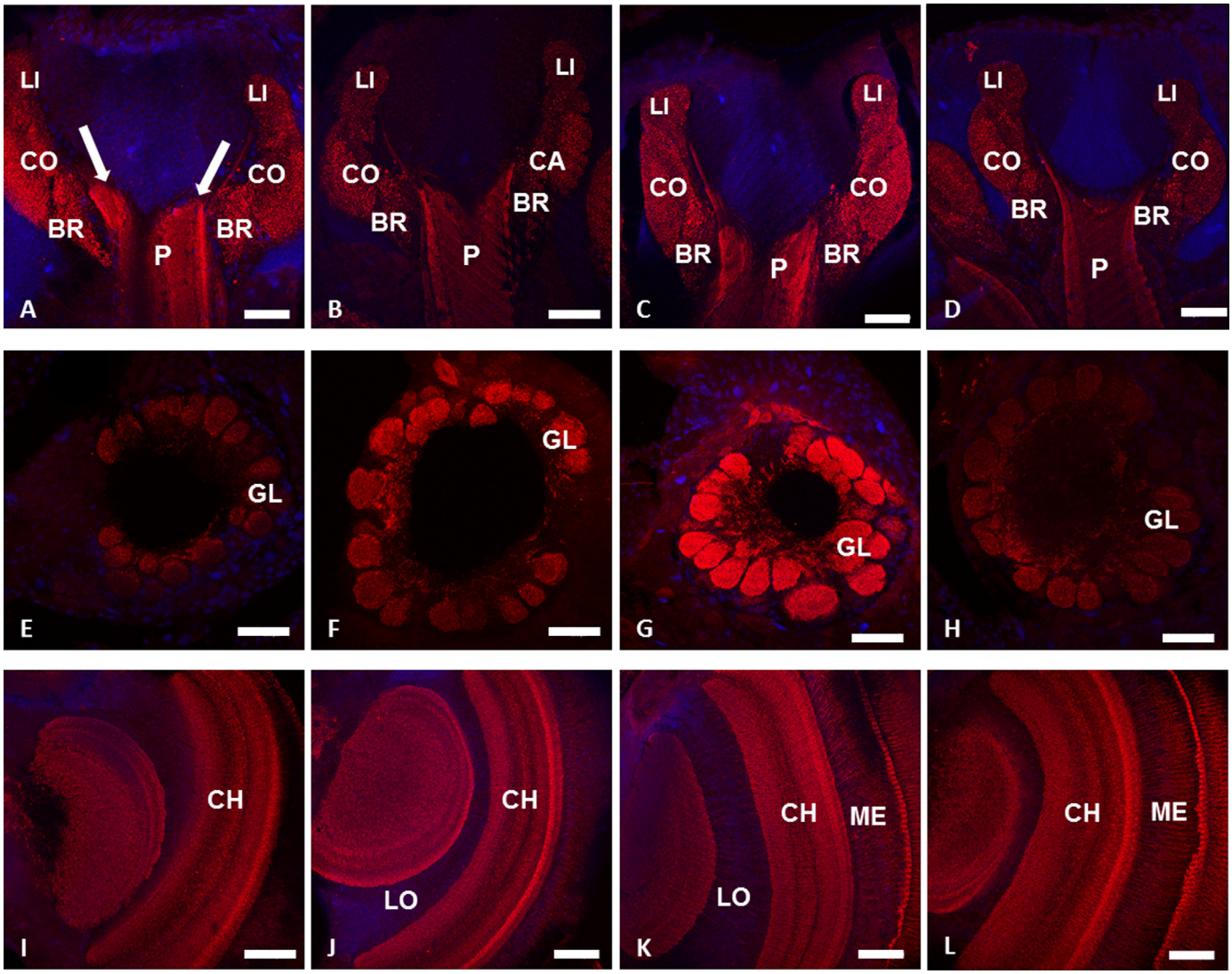

Fig. 1. Effect of thiamethoxam on the synapsin level in the brain of pupal and newly emerged honey bees exposed during the larval phase. Immunolabeling of synapsin was achieved in the mushroom bodies (MBs, A–D), antennal lobes (ALs, E–H) and optic lobes (OLs, I–L) of bees exposed or not (control) to thiamethoxam during the pupal phase. Red fluorescence, synapsin labeling. Blue fluorescence, nuclei labeling with the DAPI DNA probe. Pictures (A), (E) and (I) were respectively obtained from the MBs, ALs and OLs of control pupae. Pictures (B), (F) and (J) were obtained from pupae respectively exposed at the concentrations of 0.001, 1.44 and 0.00001 ng/μL thiamethoxam. Pictures C, G and K were respectively obtained from the MBs, ALs and OLs of control newly emerged bees. Pictures (D), (H) and (L) were obtained from newly emerged bees respectively exposed at the concentrations of 0.00001, 1.44 and 0.001 ng/μL thiamethoxam. Scale bars = 50 μm (A–H) and 100 μm (I–L). Lip (LI); collar (CO); basal ring (BR); pedunculus (P); glomeruli (GL); lobula (LO); medulla (ME).

et al., 2017). Here, the concentration of 0.001 ng/μL, which corresponds to 1 μg/kg, that caused a decrease in synapsin only in the MBs of the pupae, is within the range of those found in the bee bread. Although we cannot explain why this same concentration did not cause changes in the MBs of newly emerged bees, we cannot disregard the neurological damage that this may cause to the bees in the future, especially in the setup of neuronal network, even if the affected pupae survival.

The concentration of 1.44 ng/μL TMX affected the synapsin levels in the MBs and ALs of pupae and newly emerged bees and, although it is a high concentration, we cannot ignore the accumulation of higher levels of TMX by sequential exposure from sources contaminated by the honey bee foragers. In addition, larvae received daily food and may have been finally exposed to high amount of TMX at the end of the larval period.

The results obtained in this study do not enable identifying the mechanism that elicits the decrease in synapsin levels. It is known that the association of synapsin with synaptic vesicles occurs through their phosphorylation. When synapsin is phosphorylated through the inward flow of calcium, it mobilizes from the vesicles to the exocytic cycle. It is possible that TMX may somehow interfere with these neurophysiological mechanisms (Diegelmann et al., 2013; Hilfiker et al., 1999).

This study shows that a sublethal exposure of africanized *A. mellifera* to TMX, at larval stage, induces a decrease in synapsin in pupae and newly emerged honey bees. This decrease might lead to disorders in nervous system functions, such as olfactory learning and the control of neurotransmitter release and might, consequently, impair the behavior and survival of honey bees.

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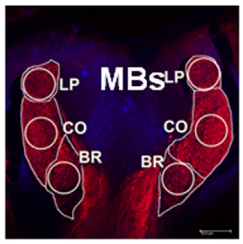

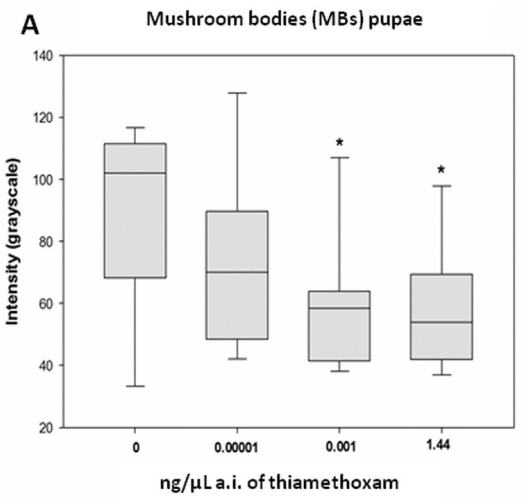

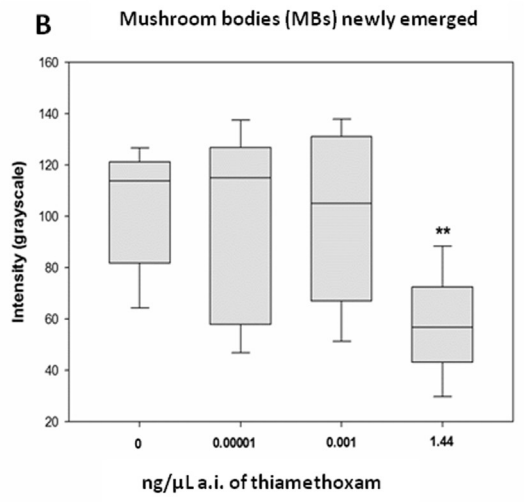

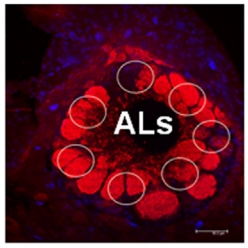

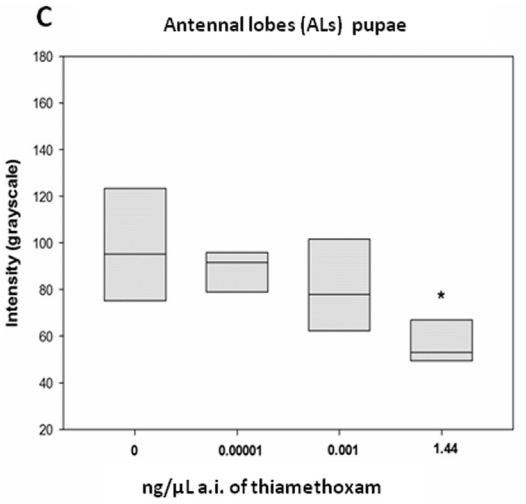

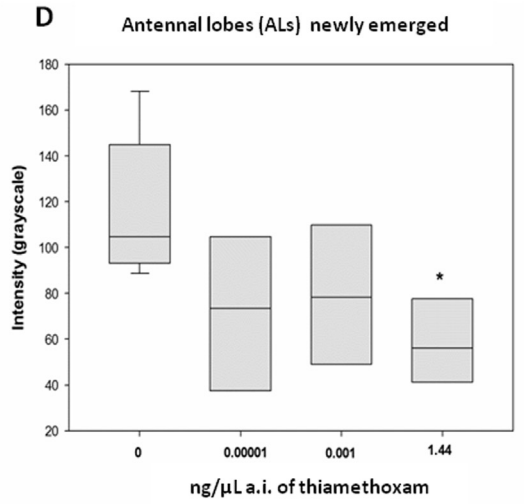

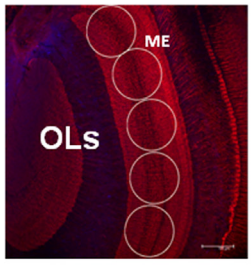

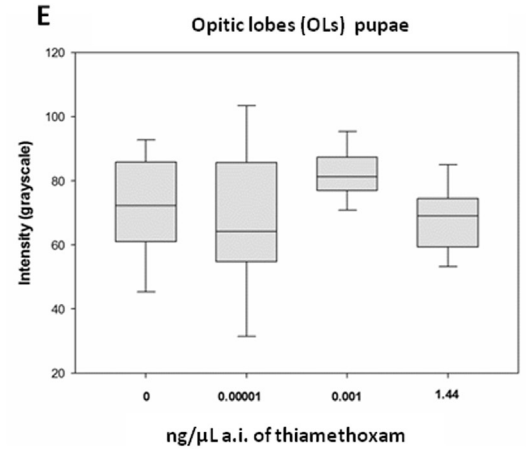

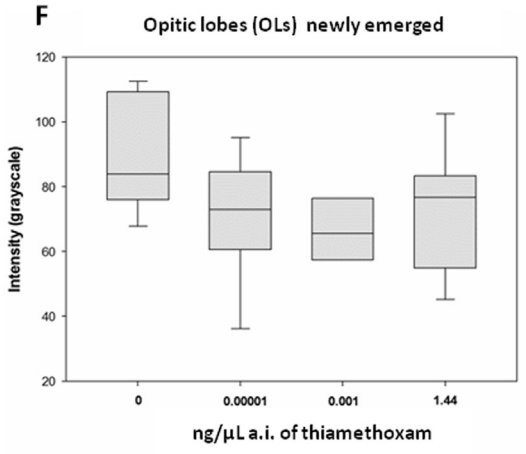

Fig. 2. Effects of thiamethoxam on the synapsin level in the brains of pupal and newly emerged bees exposed during the larval phase.
Immunolabeling of synapsin was achieved in the mushroom bodies (MBs, A–B), antennal lobes (ALs, C–D) and optic lobes (OLs, E–F) of bees exposed or not (control) to thiamethoxam during the larval phase. Fluorescence intensity was quantified in the regions of interest from 3 honey bee brains. Three structures were analyzed from each brain, and for each structure 6 measurements were made (in the MBs and LAs) and 5 measurements in the LOs. In total, 54 measurements were made in MBs and LAs and 45 in LOs. Stars * and ** denote significant differences with controls at $P < 0.05$ and $P < 0.01$, respectively. Lip (LI); collar (CO); basal ring (BR); medulla (ME).

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