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Changes in Synapsin Levels in the Millipede *Gymnostreptus olivaceus* Schubart, 1944 Exposed to Different Concentrations of Deltamethrin

Annelise Francisco,[1] Pablo H. Nunes,[2] Roberta C. F. Nocelli,[3] and Carmem S. Fontanetti[1,*]

[1]*Departamento de Biologia, Instituto de Biociências de Rio Claro, Universidade Estadual Paulista (UNESP), Bela Vista, 13.500-900, Rio Claro, São Paulo, Brazil*
[2]*Centro Interdisciplinar de Ciências da Vida e da Natureza (CICV), Instituto Latino-Americano de Ciências da vida e da Natureza (ILACVN), Universidade Federal da Integração Latino-Americana (UNILA), Avenida Silvio Américo Sasdelli, 1842, 85.866-000, Foz do Iguaçu, Paraná, Brazil*
[3]*Departamento de Ciências da Natureza, Matemática e Educação, Centro de Ciências Agrárias, Universidade Federal de São Carlos (UFSCar), Via Anhanguera, Km 174, 13.600-970, Araras, São Paulo, Brazil*

Abstract: Millipedes are ecologically important soil organisms and may also be an economically threatening species in rural and urban areas when population outbreaks occur. In order to control infestations commercial formulations of deltamethrin have been commonly applied, even though there are few studies about the effects of such insecticide on millipedes. This paper describes the effects of this insecticide on millipedes showing neurotoxic effects assessed by synapsin labeling and confocal microscopy. Deltamethrin concentrations related to the DL_{50} of the active ingredient and a field concentration were applied topically in the diplopod *Gymnostreptus olivaceus* to evaluate the behavior, mortality rate, and synapsin levels in the brain 12, 24, and 48 h after contact with deltamethin. The insecticide caused mortality at the higher concentrations employed, in which no change was observed in neurotransmission in the survivors. In contrast, at field concentrations, deltamethrin did not cause any deaths, but triggered significant changes in synapsin levels. The results obtained form the synapsin labeling provide several interpretations suggesting that the isolated application of this tool must be associated with additional tools in order to evaluate biologically induced effects of deltamethrin in an accurate way. In addition, the feasibility of chemical control of millipedes with deltamethrin is questioned.

Key words: arthropod, confocal microscopy, diplopod, olfactory glomeruli, pyrethroid

Introduction

Millipedes are terrestrial arthropods of the class Diplopoda, whose predominant life form is stratobiont, characterized by inhabiting upper layers of soil and litter (Hopkin & Read, 1992; Golovatch & Kime, 2009). Ecologically, these animals belong to the decomposer trophic level, taking part in nutrient cycling (Schubart, 1942; Petersen & Luxton, 1982; Hopkin & Read, 1992). In addition, millipedes aerate the soil while mixing the humus and mineral contents of the soil promoting its physical improvement (Romell, 1935; Fontanetti, 1989).

Despite providing benefits for soil fertility, millipedes can become agricultural and urban pests when environmental imbalances and climate changes trigger population outbreaks. Millipedes species are known to damage newly germinated seedlings, roots, and fruits in crops, leading to economic loss in rural areas worldwide (Lordello, 1954; Winder et al., 1993; Wightman & Wightman, 1994; Ebregt et al., 2004*a*, 2004*b*). In urban environments, migration and demographic explosions of millipedes are also reported (Cloudsley-Thompson, 1949; Niijima & Shinohara, 1988; Boccardo et al., 2002; Fontanetti et al., 2010). Their presence in high number in such environments is undesirable and their appearance is usually considered repulsive. Contact with their defensive glandular secretions may also result in epidermal reactions in some people (Girardin & Steveson, 2002; Arab et al., 2003; Ruppert et al., 2005).

Thus, a fast and effective means to control infestations of such organisms is demanded. Deltamethrin, a type II synthetic pyrethoid (Chen et al., 2007) has been popularly used for this purpose, even though there is no indication from the manufacturers of its effectiveness against millipedes. This insecticide class is characterized by its toxic effects on the target central nervous system (Larini, 1999).

Voltage-gated sodium channels are the main targets of pyrethroid insecticide action on invertebrates (Scharf, 2003; Zhou et al., 2011; Mccavera & Soderlund, 2012), although several secondary targets of deltamethrin action are also reported (Soderlund et al., 2002). For example, studies on honeybees (*Apis mellifera*) found an increased acetylcholinesterase activity in individuals exposed to deltamethrin doses corresponding to deltamethrin's LD_{50} and half of this LD_{50} for the species (Badiou & Belzunces, 2008; Badiou et al., 2008). Similarly, other secondary targets such as synapsin could also be affected by deltamethrin action.

Synapsins are located at presynaptic terminals of neurons. When a neuron is at rest, synapsin binds to synaptic vesicles,

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*Corresponding author. fontanet@rc.unesp.br

keeping them tethered to the actin cytoskeleton and away from the active zone, forming a reserve pool of synaptic vesicles (Hifiker et al., 1999; Cesca et al., 2010). When an action potential occurs, a cascade of events triggers the phosphorylation of synapsins. This change decreases its affinity both to synaptic vesicles and actin, resulting in the release of synaptic vesicles by exocytosis while the phosphorylated synapsins become dispersed in the cytoplasm (Huttner et al., 1983; Chi et al., 2003; Leitinger et al., 2004; Cesca et al., 2010; Benfenatti, 2011; Diegelmann et al., 2013).

This study aims to evaluate the control of millipedes by deltamethrin under experimental conditions, as well as evaluate the possible effects of this insecticide in the amount of synapsin in the somata of its olfactory glomeruli, as a way to infer neurotoxic effects. The species *Gymnostreptus olivaceus* (Spirostreptidae) was chosen for the tests since it can be found both in forests and cultivated areas. These millipedes were responsible for total loss of melon and beetroot crops in Piracicaba, São Paulo, Brazil, in 1952 (Lordello, 1954). Currently, millipedes of the species *G. olivaceus* are abundant in the region of study and may invade urban areas and damage home gardens.

Materials and Methods

Specimens of *G. olivaceus* were collected around the city of Rio Claro, São Paulo, Brazil (22°24'36" S and 47°33'36" W Gr.) from March 2013 to February 2014 and kept in a terrarium with soil, leaves, wood residues, and potato slices, periodically moistened and kept at a constant temperature of 21°C. The studies were conducted at São Paulo State University (UNESP) and all procedures involving animals were in accordance with the ethical standards of this institution.

We used plastic containers of 12cm diameter (530 mL) lined with moistened filter paper to conduct the bioassays. Potato was provided *ad libitum*. Each container received one adult millipede weighing between 1.5 and 3.5 g. Each millipede received 35 μL of deltamethrin-water solution topically. The application was made on the dorsal surface of the millipedes with a micropipette for accurate dosing. A total of 120 millipedes were used for the experiment. Individuals were divided into one control group (C_0) and four treatment groups (C_{1-3} and C_f) of 24 individuals each, kept in individual containers at 21°C in the dark throughout the bioassay.

Each treatment group was defined by the concentration of deltamethrin applied. The first group of individuals received a solution prepared at concentrations corresponding to one-fifth of the median lethal dose (LD_{50}) established by Boccardo et al. (2001), which is equivalent to 30.8 μg/g of deltamethrin (C_1); the second group was treated by the LD_{50} established in the species, whose value is 154 μg/g (C_2); and the third by a solution in which the concentration correspond to twice the LD_{50}, that is 308 μg/g of deltamethrin (C_3). A field concentration (C_f) which corresponds to the concentration based on recommendations from the manufacturer to combat infestations of other arthropods like *Ceratitis captata* (Mediterranean fruit fly) and *Agotis ipsilon* (black cutworm) was also tested. For this an application of 0.17 μg of the active ingredient per gram of animal's weight, equivalent to 0.11% of the above LD_{50}, was used. The dilutions were made from a commercial product that contains 25 g/L of deltamethrin and 886 g/L of inert ingredients. The control group (C_0) received a topical application of 35 μL of ultrapure water. Individuals were collected after 12, 24, and 48 h of exposure in order to assess the concentration effects, with three replicates collected for each concentration and time analyzed.

Millipedes were anesthesized by freezing and decapitated; the whole heads were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4 for 72 h and transferred to PBS after this period. Each head was rapidly frozen at −15°C and about 250 μm were removed from the front of the head with a Leica CM1860 cryostat (Leica Biosystems Nussloch GmbH, Germany) in order to expose the part of the brain to be examined. Following the preparation, the material was defrosted at room temperature and stored in PBS.

Synapsin labeling was performed in whole mount samples in micro-test tubes in which the material was permeabilized with a solution containing 0.3% Triton-X100 and 5% bovine serum albumin for 1h, washed twice in PBS, and incubated for 4 days at 4°C in monoclonal antibody against synaptic vesicle-associated protein synapsin 1 of *Drosophila* (1:50; SYNORF1) diluted in PBS with 0.2% Triton-X100 and 5% normal goat serum (NGS). The SYNORF1 was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA, USA.

Subsequently, the material was incubated in the secondary antibody Alexa Fluor-633 goat anti-mouse (Molecular Probes, OR, USA) (1:250 in PBS with 1% NGS) for 1h at room temperature, according to procedures described by Hoyer et al. (2005). The synapsin-marked preparations were also labeled for F-actin with Alexa Fluor 488-Phalloidin (Molecular Probes, OR, USA) for 40 min, and incubated in a solution containing DAPI (4',6-diamidino-2-phenylindole) for 10 min for nuclear staining. Finally, the material was mounted on special slides for inverted microscopy with ProLong® Gold antifade mounting medium (Molecular Probes, OR, USA).

The material was scanned at high resolution over a depth of 10 μm at 0.17 μm intervals (40x, digital zoom 2x) on a Leica Confocal Microscope TCS-SP5II (Leica Microsystems CMS, Wetzlar, Germany) with excitation performed with a 405 nm diode laser for DAPI, 488 nm argon laser for Alexa Fluor 488-Phalloidin, and 633 nm HeNe laser for Alexa Fluor-633 (synapsin). For fluorescence quantification, the conditions of laser intensity, gain and offset were also set in advance and kept at 10%, 1,100, and −20, respectively. We used the Leica Application Suite AF 2.6.0 software to process and quantify the fluorescence intensity related to synapsin labeling in the images.

A cortical region related to the olfactory lobes was chosen for quantitative assessment of synapsin expression at

the different deltamethrin concentrations described above. The quantification was performed in maximum projections from three nonoverlapping measurements of 1,008,330 pixels areas in the selected region in each half of the brain, in order to obtain a mean value for each individual. The mean value of emitted fluorescence intensity was measured in gray scale.

Three individuals were analyzed for each concentration of insecticide and exposure time, and each one was a true replica. The data obtained were organized into groups formed by the intersection of the independent variables *time* and *concentration* and statistically evaluated by SPSS Statistics (SPSS v.22; Chicago, IL, USA) software. The data were first submitted to the Shapiro–Wilk normality test and Levene homogeneity test. Meeting the normality assumptions ($p > 0.1$ in all groups), but not the homocedasticity, the data were evaluated using the one-way analysis of variance with robust test for the equality of variance F-Brown-Forsythe, followed by the *posthoc* Games-Howell test, suitable for equal variances not assumed, according to the procedures described by Marôco (2014). A significance level (α) of 0.05 was considered.

Results

Bioassays with *G. olivaceus* and Deltamethrin

The millipedes employed in the bioassay had an average body weight of 2.51 ± 0.48 g, and no difference in the average masses was found between groups ($F_{4,115} = 0.581$, $p = 0.677$). During the bioassay, the millipedes belonging to the control group (C_0) were curled under the food or under the filter paper. Food consumption was noticed in all replicas.

The group exposed to the field concentration (C_f) showed a similar behavior, except by releasing defensive secretion and gonopods eversion in one individual and more agitation of individuals than in the control group in the first 24 h. No deaths were observed in either the C_0 or C_f groups, even after the 48 h of experiment.

In groups C_1–C_3, all individuals released a defensive secretion in the first hour of the experiment. Immediately after contact with the insecticide, all individuals of C_2 and C_3 showed oscillatory body movements. After 12 h, 75% of C_1 had oscillatory and slow head movement but did not curl themselves; they remained stretched over the filter paper or turned upside down, exposing the ventral part of the body and moving the legs slowly (Fig. 1a). At C_2 and C_3, the higher concentrations, and after 24 h, such behavior was also observed, but 80% of the millipedes were rolled and reacting subtly only when stimulated by a toothpick.

After 48 h, gonopod externalization was observed in 60% of C_1-collected males and 25% of C_2-collected males. The integument became brittle in all individuals of C_2 and C_3, and three individuals of C_2 had cracks where the hemolymph was leaking (Fig. 1b). The insecticide was able to cause mortality in diplopods at C_1, C_2, and C_3, but C_3 induced the highest number of deaths during the bioassay.

Confocal Analysis of Synapsin Distribution

In median cross-sections of *G. olivaceus* protocerebrum, synapsin immunolabeling was observed in the cortical region, as shown in Figure 2. The F-actin labeling was weak in the neuropil (Fig. 2a) and was located around neuron cell bodies. These F-actin labelings resembled boundaries within which the synapsin was immuno-localized (Fig. 2b).

Among the labeled somata, the antero-proximal region was selected for synapsin analysis because it is associated with the olfactory glomeruli's neuropilar region (Francisco et al., 2015) (Fig. 2a). The labeling was in the somata of neurons, exhibiting a distinctive granular texture (Fig. 2b).

With data from the quantification of fluorescence from synapsin immunolabeling in such a region, the F-Brown-Forsythe showed a significant difference in the mean fluorescence intensity between groups ($F_{14,13,095} = 3{,}034$, $p = 0.026$), while the groups represent the different deltamethrin concentrations and times after the application. The Games-Howell *posthoc* test showed a significant difference

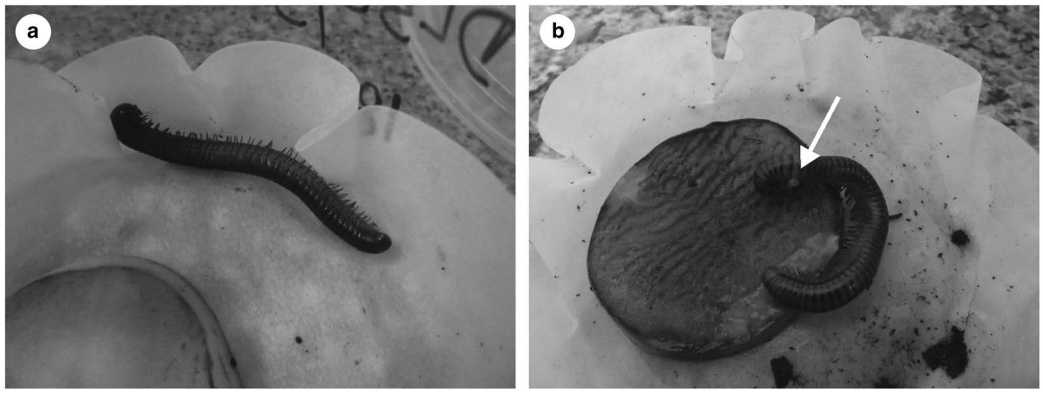

Figure 1. Millipedes exposed to deltamethrin. **a:** C_1 millipede, 12 h after deltamethrin application, twisting itself and exposing the ventral part of the body. **b:** C_2 millipede, 48 h after deltamethrin application. Arrow, crack in the integument.

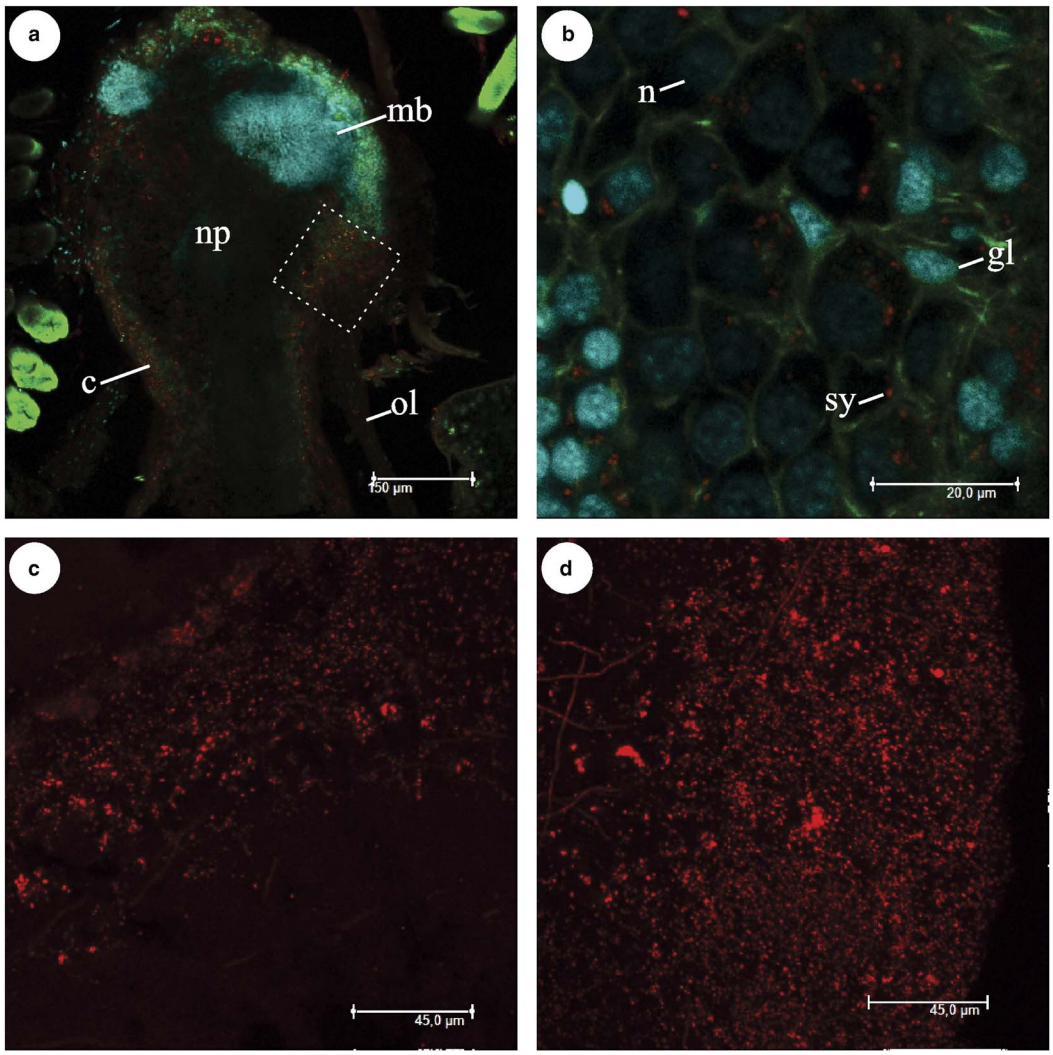

Figure 2. Maximum projections of confocal microscopy images, labeled with Anti-SYNORF1 and Alexa Fluor-633 (for synapsin) in red, Alexa Fluor 488-Phalloidin (F-actin) in green, and nuclei (DAPI) in cyan. **a:** Cross-section of half protocerebrum. The area within the square is the region selected for analysis. **b:** Synapsin labeling in the cortical cells of *Gymnostreptus olivaceus*' brain. **c:** Synapsin labeling in the control group (C_0). **d:** Synapsin labeling in the field concentration (C_f). c, cortex; gl, glial cell nucleus; mb, mushroom body; n, neuron nucleus; np, neuropil; ol, outer cell layer; sy, synapsin immunolabeling.

from control group only in C_f and C_1 after 12 h of exposure (Figs. 2c, 2d, 3, Table 1).

Discussion

In the present study, deltamethrin had a clear action on the millipede nervous system at the concentrations C_1, C_2, and C_3, causing behavioral changes and movement abnormalities. At these concentrations, the insecticide also affected the animal's integument. Thus, besides the mortality observed here, the moribundity was high, since under natural conditions such millipedes would be completely vulnerable to predators and infections.

The data reported here suggest that one-fifth of the lethal dose LD_{50} (C_1) would be the minimum recommended for chemical control of millipedes with deltamethrin. However, this concentration is higher than that recommended by the manufacturers of commercial formulations and it is also higher than the field concentration adopted in this study, which did not cause mortality or moribundity in millipedes even after 48 h.

Although chemical control of millipedes using deltamethrin is a popular practice, our results show that it would not be recommended, since only a high concentration of this product can cause mortality. These concentrations can harm nontarget organisms like honeybees. Decourtye et al. (2004) describes that a deltamethrin concentration of 500 µg/kg in syrup had a lethal effect on workers of the bee *A. mellifera*. Such syrup contamination decreased foraging activity and activity in the hive entrance. This concentration is lower than that which would be available in the environment by applying one-fifth of the LD_{50} of *G. olivaceus*.

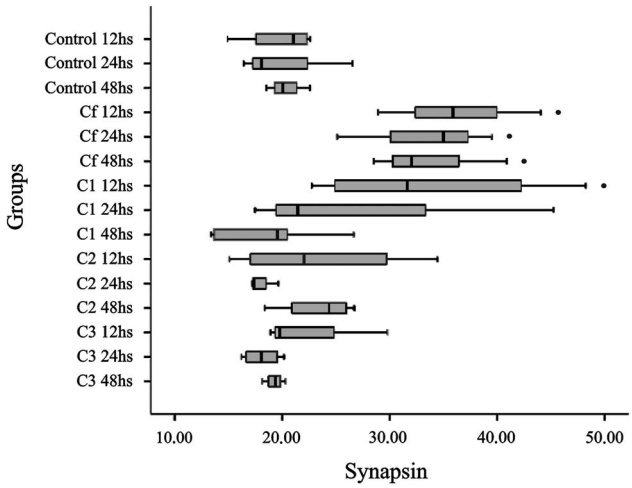

Figure 3. Box plot showing the range of means with standard deviations of the synapsin concentrations measured in the different groups. The dots indicate significant *posthoc* comparison according to the Games-Howell test ($p < 0.05$).

Deltamethrin is considered safe for mammals, however, studies using mice have shown it is capable of causing harming effects such as reduced density of muscarinic cholinergic receptors in the cerebral cortex of animals exposed to it during embryological development (Eriksson & Fredriksson, 1991) and cerebral degeneration and apoptosis in exposed adult animals (Wu & Liu, 2000*a*, 2000*b*; Chen et al., 2007). These results suggest caution in the use of this chemical.

When millipede brains were evaluated by synapsin immunolabeling, the staining pattern differed from what is usually described for other invertebrates. Anti-synapsin antibodies label synaptic vesicles (Groh et al., 2014). In most invertebrates, synapsin immunolabeling shows the presence of synapsin in neuropil regions, where synaptic contact between neurons occurs (Ott, 2008; Sombke et al., 2011). In the ant *Cardiocondyla obscurior*, the antibody against synapsin reacted strongly with synapses and was located mainly in neuropil compartments (Bressan et al., 2014). In *G. olivaceus*, in turn, synapsin was observed in cortical regions in the cell bodies of neurons. This feature suggests there are axosomatic or axodendritic synaptic contacts in these regions.

Furthermore, F-actin labeling was observed encircling neuronal cell bodies, while this labeling was weak and did not allow any distinction in the neuropil. In *A. mellifera* and in the centipede *Glomeris marginata*, phalloidin labeling occurred predominantly in axons and dendrites and allowed neuropil visualization (Groh et al., 2004; Sombke et al., 2011). Thus, the F-actin content of *G. olivaceus* is also distributed in a different way in the nervous system.

Functionally, the synapsins are responsible for keeping a reserve pool of synaptic vesicles that is not available for immediate release, and the maintenance of a dynamic balance between the reserve pool, the readily releasable pool, and the pool of vesicles engaged in the exo-endocytic cycle (Diegelmann et al., 2013). Therefore, synapsin establishes the extent of reserve pool synaptic vesicles and thus determines the availability of vesicles to exocytosis in the subsequent cycles (Hifiker et al., 1999).

Nevertheless, synapsins are not essential for synaptic transmission, even though they are important proteins for fine adjustment of synapse formation, remodeling, and plasticity (Benfenati, 2011; Humeau et al., 2011). In addition, synapsins are involved in neurotransmitter release regulation (Hifiker et al., 1999) and may be necessary for achieving appropriate associative functions (Diegelmann et al., 2013).

An increase in synapsin levels was observed after exposure of *G. olivaceus* to the lower concentration of deltametrhin used here. Considering that the antibody against synapsin labels both free and bound synapsin, this observation may lead to two hypotheses. (1) It can be supposed that an increased amount of synapsin means an increase in the reserve pool of synaptic vesicles. Synapsin tethering synaptic vesicles to the cytoskeleton ensures that the neurotransmitters present inside them are released in the subsequent cycles of exocytosis. Consequently, an increased amount of synapsin may mean abundance of synaptic vesicles available for exocytosis, with no disruption of synapsin function, but rather an increased synaptic activity. (2) Alternatively, it can be supposed that an increased amount of synapsin means a shift in the dynamic equilibrium between the different pools of vesicles because synapsins may be phosphorylated, resulting in fewer vesicles available for release and therefore less synaptic activity.

Table 1. Synapsin Average Concentration at Different Concentrations of Deltamethin and Exposure Times, with Statistical Analysis.

	Average Concentration of Synapsin					*Posthoc* Test (Games-Howell)			
Exposure Time	Control	C_f	C_1	C_2	C_3	C_f versus Control	C_1 versus Control	C_2 versus Control	C_3 versus Control
12 h	19.92 ± 1.74	36.28 ± 4.37	33.54 ± 5.63	23.38 ± 4.20	22.82 ± 3.48	0.000*	0.001*	0.876	0.822
24 h	20.36 ± 3.13	33.21 ± 4.25	28.05 ± 8.67	18.06 ± 0.79	18.10 ± 0.89	0.000*	0.656	0.966	0.938
48 h	20.40 ± 1.19	33.80 ± 3.67	18.75 ± 2.46	23.43 ± 1.81	19.26 ± 0.62	0.000*	1.000	0.727	1.000

Concentrations of synapsin are presented as mean ± standard deviation.
Control received topical application of ultrapure water; C_1 received one-fifth of the LD_{50} established, that is 30.8 μg/g of deltamethrin; C_2 received the LD_{50}, that is 154 μg/g of deltamethrin; C_3 received twice the LD_{50}, that is 308 μg/g of deltamethrin; and C_f received a field concentration, that is 0.17 μg/g of deltamethrin.
*Statistically significant at 0.05 level.

The effect of deltamethrin on synapsin may be a result of calcium-dependent ATPase inhibition, an effect described for both deltamethrin and other type II pyrethroids (Orchard, 1980; Clark & Matsumura, 1982). ATPase inhibition leads to increased intracellular calcium levels. Calcium is essential for synapsin phosphorylation, which is required to mobilize the reserve pool of synaptic vesicles and to release them by exocytosis (Cesca et al., 2010). This mechanism corroborates the increased release of neurotransmitters triggered by deltamethrin (Orchard, 1980; Clark & Matsumura, 1982; Soderlund & Bloomquist, 1989; Soderlund et al., 2002) and may be related to the greater agitation in C_f, where significantly more synapsin was detected. Thus, it can be inferred that deltamethrin caused an increase in synapsin synthesis and increased intracellular calcium levels, triggering synapsin phosphorylation and providing high neurotransmitter release, with diplopod agitation as a behavioral consequence.

This effect lasted for 12 h at C_1, the second lowest concentration. After this period, the synapsin labeling returned to levels similar to those measured in the control group. In this case, the high concentration of deltamethrin led to large amounts of intracellular calcium that phosphorylated the synapsins, which were dispersed in the cytoplasm. Synapsin functional disruption and synthesis decrease may also have occurred, causing a decrease in the amount of detectable synapsin.

A synapsin functional breakdown is expected to increase neurotransmitter release by increasing the amount of vesicles available for release. However, an inhibition of neurotransmitter release may occur if these dispersed vesicles are not held in place, but instead diffuse away from the vesicle pool in the cytoplasm and dissipate (Hifiker et al., 1999). In mutant *Drosophila* larvae lacking synapsins, synaptic vesicles are distributed over larger areas of the cytoplasm and they are not part of the reserve pool (Diegelmann et al., 2013).

In C_2 and C_3, the higher concentrations tested here, the synapsin levels were also similar to those found in the control. It should be highlighted that such concentrations, although not lethal to millipedes, are extremely high. In this sense, this absence of changes in synapsin levels can be explained by other physiological changes induced by the insecticide, which may have counterbalanced a synapsin synthesis stimulus. The lack of ATP, for example, may decrease the amount of free synapsin (Huttner et al., 1983).

Conclusion

The present study indicated an early effect on synapsin induced by the lowest concentration of deltamethrin, indicating a precocious response of the millipede brain to the insecticide exposure. Therefore, deltamethrin induced measurable changes in the millipede brain. Taken together with the availability of millipedes in almost all terrestrial environments, these observations pave the way for the use of diplopods in the assessment of contaminants neurotoxicity. However, synapsin, showed changes only at low concentrations of insecticide and provided several possibilities of physiological interpretations. In this way, synapsin is not precise about triggered effects and its use alone is not strongly justified. We should emphasize that synapsin levels were not evaluated in chronic insecticide exposure and the comments made here are valid only for acute exposure.

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