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Cytotoxic effects of permethrin in salivary glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females

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ABSTRACT

Because of the medical and veterinary importance of ticks and the wide use of synthetic chemical substances such as permethrin (active ingredient of Advantage® Max3 – Bayer)for their control, this study evaluated the effects of different concentrations (206, 1031 and 2062 ppm) of the acaricide on the salivary glands of *Rhipicephalus sanguineus* semi-engorged females. Results showed that permethrin is a potent substance that acts morpho-physiologically in the tick glandular tissue, causing changes in the acini shape intense vacuolation in acinar cells, and disruption of the tissue by cell death process, with subsequent formation of apoptotic bodies, especially at higher concentrations, thus precluding the accurate identification of different types of acini. Importantly, it is demonstrated that permethrin acts on salivary gland tissue, as well as affecting the nervous system, accelerating the process of glandular degeneration, and interfering with the engorgement process of female ticks, preventing them from completing the feeding process.

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1. Introduction

Ticks represent an arthropod group of medical and veterinary importance causing harm to hosts and transmitting pathogens to them (Walker, 1994).

Many studies, dealing mainly bio-ecology of ticks and demands for effective control methods especially those related to new vaccines are available in the literature (Kelly and Colley, 1988; Kaufman, 1989; Leal et al., 2003; Labruna, 2004). However, specific work on the cell biology of ticks are still scarce. A group of researchers from the Brazilian Central of Studies on Ticks Morphology (BCSTM) at São Paulo State University have performed several studies, primarily focusing on the morphology and histology of the main tick systems (Denardi et al., 2004; Saito et al., 2005; Oliveira et al., 2006; Nunes et al., 2006; Oliveira et al., 2007; Nunes et al., 2008; Furquim et al., 2008a,b,c, 2010; Roma et al., 2010). The relevance of structural and functional changes of *Rhipicephalus sanguineus* reproductive and glandular systems exposed to synthetic and natural chemicals has been given by Oliveira et al. (2008, 2009), Roma et al. (2009, 2010) and Arnosti et al. (2010).

Currently, field experiments have shown that the most effective method for tick control is still the use of synthetic acaricides despite their high cost, specialized labor requirements for application, and damage to the environment and public health related to the contamination induced by chemical residues (Freitas et al., 2005).

Among the synthetic acaricides widely used to control ticks, especially the dog tick *R. sanguineus*, there is permethrin (active ingredient of Advantage® Max3, Bayer), a chemical compound that causes nerve impulse disorders, as a result of disturbed sodium exchange in cell membranes. Thus, ectoparasites suffer excitement, indicated by tremors and spasms followed by paralysis and death (Mencke et al., 2003).

Literature on the direct influence of acaricides in tick systems, other than the nervous system, are still scarce. Mohamed et al. (2000) showed that permethrin would stimulate the increase of activity of salivary gland in *Hyalomma dromedary*, however, Pereira et al. (2009), studying the action of fipronil (Frontline®) in salivary glands of *R. sanguineus*, have revealed changes in this tissue, resulting in early gland degeneration.

Thus, this study aimed to analyze, using morphological and histological techniques, the action of permethrin on the salivary glands of *R. sanguineus* semi-engorged females subjected to permethrin in an attempt to provide new information to support the improvement and development of control methods' that are less aggressive to non-target organisms as well as to the environment.

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2. Material and Methods

2.1. Rhipicephalus sanguineus ticks

A total of 60 semi-engorged females of *R. sanguineus*, weighing 27 mg in average, supplied from the colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) of São Paulo State University-UNESP, at the Biosciences Institute of Rio Claro, SP, Brazil, were used throughout the experiment. The ticks were kept under controlled conditions (28 ± 1 °C, 80% humidity and 12 h photoperiod) in an Eletrolab EL 202 BOD (Biological Oxygen Demand) incubator and fed on New Zealand White rabbits (Protocol n° 5442, approved by Comitê de Ética no Uso de Animal, UNESP, de Rio Claro/ CEUA-IB-UNESP).

The laboratory feeding conditions of *R. sanguineus* ticks in the hosts were followed (Bechara et al. 1995).

2.2. Dilution assays of 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 LC_{50} of 2062 ppm determined previously in a pilot test by Roma et al. (2009). The doses correspond to 10% of the LC_{50} (206 ppm), 50% of the LC_{50} (1031 ppm) and the normal LC_{50} (2062 ppm). A control group was exposed only to distilled water.

R. sanguineus females, after being washed in a sieve with tap water, were dried on soft absorbent paper. Afterwards, 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 above. The control group was also composed of 15 females that were immersed in distilled water for the same period. Ticks were then dried on absorbent paper and placed in the BOD incubator for 7 days. The observation period was established since the effect of the acaricide is often not immediate, acting slowly on the physiology of the ticks.

2.3. Scanning Electron Microscopy (SEM)

The salivary glands of *R. sanguineus* females were removed, fixed in Karnovsky medium for 24 h and dehydrated in a graded 70–100% acetone series. The material was processed by Critical Point Drying, sputtered with gold and examined by a Philips 505 SEM.

2.4. Histology

The *R. sanguineus* females were dissected on Petri dishes containing phosphate buffered saline-PBS solution (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2), and the salivary glands removed, fixed in 4% paraformaldehyde and 0.9% NaCl in 10% phosphate buffer (0.1 M – pH 7.5), and dehydrated in an alcohol series (70, 80, 90 and 95%) at 15 min intervals. The specimens were infiltrated with Leica resin and the material embedded in plastic moulds at + 4 °C to delay pre-polymerization. The moulds with material were filled and covered with Leica resin and the polymerization completed at room temperature (about 37 °C).

Sections 4 μm thick were mounted on glass slides, stained with hematoxylin & eosin (HE), examined and photographed in a Motic BA 300 photomicroscope. This device and other equipment were supplied by the Histology Laboratory of the Biology Department at the Biosciences Institute, UNESP, Rio Claro, SP, Brazil.

3. Results

3.1. Scanning Electron Microscopy (SEM)

The salivary glands of *R. sanguineus* semi-engorged females are paired structures that extend antero-laterally on the ventral body cavity and open into the oral cavity. They have a secretory and an excretory portion, and are devoid of a secretion storage reservoir. The secretory portion is composed of spherical acini with slightly wrinkled surface, while the excretory portion is formed by a branched duct system, where the largest one is the common excretory duct. Departing from this structure, intermediate or secondary (smaller size and diameter) ducts, are distributed along the length of the gland in small canaliculi or acinar ducts (Figs. 1A and B).

In female *R. sanguineus* treated with 206 ppm of permethrin, salivary glands had the same characteristics as those of the control group, except that some acini appear broken, and the presence of apoptotic bodies can be observed (Figs. 2A–C).

In individuals subjected to 1031 ppm of permethrin, the acini lost their original shape becoming smaller and irregular, presenting extremely wrinkled surface when compared to the control group individuals (Figs. 3A and B).

Salivary glands in individuals submitted to 2062 ppm of permethrin present similar changes (e.g., presence of irregular acini) (Fig. 4A).

3.2. Histology

3.2.1. Group I (control)

Histological techniques confirm the results on the salivary gland morphology in the control group which have acini **I**, **II** and **III** with regular shape and intact acinar cells (Figs. 1C–F).

The acini type **I**, which are agranular in ticks, present different shapes ranging from oval to rounded with a larger **central** cell and strongly stained nucleus and several smaller **peripheral** cells also with smaller nuclei (Fig. 1C).

The acini type **II** (granular) are spherical and present cells **c1**, **c2**, **c3** and **c4** (Figs. 1C–E) while the acini **III** (granular), also spherical, are larger than those of type **II** and are formed of **d** and **e** cells (Figs. 1E and F).

The different cell types of salivary gland acini (**I**, **II** and **III**) of *R. sanguineus* semi-engorged females were already described by Furquim et al. (2008b). In the present study, individuals of the control group were used only as reference to demonstrate the changes caused in the salivary glands by permethrin.

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

The tick females exposed to 206 ppm of permethrin had salivary glands with a few morphologically altered acini compared to the control group.

The acini **I** lost their original form, becoming irregular with a more enlarged lumen than those in the control group (Fig. 2D).

The acini **II** were the most affected by the action of permethrin. Changes in shape as well in **c1** and **c3** cells (intense vacuolation) were observed. However, most of the cells still had the same characteristics seen in the control group, and moreover, few showed cytoplasm vacuolation (Figs. 2E and F).

The acini **III** present **d** and especially **e** cell vacuolation, and some of these acini are irregular and disrupted (Fig. 2G).

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

Individuals subjected to 1031 ppm of permethrin had salivary glands with severe morphological changes, e.g. acini transformed

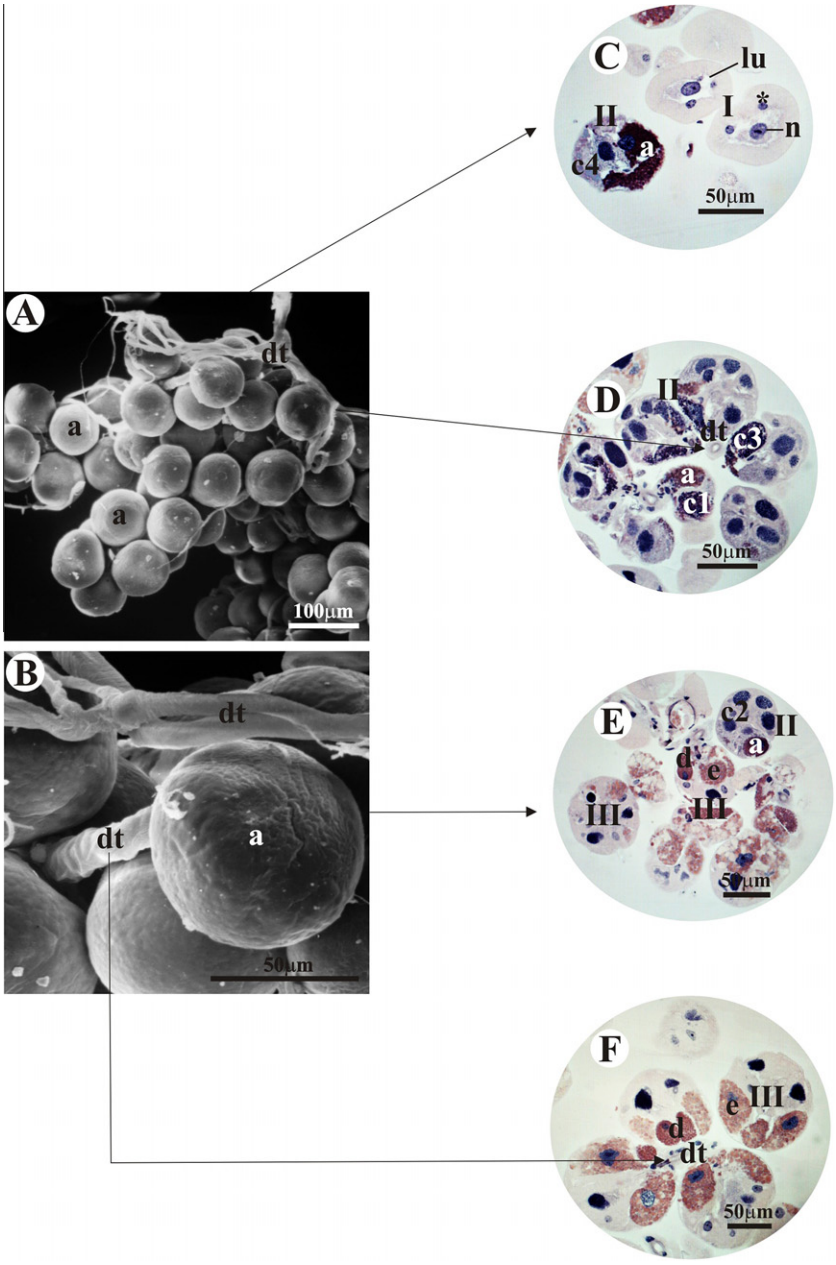

Fig. 1. (**A** and **B**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged female of the control group. (**A**) General view and (**B**) detail of the glandular acini (**a**) showing duct system (**dt**). **(C–F)** Histological sections of the *R. sanguineus* salivary glands of the control group stained with hematoxylin-eosin (HE) showing **I** (type I acinus), **II** (type II acinus) and **III** acini (type III acinus). **dt =** duct, **n** = nucleus of the central cell, ∗ = nucleus of the peripheral cells, **lu** = lumen, **a** = a cell, **c1** = c1 cell, **c2** = c2 cell, **c3** = c3 cell, **c4** = c4 cell, **d** = d cell, **e** = e cell.

into an amorphous mass revealing advanced degeneration stages (Figs. 3C–F).

Despite the many morphological changes, acini type **I** acquired an irregular shape (Figs. 3D and E). Due to the formation of this amorphous mass, identification of the other types of acini was no longer possible. These were termed **indeterminate** (Figs. 3C and D).

Degenerative processes in salivary glands, as well as acini fragmentation, resulted in the formation of numerous apoptotic bodies. The few nuclei that could still be observed were picnotic and/or fragmented (Figs. 3E and F). On the other hand, the tissue forming the glandular ducts system was not a target of permethrin action (Figs. 3E and F).

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

The salivary glands of individuals subjected to 2062 ppm permethrin also showed acini with great morphological changes, but they were less intense compared to those in the group treated with 1031 ppm.

Few acini **I** were present and they showed changes in their original shape, as well as presenting a dilated lumen (Fig. 4B). As described above, acini identification was still not possible (**indeterminate**) (Figs. 4B–E). Secretion granules were rarely observed in the **indeterminate** acini cells (Figs. 4C and D). Some nuclei showed increases in size (Figs. 4B–D). Although identification of the cells is comfounded by the action of permethrin, the acini basal membrane is preserved (Fig. 4D).

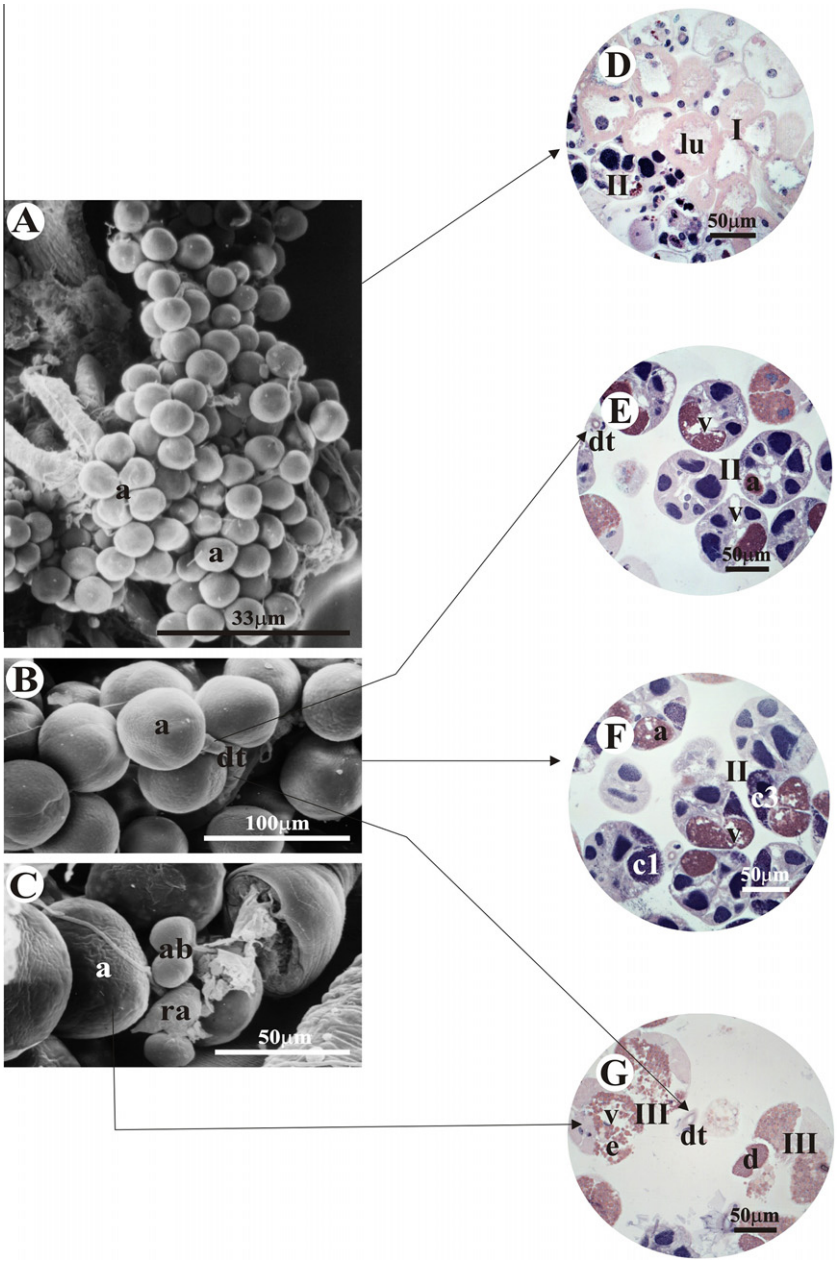

Fig. 2. (**A**–**C**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged female exposed to 206 ppm of permethrin. (**A**) General view and (**B** and **C**) detail of the acini (**a**) and duct (**dt**). Note in (**C**) the apoptotic bodies (**ab**) near ruptured acini (**ra**). (**D**–**G**) Histological sections of the *R. sanguineus* salivary glands exposed to 206 ppm of permethrin stained with hematoxylin-eosin (HE) showing **I** (type I acinus), **II** (type II acinus) and **III** acini (type III acinus). **lu** = lumen, **dt** = duct, **v** = vacuoles, **a** = a cell, **c1** = c1 cell, **c3** = c3 cell, **d** = d cell, **e** = e cell.

For comparison of results, see Fig. 5.

4. Discussion

The most effective method to control tick infestation in different hosts is the use of synthetic acaricides with a neurotoxic action (Mencke et al., 2003; Dong, 2007). However, few studies describe the changes caused by the action of these compounds in glandular and reproductive systems of ticks (Oliveira et al., 2008, 2009; Pereira et al., 2009; Roma et al., 2009, 2010).

The present study shows permethrin-induced morphophysiological changes occurs in salivary glands of *R. sanguineus* semi-engorged females. Even at lower concentrations, permethrin was able to cause glandular tissue changes compromising organ metabolism.

Individuals in the control group showed salivary glands with the characteristics described by Furquim et al. (2008b) for this same species, with organization of secretory cells in different acini and establishing the occurrence of a well defined secretory cycle. After finishing the activity cycle and glandular secretion release, gland degeneration occurred; this was observed in the later stage of feeding of individuals.

The present study showed that salivary glands in individuals subjected to 206 ppm permethrin presented acini **I** morphologically altered (irregular shape and dilated lumen) compared to the control group, corroborating data from Pereira et al. (2009), who reported that these acini in the same tick species were also affected by fipronil. These authors further suggested that the acini **I** would be osmoregulators and, through the saliva, they could remove the toxic compound from the hemolymph, as suggested here by the in-

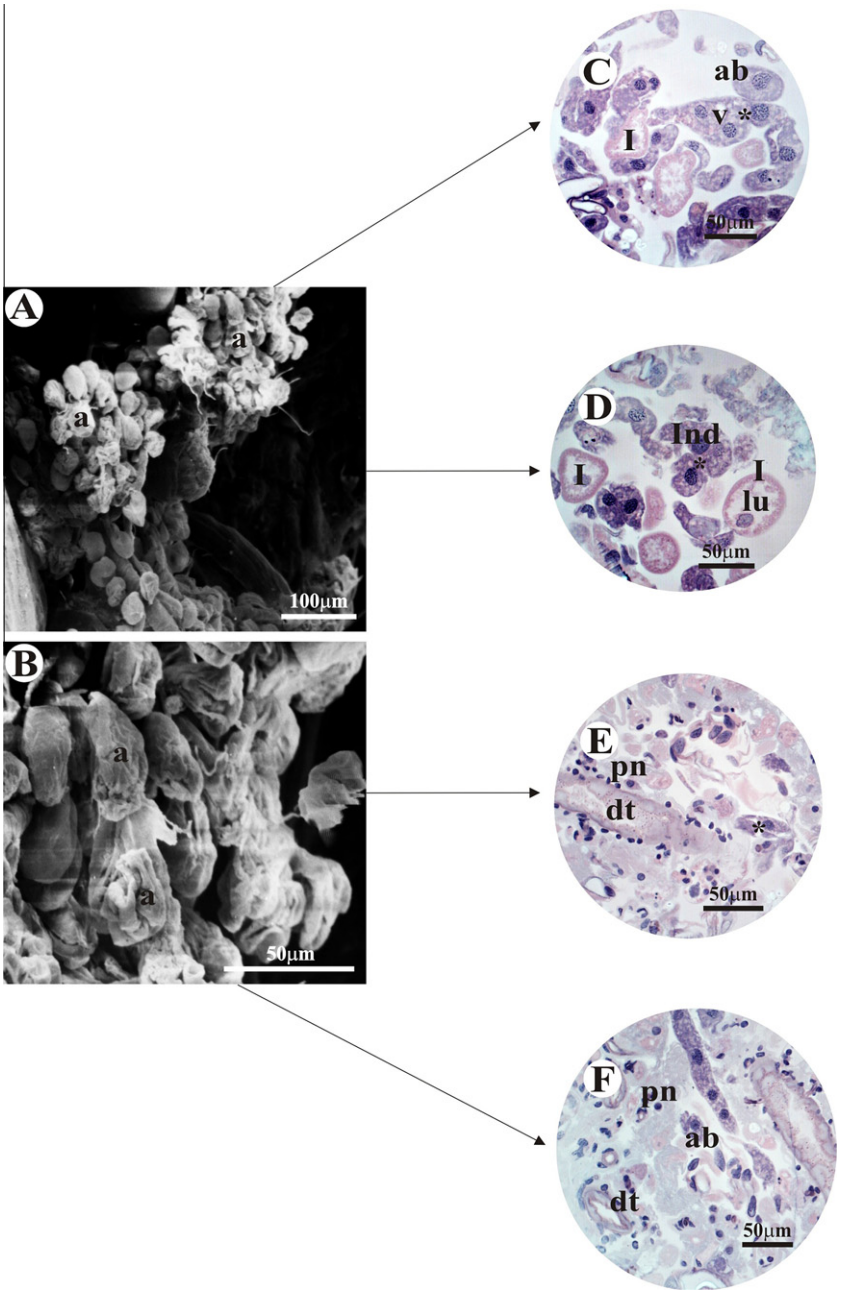

Fig. 3. (**A** and **B**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged female exposed to 1031 ppm of permethrin. (**A**) General view and (**B**) detail of the irregular acini (**a**). Note that acini seem to have "withered", thus modifying their initial morphology. **(C–F)** Histological sections of the *R. sanguineus* salivary glands exposed to 1031 ppm of permethrin stained with hematoxylin-eosin (HE) showing **I** (type I acinus) and **indeterminate** acini (**Ind**). **ab =** apoptotic body, **lu =** lumen, **dt =** duct, ∗ = acini in fragmentation process, **pn** = picnotic nucleus, **v** = vacuoles.

crease in its lumen diameter. The results here suggest that the same dynamics occur during permethrin exposure, to reduce its harmful action on tick physiology.

The acini **II** exposed to this same concentration were the most affected, especially **c1** and **c3** cells. These showed many cytoplasm vacuoles, unlike that observed in **a** cells, suggesting the occurrence of an asynchronous degeneration process, in which the **a** cells would be the last to degenerate.

The vacuoles in the cytoplasm of acinar cells are probably autophagic and they would be acting as agents in the degradation of cell organelles damaged by permethrin, which would culminate later in cell death and subsequent total tissue disorganization and degeneration.

Acini **III** cells from the salivary glands subjected to 206 ppm of permethrin showed vacuolation, especially in **e** cells. According to Walker et al. (1985) and Gill and Walker (1987), the **d** cells would be responsible for secretion of cement cone components, essential structure in the tick attachment and feeding processes. Thus, our results suggest that permethrin would act by altering the salivary glands physiology, affecting complete formation of the cement cone.

As the permethrin concentration increased (1031 ppm), changes in the salivary glands became more severe, indicating that this concentration would cause more significant damage to glandular tissue. This dose stimulated glandular tissue degeneration progress, resulting in an amorphous mass composed of acini re-

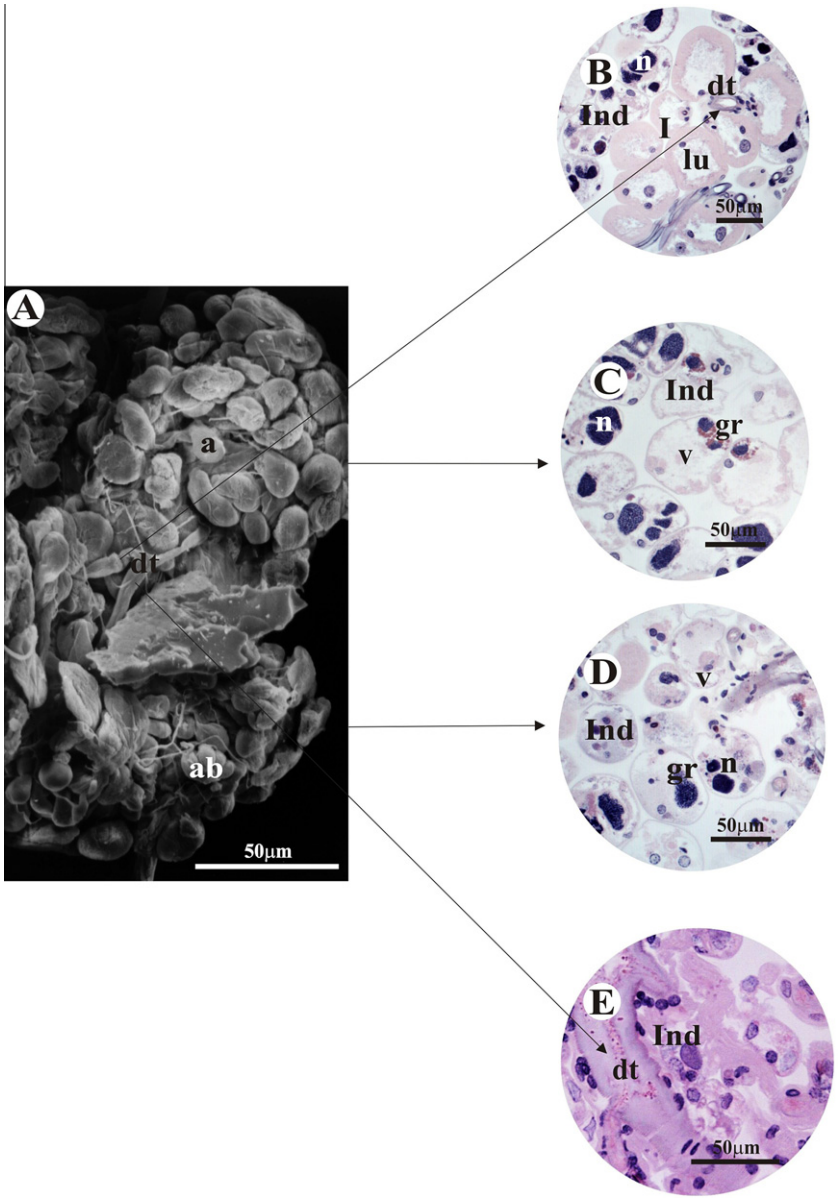

Fig. 4. (**A**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged females exposed to 2062 ppm of permethrin. (**A**) General view of the irregular acini (**a**), apoptotic body (**ab**) and ducts (**dt**). (**B**–**E**) Histological sections of the *R. sanguineus* salivary glands exposed to 2062 ppm of permethrin stained with hematoxylin-eosin (HE) showing **I** (type I acinus) with dilated lumen (**lu**), besides **indeterminate** acini (**Ind**). **dt =** duct, **gr** = granules, **n** = nucleus, **v** = vacuoles.

mains, which could no longer be identified (**indeterminate**), besides the presence of many apoptotic bodies and numerous nuclei with a fragmented and/or picnotic aspect. Similar results were found by Kerr et al. (1995), Hacker (2000) and Furquim et al. (2008b), which suggested that these morphological features could be the key indicators for the cell death by apoptosis, which would occur naturally in field situation during tick salivary gland degeneration. Our data support these authors and also show that permethrin would act by accelerating glandular tissue degeneration, which would occur through an atypical cell death process by apoptosis (fragmentation) and by autophagy (vacuolation), both happening simultaneously. The results obtained here also confirm those described by Pereira et al. (2009) who studied fipronil action on the secretory cycle of tick salivary glands in *R. sanguineus*.

At 1031 ppm permethrin only a few acini **I** were found and these still had with large morphological changes. These observations confirm those of Furquim et al. (2008b) who showed that the acini **I** would be, in the normal salivary gland deactivation process, the last to suffer degeneration that would primarily occur in acini **II** and **III**. Likewise, in individuals subjected to 2062 ppm of permethrin, only few acini **I** were identified, with the other ones classified as **indeterminate**, since they have lost their morphological and histological characteristics due to degeneration. In this case, glandular tissue characteristics such as a) loss of acini shape; b) loss of secretory cells membrane integrity; c) cytoplasm vacuolation; d) presence of a few secretory granules in the cells and e) nuclear changes (shape and size), would be earlier events that culminate in the acini disruption with the consequent formation and release of apoptotic bodies. These data corroborate Furquim et al. (2008b) and Pereira et al. (2009) in studies with salivary glands in *R. sanguineus*.

According to Lomas et al. (1998), salivary gland degeneration in tick females would be modulated by ecdysteroids. These authors suggested that the regulation would happen partly with the

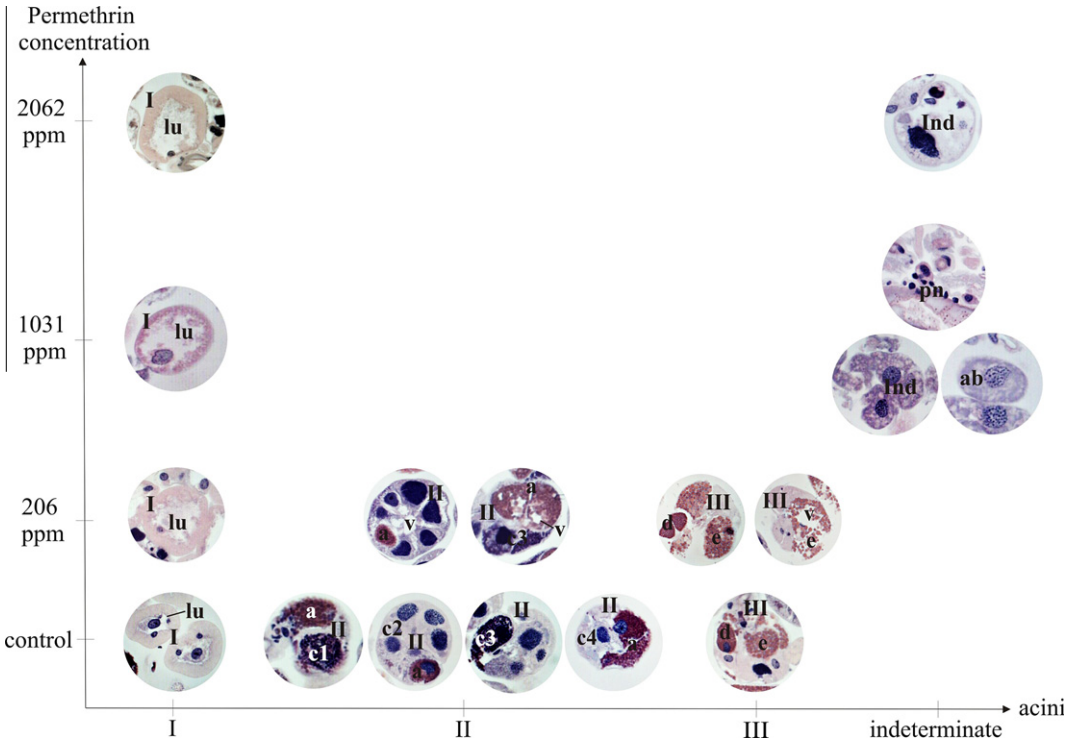

Fig. 5. Permethrin-induced morphological changes in salivary of *Rhipicephalus sanguineus* semi-engorged females. **I** = type I acinus, **II** = type II acinus, **III** = type III acinus, **Ind** = **indeterminate** acinus, **lu** = lumen, **a** = a cell, **c1** = c1 cell, **c2** = c2 cell, **c3** = c3 cell, **c4** = c4 cell, **d** = d cell, **e** = e cell, **v** = vacuoles, **pn** = picnotic nuclei, **ab =** apoptotic body.

involvement of ecdysone, a hormone that could trigger tissue degeneration (Harris and Kaufman, 1985; Lindsay and Kaufman, 1988). The synthesis and release of this hormone would occur at the beginning of the feeding period with peak production shortly after detachment of the ectoparasite from its host (Lomas, 1993).

The results obtained here confirm that permethrin, besides the proven neurotoxic action (Mencke et al., 2003), also accelerates glandular tissue degeneration, an event which would occur naturally and with greater intensity only after full female engorgement. However, this study clearly show that salivary glands of *R. sanguineus* semi-engorged females exposed to higher permethrin concentrations (1031 ppm and 2062 ppm) had an early and high rate of degeneration compared to the control group.

Our data also complement analysis performed by Roma et al. (2010) on the permethrin action in the vitellogenesis processes of *R. sanguineus* females. It is known that when female ticks are unable to complete their feeding process, vitellogenesis is impaired, just as the egg-laying processes which depend indirectly on salivary gland activity. Thus, early salivary gland degeneration caused by the acaricide would cause less blood loss in the hosts, as well as a reduced rate of pathogen transmission (via salivary glands).

Therefore, information obtained in this work confirms the need of conducting studies on tick exocrine glandular system morphology and physiology when exposed to synthetic chemicals in general, which is of great importance in providing information to help improve and develop of tick control methods that are less harmful to non-target organisms. It is also clear in this study that much smaller doses of acaricides may be necessary to inhibit the action of the ectoparasite salivary glands, and consequently its injury to the host as well as to the environment.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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