



# Neurotoxic action of permethrin in *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) female ticks. Morphological and cytochemical evaluation of the central nervous system



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## ABSTRACT

Studies on the molecular bases of the neurotoxic action of acaricides are found in the literature; but there are no studies of this action on the nervous system of ticks at the cellular level. The present study describes the morphological and cytochemical changes in the synganglion of *Rhipicephalus sanguineus* semi-engorged females exposed to different concentrations of permethrin, a pyrethroid with recognized neurotoxic action. Permethrin induced the degeneration of the synganglion through a process of apoptosis involving autophagy, characterized by the condensation and margination of the chromatin, formation of blebs in the nuclear envelope and fragmentation of the nucleus, loss of shape of neural cells and integrity of cellular membrane, cytoplasmic shrinkage, and lower levels of acid phosphatase in the nervous tissue as the concentration of permethrin increased. This study provided further evidence of the neurotoxic action of permethrin, which impairs the metabolism of *R. sanguineus* nervous systems, and consequently the physiology of other systems, dependent on the neural control. These results provide cytochemical and histological confirmation of the neurotoxic action of permethrin, previously inferred from molecular and tick behavioral evidence.

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## 1. Introduction

*Rhipicephalus sanguineus*, commonly called the brown dog tick, is one of the most widely distributed of all ticks (Estrada-Peña and Jongejan, 1999). This tick was introduced from the Afrotropical Region to many countries in the world, probably by the importation of infested

domestic dogs, its preferred host (Evans et al., 2000). For dogs, this species of tick can produce debilitating effects due to both blood loss and the transmission of infectious agents (Sexton et al., 1976). In Brazil, *R. sanguineus* is usually found on dogs in both urban and rural environments. Although the dog is the preferential host (Rey, 1973; Walker, 1994), due to the low specificity, this species can be found in other mammals, including man (Venzal et al., 2003a,b; Nebreda-Mayoral et al., 2004; Louly et al., 2006).

*R. sanguineus* is an ectoparasite of public health interest (Palmas et al., 2001), due to its capability to carry and transmit a number of pathogens to humans. Infestations of this species in humans in Brazil have been reported in the states of Goiânia, Pernambuco, São Paulo and Rio Grande do Sul

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(Guglielmo et al., 2006), which can be the cause of the increase in the incidence of anthrozooses (Fernandes et al., 2001).

Chemical control with acaricides is the most efficient method against ticks since immunological and biological controls still play a supplemental role. Among the chemical compounds is permethrin, a synthetic pyrethroid which works by quickly paralyzing the nervous system of the ticks (Mencke, 2006). Nervous impulses are disordered by prolonging sodium channels activation, keeping them open in the neuronal membranes (repetitive effect), causing the sensorial organs and nervous terminations of the ectoparasite to react in a particularly sensitive way causing a state of hyperexcitation (Dong, 2007). Permethrin, as well as other pyrethroids, interferes in the central nervous system by preventing the binding of the neurotransmitter gamma amino butyric acid (GABA) in receptor sites, blocking the entry of chloride ions into the cell, causing as well as excitement, tremors and spasms, followed by paralysis and death (Mencke, 2006; Dong, 2007).

Studies on the molecular basis of the neurotoxic action of acaricides are found in literature, but there are no studies which analyze this action at cellular level. This study aimed to describe the morphophysiological changes induced by permethrin in the synganglion of *R. sanguineus* semi-engorged females subjected to this product. In addition, we also analyze the occurrence of cell death through enzymatic changes (acid phosphatase) in this tissue, since autophagic death would be characterized mainly by an increase in the level of activity of acid hydrolases (acid phosphatase), causing consequently the destruction of the cell (Clarke, 1990; Zakeri et al., 1995).

## 2. Material and methods

### 2.1. *R. sanguineus* ticks

Semi-engorged females, weighing 27 mg on average (about 5 days of feeding) were used throughout the experiment. The specimens were supplied by the tick colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) at the São Paulo State University, Rio Claro, SP, Brazil, under controlled conditions ( $28 \pm 1$  °C, 80% relative humidity and 12 h photoperiod) in a Biological Oxygen Demand (BOD) incubator and fed on New Zealand white rabbits.

Details on feeding and maintenance of *R. sanguineus* ticks on the hosts are given by Bechara et al. (1995). Briefly, the ticks were placed inside a feeding chamber consisting of a plastic tube (2.5 cm wide and 3 cm high) glued to the shaved back of the hosts with an atoxic and harmless preparation on the day prior to feeding. Elizabethan collars were used on the rabbits to prevent grooming. In order to avoid the escape of ticks during experiments, hosts were kept in cages placed in trays surrounded by a gutter filled with water and oil.

This study was approved by Ethics Committee in the Animal Use, CEUA, UNESP, Rio Claro, SP, Brazil, Protocol n° 4093.

### 2.2. Dilution assays for permethrin (CAS n°: 52645-53-1)

Permethrin (3-phenoxybenzyl (1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) used in this study was purchased from Fersol Indústria e Comércio S/A (Mairinque, SP, Brazil). The permethrin concentrations were based on the LC<sub>50</sub> of 2062 ppm determined previously in a study by Roma et al. (2009). The doses used corresponded to 10% of the LC<sub>50</sub> (206 ppm), 50% of the LC<sub>50</sub> (1031 ppm) and the LC<sub>50</sub> (2062 ppm).

*R. sanguineus* females, after being washed in a sieve with tap water, were dried on soft absorbent paper. Subsequently, 45 females were divided into three groups of 15 females each and immersed for 5 min in Petri dishes containing the different concentrations of permethrin. A control group also consisting of 15 female ticks was immersed in distilled water for the same period. The ticks were then dried on absorbent paper and placed in the BOD incubator ( $28 \pm 1$  °C, 80% relative humidity and 12 h photoperiod) for 7 days to observe the action of permethrin on the synganglion.

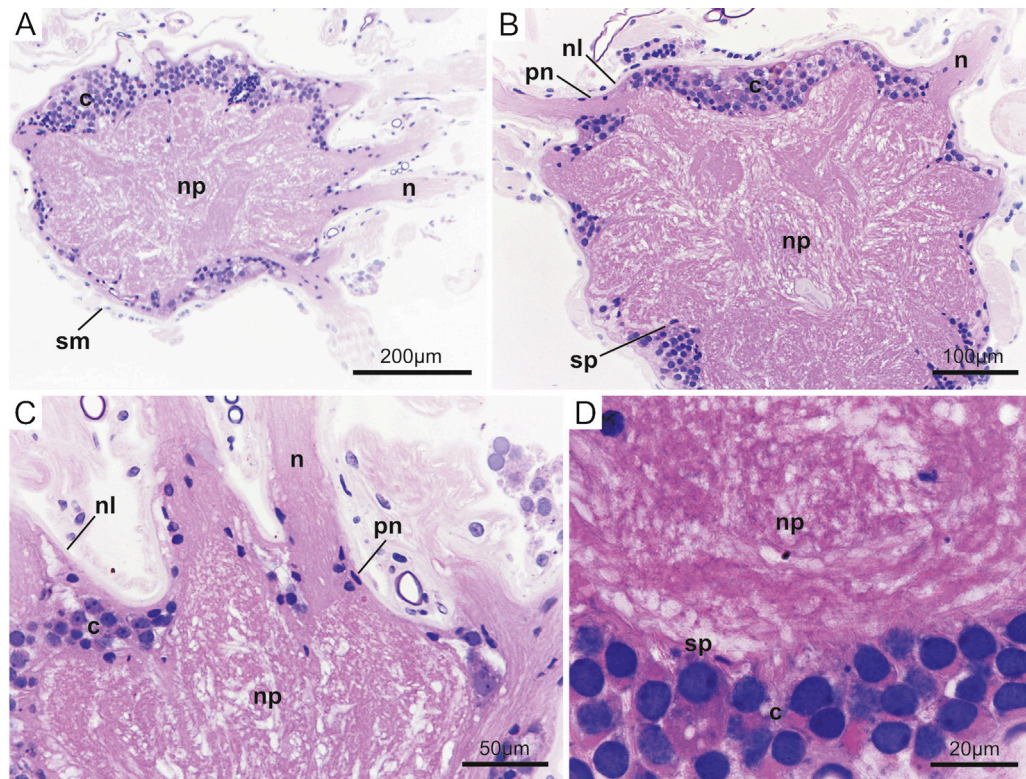
The observation period was established because frequently the effect of acaricides is not immediate, but acts slowly on the physiology.

### 2.3. Histology

Semi-engorged females were dissected in Petri dishes containing phosphate buffered saline-PBS solution (NaCl 0.13 M, Na<sub>2</sub>HPO<sub>4</sub> 0.017 M, KH<sub>2</sub>PO<sub>4</sub> 0.02 M, pH 7.2) and the synganglia were removed. The material was fixed in 4% paraformaldehyde and 0.9% NaCl in 10% phosphate buffer (0.1 M – pH 7.5), dehydrated in an alcoholic series (70, 80, 90 and 95%) at 15 min intervals. Infiltration was made with Leica histoiresin and the material embedded in plastic molds at 4 °C to delay pre-polymerization. The molds with material were filled and covered with Leica histoiresin and polymerization completed at room temperature. Sections with 3 μm thickness were mounted on glass slides, stained with hematoxylin-eosin (HE) and examined and photographed under a Leica DM 750 photomicroscope. This device and other equipments were from the Histology Laboratory of the Biology Department at the Bioscience Institute, UNESP, Rio Claro, SP, Brazil.

### 2.4. Detection of acid phosphatase

*R. sanguineus* ticks (control and treated groups) were dissected and the synganglia were removed. Afterwards, the material were fixed in 10% buffered formalin and acetone (9:1) for 90 min at 4 °C and processed according to the methods described by Hussein et al. (1990) for detection of acid phosphatase. Then, the material was dehydrated in increasing concentrations of ethanol (70, 80, 90, and 95%), for 15 min each, embedded in Leica resin, and sectioned at 7 μm thickness. Sections were placed on glass slides, counterstained with hematoxylin for 30 s and examined and photographed under a Leica DM 750 photomicroscope. In this experiment, control samples (negative control) were incubated without substrate.



**Fig. 1.** Histological sections of synganglion of semi-engorged females from *Rhipicephalus sanguineus* ticks stained with Hematoxylin-Eosin. Group I - control. (A) and (B) General view and (C) and (D) detail of the synganglion. sm: sinus membrane; nl: neural lamella; pn: perineurium; sp: subperineurium; c: cortex; np: neuropile; n: nerve trunks.

### 3. Results

#### 3.1. Histology

##### 3.1.1. Group I (control)

The morphological description of the central nervous system (synganglion) of *R. sanguineus* has already been reported by Roma et al. (2012). The present work confirmed their results in the control group (Fig. 1A–D).

##### 3.1.2. Group II (treated with 206 ppm of permethrin)

For this concentration of permethrin, the synganglion presents significant morphological changes in relation to the control group, such as:

- The cortical region cells present vacuoles all over the cytoplasm, and many of them have their size increased (cellular swelling) (Fig. 2A, C and D). In some regions these cells seem to be loose and separated by large empty spaces (Fig. 2A and B).
- Most cortex cells show their nuclei in the initial stage of chromatin margination and many of them are picnotic (Fig. 2C and D). Large accumulations of neural cells with picnotic nuclei are observed in some regions (Fig. 2D).
- The neuropile presents structural disorganization with large empty spaces among the neural fibers (Fig. 2A and B, E).

The neural lamella and the perineurium are intact in this treatment group (Fig. 2C and D).

##### 3.1.3. Group III (treated with 1031 ppm of permethrin)

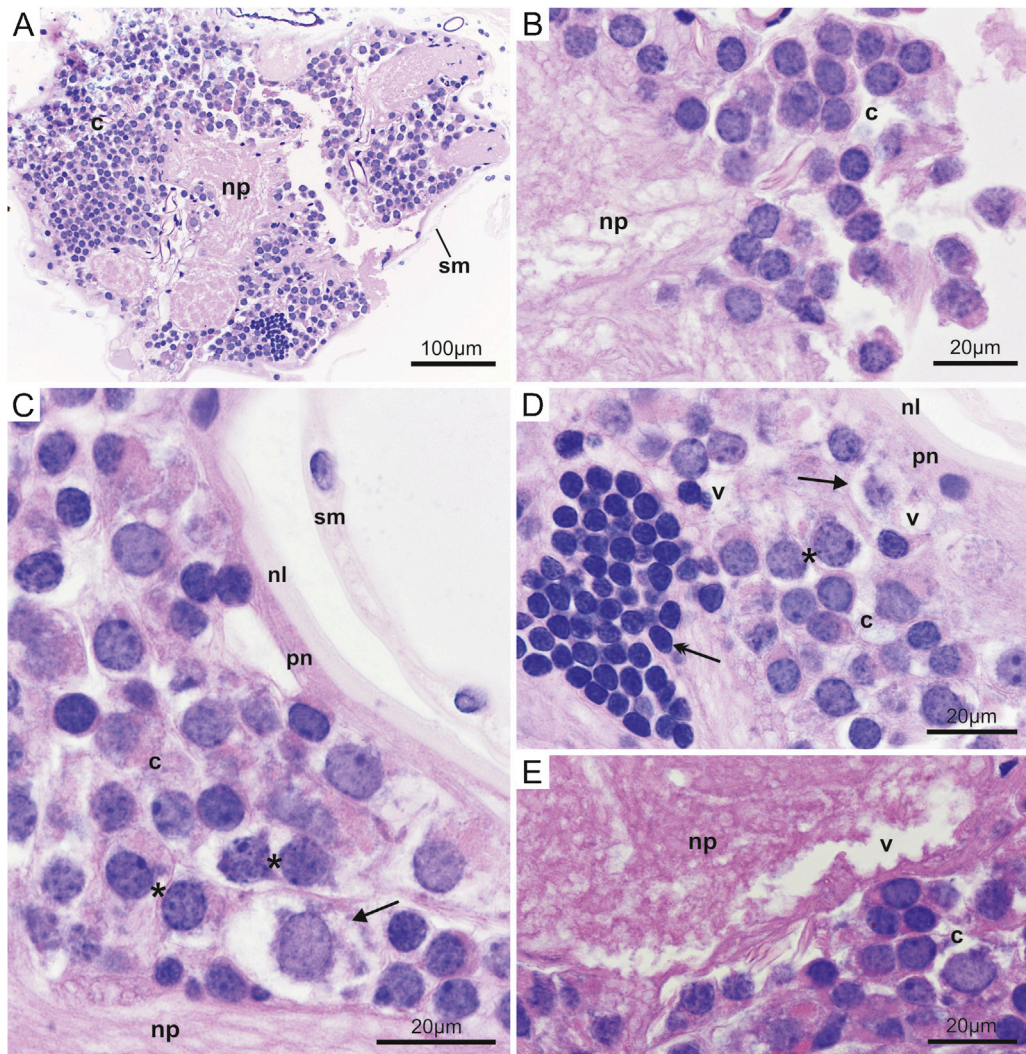
The synganglion of *R. sanguineus* females exposed to this concentration of permethrin presents an advanced stage of degeneration and more severe morphological changes in relation to the other treatment groups, mainly concerning the genetic material.

The neural lamella is intact; however, the perineurium is not visualized anymore (Fig. 3B and D). In this organ, a large region of degeneration is observed next to the cortex and neuropile (Fig. 3A and B).

The cells of the cortical region lose their integrity, and the cytoplasm looks dense (cytoplasmic shrinkage). Some vacuoles are observed only among the neural cells, which lose their original shape and their limits become difficult to be observed (loss of cell individuality) (Fig. 3B–F). Some cells present increased size, cytoplasm vacuolation and changed nucleus (Fig. 3F).

The totality of cortical region cells present nuclei with great changes in shape (irregular, fragmented or with blebs), in size (reduced) in arrangement and level of chromatin condensation (picnotic or with blebs) (Fig. 3D–F).

The neuropile shows great structural disorganization (Fig. 3A and B, G). The neural fibers acquire a denser aspect, and large empty spaces are observed among them (Fig. 3A and B, D, G).



**Fig. 2.** Histological sections of synganglion of semi-engorged females from *R. sanguineus* ticks stained with Hematoxylin-Eosin. Group II treated with 206 ppm of permethrin. (A) General view and (B)–(E) detail of the synganglion. sm: sinus membrane; nl: neural lamella; pn: perineurium; c: cortex; np: neuropile;  $\longrightarrow$ : picnotic nucleus; \*: nucleus with beginning chromatin margination;  $\rightarrow$ : cellular swelling; v: vacuoles.

#### 3.1.4. Group IV (treated with 2062 ppm of permethrin)

For this concentration of permethrin, the synganglion presents significant structural changes, but in a previous stage of degeneration considering the one found for treatment group III.

The neural lamella is preserved; however, the identification of the perineurium is no longer possible (Fig. 4A–C). The region of the cortex and the neuropile presents significant structural disorganization with large empty spaces among both the neural cells and the neural fibers (Fig. 4A–D). Between the neural lamella and the cortex a large region of degeneration can be observed (Fig. 4A and B).

The cortical region cells lose their original shape and their limits are difficult to be visualized. The cytoplasm acquires a dense aspect (cytoplasmic shrinkage) (Fig. 4B and C).

The nuclei of the cortical region cells show several changes in shape (irregular) and in the level of chromatin condensation (picnotic) (Fig. 4C).

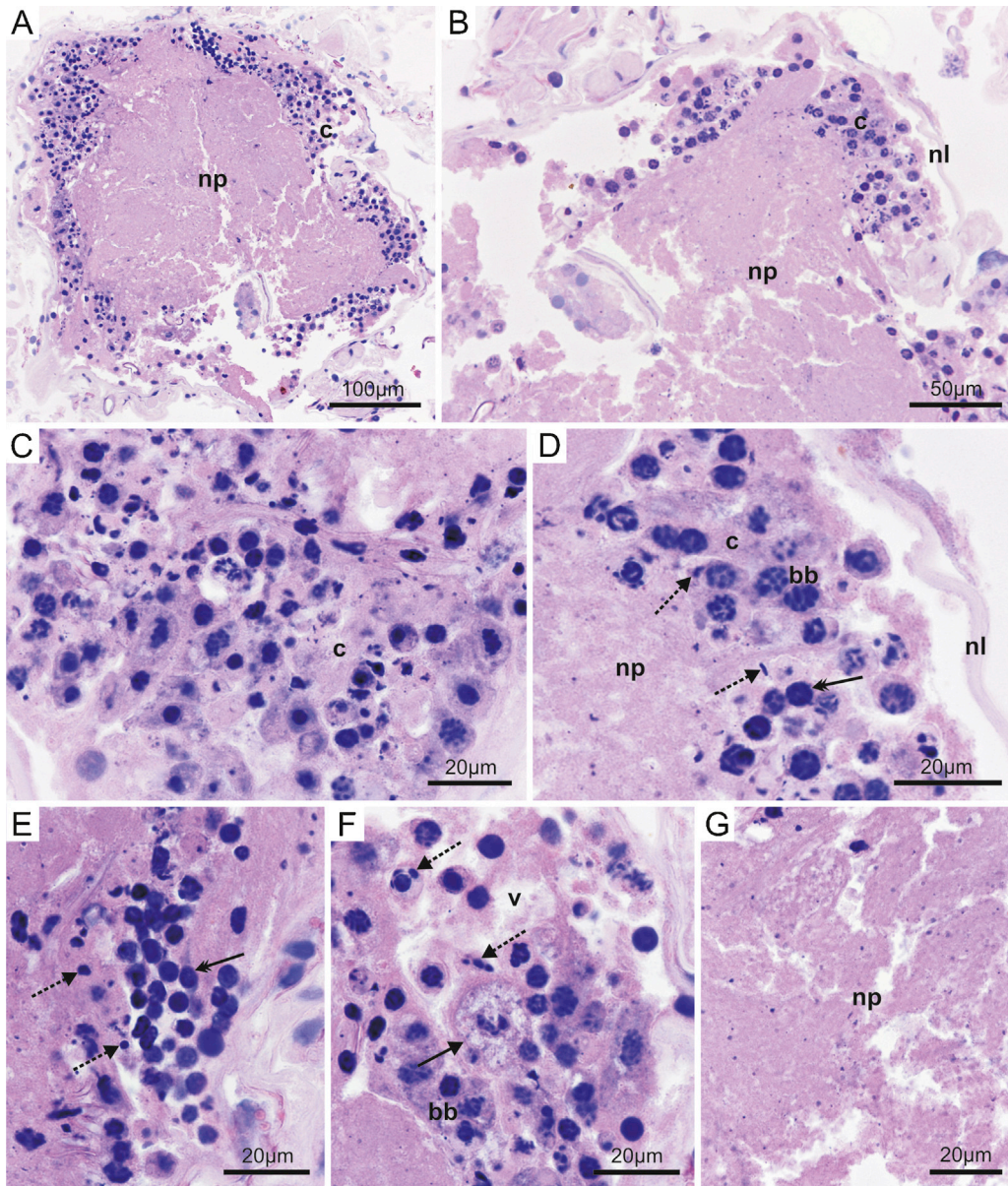
As observed in the previous concentration, the ramifications of the neural cells (neuropile), acquire a dense aspect (Fig. 4A–D).

### 3.2. Acid phosphatase

Fig. 5A and B have no marking for acid phosphatase, as they correspond to the control technique performed (negative control), demonstrating that it did not show false positive results.

#### 3.2.1. Group I (control)

The synganglion of the females from the control group shows weak positivity for acid phosphatase in the region



**Fig. 3.** Histological sections of synganglion of semi-engorged females from *R. sanguineus* ticks stained with Hematoxylin-Eosin. Group III treated with 1031 ppm of permethrin. (A) General view and (B)–(G) detail of the synganglion. Note in: (C)–(F) the cortex cells exhibiting nuclear changes. nl: neural lamella; c: cortex; np: neuropile;  $\longrightarrow$ : picnotic nucleus;  $\rightarrow$ : cellular swelling;  $\cdots\rightarrow$ : fragmented nucleus; bb: blebs; v: vacuoles.

of the cortex and neuropile. The neural lamella and the perineurium are negative to the test (Fig. 5C and D).

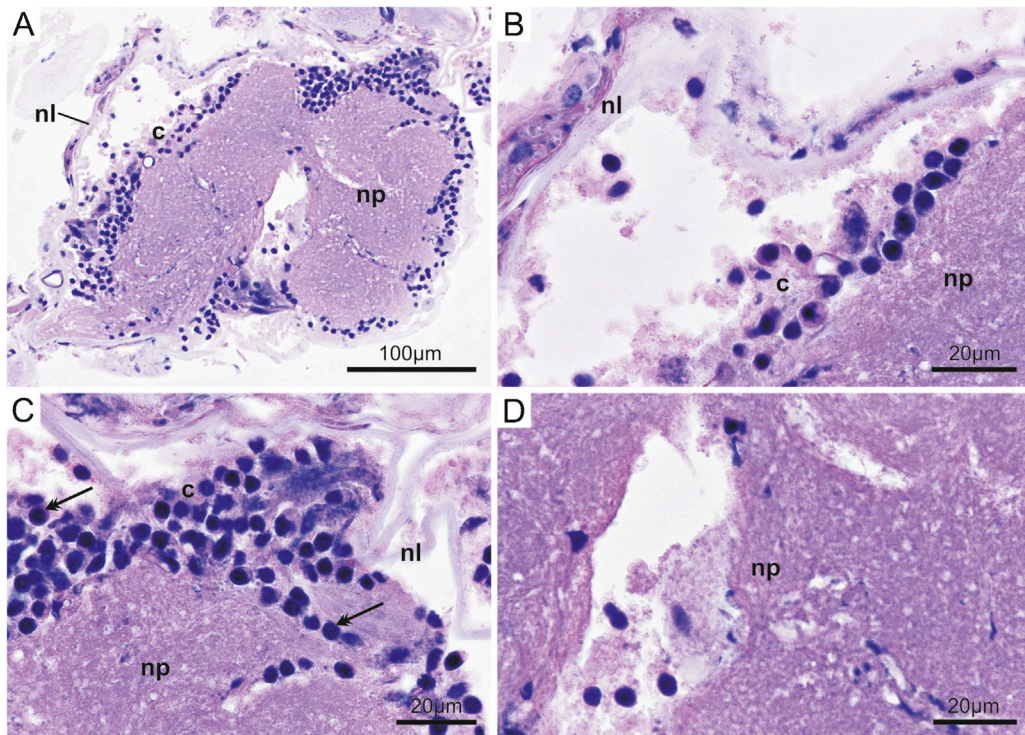
### 3.2.2. Group II (treated with 206 ppm of permethrin)

For this concentration of permethrin the synganglion shows strong positivity for acid phosphatase in the perineurium and in some regions of the cortex (degeneration regions), in which can be observed clusters of this enzyme, demonstrating an increase in the activity of acid phosphatase in relation to the control group. However, in other regions of the cortex the presence of this enzyme is not

observed. The neural lamella and the neuropile are negative to the test (Fig. 5E–G).

### 3.2.3. Group III (treated with 1031 ppm of permethrin)

The synganglion of the females exposed to this concentration of permethrin shows lower staining (moderate positivity) for acid phosphatase in the cortex, in relation to the previous treatment group. However, in other regions of the cortex the presence of this enzyme is not observed. The perineurium is not identified in this group. As observed in



**Fig. 4.** Histological sections of synganglion of semi-engorged females from *R. sanguineus* ticks stained with Hematoxylin-Eosin. Group IV treated with 2062 ppm of permethrin. (A) General view and (B)–(D) detail of the synganglion. nl: neural lamella; c: cortex; np: neuropile;  $\blackrightarrow$ : picnotic nucleus.

control group, neural lamella is negative to the test, while in the neuropile weak staining is observed (Fig. 5H–J).

#### 3.2.4. Group IV (treated with 2062 ppm of permethrin)

For this concentration, the neural lamella, cortex and neuropile are negative for acid phosphatase, demonstrating a drastic reduction in the activity of this enzyme in relation to the other treatment groups (Fig. 5K and L).

## 4. Discussion

Actually, studies on the molecular bases involved in the neurotoxic action are found in literature (Dong, 2007); however, there is no research on the action at cellular level in the nervous system of ticks. Thus, this study aimed to describe the morphological and cytochemical (enzymatic) changes in the synganglion of *R. sanguineus* female ticks exposed to different concentrations of permethrin.

The results showed that the synganglion of females from the control group presented the same morphological pattern described by Roma et al. (2012) for this species of tick. However, when this tissue was subjected to the toxic agent permethrin, it showed great structural and enzymatic changes, which resulted in the impairment of the organ's physiology.

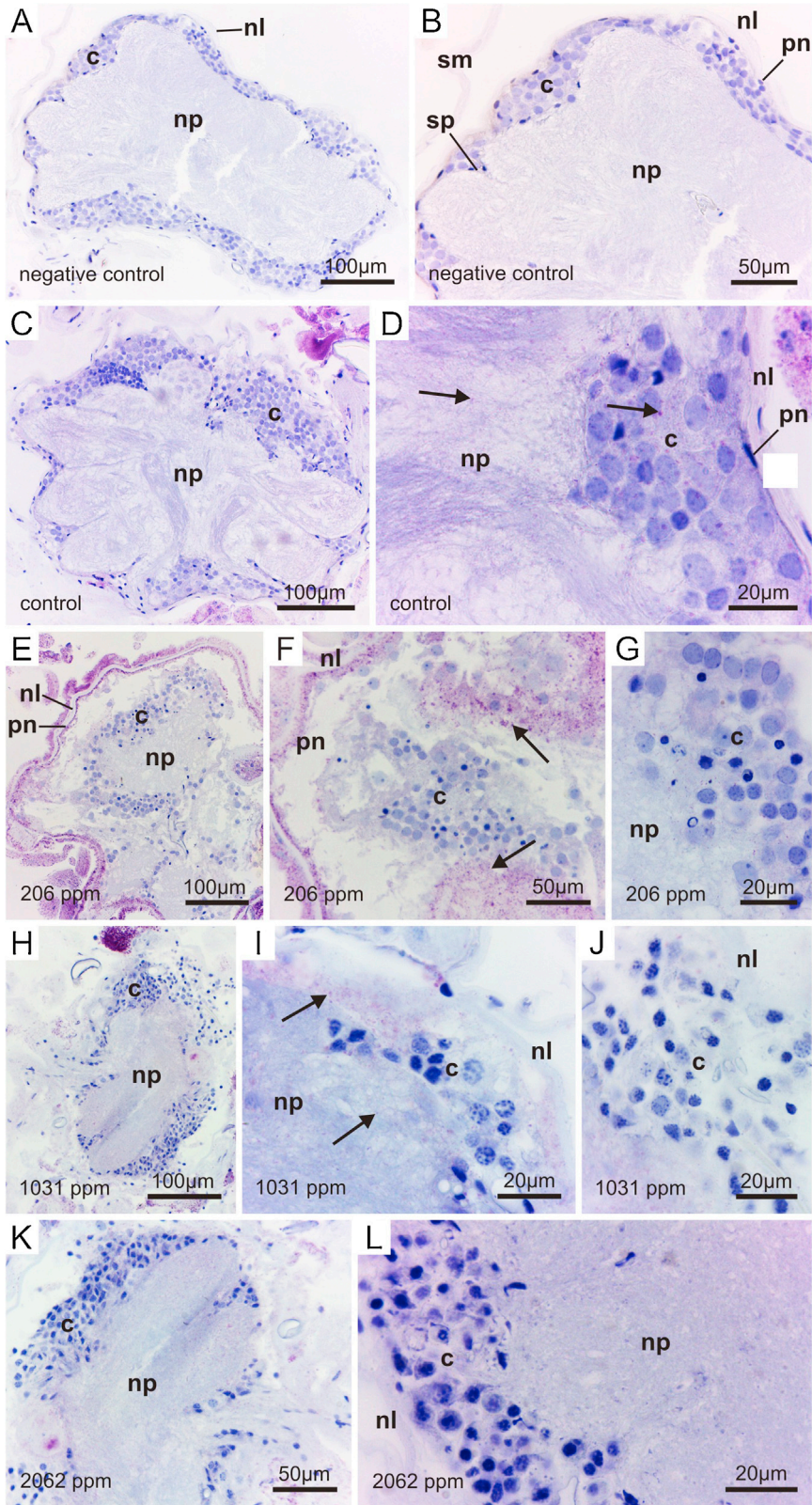
Cortical region cells with cytoplasmic vacuoles were observed in the synganglion of females exposed to 206 ppm of permethrin. This characteristic indicates that these cells are developing mechanisms of defense, probably through autophagic vacuoles, in an attempt to eliminate the cellular remains damaged by permethrin and allow the reuse

of components which would still be useful for the cell. Oliveira et al. (2008, 2009) described that acaricide fipronil, a phenylpyrazole which has a different mode of action from pyrethroids, acting as a blocker of the neural stimulation (Taylor, 2001), elicits similar histological lesions in the germ cells of females of this same species. In addition, in some cases the cortex cells were loose and separated by large empty spaces, showing that, in some regions of the synganglion, the degenerative process occurred more intensely. In this case, it can be suggested that although strategies of defense have been developed by these cells, they are not able to revert the damages caused by this product, culminating in cell death.

For the same concentration of permethrin (206 ppm) several cells of the cortical region with increased size were observed. According to MacGahon et al. (1995), the cellular swelling indicates loss of integrity (functionality) of the plasma membrane, due to changes in ion pumps caused by a degenerative process, usually found in individuals exposed to toxic agents (Dyk et al., 2007), which would certainly be happening here.

The synganglion of the females exposed to this lower concentration of permethrin presented most cortex cells with nuclei in initial stage of chromatin margination, and many of them were picnotic. According to Bowen (1993) and Furquim et al. (2008a), these nuclear changes are the result of an apoptotic process, which would be occurring here due to the cytotoxicity of permethrin for this nervous tissue.

The neuropile of the individuals exposed to 206 ppm of permethrin presented great structural disorganization



due to the changes undergone by the cortical region cells, once the neuropile is formed by the ramifications of these cells. These results show that, even at lower concentrations, permethrin is able to impair the functioning of the nervous system, by affecting the transmission of nervous impulses to the different organs of the tick.

For the females exposed to 1031 ppm of permethrin, the synganglion showed advanced stage of degeneration and more severe morphological changes in relation to the other treatment groups. For this concentration, the neural lamella is intact; however, the perineurium was no longer visible. According to Marzouk et al. (2001), the neural lamella has selective permeability to substances from the hemolymph and acts as a protective barrier to the entrance of several elements from the synganglion. Considering the results here obtained, it can be suggested that permethrin affects the permeability of this membrane, allowing damage to the nervous system, e.g., to the perineurium, which was completely degraded. As the perineurium is the first region to be in contact with the toxic agent, it is affected with more intensity, which results in the death of its cells and, consequently, in its complete elimination. According to Sonenshine (1991), this structure is formed by glial cells, which help in the control of neural activities. Thus, it can be inferred that with the destruction of the perineurium, all the functioning of the nervous system would be impaired.

In females exposed to 1031 ppm, the cortical region cells lost their integrity, presenting cytoplasmic shrinkage, vacuoles among the neural cells, loss of shape and difficult visualization of cellular limits, in addition to increased size. These data corroborate Furquim et al. (2008a, 2008b) who showed similar changes in degenerative process of salivary glands of *R. sanguineus* ticks. These characteristics are strong indicators of the degenerative processes in cells in the advanced stages of cell death.

The cortical region cells of the individuals exposed to 1031 ppm of permethrin presented nuclei with great changes in the shape (irregular, with blebs or fragmented), in the size (reduced) and in the level of chromatin condensation (picnotic or with blebs). During cell death by apoptosis, nucleus breakdown is one of the first changes observed, characterized by the compression and marginalization of the chromatin, and emergence of blebs, followed by the fragmentation of the nucleus (Bowen and Bowen, 1990; Bowen, 1993; Kerr et al., 1995; Häcker, 2000). Thus, our studies confirm that permethrin is able to cause irreversible damage to the nervous tissue cells of *R. sanguineus*, by the impairment of the genetic material, leading the nervous tissue to death by apoptosis. These data corroborate Nodari et al. (2012) who also observed similar changes in the nucleus of salivary glands cells of *R. sang-*

*uineus* females during the degeneration process induced by permethrin in this tissue.

As observed for the previous treatment group, the neuropile of the females exposed to 1031 ppm of permethrin showed great structural disorganization. However, for this intermediate concentration, the neural fibers acquired a dense aspect, and large empty spaces were observed among them. This demonstrates that the higher the concentrations of permethrin, the more severe are the changes induced by this toxic agent.

The synganglion of *R. sanguineus* females exposed to 2062 ppm of permethrin showed similar structural changes to those found at lower concentrations; but at an earlier stage of degeneration than in group III; i.e., the nuclei of the cortex cells were picnotic, while in the intermediate concentration they were already fragmented, which according to Bowen and Bowen (1990), Bowen (1993), Kerr et al. (1995) and Häcker (2000) would be the last nuclear change observed during the process of apoptosis. Nodari et al. (2011), analyzing the effects of permethrin in the glandular system of *R. sanguineus* also observed that the intermediate concentration of 1031 ppm cause more severe morphological changes than the higher dosage (2062 ppm).

The results obtained in the present study also showed that, in addition to morphological changes, permethrin was also able to induce enzymatic changes (acid phosphatase) in the synganglion of *R. sanguineus*, corroborating data from literature, stating that this pyrethroid has neurotoxic action (Mencke, 2006).

The synganglion of the females from control group showed weak positivity for acid phosphatase in the region of the cortex and the neuropile; however, the neural lamella and the perineurium were negative for the test. The presence of this enzyme in the synganglion of the ticks indicate that it would be involved in the normal metabolism of this tissue, once morphological changes were not found for this treatment group. According to literature, the activity of this enzyme is not always associated with cellular degeneration, but is part of the normal physiology of some tissues (Costa and Cruz-Landim, 2002; Britto and Caetano, 2006), in this case, the nervous tissue of *R. sanguineus*.

For the concentration of 206 ppm of permethrin the synganglion shows strong positivity for acid phosphatase in the perineurium and in some regions of cortex degeneration. The nervous tissue of females exposed to 1031 ppm of permethrin showed moderate positivity for acid phosphatase in the cortical region. According to literature, the presence of the enzyme acid phosphatase (acid hydrolases) when associated to degenerative processes, indicate the occurrence of autophagic death (Pipan and Rakovec, 1980; Cummings and Bowen, 1992; Zakeri et al., 1995). Thus, it can be suggested in this study that acid phosphatase is

**Fig. 5.** Acid phosphatase activity in the synganglion of semi-engorged females from *R. sanguineus* ticks exposed to permethrin. (A) and (B) Negative control. (C) and (D) Synganglion of the control group showing weak positivity for acid phosphatase in the cortex and neuropile region. (E)–(G) Histological sections of the synganglion of females submitted to 206 ppm of permethrin exhibiting strong positivity for acid phosphatase in the perineurium and in some regions of the cortex (degeneration region). The neural lamella and neuropile are negative. (H)–(J) Synganglion of females exposed to 1031 ppm of permethrin showing moderate positivity for acid phosphatase in the some regions of the cortex (degeneration region). The neural lamella is negative for the test. In the neuropile is observed weak staining for acid phosphatase. (K) and (L) Histological sections of the synganglion of females submitted to 2062 ppm of permethrin showing negatively for acid phosphatase. sm: sinus membrane; nl: neural lamella; pn: perineurium; sp: subperineurium; c: cortex; np: neuropile; →: staining for acid phosphatase.



involved in the removal of the cellular structures damaged by permethrin in order to recover the damaged tissue. This corroborates the histological observations, since in the regions where the degenerative process was more intense, mainly in the perineurium and cortex degeneration regions, the staining for acid phosphatase was more intense, suggesting the occurrence of apoptotic death of this tissue involving autophagy.

The synganglion of females exposed to 2062 ppm of permethrin was negative for acid phosphatase, in contrast to the results obtained for the lower concentrations. These results suggest that the pyrethroid permethrin, when applied in higher concentrations (2062 ppm) is able to affect the hydrolytic behavior of the synganglion of *R. sanguineus* females, inhibiting the action of acid phosphatase. This is extremely harmful to the nervous system, once the cellular structures damaged by permethrin are not degraded by acid phosphatase (cellular renovation), which result in the accumulation of damage, leading the tissue to death. These results differ from those described by Nodari et al. (2012) for the action of permethrin on the glandular system of *R. sanguineus* females. These authors observed an intensification of the action of acid phosphatase in higher concentrations of this pyrethroid, demonstrating that permethrin is able to induce different enzymatic changes (increase or reduction in the synthesis of acid phosphatase) in different tissues of the ticks.

Another hypothesis for the lack of a positive staining for acid phosphatase in this group would be the occurrence of rapid cell death, i.e., the peak of activity of this enzyme occurs at a time earlier than the observed in this study, probably at the beginning of the degeneration process, but additional studies will be needed for confirmation of this evidence.

Considering the morphological and enzymatic evidence found here, it can be suggested that permethrin is able to induce the degeneration of the synganglion of *R. sanguineus* females through atypical apoptosis characterized by (1) condensation and margination of the chromatin, formation of blebs in the nuclear envelope and fragmentation of the nucleus; (2) loss of shape of the neural cells; (3) loss of integrity of the cellular membrane; (4) cytoplasmic shrinkage; (5) lower concentrations of acid phosphatase in the nervous tissue as the concentrations of permethrin increased, which result in a limited degradation of the cytoplasm (vacuolation). Several authors have also described the presence of hydrolytic enzymes (acid phosphatase) during the apoptotic death in different animal tissues, and these enzymes are involved in the removal of cytoplasmic remains, and in the fragmentation of the cells (Levy and Bautz, 1985; Clarke, 1990; Lockshin and Zakeri, 1996; Jochová et al., 1997).

## 5. Conclusions

This study allowed the analysis of the neurotoxic action of permethrin at cellular level, an action which impaired the entire metabolism of the nervous system of *R. sanguineus* ticks females by several changes causing the death of the nervous tissue in all the concentrations analyzed. Thus, it can be inferred that the physiology of the other systems

of this species, such as the glandular and the reproductive, would also be impaired, since they are dependent on neural control.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

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