Degenerative Process and Cell Death in Salivary Glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) Semi-Engorged Female Exposed to the Acaricide Permethrin

ELEN FERNANDA NODARI,[1] GISLAINE CRISTINA ROMA,[1] KARIM CHRISTINA SCOPINHO FURQUIM,[2] PATRÍCIA ROSA DE OLIVEIRA,[1] GERVÁSIO HENRIQUE BECHARA,[2] AND MARIA IZABEL CAMARGO-MATHIAS[1]*

[1]*Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Avenida 24 A, 1515, CP 199, 13506-900 Rio Claro, São Paulo, Brazil*

[2]*Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, UNESP, Via de acesso Prof. Paulo Castellane, s/n, 14884-900 Jaboticabal, São Paulo, Brazil*

KEY WORDS *Rhipicephalus sanguineus*; permethrin; salivary glands; cell death; degenerative process

ABSTRACT Ticks are ectoparasites of great medical and veterinary importance around the world and synthetic chemicals such as permethrin have been used for their control. This study provides a cytochemistry analysis of both degenerative and cell death processes in salivary glands of the brown dog tick *Rhipicephalus sanguineus* semi-engorged females exposed to 206, 1,031, and 2,062 ppm of permethrin. The results presented herein demonstrate that permethrin is a potent chemical acaricide that would act on the glandular tissue's morphophysiology in this tick species by eliciting severe changes in the acinus shape, intense vacuolation of the acinar cells' cytoplasm, marked glandular tissue disorganization, culminating in an advanced degenerative stage with consequent formation of many apoptotic bodies (cell death). In addition, permethrin induced major changes in the acinar cells' nucleus, such as a change both in its shape and size, chromatin marginalization, nuclear fragmentation, and appearance of picnotic nuclei, especially when the highest concentrations of the product were used. Thus, permethrin induced early degeneration of this tissue characterized by significant changes in the structure of acinar cells and production of enzymes related to the cell death process, in addition to interfering directly in the genetic material of these cells. *Microsc. Res. Tech. 00:000–000, 2012.*

INTRODUCTION

Ticks are of great medical and veterinary importance, since they are vertebrates' parasites causing them injuries, and also transmitting pathogens to them as well as for humans (Walker, 1994).

The salivary glands are vital organs for the biological success of this animal group, since the reproductive process would be directly dependent on the normal functioning of these glands. Thus, studies addressing the morphophysiology of exocrine glandular system of ticks when exposed to chemical agents, has been a very important tool for generating information that can assist in developing control methods, making them less harmful to the non-target organisms (hosts, for example). Recent studies developed by the Brazilian Central of Studies on Ticks Morphology-BCSTM at the Universidade Estadual Paulista (UNESP), campus of Rio Claro, Brazil, showed that acaricides' doses smaller than those commercially sold and listed by the manufacturers themselves, would be effective in damaging germ cells of *R. sanguineus* ticks (Oliveira et al., 2008, 2009, Roma et al., 2009, 2010a,b,c).

Among the widely used acaricides to control ticks, especially the brown dog tick *R. sanguineus*, permethrin (active ingredient of Advantage® Max3, Bayer) is a pyrethroid which acts directly on the nervous system of these ectoparasites (Mencke et al., 2003). In addition, Nodari et al. (2011) reported that this acaricide could lead to early glandular tissue degeneration, which interferes or inhibits the ticks' feeding process.

It should be stressed that specific studies on degenerative processes of tick salivary glands are scarce (Furquim et al., 2008a,b,c; L'Amoreaux et al., 2003, Nunes et al., 2006a,b), especially those addressing both the structural and functional glandular tissue changes in ticks exposed to chemical agents, such as those by Pereira et al. (2009, 2011). In this sense, the present study aimed to perform a cytochemistry analysis of both degenerative and cell death processes in salivary glands of the brown dog tick *Rhipicephalus sanguineus* semi-engorged females exposed to permethrin acaricide.

*Correspondence to: Maria Izabel Camargo-Mathias, Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, Avenida 24 A, 1515, CP 199, 13506-900 Rio Claro, São Paulo, Brazil. E-mail: micm@rc.unesp.br

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MATERIAL AND METHODS

Rhipicephalus sanguineus Ticks

A total of 60 semi-engorged females of *R. sanguineus*, weighing 27 mg in average, supplied by the colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) of the UNESP, at the Instituto de Biociências of Rio Claro, SP, Brazil, were used throughout the experiment. The ticks were kept under controlled conditions (28°C ± 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 (Project subjected and approved by the Ethics Committee on Use of Animal-CEUA, UNESP, Rio Claro, Brazil, Protocol no 5,442).

The feeding laboratorial conditions of *R. sanguineus* ticks in the hosts were followed according to Bechara et al. (1995) technique. In summary, the ticks were placed inside a feeding chamber consisting of a plastic tube (2.5 cm of diameter and 3 cm of height) glued with a non-hazardous preparation on to the shaved backs of the hosts. 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. Daily observations were performed on some biological parameters of the female ticks.

Dilution Assays of Permethrin (CAS no: 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 (diluted in distilled water) were based on LC_{50} of 2,062 ppm determined by Roma et al. (2009). The doses correspond to 10% of the LC_{50} (206 ppm), 50% of the LC_{50} (1,031 ppm) and the normal LC_{50} (2,062 ppm). A control group was exposed only to the placebo (distilled water).

Rhipicephalus sanguineus semi-engorged females, after being washed in a sieve with tap water, were dried on soft absorbent paper. After that, 45 females were divided into three treated groups of 15 females each and immersed in Petri dishes containing the above different concentrations of permethrin, for 5 min. The control group was also composed of 15 females that had been immersed in distilled water for the same period. Ticks were then dried on absorbent paper and placed in a BOD incubator (28°C ± 1°C, 80% humidity and 12 h photoperiod) for 7 days. The observation period was established once most often the effect of the acaricide is not immediate, acting slowly on the function of the specimens analyzed (Roma et al., 2009).

Detection of Acid Phosphatase

The *R. sanguineus* ticks (control and treated groups) were dissected and the salivary glands were removed. Afterwards, the salivary glands 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 (microtome Leica RM 2255) at 7 μm thickness. Sections were placed on glass slides, counterstained with Hematoxylin for 2 min, and mounted in Canada balsam and examined and photographed in a Motic BA 300 photomicroscope.

In this experiment, control samples (negative control) were incubated without substrate.

Feulgen Reaction

The salivary glands of *R. sanguineus* females of control and treated groups were removed in a phosphate buffered saline solution (NaCl 7.5 g/L, Na_2HPO_4 2.38 g/L, and KH_2PO_4 2.72 g/L, pH 7.2), fixed in ethanol and acetic acid (3:1) at room temperature for 12 min. The material was then dehydrated in increasing concentrations of ethanol (70, 80, 90, and 95%) for 15 min each, embedded and included in Leica resin, and sectioned (microtome Leica RM 2255) at 3 μm thickness. For the Feulgen reaction (Feulgen and Rossenbeck, 1924), the slides were immersed in 4 N HCl solution for 45 min, washed in distilled water, and exposed to the Schiff's reagent for 90 min in the dark. Slides were counterstained with eosin for 5 min and mounted in Canada balsam and examined and photographed in a Motic BA 300 photomicroscope.

RESULTS

Acid Phosphatase

Figures 1A and 1B had no marking for acid phosphatase, as they corresponded to the control technique performed (negative control), demonstrating that it did not show false positive results.

Group I (Control). The acinar cells (acini I, II, and III) of the salivary glands in semi-engorged female ticks of *R. sanguineus* showed weak positivity for acid phosphatase (Figs. 1C–1E), with the exception of the acini II and III lumens, which were strongly positive to the test (Figs. 1D and 1E).

Group II (Exposed to Permethrin 206 ppm). The salivary glands of females subjected to this concentration of permethrin presented the same characteristics observed in the control group, in other words, they were weakly positive for acid phosphatase technique (Figs. 1F and 1G). However, early damages caused by the chemical in glandular tissue can already be observed in this group, such as the emergence of irregular acini (shape loss), as well as acini which identification were not possible (indeterminate acini) (Figs. 1F and 1G).

Group III (Exposed to Permethrin 1,031 ppm). The salivary glands of individuals exposed to this concentration showed, in general, strong positivity for acid phosphatase technique compared with the other treated groups. At this concentration, few acini I (slightly stained) were identified (Fig. 1H), while the other ones were classified indeterminate and strongly positive for acid phosphatase, an enzyme that is distributed throughout the acinus (Figs. 1I and 1J). In this treatment group, the salivary glands showed more pronounced morphological changes, such as large acinar cells vacuolation (Figs. 1I and 1J).

Group IV (Exposed to Permethrin 2,062 ppm). In general, in females of *R. sanguineus* subjected to this concentration of permethrin glandular tissue presented a large number of indeterminate acini with

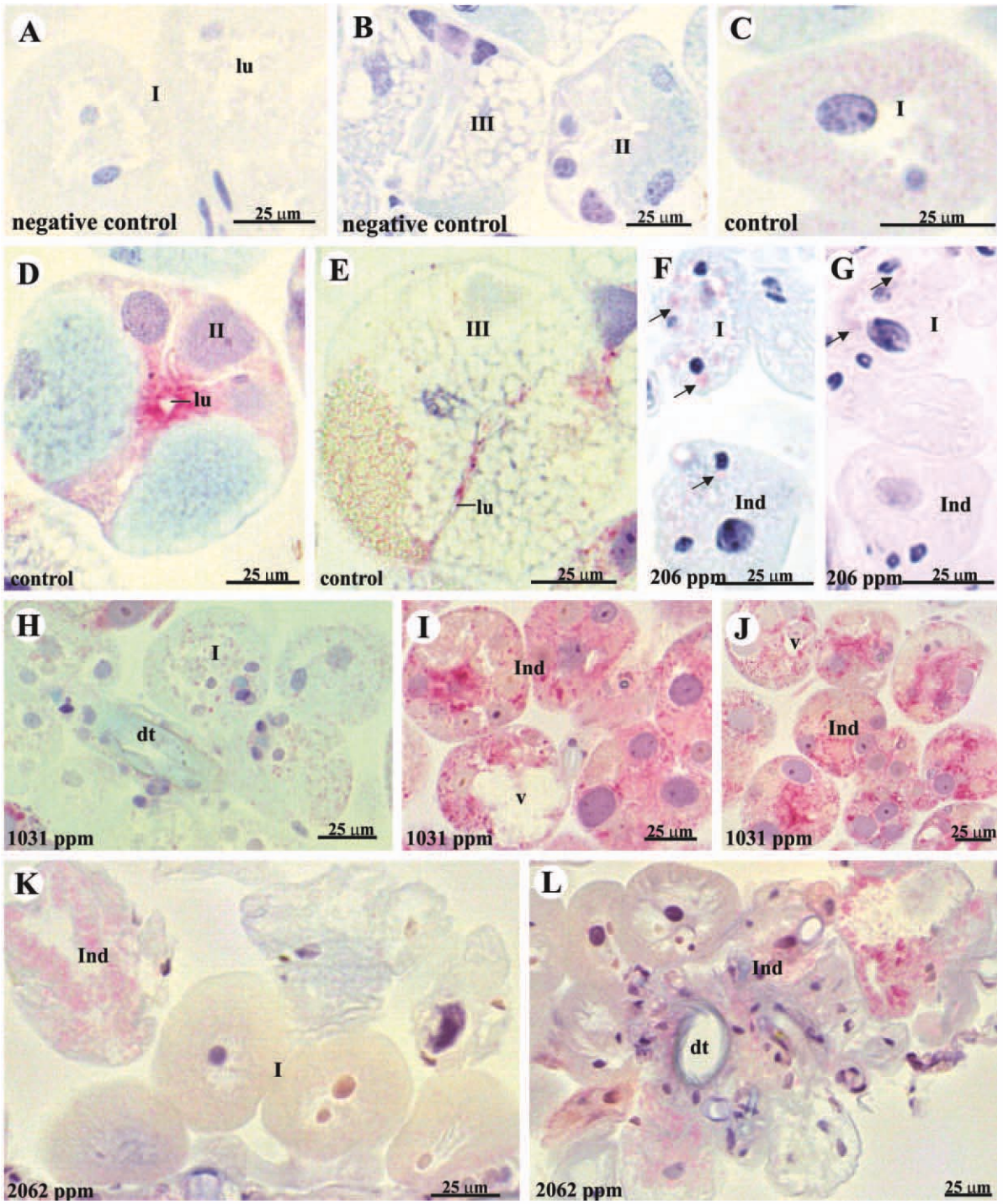

Fig. 1. Acid phosphatase activity in the salivary glands of semi-engorged females of *R. sanguineus* exposed to permethrin. **A** and **B**: Negative control. **C**–**E**: Salivary glands of the control group showing I, II, and III acini weakly positive for acid phosphatase. **F** and **G**: Histological sections of the *R. sanguineus* salivary glands exposed to 206 ppm of permethrin, which are observed only in the acini of type I (I) and indeterminate (Ind), both weakly positive. **H**–**J**: Salivary glands of *R. sanguineus* exposed to 1,031 ppm of permethrin showing type I and indeterminate (Ind) acini with weak and moderate positivity, respectively. **K** and **L**: Salivary glands of females exposed to 2,062 ppm permethrin showing type I acini weakly positive, and indeterminate (Ind) acini strongly positive for acid phosphatase. dt, duct; lu, lumen; v, vacuole; arrow, staining for acid phosphatase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regions that ranged from moderate to strongly positive for acid phosphatase technique (Figs. 1K and 1L). On the other hand, acinus I (still present) showed weak positivity (Fig. 1K). The identification of the other acini (II and III), as well as for the previous concentration, was no longer possible due to large morphological

changes induced by permethrin, such as loss of the acinar cells' limit and intense cytoplasm vacuolation (Figs. 1K and 1L) .

Feulgen

Group I (Control). The nuclei of acinar cells (acini I, II, and III) of the female *R. sanguineus* salivary glands in the control group remained intact, with rounded shape, condensed chromatin distributed throughout the nucleus length (Figs. 2A–2C).

Group II (Exposed to Permethrin 206 ppm). The salivary gland of females exposed to this concentration presented nuclei of I, II, III, and indeterminate acini cells with changes in their shape and size (Figs. 2D–2G). The acini I presented dilated lumen and cells' nuclei with marginalized chromatin (Fig. 2D). The other acini (II, III, and indeterminate) showed enlarged and irregular nuclei, when compared with the control group (Figs. 2E–2G).

Group III (Exposed to Permethrin 1,031 ppm). The salivary glands of females exposed to 1,031 ppm presented severe morphological changes, with marked disorganization of the tissue becoming the acini an amorphous mass thus revealing advanced stage of degeneration (Figs. 2H–2J) and several apoptotic bodies and picnotic nuclei also. The few acini observed were classified as indeterminate and acinar cells with fragmented nuclei (Fig. 2J).

Group IV (Exposed to Permethrin 2,062 ppm). In salivary glands of females subjected to this concentration, large morphological changes are still observed, but these were less severe than those found in the previous concentration, as some acini I were still observed, as well as the indeterminate ones (Figs. 2K–2P). In the acini I, the nuclei were enlarged and/or showed initial stages of chromatin marginalization (Fig. 2K). In the indeterminate acini, the nuclei showed increased size and irregular morphology (Figs. 2N–2P). In these acini some nuclei were fragmented and with marginalized chromatin (Figs. 2L–2P).

DISCUSSION

Currently, several methods for effective control of ticks have been tried, although the most effective is still the use of synthetic chemical acaricides. However, this method causes several damages to the environment and public health (Freitas et al., 2005). Actually, few studies describe changes caused by the toxic action of these compounds in the various systems of ticks, such as the glandular and the reproductive ones (Oliveira et al., 2008, 2009, Pereira et al., 2009, 2011, Roma et al. 2009, 2010a,b,c). In this sense, this article provides a comprehensive cytochemical study of both the degenerative and cell death processes in salivary glands of semi-engorged females of *Rhipicephalus sanguineus* ticks previously exposed to permethrin, as the preliminary data from Nodari et al. (2011) have indicated that this compound would cause severe changes in the glandular tissue, including the premature organ degeneration.

The results of this study showed enzymatic changes (acid phosphatase enzyme) as well as changes in the chromatin organization and in the nuclei function of glandular cells caused by permethrin, which contradict data in the literature showing that this chemical compound would act only as a neurotoxic agent (Mencke et al., 2003).

In the control group the salivary glands of *R. sanguineus* females showed the same characteristics (acid phosphatase and morphonucleases presence) previously described by Furquim et al. (2008a,b) for this same tick species. These data confirm the absence of glandular degeneration in this stage of development (semi-engorged females) in normal conditions, i.e., in the absence of acaricides.

Also in relation to the control group, there was intense phosphatase labeling only in the lumen of acini II and III. It can be inferred herein that the presence of this enzyme is directly related to the process of glandular secretion, in other words, it would participate in many of the components present in the female *R. sanguineus*' saliva as well as also proposed by Furquim et al. (2008b). This hypothesis is sustained by the data obtained and presented by other authors who studied different ticks' species and demonstrated histochemically that acid phosphatase was one of the components participating in the salivary gland secretion of *R. (Boophilus) microplus* (Binnington, 1978; Nunes et al., 2006a) and *R. appendiculatus* individuals (Walker et al., 1985).

In relation to the nuclei of the control individuals' acinar cells, they proved to be intact, confirming data obtained by Furquim et al. (2008a), suggesting that integrity would be due to the absence of degenerative changes in the salivary glands. It is noteworthy that the presence of acid phosphatase would not always be associated with cell death processes, but it would be often involved with secretion processes released by these glands. According to the literature, there would be hydrolytic enzymes (e.g., acid phosphatases) in ticks' salivary secretion which would present very important function for the formation of the tick feeding lesion in the host (Binnington, 1978; Brossard and Wikel, 2004; Steen, 2006; Wikel, 1996, 1999).

In this study, it was observed in the salivary glands cells of females exposed to permethrin the occurrence of several changes in the normal pattern of phosphatase labeling, as well as nuclear, which indicated the presence of severe and irreversible changes arising from the death of the glandular cells.

In the salivary glands of females exposed to 206 ppm of permethrin were observed the first signs of glandular tissue degeneration, such as the presence of cytoplasm vacuolation. These data corroborate the studies performed by Nodari et al. (2011) for the same tissue exposed to the same acaricide. Moreover, in the nuclei of acinar cells, changes in shape (irregular), size (increased), and chromatin disposition (early marginalization) were also observed. However, it was found that for the permethrin 206 ppm concentration, no change in the phosphatase labeling occurred, in relation to those ones from the control group.

Thus, although morphological and cytochemical changes occur respectively in the cytoplasm and nucleus of the salivary glands cells exposed, no changes were observed for acid phosphatase labeling, which led the authors to suggest that exposure to permethrin in a 206 ppm concentration would only affect, in a first moment, the cytoplasm organization in glandular cells (cytoplasmic vacuoles) and the physiological state of

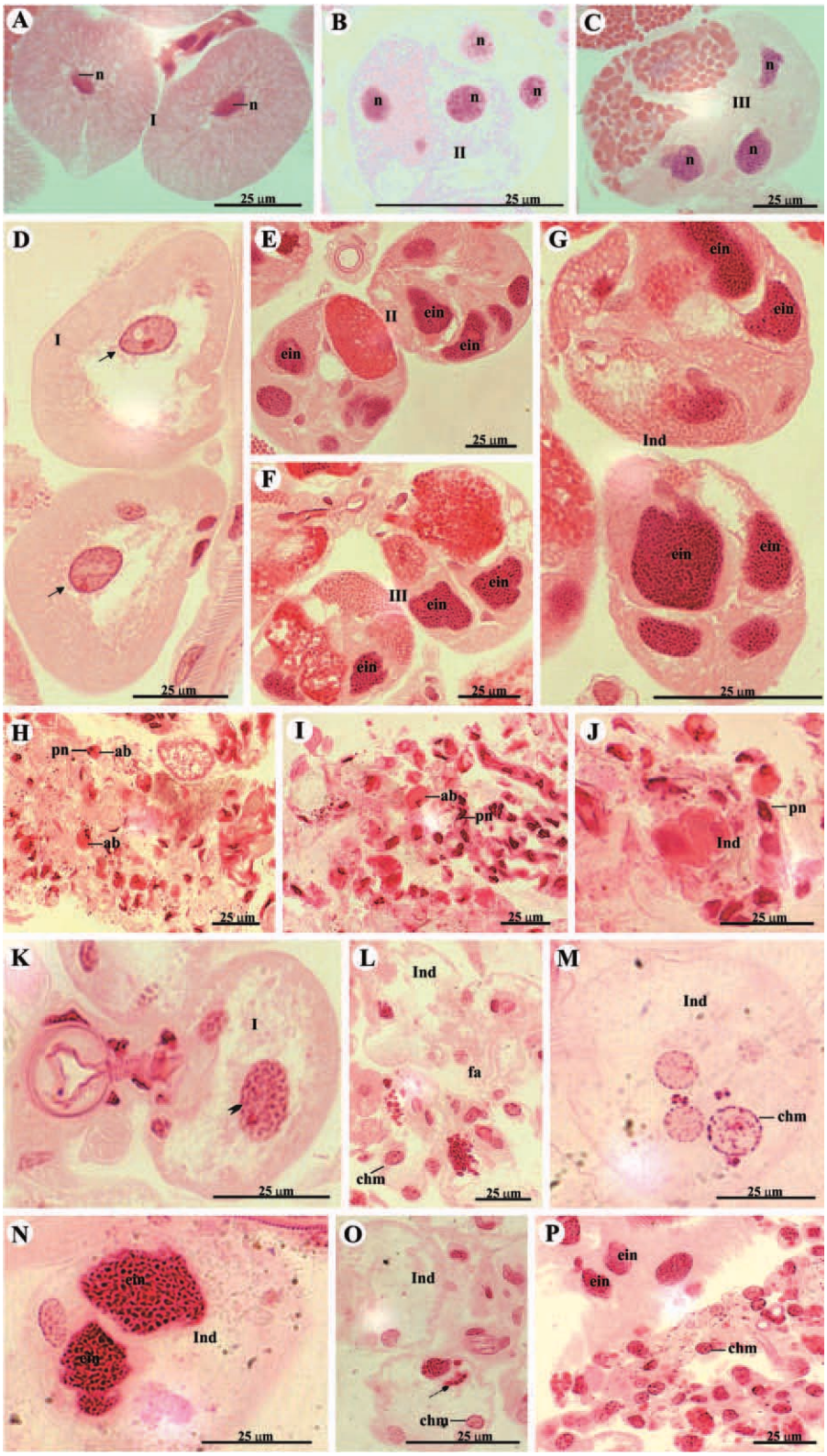

Fig. 2. Histological sections of the salivary glands of semi-engorged females of *R. sanguineus* (control groups and exposed to permethrin) subjected to the Feulgen reaction. **A–C**: Control group; observe intact nuclei (n) of cells from I (I), II (II), and III (III) acini. **D–G**: Salivary glands of the ticks exposded to 206 ppm of permethrin; note nuclei of cells from I (I), II (II), III (III) and indetermined (Ind) acini with changes in relation to the control group. **H–J**: Salivary glands of individuals exposed to 1,031 ppm of permethrin: showing indeterminate (Ind) acini, apoptotic bodies (ab), and picnotic nuclei (pn). **K–P**: Salivary glands of females exposed to 2,062 ppm of permethrin, which are observed in the I and indeterminate (Ind) acini with many morphological changes. n, intact nucleus; ein, enlarged and irregular nucleus; arrow, enlarged nucleus with chromatin margination; arrow head, enlarged nucleus with beginning chromatin margination; pn, picnotic nucleus; dashed arrow, fragmented nucleus. fa, fragmenting acini; chm, normal sized nucleus and with chromatin margination. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the same nucleus, but would not change the cellular hydrolytic behavior (acid phosphatase action).

At higher permethrin concentrations (1,031 and 2,062 ppm) severe changes were observed for both acid phosphatase labeling—which has intensified—and nucleus, which began to show changes in size (increased) in shape (irregular or fragmented) and in degree of chromatin condensation (condensed and/or marginalized). These data corroborate the morphological analysis previously performed by Nodari et al. (2011), which showed increased incidence of the degenerative process in the glandular tissue obtained from individuals exposed to higher permethrin concentrations.

The data so far discussed in this study may suggest that the synthesis of acid phosphatase, related to the death process of glandular cells, would be intensified by higher permethrin concentrations, demonstrating that the same action would be primarily related to morphological changes of the glandular cells and only later would act in cellular hydrolytic behavior. According to Nodari et al. (2011), permethrin could accelerate the degeneration of salivary glands, without, however, change the way this process occurs, which according to Furquim et al. (2008b), under normal conditions would happen by atypical apoptosis. According to these authors, acid phosphatase participates in this process finalization, removing cytoplasm debris and helping cell fragmentation. Thus, the increase in phosphatase labeling forward to permethrin exposure would possibly cause the acceleration of the gland degenerative process, which in normal conditions would occur only at the end of the feeding process (Furquim et al., 2008b).

Furthermore, nuclear changes detected here (picnotic nuclei, chromatin marginalization and fragmentation) also characterized the apoptosis occurrence. In fact, according to Bowen (1993), Bowen and Bowen (1990), Furquim et al. (2008a), Häcker (2000), Lockshin and Zakeri (1996), and Zakeri and Ahuja (1997), those events were the result of biochemical and morphological changes occurring in the nucleus as result of the apoptotic process. Moreover, Furquim et al. (2008a) argued that the presence of increased nuclei in cells of the glandular acini, as also observed here, would suggest a chromatin breakdown—characteristic of the processes observed during apoptosis (Bowen, 1993; Bowen and Bowen, 1990; Häcker, 2000; Lockshin and Zakeri, 1996; Zakeri and Ahuja, 1997).

On the basis of these considerations, it can be concluded that permethrin, in addition to the already proven its neurotoxic action (Mencke et al., 2003), it acts to accelerating the salivary glands degenerative process of *R. sanguineus* semi-engorged females, which can be confirmed by the observation of significant changes in the glandular cells nuclei, as well as in the increased hydrolytic labeling (action group of the enzyme acid phosphatase) of glandular cells analyzed here.

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