International Journal of Agriculture Innovations and Research
Volume 6, Issue 3, ISSN (Online) 2319-1473
Manuscript Processing Details (dd/mm/yyyy):
Received: 12/11/2017 | Accepted on: 18/11/2017 | Published: 29/11/2017

Toxicological and Morphological Comparative Studies of Insecticides Action in Leaf-cutting Ants

Gabriela Ortiz[1], Alexsandro S. Vieira[1]* and Odair C. Bueno[1]
[1]Centro de Estudos de Insetos Sociais, Instituto de Biociências, UNESP - Univ Estadual Paulista, Campus Rio Claro-São Paulo, Brazil, Av. 24A, 1515 - Bela Vista, 13506-900.
*Corresponding author email id: alexsvieira@yahoo.com.br

Abstract **– Leaf-cutting ants are considered pests in agriculture, causing great economic losses. The aim of this study was to evaluate the effects of the insecticides Fipronil and Thiamethoxam on *Atta sexdens rubropilosa* workers through toxicological and morphological bioassays. The workers were submitted to applications of the 1 µl Fipronil and Thiamethoxam at sublethal doses: LD_{50}, $LD_{50/10}$ and $LD_{50/010}$ (acetone was used as control). The results obtained by the confocal microscopy showed loss of fluorescence in the postpharyngeal glands, midgut, and Malpighian tubules during treatment with both insectides, which revealed the cytoskeleton degradation. However, during treatment with Thiamethoxam, was observed that loss was not as evident as in the groups treated with Fipronil. In the histological analysis during treatment with both insecticides, there was a notable difference in the structure of the organs where characteristics indicative of cell death were observed. The combination of morphological data with data from bioassays of toxicity suggests that Fipronil acts directly on the organs analyzed, with a higher efficiency than Thiamethoxam and can therefore be considered more toxic in this study.**

Keywords **– Postpharyngeal gland, Insecticides, morphological changes, Cell death, Midgut, Malpighian tubules, Confocal microscopy.**

I. INTRODUCTION

Leaf-cutting ants are the dominant species in both natural and human-disturbed settings where they occur, and are considered to be a keystone species because of their influence on the environment; these ants contribute largely to environmental diversity, productivity, and nutrient and energy flow [1]-[2]. More recently, leaf-cutting ants have been regarded as ecosystem engineers because they modulate, directly and indirectly, resource availability to other species by changing the physical state of biotic and abiotic materials [3]-[4]-[5]-[6]-[7]. Their activity modifies soil properties by improving aeration, drainage and root penetration, and by increasing organic matter and nutrient mineralization and availability [8]-[9]-[10]-[11]-[12]-[13]. Secondary seed dispersal and improved germination by manipulation have also been associated with the activity of leaf-cutting ants [14]-[15] and their symbiotic relationships have led to recent antibiotic discoveries, including candicidin and dentigerumicin [16]-[17] In addition, leaf-cutting ant colonies are considered to be models for designing improved agricultural management for sustainable production.

The notoriety of leaf-cutting ants as pests is in startling contrast to their environmental importance and benefits. This apparent paradox is not difficult to understand in areas where leaf-cutting ants are native, however, in human-altered environments (e.g. agriculture and reforestation fields) these ants are considered important pests. In silviculture, agriculture, and pasture in Brazil because they cause severe defoliation of plants [18]-[19].

Cultural, biological, and chemical approaches have been studied to control these ants [20], but the only large-scale technology available is based on chemical control. Chemical strategies differ in their formulation and mode of application, and fogging and toxic baits are the most efficient strategies for ant control [21]. The incorporation of toxic baits in the food cycle of ant colonies is efficient and allows the insecticide to act after ingestion [22]. Nevertheless, this system requires slow toxic activity of the active ingredient present in the bait so that it can be widely distributed throughout the colony before the first symptoms of poisoning develop [23]-[24]. Recent toxicological analyses of several active ingredients used in pest control revealed that cellular respiration inhibitors yield satisfactory results for use in the control of leaf-cutting ant [25]-[26]-[27].

Insects, weeds and diseases are major biotic factors that cause losses in agricultural production. It makes necessary the continuous development of new molecules [28] with selective properties and specific action [29] is necessary for their control. To be launched in the market, insecticides are subjected to a battery of ecotoxicological tests to ensure that they are safe for the environment, including the pollinators [30]. Fipronil is an insecticide that is registered for use in Brazil. It is a neurotoxic insecticide from the phenylpyrazole group, which acts as a blocker from the gamma-aminobutyric acid receptor (GABA), resulting in non-inhibition of nerve impulses. It results in hyperexcitation of the nervous system, paralysis and death of the organism [31]. Fipronil is recommended for insects control in various crops such as sugarcane, soybeans, corn, eucalyptus and others [32]. However, beyond the target insects, Fipronil has demonstrated high toxicity on insects no target [33].

Thiamethoxam is a systemic insecticide in the class of neonicotinoids. It has a broad spectrum of activity against many types of insects. Thiamethoxam is a broad-spectrum, systemic insecticide, which means it is absorbed quickly by plants and transported to all of its parts, including pollen, where it acts to deter insect feeding. An insect can absorb it in its stomach after feeding, or through direct contact, including through its tracheal system. The compound gets in the way of information transfer between nerve cells by interfering with nnicotinic acetylcholine receptors in the central nervous system, and eventually paralyzes the muscles of the insects [34]. Although several studies have

evaluated the toxic activity of chemicals in ants, only a few have described the morphological alterations in insect tissues caused by these compounds [35].

The postpharyngeal glands is part of salivary system of ants and regardless of sex, caste or mode of life are present in pairs and are located dorsally in the transition between the pharynx and esophagus [36]-[37]. They originate from the ectoderm and emerge during the post-embryonic development (final phases of pre-pupa) from two dorsal evaginations of the pharyngeal epithelium [38]-[39]. We believe that the postpharyngeal glands of Formicidae, the functions of which remain unclear, may exhibit morphological alterations caused by toxic compounds present in the hemolymph.

The midgut and the Malpighian tubules are involved in the absorption and excretion of chemical compounds, respectively. Morphological analysis of these organs may reveal ultra-structural alterations caused by toxic substances [40]-[41]. According to Sumida et al. [35], a chemical compound that is absorbed by the gut, but not properly excreted by the Malpighian tubules, may remain in the hemolymph and indirectly interfere with the absorption of glandular product precursors from the hemolymph by the glands.

Cintra-Socolowski et al. [42] verified the neurotoxic action of sublethal doses of fipronil on the mushroom bodies of brains from the leaf-cutting ant *Atta sexdens rubropilosa* through immunocytochemistry analysis for the protein synapsina and concluded that sublethal doses of the insecticide fipronil intensified synapsin immunostaining, suggesting an increased release of neurotransmitters, which may be linked to neurotoxicity and overexcitation. Decio et al.[43] already evaluated the effects of hydramethylnon on *Atta sexdens rubropilosa* workers through toxicological bioassays and morphological analysis of the post-pharyngeal glands, midgut, and Malpighian tubules; the results revealed morphological alterations in the midgut and Malpighian tubules of workers treated with HA, whereas alterations of the post-pharyngeal glands were observed in the HAO-treated group indicating that the presence of soy oil provided an alternate route for the ingestion of the formicide's active ingredient.

When deciding which type of microscopy to use, one may ask: "Why confocal microscope?" Transmission electron microscopy offers superb resolution, however it is damaging to living specimens and suffers from fixation and sectioning artifacts. Conventional light microscopy allows examination of living and fixed cells with a variety of imaging modes including fluorescence. However, ultrastructural details cannot be obtained because of the relatively low resolution of the light microscope. Another difficulty with conventional light microscopy is that it suffer from out-of-focus information, which often blurs the image. Video image processing can help "clean up" the images by improving contrast and detection, but does not completely eliminate the problem [44].

Therefore, the aim of this study was to evaluate the toxicity of Fipronil and Thiamethoxam on *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae) workers, as well as to perform morphological analysis of the postpharyngeal glands, midgut and the Malpighian tubules in order to verify the toxic effects of these insecticide on cellular level.

II. Material and Methods

1) *Bioassays*

The *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae) workers used in the assays were randomly collected from laboratory nests on the São Paulo State University (UNESP) campus in Rio Claro, São Paulo State, Brazil. Their body mass was 20–25 mg. Prior to initiation of the assays, the nests were supplied daily with leaves of Eucalyptus alba, oat seeds and occasionally with the leaves of other plants such as *Hibiscus* sp., *Ligustrum* sp. or rosebush petals. Forty ants were taken from the nests and put into four Petri dishes (ten ants each) for each treatment. During the assays, the ants were maintained on an artificial diet prepared with glucose (50 g L^{-1}), bacto-peptone (10 g L^{-1}), yeast extract (1.0 g L^{-1}) and agar (15 g L^{-1}) in distilled water (100 mL) [45].

The ants were maintained in na incubator for 24 h at a temperature of 25 ± 1º C and a relative humidity ranging between 70 and 80%. The active ingredient fipronil used in the tests had greater than 99% purity, from a test batch kindly provided by the insecticide industry. The main solutions, from which all other concentrations were made, was prepared by diluting 0.001 g of active ingredient in 100 mL of acetone, forming a concentration of 10 µgmL^{-1}. The solution was kept in amber glass and cold (3.0º C) until use. For Fipronil the LD_{50} value was established as 1.42 ng ant^{-1}, and the sublethal doses used were $LD_{50/10}$ 0.142 ng ant^{-1} and $LD_{50/100}$ 0.0142 ng ant^{-1} [42]. For Thiamethoxam the LD_{50} value was established as 9.31 ng ant^{-1}, and the sublethal doses used were $LD_{50/10}$ 0.931 ng ant^{-1} and $LD_{50/100}$ 0.0931 ng ant^{-1}.

The ants of the experimental group received a topical application (with the aid of a micropipette) of 1 mL of solution containing the insecticide to be tested on the dorsal thorax and were then transferred to Petri dishes. The ants of the acetone control group received 1 mL of acetone, and the ants of the control group were only placed in Petri dishes without any contact with acetone or insecticide. After application of the solutions containing the active ingredient, the deadants were counted for each concentration after 4h, 24 h and 48h.

2) *Postpharyngeal glands, midgut and the Malpighian tubules preparation, immunofluorescence and nuclear staining.*

The organs were extracted from the samples 4h, 24 h and 48h after application of 0.42, 0.142 and 0.0142 ng ant^{-1}. The ants were anaesthetised at 0º C for 3 min and decapitated. The organs were immediately dissected in phosphate-buffered saline (PBS). After dissection, were immediately fixed in 4.0% paraformaldehyde for 2 h. After fixation, two rinses in PBS were performed, followed by permeabilisation with 0.2% Triton X-100 (in PBS) for 30 min. After two additional rinses in PBS, the material was incubated overnight with a mouse monoclonal antibody (MOUSE) against the α-tubulin and β-tubulin. After

washing in PBS, preparations were incubated with a secondary antibody, a goat antimouse antibody conjugated to Cy5 (1:1000 in PBS), for 2 h at room temperature, then washed 2 times with PBS (5 min each). For the labeling actin material was incubated with Alexa solution Fluor 488 Phalloidin for 30 min at room temperature and in the dark, then was stained with DAPI to mark nuclei for 10 min, washed 2 times with PBS (5 min each). The slides were mounted using Prolong gold antifade reagente. The analysis was performed by laser scanning confocal microscope (ZEISS - LSM780).

3) *Statistical Analysis*

The intensity of the fluorescence emitted (grayscale) both for actin as the tubulin was quantified using the ImageJ software. The Shapiro-Wilk test was carried out to assess normal distribution of data using the statistical software BioEstat, and then the parametric statistical test one-way ANOVA was performed with a significance level of 95%. In cases where significant differences were observed, a pairwise Tukey test was used to check which groups were different from each other, using the software Statistica 7.

4) *Morphological Analysis*

4.1) Harris hematoxylin and aqueous eosin staining [46]

During the assays, the ants were maintained on an artificial diet prepared with glucose (50 g L^{-1}), bacto-peptone (10 g L^{-1}), yeast extract (1.0 g L^{-1}) and agar (15 g L^{-1}) in distilled water (100 mL) [45]. The ants of the experimental group received a topical application (with the aid of a micropipette) of 1 mL of solutions containing the insecticides to be tested (LD_{50}, $LD_{50/10}$ and $LD_{50/100}$) on the dorsal thorax and were then transferred to Petri dishes. The ants of the acetone control group received 1 mL of acetone.

The ants were anaesthetised at 4° C for 3 min and decapitated. To extract the PPGs, midgut and the Malpighian tubules dissection was performed in Petri dishes containing saline solution (20 mM Na^2HPO^4/KH^2PO^4, pH 7.4, and 130 mM NaCl) under a Zeiss stereomicroscope using dissecting forceps. The organs were extracted from the samples 4h, 24 h and 48h after application. The organs were fixed in paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 2h at room temperature, then washed in PBS, dehydrated in a graded alcohol series (70–100%), transferred to the embedding resin, and stored overnight at 4ºC. After embedding in resin, the tissues were immersed in historesin, and 5-µm sections were cut on a microtome. The sections were stained with hematoxylin-eosin, mounted in Canada Balsam, and subsequently were examined under a light microscope (Olympus BX51; Olympus America, Center Valley, PA). Micrographs were obtained using a digital camera (Olympus DP-71) that was fitted to the microscope and coupled to a Dell computer. DP Controller software (Olympus America) was used for image acquisition.

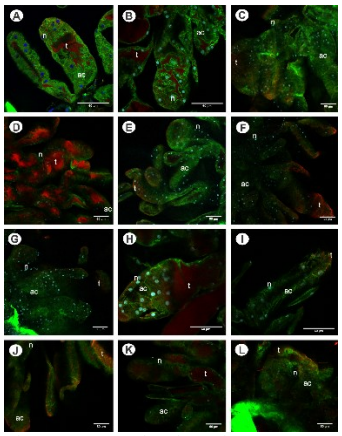

Fig. 1. Confocal microscopy showed loss of fluorescence in the postpharyngeal glands during treatment with Fipronil, which showed a greater fluorescence in control, marking the actin (ac) in green, the tubulin (t) in red and nucleus (n) in blue. - A: control 4h, B: control 24h, C: control 48h; D: $LD_{50/100}$ 4h, E $LD_{50/100}$ 24h, F: $LD_{50/100}$ 48h; G: $LD_{50/10}$ 4h, H: $LD_{50/10}$ 24h; I: $LD_{50/10}$ 48h; J: LD_{50} 4h, K: LD_{50} 24h, L: LD_{50} 48h.

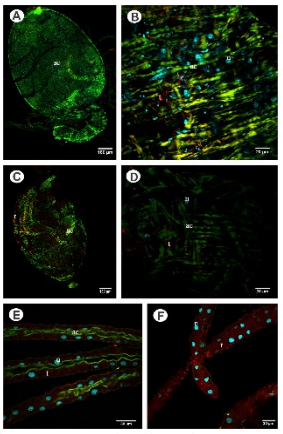

Fig. 2. Confocal microscopy showed loss of fluorescence in the midgut and Malpighian tubules during treatment with Fipronil, which showed a greater fluorescence in control, marking the actin (ac) in green, the tubulin (t) in red and nucleus (n) in blue. – A.B: control 4h. C.D: LD_{50} 48h. E.F. Malpighian tubules – E: control 4h, F: LD_{50} 48h.

III. Results

Using the program laser scanning confocal microscope (ZEISS - LSM780) it was possible to measure the intensity of the emitted fluorescence in the the postpharyngeal glands, midgut and the Malpighian tubules.

The intensity of fluorescence emitted to actin and tubulin was measured using the ImageJ software and applied parametric statistical test one-way ANOVA (BioEstat). For the ratio control group C 48h x groups submitted to treatment in sub-lethal dose of Fipronil $LD_{50/100}$ 48h, $LD_{50/10}$ 48h and LD_{50} 48h there was a decrease of cytoskeleton fluorescence intensity for post pharyngeal gland that showed significant actin differences ($F = 44.2616$, $p < 0.0001$) and tubulin ($F = 8.1588$, $p < 0.0019$), for midgut, actin ($F = 57.3034$, $p < 0.0001$) and tubulin ($F = 69.2708$, $p < 0.0001$) and for Malpighian tubules, actin ($F = 33.1284$, $p < 0.0001$) and tubulin ($F = 45.6081$, $p < 0.0001$) (Table I). For relative control group C 48h x groups submitted to treatment in sublethal dose of Thiamethoxam $LD_{50/100}$ 48h, $LD_{50/10}$ 48h and LD_{50} 48h can also be observed a decrease in cytoskeletal fluorescence intensity for post-pharyngeal gland revealed significant differences actin ($F = 100.0791$, $p < 0.0001$) and tubulin ($F = 14.8226$, $p < 0.0002$), for midgut actin, $F = 44.2578$, $p < 0.0001$) and tubulin ($F = 18.8794$, $p < 0.0001$) and for Malpighian tubules, actin ($F = 31.9036$, $p < 0.0001$) and tubulin ($F = 18.9105$, $p < 0.0001$) (Table II).

The results obtained by the confocal microscopy showed loss of fluorescence in the postpharyngeal glands, midgut and the Malpighian tubules during treatment with the insecticides, which showed a greater fluorescence in control, marking the actin in green, the tubulin in red and nucleus in blue.

International Journal of Agriculture Innovations and Research
Volume 6, Issue 3, ISSN (Online) 2319-1473

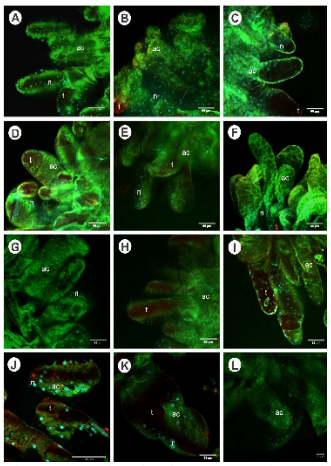

Fig. 3. Confocal microscopy showed loss of fluorescence in the postpharyngeal glands during treatment with Thiamethoxam, which showed a greater fluorescence in control, marking the actin (ac) in green, the tubulin (t) in red and nucleus (n) in blue. - A: control 4h, B: control 24h, C: control 48h; D: $LD_{50/100}$ 4h, E $LD_{50/100}$ 24h, F: $LD_{50/100}$ 48h; G: LD50/10 4h, H: LD50/10 24h; I: $LD_{50/10}$ 48h; J: LD_{50} 4h, K: LD_{50} 24h, L: LD_{50} 48h.

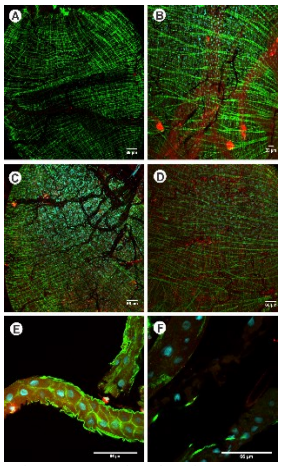

Fig. 4. Confocal microscopy showed loss of fluorescence in the midgut and Malpighian tubules during treatment with Thiamethoxam, which showed a greater fluorescence in control, marking the actin (ac) in green, the tubulin (t) in red and nucleus (n) in blue. – A.B: control 4h. C.D: LD_{50} 48h. E.F. Malpighian tubules – E: control 4h, F: LD_{50} 48h.

The fluorescence of the tissue has been gradually decreasing during treatment with Fipronil and showed weaker still at sublethal dose LD_{50}, which revealed the cytoskeleton degradation (Fig. 1 and 2). During treatment with Thiamethoxam, it can also be observed loss of fluorescence in the organs, but when comparing the control groups with treatment groups is noted that the loss was not as evident as in groups treated with Fipronil, and this loss became more evident only in sublethal dose LD_{50}, corroborating the data obtained with the previously midgut and Malpighian tubules (Fig. 3 and 4).

Fig. 5 and 7 shows the postpharyngeal glands of control ants. The glandular lobular epithelium had cuticles on the apical portion that aligns the lumen, and in the nuclei of gland cells the chromatin appears decondensed with visible nucleoli (Fig. 5A-C, 7A-C). A thicker glandular epithelium was observed in glands in the groups treated with Fipronil (Fig. 5D-I) and Thiamethoxam (7D-I), mainly in sublethal dose LD_{50} (Fig. 5J-L, 7J-L). Pyknotic nuclei and nuclei with marginalized chromatin were observed in all treatment groups with both insecticides, which also displayed vacuolated cytoplasmic regions (Fig. 5D-L, 7D-L).

Fig. 6A, B and 8A, B shows the midguts of ants from the control group, where no obvious morphological alterations. Large numbers of secretory vesicles were found in the midgut cells along the entire epithelium. Moreover, the nuclei of cells were decondensed.

About the morphological alterations caused by both insecticides in the midgut shown was observed: nuclear pyknosis cells, drastically reducing the amount of secretory vesicles in the epithelium and the large amount of vacuolization of the cytoplasm of cells (Fig. 6C-E, 8C, D). The vacuoles may reflect programmed cell death autophagic type.

Fig. 6F, G and 8E shows Malpighian tubules from control ants. In the ventricular region where the tubules inserted,

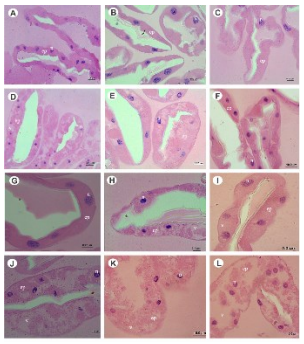

Fig. 5. Histological sections of postpharyngeal glands of the groups treated with Fipronil - A: control 4h, B: control 24h, C: control 48h; D: $LD_{50/100}$ 4h, E: $LD_{50/100}$ 24h, F: $LD_{50/100}$ 48h; G: $LD_{50/10}$ 4h, H: $LD_{50/10}$ 24h, I: $LD_{50/10}$ 48h, J: LD_{50} 4h, K: LD_{50} 24h, L: LD_{50} 48h; where: ep: epithelium; n: nucleus; v: vacuolated cytoplasmic regions.

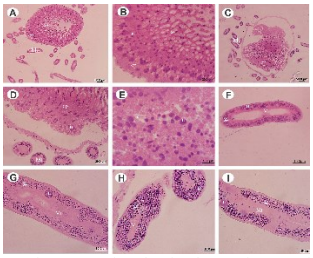

Fig. 6. Histological sections of midgut of the groups treated with Fipronil – A.B: control 4h. C.D.E: LD_{50} 48h. F.G.H.I. Malpighian tubules – F.G: control 4h, H.I: LD_{50} 48h; where: ep: epithelium; n: nucleus; v: vacuolated cytoplasmic regions; sv: secretory vesicles, Mt: Malpighian tubules; gr: basophilic granules.

excretory tubular cells featured decondensed nuclei with visible nucleoli and large amounts of basophilic granules. The luminal region of the Malpighian tubules is shown excretory cells with regions containing large amounts of basophilic granules and regular nuclei.

For the data obtained for the Malpighian tubules for the ants of treatment groups for both insecticides, it was found again vacuolated cytoplasmic regions, and pyknotic nuclei and an apparent decrease in the amount of granules mineralized groups compared to control groups, although it is difficult to quantify these granules (Fig. 6H, I; 8F).

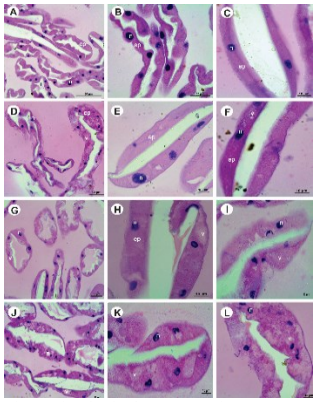

Fig.7.Histological sections of postpharyngeal glands of the groups treated with Thiamethoxam - A: control 4h, B: control 24h, C: control 48h; D: $LD_{50/100}$ 4h, E: $LD_{50/100}$ 24h, F: $LD_{50/100}$ 48h; G: $LD_{50/10}$ 4h, H: $LD_{50/10}$ 24h, I: $LD_{50/10}$ 48h, J: LD_{50} 4h, K: LD_{50} 24h, L: LD_{50} 48h; where: ep: epithelium; n: nucleus; v: vacuolated cytoplasmic regions.

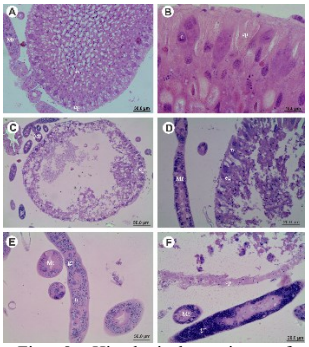

Fig. 8. Histological sections of midgut of the groups treated with Thiamethoxam – A.B: control 4h. C.D: LD_{50} 48h. E. F. Malpighian tubules – E: control 4h, F: LD_{50} 48h; where: ep: epithelium; n: nucleus; sv: secretory vesicles, Mt: Malpighian tubules; gr: basophilic granules.

IV. Discussion

The notoriety of leaf-cutting ants as pests is not evident in areas where they are native (American rain-forest

ecosystems); however, these ants are recognised as pests in human-altered environments (e.g. agriculture and reforestation fields), but not in natural environments [21].

In agricultural settings, where it is necessary to control the populations of *A. sexdens*, the medium dose indicated for the control of this ant in cane sugar is 600 ngμL−1 ha−1 [47] above the LD_{50} values established. However, there are no developed application systems to ensure that the doses reach the insects to be controlled, leading to the use of the molecule at higher doses than necessary to control the population. The use of great quantities of agrochemicals can be considered as prejudice over other species of ants that do not affect agriculture or other beneficial insects, such as bees. Populations of agricultural interest in continuous contact with these lethal or even sublethal doses may develop resistance and require new control methods.

Because these ant species are treated as pests, morphological studies that consider the effects of insecticides are rare. The majority of the studies found applied tests that involved attractiveness, control efficacy and treatment with granulated baits based on citric pulp and different active ingredients.

The results showed that occur detectable morphological alterations in the of ants exposed to Fipronil and Thiamethoxam compared with the control groups. It is interesting to note that morphological analyses, in the present study, revealed that the following features typical of dying cells were observed with more intensity in the glands of insecticides treated ants: nuclear pyknosis and nuclei with marginalized chromatin, which is indicative of cell death [48]-[49]; and regions of cytoplasmic vacuolization, as well as the presence of autophagic vacuoles, which are indicative of macroautophagy [50]. In the midgut, where more dramatic histological alterations were observed in ants treated with the both insectides, including a decrease in the secretory activity of digestive cells reflected by the decreased number of secretory vesicles. As in the midgut as in the Malpighian tubules, ants treated with the insecticides exhibited clearer morphological changes. Pyknotic nuclei and vacuolated regions of cytoplasm were observed in treated ants and an apparent decrease in the amount of granules mineralized was observed compared to control groups, although it is difficult to quantify these granules.

According to Caetano and Cruz-Landim [51], these granules are composed of metabolic residues from hemolymph filtrates. As the vast amount and degree of development of basophilic granules indicate high cellular metabolic activity [52], the reduction of concentric rings inside the granules observed in ants treated with Fipronil ants, suggests that fipronil treatment could have decreased the secretory activity of the Malpighian cell. Decio et al. [43] obtained the same results when studying PPGs, midgut and Malpighian tubules in exposed ants to Hydramethylnon pesticide and found that he toxicity of hydramethylnon was significantly more acute in the presence of soy oil, the evidenced by the highest mortality rate HAO-treated in ants.

On ants exposed to insecticides sublethal doses, the intensities of the emitted immunofluorescence were different between the control group and treatment groups with $LD_{50/100}$, $LD_{50/10}$ and LD_{50}. For the treated groups, the measured intensity was almost twice as intense when compared with the control group. The utilization of immonustaining as an important tool for comparing the level of preservation of the cytoskeleton after applying the inseticides.

Thus, toxic substances, such as those presented here, may have different effects on different castes and stages of development, affecting the entire colony. The results obtained show an action of sublethal doses precisely in tissue, directly related to the effects of insecticides. Fipronil works by blocks the passage of chlorine ions by interacting with gamma-aminobuteric acid (GABA)-gated chloride channels on nerve cell membranes [53]. Thiamethoxam acts on the nicotinic acetylcholine receptor of insects where it mimics the messenger chemical acetylcholine and binds to the receptor site, irreparably damaging the insects nervous system and eventually leading to insect death.

V. Conclusion

The combination of data obtained through the toxicological bioassays with morphological data showed that the both insectides acts directly in the analyzed organs in this study. The results of toxicity bioassays and light microscopy suggests that the highest sublethal doses as well as longest time of exposure to the insecticides cause greater damage to the cells. However toxicological data, corroborated by morphological suggest that Fipronil insecticide acts directly in the analyzed organs, as well as the ants mortality rate, with greater efficiency than the Thiamethoxam insecticide and can therefore be considered more toxic in this study. It was also observed that the highest sublethal doses as well as longest time of exposure to the insecticides cause greater damage to the cells, may cause cell death. Studies like this are important to understand how pesticides act on these organisms, and thereby develop bait for controlling ants more effective.

Table I. Statistical analysis of the actin and tubulin in postpharyngeal glands, midgut and mapighi tubules of *Atta sexdens rubropilosa* during treatment with Fipronil.

Organ	ANOVA	Tukey Test					
Postpharyngeal gland		Mean (1 from to 2)	Mean (1 from to 3)	Mean (1 from to 4)	Mean (2 from to 3)	Mean (2 from to 4)	Mean (3 from to 4)
Actin[1]	F= 14.1241, p<0.0002	p< 0.01	p< 0.05	p< 0.01	ns	ns	ns
Actin[2]	F= 8.2349, p<0.0018	ns	p< 0.05	p< 0.05	p< 0.05	p< 0.05	ns
Actin[3]	F= 44.2616, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	ns	ns	ns
Tubulin[1]	F=9.8232, p< 0.0018	ns	ns	p< 0.01	ns	p< 0.01	ns
Tubulin[2]	F=3.825, p< 0.0387	ns	p< 0.05	ns	ns	ns	ns
Tubulin[3]	F=8.1588, p< 0.0019	ns	ns	p< 0.01	ns	p< 0.01	p< 0.05
Midgut							
Actin[1]	F=69.6223, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	p< 0.01	p< 0.01	ns
Actin[2]	F=64.4467, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	p< 0.01	p< 0.01	ns
Actin[3]	F=57.3034, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	p< 0.05	p< 0.01	ns
Tubulin[1]	F=53.605, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	p< 0.05	p< 0.01	p< 0.01
Tubulin[2]	F=30.4479, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	ns	p< 0.01	p< 0.01
Tubulin[3]	F=69.2708, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	p< 0.01	p< 0.01	p< 0.05
Malpighi tubulus							
Actin[1]	F=21.677, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	ns	p< 0.05	ns
Actin[2]	F=15.7004, p< 0.0001	ns	p< 0.01	p< 0.01	ns	p< 0.01	ns
Actin[3]	F=33.1284, p< 0.0001	p< 0.05	p< 0.01	p< 0.01	ns	p< 0.01	p< 0.05
Tubulin[1]	F=25.2709, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	ns	p< 0.01	ns
Tubulin[2]	F=20.266, p< 0.0001	p< 0.05	p< 0.01	p< 0.01	ns	p< 0.01	ns
Tubulin[3]	F=45.6081, p< 0.0001	p< 0.05	p< 0.01	p< 0.01	p< 0.01	p< 0.01	ns

Actin[1]= C 4 h, LD50/100 4 h, LD50/10 4 h, LD50 4 h; Actin[2]= C 24 h, LD50/100 24 h, LD50/10 24 h, LD50 24 h; Actin[3]= C 48 h, LD50/100 48 h, LD50/10 48 h, LD50 48 h; Tubulin[1]= C 4 h, LD50/100 4 h, LD50/10 4 h, LD50 4 h; Tubulin[2]= C 24 h, LD50/100 24 h, LD50/10 24 h, LD50 24 h; Tubulin[3]= C 48 h, LD50/100 48 h, LD50/10 48 h, LD50 48 h. 1= Group Control; 2= Group LD50/100; 3= Group LD50/10; 4= Group LD50.

Table II. Statistical analysis of the actin and tubulin in postpharyngeal glands, midgut and mapighi tubules of *Atta sexdens rubropilosa* during treatment with Thiamethoxam.

Organ	ANOVA-one way	Tukey Test					
Postpharyngeal gland		Mean (1 from to 2)	Mean (1 from to 3)	Mean (1 from to 4)	Mean (2 from to 3)	Mean (2 from to 4)	Mean (3 from to 4
Actin[1]	F=27.0408, p<0.0001	ns	ns	p< 0.01	p< 0.05	p< 0.01	p< 0.01
Actin[2]	F=56.2821, p<0.0001	p< 0.01	p< 0.01	p< 0.01	p< 0.05	p< 0.01	ns
Actin[3]	F=100.0791, p<0.0001	ns	p< 0.01	p< 0.01	p< 0.01	p< 0.01	ns
Tubulin[1]	F=17.7118, p<0.0001	ns	p< 0.01	p< 0.01	p< 0.01	p< 0.01	ns
Tubulin[2]	F=10.9493, p<0.0006	p< 0.05	p< 0.01	p< 0.01	ns	ns	ns
Tubulin[3]	F=14.8226, p<0.0002	ns	p< 0.05	p< 0.01	ns	p< 0.01	p< 0.01
Midgut							
Actin[1]	F=18.8533, p<0.0001	p< 0.01	p< 0.01	p< 0.01	ns	ns	ns
Actin[2]	F=31.2087, p<0.0001	p< 0.05	p< 0.01	p< 0.01	p< 0.01	p< 0.01	ns
Actin[3]	F=44.2578, p<0.0001	p< 0.01	p< 0.01	p< 0.01	ns	p< 0.01	ns
Tubulin[1]	F=6.4062, p<0.0049	p< 0.05	p< 0.05	p< 0.01	ns	ns	ns
Tubulin[2]	F=8.272, p<0.0018	ns	p< 0.05	p< 0.05	p< 0.05	p< 0.01	ns
Tubulin[3]	F=18.8794, p<0.0001	p< 0.01	p< 0.01	p< 0.01	ns	ns	ns
Malpighi tubulus							
Actin[1]	F=30.3883, p< 0.0001	p< 0.01	ns	p< 0.01	p< 0.01	p< 0.01	p< 0.01
Actin[2]	F=11.5162, p< 0.0005	ns	ns	p< 0.01	ns	p< 0.01	p< 0.01
Actin[3]	F=31.9036, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	ns	p< 0.01	ns
Tubulin[1]	F=14.2752, p< 0.0002	p< 0.05	p< 0.01	p< 0.01	ns	p< 0.05	ns
Tubulin[2]	F=14.0254, p< 0.0002	ns	p< 0.01	p< 0.01	ns	p< 0.01	ns
Tubulin[3]	F=18.9105, p< 0.0001	p< 0.01	p< 0.01	p< 0.01	ns	ns	ns

Actin[1]= C 4 h, LD50/100 4 h, LD50/10 4 h, LD50 4 h; Actin[2]= C 24 h, LD50/100 24 h, LD50/10 24 h, LD50 24 h; Actin[3]= C 48 h, LD50/100 48 h, LD50/10 48 h, LD50 48 h; Tubulin[1]= C 4 h, LD50/100 4 h, LD50/10 4 h, LD50 4 h; Tubulin[2]= C 24 h, LD50/100 24 h, LD50/10 24 h, LD50 24 h; Tubulin[3]= C 48 h, LD50/100 48 h, LD50/10 48 h, LD50 48 h. 1= Group Control; 2= Group LD50/100; 3= Group LD50/10; 4= Group LD50.

ACKNOWLEDGMENT

This work was supported by FAPESP (São Paulo Research Foundation - grant 2013/20375-9) to Gabriela Ortiz, and CNPq (National Council for Scientific and Technological Development – grant 157837/2015-7) to Alexsandro Santana Vieira. We are grateful to CEIS (Center for the Study of Social Insects) and Biophotonic Laboratory – INFABIC – UNICAMP (Mariana Ozello Baratti and Vitor Bianchin Pelegati) for the technical support.

REFERENCES

[1] H. G. Fowler, M. I. Pagani, A. O. Silva, L. C. Forti, V. Pereira-da-Silva and H. L. Vasconcelos, "A pest is a pest? The dilemma of Neotropical leafcutting ants: keystone taxa of natural ecosystems," *Environ Manag*. v.13, 1989, pp. 671–675.

[2] I. Perfect and J. Vandermeer, "Distribution and turnover rate of a population of *Atta cephalotes* in a tropical rain forest in Costa Rica," *Biotropica* v.25, 1993, pp. 316–321.

[3] C. G. Jones, J. H. Lawton and M. Shachak, "Organisms as ecosystem engineers," *Oikos*. v.69, 1994, pp. 373–386.

[4] C. G. Jones, J. H. Lawton and M. Shachak, "Positive and negative effects of organisms asphysical ecosystem engineers," *Ecology*. v.78, 1997, pp. 1946–1957.

[5] R. Wirth, H. Herz, R.J. Ryel, W. Beyschlag and B. Hölldobler, *Herbivory of Leaf-cutting Ants*, Springer-Verlag, New York-Heidelberg-Berlin, 2003, p. 230.

[6] V. Rico-Gray and O.S. Oliveira, *the Ecology and Evolution of Ant–Plant Interactions* University of Chicago Press, Chicago, 2007, p. 320.

[7] A. G. Farji-Brener, S. Amador, F. Chinchillaa, S. Escobar, S. Cabrera and I. Herrera, "Information transfer in head-on encounters between leafcutting ant workers: food, trail condition or orientation cues?," *Anim Behav*. v.79, 2010, pp. 343–349.

[8] J. M. Coutinho, "Aspectos ecológicos da saúva no cerrado. A saúva, as queimadas e sua possível relação com a ciclagem de nutrientes minerais (Ecological aspects of saúva in the cerrado. Saúva, burnings and their possible relationship with mineral nutrient cycling)," *Biol Zool da USP*. v. 8, 1984, pp. 1–9.

[9] A. G. Farji-Brener and J. Silva, "Leaf-cutting ant nests and soil fertility in a well drained savanna in western Venezuela," *Biotropica*. v. 27, 1995, pp. 250–253.

[10] A. G. Farji-Brener and A. E. Illes, "Do leaf-cutting ant nests make 'bottomup gaps' in Neotropical rain forest? A critical review of the evidence," *Ecol Lett*. v. 3, 2000, pp. 219–227.

[11] P. Moutinho, D. C. Nepstad, and E. A. Davidson, "Influence of leaf-cutting ant nests on secondary forest growth and soil properties in Amazonia," *Ecology*. v. 84, 2003, pp. 1265–1276.

[12] L. Sternberg, M. C. Pinzon da Silva, M.Z. Moreira, P. Moutinho, E. I. Rojas and E. A. Herre, "Plants use macronutrient accumulated in leaf-cutting ant nests," *Proc R Soc Lond B*. v. 274, 2007, pp. 315–321.

[13] L. Sousa-Souto, J. H. Shoereder, C. E. G. Schaefer and W. L. Silva, "Ant nests and soil nutrient availability: The negative impact of fire," *J Trop Ecol*. v. 24, 2008, pp. 639–646.

[14] I. R. Leal and P. S. Oliveira, "Interactions between fungus growing ants (Attini), fruits and seeds in Cerrado vegetation in Southeast Brazil," *Biotropica*. v. 30, 1998, pp. 170–178.

[15] E. F. O. Peternelli, T. M. C. Della Lucia, L. A. Peternelli, S.V. Martins and E.E. Borges, "The interaction among workers of the genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae) and seeds of *Mabea fistulifera* (Euphorbiaceae) a pioneer tree species in Brazil," *Sociobiology*. v. 42, 2003, pp. 597–603.

[16] S. Haeder, R. Wirth, H. Herz and D. Spiteller, "Candicinin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*," *Proc Natl Acad Sci. USA*. v. 106, 2009, pp. 4742–4746.

[17] D. C. Oh, M. Poulsen, C. R. Currie and J. Clardy, "Dentigerumicin a bacterial mediator of an ant-fungus symbiosis," *Nat Chem Biol*. v. 5, 2009, pp. 391–393.

[18] J. M. Cherrett, "The foraging behaviour of *Atta cephalotes* (L.) (Hymenoptea: Formicidae). Foraging pattern and plant species attacked in tropical rain forest," *J Anim Ecol*. v. 37, 1968, pp. 387–402.

[19] R. Zanetti, J. C. Zanuncio, A. J. Mayhé-Nunes, A. G. B. Medeiros and A. Souza-Silva, "Combate sistemático de formigas-cortadeiras com iscas granuladas em eucaliptais com cultivo mínimo (Systematic combat of granulated bait-cutter ants in minimally cultivated eucalyptus)," *Revista Árvore (Tree Magazine)*. v. 27, 2003, pp. 387–392.

[20] T. M. C. Della Lucia, *As formigas cortadeiras*. Folha de Viçosa, Viçosa, 1993, pp. 63-190.

[21] T. M. C. Della Lucia, L. C. Gandra and R. N. C. Guedes, "Managing leaf-cutting ants: peculiarities, trends and challenges," *Pest Manag Sci*. v. 70, 2014, pp. 14–23.

[22] A. E. Loeck and O. Nakano, "Efeito de novas substâncias visando o controle de sauveiros novos de *Atta laevigata* (Effect of new substances aiming at the control of new Atta laevigata sauveiros) (Smith, 1858) (Hymenoptera: Formicidade)," *O Solo*. v. 76, 1984, pp. 25–30.

[23] O. C. Bueno and A. E. C. Farinha, *As formigas domésticas. In: Insetos e outros invasores de residências (Insects and other home invaders)*, ed. by Mariconi, F.A.M, FEALQ, Piracicaba, 1999, pp. 135-180.

[24] P. Etheridge and F. T. Phillips, "Laboratory evaluation of new insecticides and bait matrices for the control of leaf-cutting ants (Hymenoptera, Formicidae)," *Bull Entomol Res.* v. 66, 1976, pp. 569–578.

[25] F. C. Bueno, *Seleção de ingredientes ativos para uso em iscas no controle de formigas cortadeiras (Selection of active ingredients for use in litter control of leaf-cutting ants) (Hymenoptera: Formicidae)*. Dissertação, Universidade Estadual Paulista, (Dissertation, Paulista State University,) Botucatu, São Paulo, 2005, 79p.

[26] O. C. Bueno, *Filtro infrabucal e glândulas pós-faríngeas da saúva-limão Atta sexdens rubropilosa Forel (Lemongrass infrared filter and post-pharyngeal glands Atta sexdens rubropilosa Forel) (Hymenoptera, Formicidae)* Dissertação (Dissertation), Universidade Estadual Paulista (Paulista State University), Rio Claro, São Paulo, 2005,107p.

[27] N. S. Nagamoto, L. C. Forti, A. P. P. Andrade, M. A. C. Boaretto and C. F. Wilcken, "Method for the evaluation of insecticidal activity over time in *Atta sexdens rubropilosa* workers (Hymenoptera: Formicidae)," *Sociobiology*. v. 44, 2004, pp. 413–431.

[28] D. Hainzl and J. E. Casida, "Fipronil insecticide: novel photochemical desulfinylation with retention of neurotoxicity," *Proc Natl Acad Sci U S A*. v. 93, 1996, pp. 12764–12767.

[29] I. Ishaaya, "Biochemical processes related to insecticide action: An overview", in *Biochemical sites of insecticide action and resistance*, ed. by Ishaaya, I, Springer, Berlin, pp. 1–10. 2001.

[30] A. Rortais, G. Arnold, M.P. Halm and F. Touffet-Briens, "Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by diferente (different) categories of bees" Apidologie. v. 36, 2005, pp. 71–83.
[31] P. Caboni, R. E. Sammelson and J. E. Casida, "Phenylpyrazole insecticide photochemistry, metabolism, and GABAergic action: ethiprole compared with fipronil," *J Agric Food Chem.* v. 51, 2003, pp. 7055–7061.
[32] C. C. Tingle, J. A. Rother, C. F. Dewhurst, S. Lauer and W. J. King, "Fipronil environmental fate, ecotoxicology and human health concerns," *Rev Environ Contam Toxicol.* v. 176, 2003, pp. 1–66.
[33] A. K. E. El Hassani, M. Dacher, M. Gauthier and C. Armengaud, "Effects of sublethal doses of fipronil on the behavior of the honeybee *Apis mellifera,*" *Pharmacol Biochem Behav.* v. 82, 2005, pp. 30–39.
[34] Y. J. Fan, X. Y. Shi and Xi-wu Gao, "Research progresses on the metabolism of neonicotinoids imidacloprid and thiamethoxam", *Chin J Pestic Sci.* v. 14, 2012, pp. 587-596.
[35] S. Sumida, E. C. M. Silva-Zacarin, P. Decio, O. Malaspina, F. C. Bueno and O. C. Bueno, "Morphological changes in organs of *Atta sexdens rubropilosa* (Formicidae: Hymenoptera) treated with boric acid in toxicological bioassays," *J Econ Entomol.* v. 103, 2010, pp. 676–690.
[36] F. H. Caetano, *Aspectos ultramorfológicos, ultra-estruturais e enzimológicos da glândula pós-faríngea de Dinoponera australis (Ultramorphological, ultrastructural and enzymatic aspects of the post-pharyngeal gland of Dinoponera australis) (Formicidae: Ponerinae)* Dissertação, Universidade Estadual Paulista (Dissertation, Paulista State University), Rio Claro, São Paulo, 137p. 1998.
[37] B. Hölldobler and E.O. Wilson*, the Ants,* Springer, London, 1990, 746p.
[38] V. Gama, "O sistema salivar de *Camponotus* (Myrmothrix) rufipes (Fabricius, 1775), (Hymenoptera Formicidae)," *Rev Bras Biol.* v. 45, 1985, pp. 317–359.
[39] C. Janet, *Anatomie de la tête du Lasius niger (Anatomy of the Lasius Niger)*. Limoges Impremeire-libraire. Paris: Ducourtieux et Gout, 40p. 1905.
[40] B. Braeckman, K. Brys, U. Rzeznik and H. Raes, "Cadmium pathology in an insect cell line: ultrastructural and biochemical effects," *Tissue and Cell*. v. 31, 1999, pp. 45–53.
[41] J. Sorour, "Ultrastructural variations in *Lethocerus niloticum* (Insecta: Hemiptera) caused by pollution in Lake Mariut, Alexandria," *Egypt Ecotoxicol Environ Saf.* v. 48, 2001, pp. 268–274.
[42] P. Cintra-Socolowski, T. C. Roat, R. C. F. Nocelli, P. H. Nunes, R. A. C. Ferreira, O. Malapina and O. C. Bueno, "Sublethal doses of fipronil intensify synapsin immunostaining in *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) brains," *Pest Manag Sci*. v. 72, 2015, pp. 907-912.
[43] P. Decio, E. C. M. Silva-Zacarin, F. C. Bueno and O. C. Bueno, "Toxicological and histopathological effects of hydramethylnon on *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) workers," *Micron.* v. 45, 2013, pp. 22-31.
[44] B. Matsumoto, "Cell Biological Applications of Confocal Microscopy", in *Methods in Cell Biology*, ed. by Wilson L and Matsudaira P, Academic Press, San Diego California, 2002, pp 2-501.
[45] O. C. Bueno, M. S. C. Morini, F. C. Pagnocca, M. J. A. Hebling and A. O. Silva, "Sobrevivência de operárias de *Atta sexdens rubropilosa* (Survival of Atta sexdens rubropilosa workers) Forel (Hymenoptera, Formicidae) isoladas do formigueiro e alimentadas com dieta artificial (isolated from anthill and fed artificial diet)," *Ann Soc Entomol Brasil.* v. 26, 1997, pp. 107–113.
[46] L. C. U. Junqueira and L. M. M. S. Junqueira, *Técnicas básicas de citologia e histologia (Basic cytology and histology techniques)*. Livraria Editora Santos, São Paulo, 1983, p. 50-75.
[47] AGROFIT, Sistema de Agrotóxicos Fitossanitarios (Phytosanitary Pesticide System). [Online]. Ministério da Agricultura, Pecuária e Abastecimento (Ministry of Agriculture, Livestock and Supply), Brasilia, Brazil. 2010. Available: http://extranet.agricultura.gov.br/agrofit_cons/principal_agrofit_cons [12 November 2013].
[48] I. D. Bowen and S. M. Bowen, *Programmed cell death in tumours and tissues*. Chapman and Hall, London, 1990, 268p.
[49] I. D. Bowen, S. M. Bowen and A. H. Jones, *Mitosis and Apopotosis: Matters of Life and Death*. Chapman and Hall, London, 1998, 182p.
[50] E. C. M. Silva-Zacarin, "Authophagy and apoptosis coordinate physiological cell death in larval salivary glands of *Apis mellifera* (Hymenoptera: Apidae)," *Autophagy.* v. 3, 2007, pp. 516–518.
[51] F. H. Caetano and C. Cruz-Landim, "Ultra-estrutura das células colunares do ventrículo de *Camponotus arboreus* (Hymenoptera, Formicidae)," *Naturalia*. v. 8, 1983, pp. 91–100.
[52] A. Arab and F. H. Caetano, "Estudo ultramorfológico dos túbulos de Malpighi de operárias de *Solenopsis* sp. (Hymenoptera, Formicidae)," *Naturalia*. v. 24, 1999, pp. 151–153.
[53] C. R. O. Jacob, H. M. Soares, R. C. F. Nocelli and O. Malaspina, "Impact of Fipronil on the mushroom bodies of the stingless bee *Scaptotrigona postica,*" *Pest Manag Sci*. v. 71, 2015, pp. 114-122.

AUTHORS' PROFILES

Gabriela Ortiz - Graduation in Biological Sciences from São Paulo State University – UNESP (2002), PhD (2007) and PhD in Cell and Molecular Biology from São Paulo State University - UNESP (2011). Currently developing a postdoctoral project at the Center for the Study of Social Insects (CEIS) of the Unesp of Rio Claro, evaluating the effects of pesticides, as well as other environmental contaminants used in sugar cane *Atta sexdens rubropilosa* species.

Alexsandro Santana Vieira - Graduation in Biological Sciences from State University of Mato Grosso do Sul (2005), Master in Entomology and Biodiversity Conservation from Federal University of Grande Dourados (2008) and PhD in Cell and Molecular Biology from São Paulo State University – UNESP (2012). Postdoctoral stage in Biological Sciences by São Paulo State University - UNESP in the Center for the Study of Social Insects, UNESP (2012-2017). He has experience in Entomology, Cellular and Molecular Biology, and mainly works in subjects related to social insects and microbiome.

Odair Correa Bueno - Graduation in Biological Sciences from São Paulo State University – UNESP, Masters in Biological Sciences (Zoology) from the University of São Paulo and PhD in Biological Sciences (Zoology) from the University of São Paulo. He is currently an Adjunct Professor at the Department of Biology and Researcher at the Center for the Study of Social Insects of the Institute of Biosciences at UNESP, Rio Claro-SP. Has experience in the field of Entomology, with emphasis on Social Insect Biology and Biology, Behavior and Control of Invasive Ants.