UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS - RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR E MOLECULAR)

ESTUDO DAS GLÂNDULAS SALIVARES DE FÊMEAS E DE MACHOS DE CARRAPATOS *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE): CARACTERIZAÇÃO DO CICLO SECRETOR COM ÊNFASE NO PROCESSO DE DEGENERAÇÃO

KARIM CHRISTINA SCOPINHO FURQUIM

Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular).

Rio Claro
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Orientadora: Profa. Dra. Maria Izabel Camargo-Mathias

Co-Orientador: Prof. Dr. Gervásio Henrique Bechara

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Dedico esta tese...

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(Batuíra, trecho do livro "Conviver e Melhorar")

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Resumo e Abstract

RESUMO

As glândulas salivares de carrapatos fêmeas: em jejum, com dois e quatro dias de alimentação (em ingurgitamento), alimentadas (ingurgitadas), com três e sete dias pós-alimentação (pós-ingurgitamento); e de machos: em jejum, com dois, quatro e sete dias de infestação e com três e sete dias pós-remoção do hospedeiro, da espécie *Rhipicephalus sanguineus*, foram analisadas morfológica, histoquímica e citoquimicamente.

Nas fêmeas elas são compostas pelos ácinos I (agranulares), II e III (granulares), e nos machos pelos ácinos I (agranulares), II, III e IV (granulares). Em ambos os sexos também foram observados ácinos **Indeterminados**, assim chamados devido ao processo degenerativo onde perderam suas características e não puderam ser identificados.

Histologicamente os ácinos do tipo I sempre apresentaram uma célula **central** maior e várias **periféricas** menores, os do tipo II nas fêmeas apresentaram células "**indiferenciadas**", **indefinidas 1** e **2**, **a**, **b** e **c1** a **c6** e nos machos as "**indiferenciadas**", **indefinidas 1** e **2**, **a**, **b** e **c1** a **c8**, tendo sido as "**indiferenciadas**", as **indefinidas 1** e **2** e as **c5** a **c8** descritas pela primeira vez. As células aqui denominadas de **indeterminadas** foram observadas nos ácinos II em estágio de degeneração. Os ácinos do tipo III apresentaram as células **d**, **e** e **f** e os do tipo IV células **g**.

Quanto ao estágio, durante todo o processo de alimentação, tanto nas fêmeas quanto nos machos, os ácinos I sofreram apenas alterações no tamanho.

Nos ácinos II de fêmeas em jejum apenas as células "**indiferenciadas**", **indefinidas 1** e **2** e **a**, **c1** e **c3** foram observadas; nos ácinos III os três tipos descritos estavam presentes.

Nos machos em jejum os ácinos II e III tinham as mesmas características observadas nas fêmeas em jejum. Nos ácinos IV todas as células (**g**) estavam pouco ativas.

Com o iniciar da alimentação, as glândulas passaram a secretar ativamente, sendo que nas fêmeas com dois dias de alimentação observaram-se nos ácinos II todos os tipos celulares, exceto as células "**indiferenciadas**" e **indefinidas 1 e 2**, não ocorrendo alteração nos III, exceto pela presença de secreção nas células **f**.

Nos machos com dois dias de infestação, os ácinos II apresentaram todos os tipos de células, exceto as "**indiferenciadas**" e **indefinidas 1** e **2**, além das **f** estarem com citoplasma reduzido. Nos ácinos IV todas as células estavam repletas de secreção.

Nos ácinos II de fêmeas com quatro dias de alimentação as células **c5** não foram mais observadas e nos III as **f** mudaram de forma e função.

Nos ácinos II de machos com quatro dias de infestação as células **c6** não foram mais observadas, bem como as **f** dos III.

Nas fêmeas alimentadas todas as células dos ácinos granulares estavam em processo de morte, porém, ainda foi possível identificar nos ácinos II as células **a**, **c1** e **c3** e nos III as **d**, **e** e **f**. Naquelas com três dias pós-alimentação nos II as **a** e **c3**. Fêmeas com sete dias pós-alimentação tinham suas glândulas completamente degeneradas.

Nos machos com sete dias de infestação os ácinos granulares estavam em degeneração, embora os II apresentassem ainda algumas células **c1** e **c8** íntegras. Nos machos com três dias pós-remoção do hospedeiro todas as células estavam em degeneração e as **f** não foram mais observadas. A mesma situação aconteceu com aqueles com sete dias pós-remoção, porém nos ácinos II apenas as **c2** não foram mais observadas, permanecendo os III igual ao descrito para o estágio anterior.

Os resultados mostraram que durante a degeneração das glândulas salivares de *R. sanguineus* as células morrem por apoptose atípica, sendo que as alterações celulares ocorrem na seguinte ordem: a) presença de poucos grânulos, rompimento ou ainda ausência dos mesmos, b) aumento de RNA, c) alterações nucleares quanto à forma, tamanho, grau de condensação e disposição da cromatina, d) retração citoplasmática e perda da forma celular, e) queda da atividade ATPásica, f) perda da integridade da membrana celular, g) perda dos limites celulares, h) aumento da atividade fosfatásica e i) vacuolização citoplasmática, tudo isso provocando a desorganização e o rompimento dos ácinos, com conseqüente liberação de corpos apoptóticos.

ABSTRACT

The salivary glands of the tick *Rhipicephalus sanguineus* were analyzed morphologically, histochemically and cytochemicallt at the following conditions: unfed, two and four-day fed, engorged females and females at day three and seven post-engorgement; and unfed males and males at day two, four and seven days post-attachment, and at day three and seven post-detachment from the rabbits.

In females, these glands consist of types I (agranular), II and III (granular) acini and in males, of types I (agranular), II, III and IV (granular) acini. In both sexes, **Indeterminate** acini were also observed, which due to the degeneration process, have lost their characteristics and could not be identified.

Histologically, type I acinus always exhibited a large **central** cell and several smaller **peripheral** ones. In females, type II acini are composed of "**undifferentiated**", **undefined 1** and **2**, **a**, **b** and **c1–c6** ; and in males, "**undifferentiated**", **undefined 1** and **2**, **a**, **b** and **c1–c8**, with "**undifferentiated**", **undefined 1** and **2**, and **c5–c8** being described here for the first time. The cells termed in this study as **indeterminate** were observed in degenerating type II acini. Type III acinus exhibited cells **d**, **e** and **f**, while type IV acini, cells **g**.

Regarding the feeding stage, throughout the entire process, in females as well as males, type I acinus only underwent changes in size.

In type II acinus of unfed females, only "**undifferentiated", undefined 1** and **2** and **a**, **c1**, and **c3** cells were observed, while in type III acinus, the three cell types described were present.

In unfed males, type II and III acini exhibited the same characteristics observed in unfed females. In type IV acinus, all cells (**g**) were little active.

With the start of feeding, glands began to secrete actively. In two-day fed females, all cell types were observed in type II acinus, except cells types "**undifferentiated"**, **undefined 1** and **2**. Type III acinus did not exhibit changes, except by the presence of secretion in cells **f.**

In males at day two post-attachment, type II acinus presented all cell types, except "**undifferentiated"**, **undefined 1** and **2**; also cells **f** exhibited reduced cytoplasm. In type IV acinus, all cells were filled with secretion.

In type II acinus of four-day fed females, cells **c5** were no longer observed; and in type III acinus, changes in shape and function were observed in cells **f**.

In males at day four post-attachment, cells **c6** of type II acinus were no longer observed, as well as cells **f** of type III acinus.

In engorged females, all cells of granular acini were undergoing cell death. However, cells **a**, **c1**, and **c3** of type II acinus, and **d**, **e**, and **f** of type III acinus could still be identified. This was also possible for cells **a** and **c3** of type II acinus of females at day three post-engorgement. At day seven post-engorgement, female salivary glands were completely degenerated.

In males at day seven post-attachment, granular acini were undergoing degeneration, although type II acinus still exhibited some intact **c1** and **c8** cells. In males at day three post-detachment from the host, all cells were undergoing degeneration and cells **f** were no longer observed. The same was observed in males at day seven post-detachment. In type II acinus of the latter, however, only cells **c2** were no longer observed, while type III acinus remained unchanged compared to the previous stage.

The results showed that during the degeneration of salivary glands of *R. sanguineus*, cells die by atypical apoptosis, with cell changes occurring in the following order: a) presence of few granules, rupture or absence of granules, b) increase in RNA, c) nuclear changes in: shape, size, arrangememt and condensation of chromatin, d) cytoplasmic shrinkage and loss of cell shape, e) decrease in ATPase activity, f) loss of integrity of the cell membrane, g) loss of cell boundaries, h) increase in phosphatase activity and i) cytoplasmic vacuolation; all causing disorganization and breakdown of acini with consequent release of apoptotic bodies.

Introdução Geral

I. INTRODUÇÃO GERAL

Os carrapatos *Rhipicephalus sanguineus*, popularmente conhecidos como carrapatos do cão, são cosmopolitas das regiões tropicais e temperadas (REY, 1973; WALKER, 1994) e têm ampla distribuição geográfica pelas Américas, Europa, África, Ásia e Austrália (RIBEIRO et al., 1996). Muito embora o cão seja seu hospedeiro principal (REY, 1973; WALKER, 1994), ele pode também ser encontrado em outros mamíferos, inclusive no homem (REY, 1973).

A espécie *R. sanguineus* é caracterizada por ser trioxena (ciclo biológico desenvolvido em três hospedeiros), parasitando um novo hospedeiro em cada fase da vida (larva, ninfa e adulto) e voltando ao solo sempre que completado seu repasto sanguíneo. As fêmeas em quatro ou cinco dias depois da alimentação começam a ovipositar, podendo colocar até três mil ovos no período de 15 dias, os quais eclodirão em três semanas. Em quatro ou cinco dias as larvas estarão aptas a instalar-se no seu primeiro hospedeiro e o ciclo de vida se completará em dois ou três meses, exceto nas regiões temperadas, onde pode haver hibernação na fase ninfal ou na adulta. A longevidade dos adultos é de aproximadamente um ano (REY, 1973).

No processo de alimentação os carrapatos primeiramente caminham sobre a pele do hospedeiro, tocando-a com a extremidade dos palpos maxilares, onde localizam-se estruturas sensoriais. Assim que é encontrado o ponto adequado, prendem-se firmemente e forçam o hipostômio, que possui fileiras de dentes quitinosos dirigidos para trás contra a pele do hospedeiro, penetrando-a lentamente e funcionando como um órgão de fixação ao animal durante todo o repasto sanguíneo. As mandíbulas também penetram na pele e com movimentos cortantes dilaceram-na (REY, 1973).

As glândulas salivares dos ixodídeos (família Ixodidae) são órgãos vitais para o sucesso biológico deste grupo, pois apresentam grande diversidade de funções, como a produção de substâncias necessárias à fixação e à alimentação dos parasitas (BINNINGTON, 1978; WALKER et al., 1985; GILL; WALKER, 1987).

A presença destes órgãos e sua atuação nos hospedeiros dão hoje a estes parasitas o "status" de um dos mais importantes grupos dentro dos artrópodos. As glândulas salivares são também responsáveis pela transmissão de agentes infecciosos a outros grupos de animais, atribuindo a esses parasitas grande importância médico-veterinária (BALASHOV, 1983). A própria alimentação dos carrapatos causa no hospedeiro perda de sangue, refletindo em prejuízo econômico, principalmente para as cadeias de produção do leite e da carne, no caso dos bovinos.

Segundo Sonenshine (1991), a saliva é uma mistura complexa atuando numa variedade de funções durante os períodos de parasitismo e não parasitismo:

1. Aumentando o fluxo sanguíneo (circulação) na região da picada no hospedeiro, através da secreção de agentes vasoativos (FAWCETT et al, 1986; SAUER et al., 2000);
2. Introduzindo anticoagulantes que possibilitam que o sangue do hospedeiro permaneça fluido (RIBEIRO et al., 1985; FAWCETT et al., 1986; SAUER et al., 2000);
3. Inibindo o processo inflamatório no hospedeiro (RIBEIRO et al., 1985; FAWCETT et al., 1986; SAUER et al., 2000)
4. Imunossuprimindo o hospedeiro e possibilitando aos carrapatos a fixação ao hospedeiro sem que este último desenvolva rejeição (RIBEIRO et al., 1985; WIKEL, 1999; SAUER et al., 2000);
5. Fixando o carrapato à pele do hospedeiro através da secreção de cemento para formação do cone (FAWCETT et al, 1986);
6. Excretando o excesso de água e íons provenientes da alimentação (sangue) (SAUER et al., 2000);
7. Secretando solução higroscópica, a qual fica depositada na região bucal e absorve a água atmosférica, hidratando o parasita nos períodos de não parasitismo (SAUER et al., 2000; BOWMAN; SAUER, 2004);
8. Produzindo secreções que lubrificam o espermatóforo durante sua transferência na cópula (FELDMAN-MUHSAM et al., 1970 apud FAWCETT et al., 1986);

9. Liberando toxinas que causam paralisia no hospedeiro (FAWCETT et al., 1986);
10. Liberando antígenos (FAWCETT et al, 1986);
11. Veiculando agentes patogênicos ao hospedeiro (FAWCETT et al., 1986; WIKEL, 1999; SAUER et al., 2000; BOWMAN; SAUER, 2004);

As glândulas salivares, tanto dos machos quanto das fêmeas dos carrapatos, são estruturas pares (SCHUMAKER; SERRA FREIRE, 1991; SONENSHINE, 1991) que se estendem antero-lateralmente na porção ventral da cavidade corpórea, desembocando na cavidade oral (OLIVIERI; SERRA-FREIRE, 1992; TILL, 1961; WALKER et al., 1985).

Elas são constituídas por uma porção secretora e uma excretora, sendo desprovidas de um reservatório para armazenamento da secreção. A porção secretora é formada por diferentes tipos de ácinos, I, II, III e IV, este último presente só nos machos (BINNINGTON, 1978; WALKER et al., 1985; FAWCETT et al., 1986; GILL; WALKER, 1987; SONENSHINE, 1991; OLIVIERI; SERRA-FREIRE, 1992; SERRA-FREIRE; OLIVIERI, 1993). Nas fêmeas de *R. appendiculatus* foi registrado um total de 1400 ácinos/glândula, sendo aproximadamente 250 do tipo I, 300 do II e 850 do III. Nos machos um total de 1350 ácinos/glândula, sendo 150 do tipo I, 200 do II, 600 do III e 400 do IV (WALKER et al., 1985). Segundo Sonenshine (1991), os ácinos I são agranulares e os II, III e IV granulares por conterem secreção na forma de grânulos no citoplasma de suas células.

A porção excretora é composta por um sistema de dutos ramificados, havendo um principal ou excretor comum, tubo central longo de maior calibre, que leva a secreção para a cavidade bucal do carrapato. Deste partem dutos intermediários ou secundários (calibre menor), que se subdividem ao longo do comprimento da glândula em pequenos canalículos ou dutos acinares que coletam diretamente do ácino a secreção nele produzida (TILL, 1961; BALASHOV, 1979; WALKER et al, 1985; FAWCETT et al, 1986; NUNES et al., 2005). Todos os dutos são histologicamente semelhantes (OLIVIERI; SERRA-FREIRE, 1992).

Os ácinos distribuem-se de forma regular ao longo do sistema de dutos. Os do tipo I estão ligados na porção anterior e mediana do duto principal. Os II, conectados aos dutos intermediários, distribuem-se nas regiões anterior e mediana da glândula. Os III estão ligados à extremidade das ramificações dos dutos intermediários e distribuem-

se na região mediana-periférica da glândula (OLIVIERI; SERRA-FREIRE, 1992). Os IV, presentes somente nos machos, encontram-se próximos aos III (SONENSHINE, 1991; WALKER et al., 1985; GILL; WALKER, 1987).

Vários autores, por meio da utilização de técnicas de microscopia, atestaram a complexidade dos ácinos das glândulas salivares de carrapatos por meio da utilização de técnicas histológicas associadas às histoquímicas, as quais possibilitam a identificação dos mesmos (TILL, 1961; BINNINGTON, 1978; BALASHOV, 1983; WALKER et al., 1985; FAWCETT et al., 1986; GILL; WALKER, 1987; SONENSHINE, 1991; MARZOUK; DARWISH, 1994). Uma das técnicas mais utilizadas é a reação do PAS, que além de mostrar o tamanho e a forma dá aos grânulos secretores diferentes intensidades de coloração.

De acordo com a classificação dos ácinos, os agranulares estão envolvidos com o balanço hídrico do animal e os granulares com a alimentação (OLIVIERI; SERRA-FREIRE, 1992) e com a osmorregulação do carrapato na fase de grande consumo de sangue (KAUFMAN; SAUER, 1982; FAWCETT et al., 1986; SONENSHINE, 1991).

O ácino do tipo I, agranular, é responsável pela eliminação do excesso d'água vindo do sangue na alimentação, além de secretar, em períodos de não parasitismo, soluções higroscópicas (BINNINGTON,1978; WALKER et al., 1985), e é composto por uma grande célula **central**, rodeada por várias **periféricas** menores (BINNINGTON, 1978; FAWCETT et al., 1986; WALKER et al., 1985; GILL; WALKER, 1987; OLIVIERI; SERRA-FREIRE, 1992; SERRA-FREIRE; OLIVIERI, 1993).

O do tipo II, granular, segundo a literatura, é constituído por diferentes tipos de células secretoras: **a**, **b**, **c1**, **c2**, **c3** e **c4** (BINNINGTON, 1978). Sabe-se que as **a** estão envolvidas com a secreção do cemento para construção do cone de fixação (BINNINGTON, 1978; WALKER et al., 1985; FAWCETT et al., 1986; GILL; WALKER, 1987), e as **b** e **c** com as várias funções que têm sido atribuídas à saliva na manipulação da resposta do hospedeiro (BINNINGTON, 1978; WALKER et al., 1985).

O do tipo III, também granular, é formado por três tipos celulares, **d**, **e** e **f** (BINNINGTON, 1978; WALKER et al., 1985; GILL; WALKER, 1987; MARZOUK; DARWISH, 1994). As células **d** e **e** secretam componentes do cemento durante a fixação (BINNINGTON, 1978; WALKER et al., 1985; GILL; WALKER, 1987). As

células **f** têm duas funções: secretora e osmorreguladora, juntamente com as células epiteliais abluminais (BINNINGTON, 1978; WALKER et al., 1985; COONS; LAMOREAUX, 1986; GILL; WALKER, 1987).

O ácino IV, granular, exclusivo dos machos, é constituído por um único tipo de célula, o **g**. Em alguns ixodídeos seu produto participa da secreção do cemento (FAWCETT et al., 1986) e pode também produzir outra secreção importante na transferência do espermatóforo para a fêmea (FELDMAN-MUHSAM et al., 1970 apud FAWCETT et al., 1986).

As glândulas salivares, como qualquer outro órgão envolvido na produção de secreção, apresentam ciclo secretor bem definido, marcado por uma fase de produção e outra de liberação da secreção, seguida posteriormente pela degeneração do órgão. Esse ciclo secretor é determinado pelo estado fisiológico do carrapato, que pode ser classificado como jejum, em alimentação (semi-ingurgitado) e alimentado (ingurgitado). O estudo do ciclo secretor das glândulas salivares já foi realizado em carrapatos de outros gêneros e espécies, mas não em *R. sanguineus*.

As glândulas salivares de carrapatos adultos machos e fêmeas apresentam diferenças, que se tornam mais pronunciadas à medida que se dá a alimentação (TILL, 1961), provavelmente devido ao fato de haver diferenças comportamentais entre ambos os sexos (BINNINGTON, 1978). As fêmeas se fixam e se alimentam somente uma vez, já os machos se fixam e alimentam-se várias vezes (BINNINGTON, 1978).

Durante a alimentação, o tecido glandular sofre rápida transformação estrutural e funcional. Os ácinos do tipo I, em ambos os sexos, sofrem apenas mudanças no tamanho. Os do tipo II aumentam no tamanho e na atividade secretora, sendo que nas fêmeas são os ácinos dominantes na produção de secreção no final do estágio alimentar (WALKER et al., 1985). Os dos tipos III e IV sofrem mudanças significativas (FAWCETT et al., 1986). Os IV nos machos em jejum são inclusive denominados de "indiferenciados" (BINNINGTON, 1978; WALKER et al., 1985; FAWCETT et al., 1986; GILL; WALKER, 1987; SERRA-FREIRE; OLIVIERI, 1993). Após a fixação dos carrapatos as células glandulares hipertrofiam, deixando estes ácinos maiores que os II e III (FAWCETT et al., 1995).

Nos carrapatos em jejum as glândulas salivares se encontram em fase pré-secretora. A fixação desses animais ao hospedeiro é o estímulo para que elas iniciem

seu desenvolvimento, que só se completa quando o carrapato inicia a alimentação (WALKER et al., 1985). Assim, durante o repasto sanguíneo as glândulas se encontram em plena atividade secretora. No caso das fêmeas, após o final da alimentação e desprendimento do hospedeiro, as glândulas em degeneração vão diminuindo gradualmente sua capacidade secretora até a oviposição da fêmea, quando o órgão já estará completamente desativado, restando somente o sistema de dutos (TILL, 1961; SONENSHINE, 1991). Nos machos, devido aos comportamentos de fixação e de desprendimento várias vezes do hospedeiro, as glândulas salivares conseguem ainda manter-se ativas (WALKER et al., 1985).

Na literatura, trabalhos específicos sobre o processo de degeneração das glândulas salivares de carrapatos são escassos. L'Amoreaux et al. (2003) e Nunes et al. (2006a, b) realizaram um estudo aprofundado e específico da degeneração da glândula salivar de fêmeas de *Dermacentor variabilis* e *R. (Boophilus) microplus*, respectivamente, revelando indícios de morte apoptótica nas células glandulares.

Segundo Lomas et al. (1998), a degeneração das glândulas salivares é controlada hormonalmente por um esteróide. Estes autores sugeriram que a regulação se daria em parte pela ecdisona, hormônio este que provocaria a degeneração deste tecido (HARRIS; KAUFMAN, 1985; LINDSAY; KAUFMAN, 1988). Sua síntese e liberação iniciar-se-iam no começo do período alimentar com um pico de produção logo após o desprendimento (LOMAS, 1993). A liberação de ecdisteróides para a hemolinfa, até onde se sabe, teria relação com dois fatores: o chamado "peso crítico" atingido pelo carrapato (WEISS; KAUFMAN, 2001) e o "fator de macho/ingurgitamento", liberado pelas gônadas do macho, o qual seria transferido para a fêmea durante a cópula (WEISS; KAUFMAN, 2004).

A degeneração das glândulas salivares das fêmeas, além de ser um processo hormonalmente controlado (LOMAS et al., 1998; HARRIS; KAUFMAN, 1985; LINDSAY; KAUFMAN, 1988), seria também programado (BOWMAN; SAUER, 2004). Segundo Nunes et al. (2006b), proporcionaria aos carrapatos uma economia energética, visto que estas estruturas não lhes seriam mais necessárias depois de finalizada a alimentação.

Segundo a literatura, no processo de degeneração de forma geral ocorreriam dois tipos de morte celular geneticamente programados: a apoptose e a morte celular

autofágica (CLARKE, 1990; BOWEN, 1993; JIANG et al., 1997; ZAKERI; AHUJA, 1997), ambas com características morfológicas e citoquímicas típicas, podendo, no entanto, haver a sobreposição de eventos nos dois tipos, mesmo que em momentos diferentes (ZAKERI et al., 1995).

A apoptose seria caracterizada pelo colapso nuclear precoce (KERR et al., 1995; ZAKERI et al., 1995; LOCKSHIN; ZAKERI, 1996), onde o DNA, através da ação de endonucleases, seria clivado nas regiões internucleossômicas (BOWEN, 1993; ZAKERI et al., 1995; LOCKSHIN; ZAKERI, 1996; ZAKERI; AHUJA, 1997; HÄCKER, 2000). Segundo Häcker (2000), esta clivagem do DNA condensaria e marginalizaria a cromatina, além de agir na formação das bolhas do envoltório nuclear (KERR et al., 1995; ZAKERI et al., 1995; HÄCKER, 2000). Também na apoptose ocorreria retração citoplasmática, devido à perda de água (CLARKE, 1990; BOWEN, 1993; KERR et al., 1995; ZAKERI; AHUJA, 1997) e formação de corpos apoptóticos, resultado da fragmentação celular, os quais, porém, ainda permaneceriam interligados por membranas, sendo a seguir fagocitados (BOWEN; BOWEN, 1990; KERR et al., 1995; LOCKSHIN; ZAKERI, 1996; ZAKERI; AHUJA, 1997; HÄCKER, 2000).

De acordo com Bowen e Bowen (1990), a apoptose seria um processo dependente de ATP, por isto as bombas de íons da membrana plasmática continuariam funcionando. Contudo, no estágio final da apoptose, após a formação dos corpos apoptóticos, observar-se-ia queda de ATP e da atividade da ATPase, com conseqüente perda da integridade (funcionamento) da membrana plasmática (BOWEN; BOWEN; 1990; KERR et al., 1995; MCGAHON et al., 1995).

Segundo Clarke (1990) e Zakeri et al. (1995), alguns tecidos, durante o processo apoptótico, poderiam sofrer a ação de hidrolases (autofagia), justificando as discussões e controvérsias sobre qual seria o real papel exercido pelas hidrolases ácidas durante a apoptose (BOWEN; BOWEN, 1990; BOWEN, 1993).

A morte autofágica, comumente discutida em insetos, principalmente durante a metamorfose, seria caracterizada primariamente pelo aumento no nível da atividade de hidrolases ácidas (fosfatase ácida) e surgimento de extensos e numerosos vacúolos autofágicos, causando por conseqüência a destruição da célula (PIPAN; RAKOVEC, 1980; ARMBRUSTER et al., 1986; CLARKE, 1990; CUMMINGS; BOWEN, 1992; ZAKERI et al., 1995; LOCKSHIN; ZAKERI, 1996; JOCHOVÁ et al., 1997;

GREGORC et al., 1998). Outras características da morte autofágica seriam a ocorrência tardia de colapso nuclear (BOWEN, 1993; ZAKERI et al., 1995; LOCKSHIN; ZAKERI, 1996), bem como a remoção dos restos celulares por heterofagia (PAUTOU; KIENY, 1971 apud CLARKE, 1990; KRSTIC; PEXIEDER, 1973 apud CLARKE, 1990).

Embora existam amplos estudos dos processos de morte celular, nem sempre é possível determinar o tipo exato que ocorre num tecido, pois as alterações celulares não são exclusivas de nenhum dos tipos conhecidos ou ainda pode haver sobreposição de alterações num mesmo tecido, caracterizando por exemplo morte apoptótica com envolvimento de hidrolases (CLARKE, 1990; ZAKERI et al., 1995; YAMAMOTO et al., 2000).

Nos invertebrados a caracterização e classificação dos processos de morte celular é ainda muito mais complexa que nos vertebrados. A literatura disponível relata diferentes formas de morte, das quais algumas apresentam alterações comuns à apoptose e à autofagia (BOWEN et al., 1996; LEVY; BAUTZ, 1985; GREGORC; BOWEN, 1997; DAÍ; GILBERT, 1997; JIANG et al., 1997; JONES; BOWEN, 1993; JOCHOVÁ et al., 1997), características de apoptose atípica e de necrose (SILVA de MORAES; BOWEN, 2000), bem como características de apoptose, de autofagia e de necrose (FURQUIM et al., 2004). Nos insetos, por exemplo a morte celular é do tipo autofágica (ZAKERI et al., 1995; GREGORC et al., 1998; PIPAN; RAVOC, 1980; JOCHOVÁ et al., 1997) ou apoptótica atípica, com envolvimento de autofagia (DAÍ; GILBERT, 1997; BOWEN et al., 1996; GREGORC; BOWEN, 1997; JIANG et al., 1997; LEVY; BAUTZ, 1985; ZAKERI et al., 1995).

No processo de morte celular por apoptose nem sempre todas as características deste tipo estão presentes, podendo, portanto, haver ainda outras formas de morte celular, como por exemplo a morte induzida experimentalmente, onde surgem as diferenças fenotípicas dependendo do estímulo ou tratamento recebido pela célula (HÄCKER, 2000).

Objetivos

II. OBJETIVOS

O presente trabalho teve por objetivos estudar nas glândulas salivares de fêmeas e machos de *Rhipicephalus sanguineus*:

a) o ciclo secretor, identificando os diferentes tipos de células presentes em cada tipo de ácino, bem como estabelecer quando e como cada um atuaria durante a alimentação destes carrapatos;

b) o processo de degeneração glandular, determinando o momento em que este se inicia, considerando os diversos estados de alimentação aos quais os indivíduos analisados foram submetidos (jejum, em alimentação e alimentados);

b) o tipo de morte celular que estaria ocorrendo no tecido glandular;

c) em qual tipo de ácino, de célula e em que seqüência a degeneração ocorreria;

Material e Métodos

III. MATERIAL E MÉTODOS

III.1. MATERIAL

Para a realização deste trabalho foram utilizados machos e fêmeas adultos de carrapatos *Rhipicephalus sanguineus* submetidos ao jejum, a alimentação e pós-alimentação. Os indivíduos em jejum foram cedidos pelo Prof. Dr. Gervásio Henrique Bechara do Departamento de Patologia Veterinária da UNESP *campus* de Jaboticabal (SP), obtidos a partir de colônia mantida em laboratório em condições controladas (29^{o} C, 80% de umidade e fotoperíodo de 12 horas) em estufa BOD. Parte dos indivíduos, em jejum, foi utilizada para realização das diferentes metodologias, e parte foi depositada no hospedeiro (coelho), ou seja, utilizada para a infestação.

Para o desenvolvimento deste trabalho foram realizadas 12 infestações, segundo o procedimento abaixo, de acordo com a técnica descrita por Bechara et al. (1995):

III.1.1. Construção da Câmara Alimentadora (BECHARA et al., 1995)

Um círculo de borracha fina de 9 cm de diâmetro foi cortado e revestido com tecido de algodão (ficou em contato com a pele do hospedeiro). Em seguida um círculo de 3,5 cm de diâmetro foi retirado do centro do círculo de 9 cm de diâmetro. Na borda deste foi fixado com cola plástica um tubo plástico de 2 cm de altura, que foi vedado internamente também com a mesma cola e externamente com esparadrapo. Esse tubo plástico recebeu uma tampa com três furos revestidos internamente com tela de nylon, para que os carrapatos fossem supridos com ar e não escapassem pelos orifícios.

III.1.2. Fixação da Câmara Alimentadora no Hospedeiro (BECHARA et al., 1995)

O hospedeiro teve uma área da região dorsal tosada, a qual recebeu uma camada de cola atóxica (Britannia Adhesives-Unit 4, Inglaterra). Da mesma forma a câmara alimentadora (a região revestida com tecido de algodão) recebeu uma camada desta cola, a qual foi fixada na pele do coelho. A fixação foi reforçada com esparadrapo, que cobriu parte da câmara e da região tosada.

Depois de fixada a câmara alimentadora permaneceu 24 horas destampada para eliminar o odor da cola, para então serem depositados os carrapatos.

III.1.3. Alocação dos Casais de *Rhipicephalus sanguineus* na Câmara Alimentadora (BECHARA et al., 1995)

Este procedimento, bem como toda a observação (em média sete dias) deram-se na residência do pesquisador em Rio Claro (SP) responsável pelo desenvolvimento do projeto.

Após decorridas 24 horas da fixação da câmara, os 20 casais de carrapatos foram colocados no interior da câmara.

A primeira observação realizou-se 8 horas após (tempo necessário para a acomodação dos parasitas), e a partir daí as seguintes deram-se a cada 3 horas. As fêmeas se fixaram e não se desprenderam mais até o final da alimentação (em média sete dias), já os machos se fixaram, se alimentaram por um período pequeno e se desprenderam, repetindo este comportamento muitas vezes, para poderem nos intervalos da alimentação copular com as fêmeas.

Visto que o termo "alimentação" não é válido para os machos, pois estes não se alimentam continuamente, sua presença no hospedeiro se alimentando foi denominada de "infestação". As fêmeas foram analisadas em jejum, com dois e quatro dias de alimentação (em ingurgitamento), alimentadas (ingurgitadas) e com três e sete dias pós-alimentação (pós-ingurgitamento). Já os machos foram analisados em jejum, com dois, quatro e sete dias de infestação e com três e sete dias pós-remoção do hospedeiro.

Depois disso, nas dependências do Laboratório de Histologia do Departamento de Biologia da UNESP *campus* de Rio Claro, machos e fêmeas foram anestesiadas através de choque térmico e, então, as glândulas salivares foram retiradas em solução salina (7,5 g de NaCl + 2,38 g de Na_2HPO_4 + 2,72 g de KH_2PO_4 + 1000 mL de água destilada) para a aplicação das diferentes técnicas.

III.2. MÉTODOS

III.2.1. Análise Morfológica

III.2.1.1. Técnica da Hematoxilina de Harris-Eosina Aquosa (JUNQUEIRA, 1983)

Para realização dessa técnica a fixação das glândulas salivares deu-se em formalina neutra tamponada 10% (pH 7- 7,4) e acetona, na proporção de 9:1, durante 1 hora e 30 minutos, a 4^o C. Então o material foi desidratado em concentrações crescentes de álcool (70%, 80%, 90% e 95%), banhos de 15 minutos cada, transferido para resina de embebição, incluído e seccionado. A embebição e a inclusão foram efetuadas em resina Leica. Os cortes, com espessura de 3 μm, foram recolhidos em lâminas de vidro, reidratados em água destilada por 1 minuto e corados, por 10 minutos, em Hematoxilina e lavados em água. Na seqüência, foram corados com Eosina por 10 minutos, novamente lavado e as lâminas foram secas. A montagem final deu-se em bálsamo do Canadá com posterior observação ao microscópio de luz.

III.2.2. Análise Histoquímica

III.2. 2.1. Reação pelo PAS (Ácido Periódico- Schiff) (McManus, 1946) e Contra-Coloração com Verde de Metila

Para realização deste procedimento as glândulas foram fixadas em formalina neutra tamponada 10% (pH 7- 7,4) e acetona, na proporção de 9:1, durante 1 hora e 30 minutos, a 4^o C. Na seqüência foram desidratadas em concentração crescente de álcool (70%, 80%, 90% e 95%), banhos de 15 minutos cada, transferidas para resina de

embebição, incluídas em resina Leica e seccionadas. Os cortes, com 3 μm, foram recolhidos em lâminas de vidro e reidratados por 1 minuto em água destilada para então serem transferidos para solução de ácido periódico por 10 minutos. Novamente foram lavados em água destilada por 1 minuto e na seqüência colocados, por 1 hora, no reagente de Schiff. A seguir foram lavados, por 30 minutos, em água corrente e contra-corados, por 20 segundos com Verde de Metila, lavados, secos e montados em Bálsamo do Canadá para posterior observação ao microscópio de luz.

III.2.3. Análise Citoquímica

III.2.3.1. Detecção da Atividade da ATPase (HUSSEIN et al., 1990)

Para realização dessa técnica as glândulas foram fixadas em glutaraldeído 0,5% em tampão cacodilato de sódio (0,2M, pH 7,2), a 4 ^{o}C durante 1 hora. Na seqüência foram lavadas em tampão cacodilato de sódio (0,2 M, pH 7,2) a 4^{o} C e incubadas por 45 minutos a 37^{o} C no seguinte meio: Tris-Maleato (200mM, pH 7,2), ATP (5mM), $MgSO_4$ (5mM), KCl (15mM), $CaCl_2$ (10mM), acetato de chumbo (4mM) e sacarose (160mM). O acetato de chumbo foi dissolvido no tampão Tris-Maleato com o auxílio do ultra-som, adicionou-se o restante dos reagentes, acrescentou-se o ATP (no momento da incubação), e então completou-se com a outra parte do tampão Tris-Maleato.

Após a incubação o material foi lavado em tampão Tris-Maleato (200 mM, pH 7,2) a 4^{o} C e pós-fixado em formalina neutra tamponada 10% (pH 7- 7,4) e acetona, na proporção de 9:1, por 40 minutos, a 4^{o} C. O controle foi realizado excluindo-se o substrato (ATP, 5 Mm) do meio de incubação.

Procedeu-se a desidratação em concentrações crescentes de álcool (70%, 80%, 90% e 95%), com banhos de 15 minutos cada, e transferiu-se para resina de embebição para posterior inclusão em resina Leica e secção dos blocos. Os cortes, com 7 μm, foram recolhidos em lâminas de vidro, reidratados por 1 minuto em água destilada e lavados por 4 minutos em solução de sulfeto de amônia 1%, para revelação do produto da reação da ATPase com o ATP (substrato).

Então as lâminas foram lavadas rapidamente em água destilada, coradas por 2 minutos com Hematoxilina de Harris, secas e montadas em Bálsamo do Canadá para posterior observação ao microscópio de luz.

III.2.3.2. Análise da Viabilidade Celular e Detecção de Células Apoptóticas e/ou Necróticas (MACGAHON et al., 1995)

Para realização dessa técnica as glândulas salivares foram depositadas sobre lâminas de vidro e receberam duas gotas da mistura de de Acridine Orange (100 μg/mL) e de Brometo de Etídio (100 μg/mL), ambos diluídos em PBS. As lâminas foram cobertas com lamínula, mantidas no escuro e imediatamente observadas ao microscópio de fluorescência com filtro de excitação de 488 nm.

O Acridine Orange e o Brometo de Etídio têm afinidade com DNA e RNA, o Acridine Orange cora DNA em verde e o RNA em vermelho alaranjado, e o Brometo de Etídio DNA em laranja e RNA em vermelho. A membrana celular é permeável apenas ao Acridine Orange.

Desta forma, as células integras apresentaram coloração verde homogênea (citoplasma e núcleo) ou núcleo verde homogêneo e citoplasma vermelho alaranjado; as apoptóticas iniciais apresentaram citoplasma verde ou vermelho alaranjado e núcleo verde com blocos de cromatina condensada em verde brilhante; as células apoptóticas tardias, citoplasma vermelho e o núcleo com áreas de cromatina condensada em laranja brilhante e as necróticas citoplasma vermelho e núcleo laranja homogêneo.

III.2.3.3. Detecção da Atividade da Fosfatase Ácida (HUSSEIN et al., 1990)

Para realização dessa técnica as glândulas foram fixadas em formalina neutra tamponada 10% (pH 7- 7,4) e acetona, na proporção de 9:1, durante 1 hora e 30 minutos, a 4º C. Na seqüência foram lavadas em tampão acetato de sódio (0,05M, pH 4,8) e incubadas por 45 minutos a 37º C no seguinte meio: naftol AS-TR fosfato, DMSO (dimetil sulfoxido), tampão acetato de sódio (0,05M, pH 4,8), $MnCl_2.4H_2O$ 10% e sal vermelho violeta.

Para o preparo do meio de incubação foram dissolvidos 3 mg do substrato naftol AS-TR fosfato em duas gotas de DMSO e, em seguida, adicionados 10 mL de tampão acetato de sódio. Então acrescentou-se 0,2 mL de cloreto de manganês 10% mais 6 mg do sal vermelho violeta e, para finalizar, a solução final foi vigorosamente misturada.

O controle foi realizado excluindo-se o substrato (3 mg de naphtol AS-TR fosfato) do meio de incubação.

O material foi desidratado em concentrações crescentes de álcool (70%, 80%, 90% e 95%), banhos de 15 minutos cada, transferido para resina de embebição, incluído em resina Leica e seccionado. Os cortes de 7 μm foram recolhidos em lâminas de vidro e reidratados por 1 minuto em água destilada, contracorados por 1 minuto com Hematoxilina de Harris, secos e montados em Bálsamo do Canadá para observação ao microscópio de luz.

Após a desidratação procedeu-se a montagem total de algumas glândulas, as quais foram observadas ao microscópio de luz.

III.2.3.4. Reação de Feulgen (FEULGEN e ROSSENBECK, 1924)

Para realização dessa técnica o material foi fixado em mistura de álcool etílico e ácido acético na proporção de 3:1 por 12 minutos, desidratado em concentrações crescentes de álcool (70%, 80%, 90% e 95%), banhos de 15 minutos cada, transferido para resina de embebição e incluído. A embebição e a inclusão foram efetuadas em resina Leica. O material foi seccionado com espessura de 3 μm e os cortes recolhidos em lâminas de vidro.

Então, as lâminas contendo as secções permaneceram por 11 minutos em solução de HCl 1N a 60^{o} C. Na seqüência o material foi lavado em água destilada e colocado no reativo de Schiff por 2 horas. Então o material foi lavado por 5 minutos em água corrente.

Em seguida os cortes foram contra-corados com Eosina aquosa durante 5 minutos, lavados em água corrente, secos e montados em Balsamo do Canadá, para posterior observação ao microscópio de luz.

III.2.3.5. Técnica da Variante da Concentração Crítica de Eletrólitos (CEC) (MELLO et al., 1993)

Para realização dessa técnica o material foi fixado em mistura de álcool etílico e ácido acético na proporção de 3:1 por 12 minutos, desidratado em concentração crescente de álcool (70%, 80%, 90% e 95%), banhos de 15 minutos cada, transferido para resina de embebição e incluído em resina Leica. O material foi seccionado com 3 μm e os cortes recolhidos em lâminas de vidro.

As lâminas foram coradas com solução de Azul de Toluidina 0,025% em tampão McIlvane (pH 4,0) durante 20 minutos à temperatura ambiente. Na seqüência foram levadas em solução aquosa de $MgCl_2$ 0,05M, onde permaneceram diferentes tempos, 5, 7 e 10 minutos, então foram lavadas em água destilada, secas e montadas em Entellan, para que a coloração do meio de montagem não interferisse na coloração da técnica. Posteriormente foram observadas ao microscópio de luz.

Resultados

IV. RESULTADOS

Os resultados obtidos no presente estudo são apresentados na forma de artigos submetidos para publicação em revistas especializadas:

Capítulo 1: "Morpho-histochemical changes in salivary glands of female ticks of *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae) during feeding. Description of new cell types." Artigo submetido à *Journal for Parasitology* em Abril de 2007.

Capítulo 2: "Salivary glands of females of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae). Degenerative morphological changes detected at the end and after the feeding period." Artigo submetido à *Journal for Parasitology* em Abril de 2007.

Capítulo 3: "Death by apoptosis in salivary glands of females of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae)." Artigo submetido à *Veterinary Parasitology* em Abril de 2007.

Capítulo 4: "Morpho-histochemical characterization of salivary gland cells of males of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae) at different feeding stages. Description of new cell types." Artigo submetido à *Veterinary Parasitology* em Abril de 2007.

Capítulo 5: "Degeneration of salivary glands of males of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae)." Artigo submetido à *Experimental Parasitology* em Abril de 2007.

Capítulo 6: "The process of cell death in salivary glands of males of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae)." Artigo submetido à *Parasitology International* em Abril de 2007.

Capítulo 7: "Cytoplasmic and nuclear changes detected cytochemically during the degeneration of salivary glands of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae)." Artigo submetido à *Micron* em Abril de 2007.

Capítulo 1

CAPÍTULO 1

TITLE: Morpho-histochemical changes in salivary glands of female ticks of *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae) during feeding. Description of new cell types.

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RESUMO

Fêmeas do carrapato *Rhipicephalus sanguineus* em jejum, com dois e quatro dias de alimentação tiveram suas glândulas salivares analisadas histológica e histoquimicamente. Os resultados obtidos demonstraram as alterações pelas quais estes órgãos passaram durante o período de alimentação, em comparação com os indivíduos em jejum. Nas glândulas destas fêmeas foram encontrados todos os tipos celulares descritos na literatura, ou seja, no ácino I, a célula **central** e as **periféricas**, no II as células **a**, **b**, **c1** à **c4** e no III as células **d**, **e** e **f**, porém o presente estudo vem ainda descrever novos tipos de células que foram classificados como: **indefinidas 1** e **2** presentes nos ácinos II de fêmeas em jejum e **c5** e **c6** nos ácinos II de fêmeas em alimentação. Os dados mostraram que com o início da alimentação há um desenvolvimento das glândulas salivares, com intensas modificações apenas nos ácinos II e III, onde células antes indiferenciadas nas fêmeas em jejum sofreram severas modificações, tais como aumento dos grânulos de secreção. Especificamente no ácino II das fêmeas em jejum apenas algumas células (**a**, **c1** e **c3**) estavam desenvolvidas e permaneceram ativas nas fêmeas com dois dias de alimentação juntamente com as células **b**, **c2**, **c4**, **c5** e **c6**. No ácino II, nas fêmeas com quatro dias de alimentação, as células **c6** se tornaram inativas. O ácino III das fêmeas em jejum apresentou as células **d** e **e** desenvolvidas e as **f** indiferenciadas e apenas nas fêmeas com dois dias de alimentação, estas últimas se desenvolveram e apresentaram grânulos de secreção, os quais não foram mais observados nas fêmeas com quatro dias de alimentação.

PALAVRAS-CHAVE: *Rhipicephalus sanguineus*; carrapatos; fêmea; glândula salivar; morfologia; histoquímica; ciclo secretor; novos tipos celulares.

ABSTRACT

The salivary glands of unfed, two and four-days fed females of the tick *Rhipicephalus sanguineus* were examined histologically and histochemically. The results describe the changes undergone by these organs during feeding. All cell types described in the literature were observed in the glands of the examined females: In type I acinus, **central** and **peripheral** cells; in type II, **a**, **b**, **c1-c4** cells**;** and in type III acinus, **d**, **e** and **f** cells. This study also describes new cell types here termed: **undefined 1** and **2** present in type II acini of unfed females, and **c5** and **c6** in type II acini of two and four-days fed females. The data show that as the tick starts to feed, the salivary glands develop; only type II and III acini undergo remarkable changes, as their cells, undifferentiated in unfed females, undergo important changes with increase in the number of secretion granules. Especially in type II acini of unfed females, only few cells (**a**, **c1** and **c3**) exhibited secretion granules and remained active in two-days fed females along with cells **b**, **c2**, **c4**, **c5** and **c6**. In four-days fed females, **c5** cells in type II acinus become inactive. Type III acinus of unfed females exhibited **d** and **e** cells filled with granules, and undifferentiated **f** cells. The latter exhibited secretion granules only in two-days fed females; in four-days ones granules were no longer observed.

KEY WORDS: *Rhipicephalus sanguineus*; ticks; female; salivary gland; morphology, histochemistry; secretory cycle; new cells types.

INTRODUCTION

The brown dog tick, *Rhipicephalus sanguineus*, is a species widely distributed in tropical and temperate regions (Walker, 1994) and is found in the Americas, Europe, Africa and Australia (Ribeiro et al., 1996).

The salivary glands are vital organs to the biological success of ixodid ticks, producing several compounds, mainly substances involved in the attachment and feeding of these parasites (Binnington, 1978; Gill and Walker, 1987; Walker et al., 1985). They consist of an excretory and a secretory portion, and lack a reservoir to store its secretion. The excretory portion is composed of a duct system that includes a common excretory duct, intermediary and acinar ducts (Binnington, 1978; Balashov, 1983; Fawcett et al., 1986; Till, 1961; Walker et al., 1985). In females, the secretory portion comprises three types of acini: I, II and III. Type I exhibits a **central** cell surrounded by smaller **peripheral** cells (Fawcett et al., 1986; Gill and Walker, 1987; Marzouk and Darwish, 1994; Olivieri and Serra-Freire, 1992). Type II consists of secretory cells, termed **a**, **b**, **c1**, **c2**, **c3**, **c4** (Binnington, 1978; Fawcett et al., 1986; Sonenshine, 1991). Type III comprises three cell types, **d**, **e**, **f**, (Binnington, 1978; Gill and Walker, 1987; Marzouk and Darwish, 1994; Walker et al., 1985).

The morphological variation found in the salivary glands of ticks reflects their functional complexity. Type I acini are involved in water balance during non-parasitic periods (McMullen et al., 1976 apud Fawcett et al., 1986; Rudolph and Knülle, 1974, 1978 apud Fawcett et al., 1986), while types II and III play a role in the production and secretion of substances that manipulate host responses, such as: increase in vascular permeability, inhibition of inflammatory processes and blood coagulation (Fawcett et al., 1986), and immunosuppression (Wikel, 1981), as well as the production of the cement cone that attaches the tick to the host skin (Binnington, 1978; Fawcett et al., 1986; Walker et al., 1985), allowing it to feed.

The salivary glands of ticks present a well-defined secretory cycle that parallels a sequence of events: attachment to the host, formation of the tick feeding lesion, feeding, matting, and loss of characteristics associated to the parasitic phase (Sonenshine, 1991). Thus, before attachment or feeding, salivary gland cells of adult ticks are still inactive. Salivary gland development starts with tick attachment to the host

and it is completed with feeding (Walker et al., 1985), during this process, glands exhibit high secretory activity and undergo rapid structural and functional transformations (Binnington, 1978; Gill and Walker, 1987; Marzouk and Darwish, 1994; Walker et al., 1985).

The identification and classification of cells of salivary glands of ticks have been a subject of great controversy (Binnington, 1978). This study aims at describing the morpho-histochemical changes undergone by these glands, as well as identifying and characterizing their cell types in female ticks of *Rhipicephalus sanguineus*.

MATERIAL AND METHODS

In this study, were utilized unfed, two and four-days fed females of the tick *Rhipicephalus sanguineus*. Unfed ticks were provided by Dr. Gervásio Henrique Bechara of the Department of Animal Pathology, UNESP, Jaboticabal (SP) campus, from a laboratory colony maintained under controlled conditions (29^{o} C, 80% humidity, and 12h photoperiod) in BOD incubators. Ticks were separated into two groups. One consisted of unfed ticks, was subjected to histological procedures. The second was placed with males inside a feeding chamber previously glued with an atoxic and non-lesive preparation (Britannia Adhesives-Unit 4, UK) to the shaved back of the host (rabbit) according to technique described elsewhere (Bechara et al., 1995) for monitoring the feeding process (observations every three hours).

Following the feeding period, ticks were collected and salivary glands were removed in saline solution, fixed in a 10 % neutral buffered formalin and acetone solution (9:1) for one hour and thirty minutes at 4^{o} C. After fixation, the material was dehydrated in a series of increasing concentrations of alcohol (70%, 80%, 90% and 95%), embedded in resin (Leica), and sectioned at 3 μm. Sections were placed on glass slides and stained with Hematoxylin-Eosin and PAS (Periodic Acid Schiff), McManus (1946), for detection of polysaccharides, and counterstained with Methyl Green. Slides were mounted with Canada balsam and examined under light microscope.

RESULTS

The cell types of acini in salivary glands of *Rhipicephalus sanguineus* were described following the system developed and adopted by Binnington (1978) for *Boophilus microplus*. In addition, we described new cell types not previously observed by this author.

For a better comparison, tables **1**, **2** and **3** summarize the results obtained.

Table 1: Morpho-histochemical results obtained for the salivary glands of unfed females ticks of *Rhipicephalus sanguineus*, including the new cell types observed, as well as those described by Binnington (1978).

Acinus	Cell		Cell Characteristics		
			Localization	**Shape**	**Cytoplasm**
I			- one **central** cell (Fig. 1A); - several **peripheral** cells (Fig. 1A);	- * (Fig. 1A);	- fibrillar PAS +++ (Fig. 2A);
II	**undifferentiated**		- ? (Fig. 1B);	- cubic (Fig. 1B);	- scarce, no granules (Fig. 2B);
	undefined	**1**	- beside **a** cells (Fig. 1C);	- cubic (Fig. 1C);	- larger granules PAS - (Fig. 2C);
		2	- near to **a** cells (Fig. 1D);	- cubic (Fig. 1D);	- smaller granules PAS + (Fig. 2C);
	a		- hilus of the acinus (Fig. 1E);	- cubic (Fig. 1E);	- granules PAS - (Fig. 2B);
	b		- ∅;	- ∅;	- ∅;
	c1		- near to **a** cells (Fig. 1F);	- cubic (Fig. 1F);	- granules PAS +++ (Fig. 2B);
	c2		- ∅;	- ∅;	- ∅;
	c3		- fundus of the acinus (Fig. 1G);	- cubic (Fig. 1G);	- larger granules than those of **c1** cells PAS +++ (Fig. 2D);
	c4		- ∅;	- ∅;	- ∅;
	c5		- ∅;	- ∅;	- ∅;
	c6		- ∅;	- ∅;	- ∅;
III	**d**		- hilus of the acinus (Fig. 1H);	- cubic (Fig. 1H);	- granules PAS - (Fig. 2E);
	e		- beside **d** cells (Fig. 1I);	- cubic (Fig. 1I);	- larger granules than those of **d** cells PAS - (Fig. 2E);
	f		- fundus of the acinus (Fig. 1I);	- cubic (Fig. 1I);	- scarce, no granules (Fig. 1I);

☐ new cell types described in this study; *****: cell boundaries not evident; **?**: location not defined; ∅: cell type not observed; **Undifferentiated**: undifferentiated cells still without secretion granules; **Undefined**: cells exhibiting immature and undefined secretory granules (atypical);

Table 2: Morpho-histochemical results obtained for the salivary glands of two-days fed females ticks of *Rhipicephalus sanguineus*, including the new cell types observed, as well as those described by Binnington (1978).

Acinus	Cell		Cell Characteristics		
			Localization	**Shape**	**Cytoplasm**
I			- one **central** cell (Fig. 1J); - several **peripheral** cells (Fig. 1J);	- * (Fig. 1J);	- fibrillar PAS + (Fig. 2F);
II	**undifferentiated**		- ∅;	- ∅;	- ∅;
	undefined	**1**	- ∅;	- ∅;	- ∅;
		2	- ∅;	- ∅;	- ∅;
	a		- hilus of the acinus (Fig. 1K);	- cubic (Fig. 1K);	- granules PAS - (Fig. 2G);
	b		- beside **a** cells (Fig. 1L);	- cubic (Fig. 1L);	- elliptic and heterogeneous granules PAS+ and PAS ++ (Fig. 2H);
	c1		- near **a** cells (Fig. 1M);	- cubic (Fig. 1M);	- granules PAS +++ (Fig. 2I);
	c2		- near **a** cells (Fig. 1N);	- cubic (Fig. 1N);	- granules PAS + (Fig. 2J);
	c3		- fundus of the acinus (Fig. 1K);	- cubic (Fig. 1K);	- larger granules than those of **c1** cells PAS +++ (Fig. 2I);
	c4		- fundus of the acinus (Fig. 1O);	- cubic (Fig. 1O);	- elliptic granules PAS - (Fig. 2K);
	c5		- near **a** cells (Fig. 1O);	- cubic (Fig. 1O);	- smaller granules than those of **a** cells and larger than those of **c3** cells PAS ++ (Fig. 2L);
	c6		- near **c5** cells (Fig. 2L);	- cubic (Fig. 2L);	- fine granules PAS ++ (Fig. 2L);
III	**d**		- hilus of the acinus (Fig. 1P);	- cubic (Fig. 1P);	- granules PAS - (Fig. 2M);
	e		- beside **d** cells (Fig. 1P) ;	- cubic (Fig. 1P);	- larger granules than those of **d** cells PAS - (Fig. 2M);
	f		- fundus of the acinus (Fig. 1Q);	- cubic (Fig. 1Q);	- smaller granules than those of **d** cells PAS + (Fig. 2M);

new cell types described in this study; *: cell boundaries not evident; ∅: cell type not observed; **Undifferentiated**: undifferentiated cells still without secretion granules; **Undefined**: cells exhibiting immature and undefined secretory granules (atypical);

Table 3: Morpho-histochemical results obtained for the salivary glands of four-days fed females ticks of *Rhipicephalus sanguineus*, including the new cell types observed, as well as those described by Binnington (1978).

Acinus	Cell	Cell Characteristics		
		Localization	Shape	Cytoplasm
I		- one **central** cell (Fig. 1R); - several **peripheral** cells (Fig. 1R);	- * (Fig. 1R);	- fibrillar PAS + (Fig. 2N);
II	**undifferentiated**	- ∅;	- ∅;	- ∅;
	undefined 1	- ∅;	- ∅;	- ∅;
	undefined 2	- ∅;	- ∅;	- ∅;
	a	- hilus of the acinus (Fig. 1S);	- cubic (Fig. 1S);	- granules PAS - (Fig. 2O);
	b	- beside **a** cells (Fig. 1S);	- cubic (Fig. 1S);	- elliptic and heterogeneous granules PAS+ and PAS ++ (Fig. 2P);
	c1	- near **a** cells (Fig. 1T);	- cubic (Fig. 1T);	- granules PAS +++ (Fig. 2O);
	c2	- near **a** cells (Fig. 1S);	- cubic (Fig. 1S);	- granules PAS + (Fig. 2Q);
	c3	- fundus of the acinus (Fig. 1U);	- cubic (Fig. 1U);	- larger granules than those of **c1** cells PAS +++ (Fig. 2R);
	c4	- fundus of the acinus (Fig. 1U);	- cubic (Fig. 1U);	- elliptic granules PAS - (Fig. 2O);
	c5	- ∅;	- ∅;	- ∅;
	c6	- near **a** cells (Fig. 1V);	- cubic (Fig. 1V);	- fine granules PAS + (Fig. 2S);
III	**d**	- hilus of the acinus (Figs. 1W, X);	- cubic (Fig. 1W) and squamous (Fig. 1X);	- small and compact granules PAS - (Figs. 2T, W);
	e	- beside **d** cells (Figs. 1W, X);	- cubic (Fig. 1W) and squamous (Fig. 1X);	- granules of different sizes PAS - (Figs. 2U, W);
	f	- fundus of the acinus (Figs. 1W, X);	- cubic (Fig. 1W) and squamous (Fig. 1X);	- PAS +++ (Fig. 2V, W);

▢ new cell types described in this study; *****: cell boundaries not evident; ∅: cell type not observed; **Undifferentiated**: undifferentiated cells still without secretion granules; **Undefined**: cells exhibiting immature and undefined secretory granules (atypical);

FIGURES

FIGURE 1:

Histological sections of the acini I, II and III of salivary glands of unfed, two and four-days fed females of *Rhipicephalus sanguineus*, stained with Hematoxylin-Eosin.

A-I. Unfed females.
J-Q. Two-days fed females.
R-X. Four-days fed females.

I-III: types acini; **rc: undifferentiated** cell; **nc1: undefined** cell **1**; **nc2: undefined** cell **2**; **a: a** cell; **b: b** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **c6: c6** cell; **d: d** cell; **e: e** cell; **f: f** cell; **c:** cytoplasm; **cn: central** cell nucleus; **pn: peripheral** cell nucleus; **n:** nucleus; **dt:** duct; **lu:** lumen; **s:** secretion; **iv:** intraacinar valve.

Bars: M= 10 μm; **A-L** and **N-X=** 20 μm.

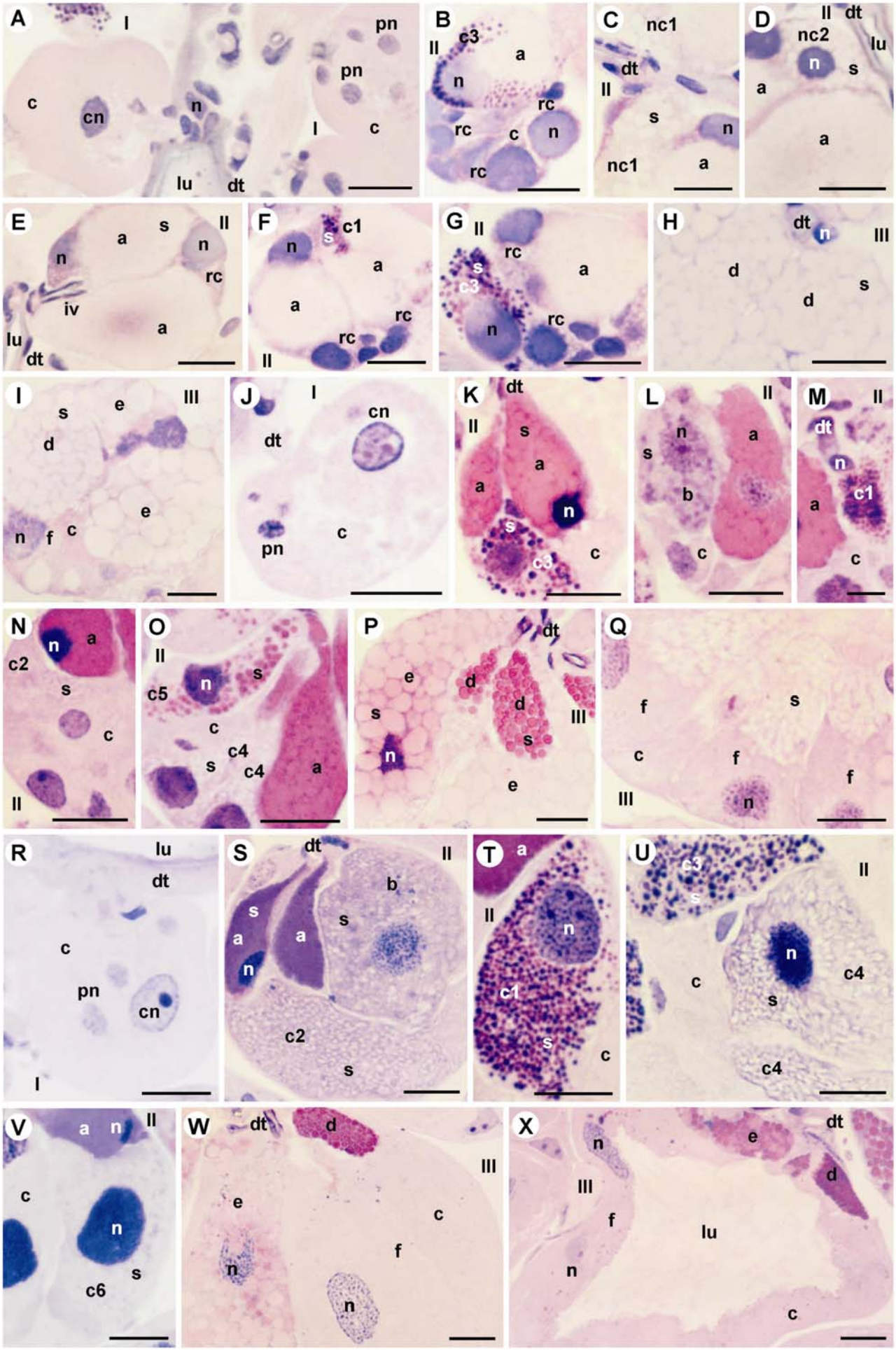
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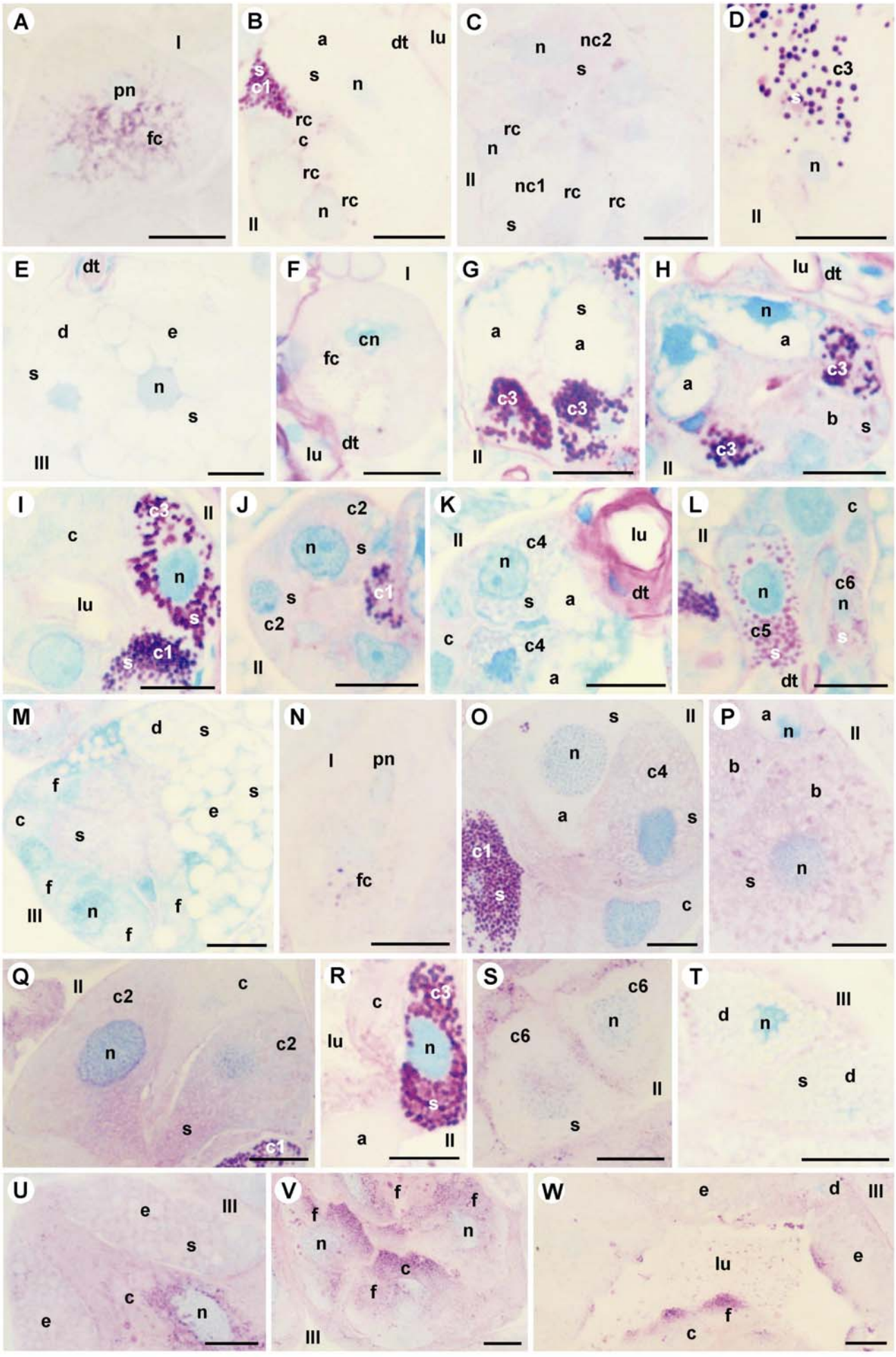
FIGURE 2:

Histological sections of the acini I, II and III of salivary glands of unfed, two and four-days fed females of *Rhipicephalus sanguineus*, stained with PAS and counterstained with Methyl Green.

A-E. Unfed females.
F-M. Two-days fed females.
N-W. Four-days fed females.

I-III: types acini; **rc: undifferentiated** cell; **nc1: undefined** cell **1**; **nc2: undefined** cell **2**; **a: a** cell; **b: b** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **c6: c6** cell; **d: d** cell; **e: e** cell; **f: f** cell; **fc:** fibrillar cytoplasm; **pn: peripheral** cell nucleus; **n:** nucleus; **c:** cytoplasm; **dt:** duct; **lu:** lumen; **s:** secretion; **cn: central** cell nucleus;

Bars: A-W= 20 μm.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DISCUSSION

The present study on salivary glands of females of *Rhipicephalus sanguineus* confirmed the information available in the literature for ixodid ticks, and described the new cell types found in these organs.

According to Fawcett et al. (1986), the different salivary gland cells observed in ticks are distinguished under light microscope in accordance to morphological characteristics and metachromasia of secretion granules. We followed this methodology, using the same morphological characteristics described for the glands of *Boophilus microplus* (Binnington, 1978). The results revealed the presence of new cell types in type II acini, termed: **undefined 1** and **2** in unfed females; and **c5** and **c6** in two and four-days fed females.

The size and morphology of type I acini were similar in unfed and two and four-days fed females, as observed by Binnington (1978) and Walker et al. (1985). According to these authors the morphology of type I acini remained the same throughout the entire feeding process. Needham et al. (1983) and Barker et al. (1984), however, reported an increase in the size of type I acini during early stages of tick feeding, although during rapid feeding, acini reduced to the size of those of unfed ticks.

In type II acini of *R. sanguineus,* several changes were observed in the three feeding stages assessed in our study. We identified in unfed females **a**, **c1** and **c3** cells already described in the literature; "**undifferentiated"** cells probably programmed to develop with the onset of feeding; as well as **undefined cells 1** and **2** with unstained cytoplasmic granules. These did not persisted in two and four-days fed females, undergoing changes characterized by decrease in size and condensation of granule content, suggesting a maturation process (Junqueira and Carneiro, 2004).

As feeding progressed, all cells of type II acini of *R. sanguineus* exhibited intense activity of synthesis and secretion, and granules containing substances involved in tick attachment, maintenance, and consumption of blood. This corroborates with the observed by Walker et al. (1985) that suggested that salivary glands are not completely active until ticks start to feed.

In two-days fed females, cells in type II acini were filled with secretion granules, unlike the observed in unfed females. However, there was no difference in size between

acini of these groups. Also, we observed eight cell types (**a**, **b**, **c1** to **c6**) in two-days fed females instead of the six described in the literature. In four-days fed females, type II acini were larger compared to those of two-days fed females, due to accumulation of secretion granules in cells, indicating henceforth an increase in gland activity associated with the consumption of blood by the parasite. In this group, **c5** cells were not observed, suggesting that they might no longer be functional and atrophied.

In unfed females of *R. sanguineus*, **a** cells in type II acini were already filled with secretion granules, as also observed in two-days fed females, despite changes in staining patterns which suggests that granules might be undergoing a maturation process (Junqueira and Carneiro, 2004). In four-days fed females, these cells became even more active, indicated by the increase in size compared to those of previous groups. This finding partially confirms the observed by Binnington (1978) and Walker et al. (1985) in *B. microplus* and *R. appendiculatus,* respectively. Both authors reported granules in **a** cells of unfed females, however, Binnington (1978) observed that, unlike the obtained in our study, these cells became non-functional 72 hours after attachment to the host. This period of time coincides with the time necessary for the formation of the cement cone. On the other hand, Walker et al. (1985) found that the same cells became less active, although still functional as feeding progressed.

The secretion granules of **a** cells of *R. sanguineus* exhibited the same staining pattern in all groups, although they were stained more intensely in two and four-days fed females. The secretion was not stained by PAS, indicating the absence of polysaccharides in its composition, but it was stained by Eosin, confirming the observed by Binnington (1978), Walker et al. (1985), and Sonenshine (1991). The two latter studies reported the presence of basic proteins in the composition of granules, although according to Binnington (1978), granules contain lipoproteins.

Our results suggest that **a** cells of *R. sanguineus* play a role in the production of cement precursors, as proposed by Binnington (1978), Walker et al. (1985), Fawcett et al., (1986), Sonenshine (1991). However, *R. sanguineus* species, these cells remained functional for a longer period than the described for *B. microplus* (Binnington, 1978). This might indicate that the time necessary for the formation of the cement cone in *R. sanguineus* is over 72 hours (Binnington, 1978).

In type II acinus, **b** cells were not active in unfed females; two-days fed females exhibited cells with few granules, while cells were filled with granules in four-days fed females, as observed by Walker et al. (1985) in *R. appendiculatus*. These authors have shown that these cells are functional in early stages of tick feeding, decreasing their activity in seven-days fed females. Binnington (1978), however, reported that in *B. microplus* these cells were active in all feeding stages, and granules were already present in unfed females. After 72 hours of feeding, most granules were eliminated.

In *B. microplus* as feeding progressed, the composition of the secretion of **b** cells remained unchanged, maintaining the same staining patterns in two and four-days fed females. Granules exhibited PAS stained and not stained regions, as well as portions of acidic nature. Binnington (1978) and Walker et al. (1985) demonstrated that the secretion of these cells consisted of glycoproteins, and suggested they play a role in the manipulation of the host response. In *R. sanguineus*, although these cells were active only after the onset of feeding (two days), they became important especially in four-days fed females for the continuity of the feeding process and/or maintenance of the tick on the host.

In this study, type **c** cells were classified in six subtypes, from **c1** to **c6**; **c5** and **c6** were described for the first time. Only **c1** and **c3** were active in unfed females. In two-days fed females, all six cell types were active, and **c1**, **c3**, **c5** and **c6** contained the largest amounts of secretion granules. In four-days fed females, **c1**, **c2**, **c3**, **c4** and **c6** cells remained active, but **c1**, **c2** and **c4** increased in size as a consequence of larger amounts of granules. The aspect of the cytoplasm of **c3** cells remained the same during the secretory cycle, while **c6** cells exhibited fewer granules, indicating a lower activity. Cells **c5** were no longer observed in four-days fed females, suggesting a loss of functionality.

Cells type **c** were associated with the manipulation of host response (Walker et al., 1985), and some of them secrete glycoproteins acting as anticoagulants (Sonenshine, 1991).

The results for **c1** cells partially confirm those obtained by Binnington (1978). It was observed the presence of secretion granules in unfed females, however the period of cell activity differs from the obtained by Binnington (1978), which suggested that these cells remain functional for approximately 72 hours of feeding. According to

Walker et al. (1985), these cells are inactive in unfed females, and as observed in the present study, develop during feeding.

Cells **c1** of salivary glands of *R. sanguineus* played a major role in all phases of the secretory cycle, as they were already active before the first contact between parasite and host (unfed females), and remained active as feeding progressed. It is worth pointing out that these cells were most active in four-days fed females, when blood consumption increased.

Our results on **c2** cells confirm those obtained by Walker et al. (1985). These authors observed that these cells were active in early stages of tick feeding. As feeding progressed, cells exhibited a moderate increase in size and amount of granules. Binnington (1978) observed few granules in **c2** cells of unfed females, and an increase in the number of granules after 12-24 hours of feeding. These cells remained functional during the 24-72 hour period after the attachment of the tick to the host. In *R. sanguineus*, these cells were inactive in unfed females and filled with granules in four-days fed females. Binnington (1978) proposed that **c2** cells play a role in blood consumption. This might also occur in *R. sanguineus* females, as these cells developed in parallel with the increase in blood consumption (four days).

Regarding **c3** cells, we observed the presence of secretion granules in unfed females confirming the obtained by Binnington (1978). However, according to this author, these cells remained active for 72 hours after attachment, which was not observed in our study. Binnington (1978) demonstrated that **c3** cells play a general role in the feeding process. This could also be the case in *R. sanguineus*, as these cells were functional in all phases assessed and exhibited granules with contents similar to those observed in *B. microplus*.

Cells **c4** exhibited low activity in unfed females, as opposed to the observed in four-days fed females. This result differs from the obtained by Binnington (1978) that reported secretion granules in cells of unfed females, which were released after 72 hours of feeding. This author also suggested that in *B. microplus*, **c4** cells might play a role in enzyme secretion. Considering this information and the histochemical similarities, this could occur in **c4** cells of *R. sanguineus*.

The composition of secretion granules of **c** cells in the stages examined in *R. sanguineus* remained the same. Cells **c1**-**c3** contained acids and polysaccharides,

confirming the observed by other authors (Binnington, 1978; Walker et al., 1985, Fawcett et al., 1986; Sonenshine, 1991). According to some studies, polysaccharides might also form complexes with proteins (glycoproteins) (Binnington, 1987; Walker et al., 1985; Sonenshine, 1991).

The granules of **c4** cells were not stained by the dyes used in this study and according to Binnington (1978), despite chromophobia, these cells play a role in enzyme secretion.

Cells **c5** and **c6** of *R. sanguineus*, described for the first time in this study, exhibited granules containing mainly polysaccharides. These cells were essential during the early phases of the secretory cycle in *R. sanguineus*. Cells **c6** exhibited few granules in four-days fed females, but their highest activity was observed during the early phases of the secretory cycle. Cells **c5** were observed only in two-days fed females and atrophied in the following stage. These results suggest that the secretion of **c5** and **c6** cells might act as inhibitors of host response, evading any type of specific resistance of dogs against the attachment of ticks. This possible inhibitor may not be present in other hosts or still does not affect other tick species, as these cells types (**c5** and **c6**) were not describe previously.

Fawcett et al. (1986), studying *R. appendiculatus*, reported that cells of type III acinus undergo rapid structural and functional changes during the feeding period, as observed in this study. In *R. sanguineus*, acini gradually increased in size as feeding progressed, and cells of two and four-days fed females stained more intensely. In the latter group, some type III acini exhibit cells that had already released most of their granules, their morphology changed from pyramidal to squamous, consequently increasing the lumen of the acinus, as also observed by other authors in different species (Binnington, 1978, Walker et al., 1985, Sonenshine, 1991).

In type III acini of *R. sanguineus*, **d** and **e** cells were filled with secretion granules in unfed females, as also observed in two and four-days fed females. As feeding progressed, granules stained more intensely, suggesting they might be undergoing a maturation process, characterized by, according to Junqueira and Carneiro (2004), condensation of contents and decrease in size. Thus in four-days fed females, granules might be ready to be released, concentrating in the apical region of these cells. This was clearly evident in **e** cells, in addition to immature granules present in the basal

region. These findings confirm the observed by Binnington (1978), Walker et al. (1985) and Fawcett et al. (1986), regarding the presence of granules already in unfed females. Our results, however, differ from those of Binnington (1978), Fawcett et al. (1986) and Sonenshine (1991) regarding the period when granules were present in the cells. According to Binnington (1978), these cells release most of their granules after 72 hours of feeding, while the other authors report that granules are released for the formation of the cement cone after tick attachment, and cells atrophy as feeding progresses.

Cells **d** and **e** in ticks are thought to secrete cement precursors (Binnington, 1978; Fawcett et al., 1986; Walker et al., 1985; Sonenshine, 1991; Bishop et al., 2002). According to Walker et al. (1985), these cells secrete aminopeptidase, which participates in the infiltration of cement in the host's skin; while Binnington (1978) suggests that phenol and phenol oxidase might play a role in the process of cement hardening. In *R. sanguineus*, these cells might play the same roles, as the presence of granules before attachment to the host and the composition of granules support the observed by Binnington (1978), Walker et al. (1985), Fawcett et al. (1986) and Sonenshine (1991), despite the longer period of cell activity observed in this study.

In type III acini of *R. sanguineus*, **f** cells did not exhibit granules in unfed females, unlike the observed in two-days fed females, which presented granules mainly in the apical region. In four-days fed females, all granules observed in the previous stage were released, pyramidal cells became squamous, and the cytoplasm was strongly stained by PAS, as also observed by other authors. According to Binnington (1978) and Coons and L'amoreaux (1986), cells became active and filled with granules in a period of 12-24 hours following attachment to the host. After approximately 72 hours of attachment, granules were released, and cells lost their secretory function. Fawcett et al. (1986) and Sonenshine (1991) also report that granules are first observed in **f** cells of other species only after ticks start feeding, and cells actively secrete their products during a period of two days. After this period, organelles undergo autophagy, consequently exhibiting changes in cell structure. These cells then loose their secretory function and transport fluids from the hemolymph to the saliva (Binnington, 1978; Coons and L'amoreaux, 1986; Fawcett et al., 1986; Sonenshine, 1991).

In this study, **f** cells exhibited granules containing polysaccharides, as observed by Binnington (1978) in *B. microplus* females, and according to this author, the role of

these cells would be associated with the consumption of blood by the tick, as high activity was observed in the glands of adult females of *B. microplus*. Thus, considering the period of activity (early stages of feeding) of these cells in some species (Binnington, 1978; Fawcett et al., 1986; Sonenshine, 1991), as well as in *R. sanguineus*, the participation of **f** cells was clearly evident in early stages of blood consumption, as they were no longer active in four-days fed females. According to Walker et al. (1985), this is a transitory role played by these cells in early stages of feeding; following this period, they begin to absorb fluids from the hemolymph and eliminate them through the saliva (Kaufman and Sauer, 1982; Walker et al., 1985, Coons and L'amoreaux, 1986; Sonenshine, 1991).

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REFERENCES

Balashov, Y.S., 1983. Salivary glands. In: Raikhel, A.S., Hoogstraal, I.I. (Eds.), An atlas of ixodid tick ultrastructure. Entomological Society of America, Lanham

Barker, D.M., Ownby, C.L., Krolak, J.M., Claypool, P.L., Sauer, J.R., 1984. The effects of attachment, feeding and mating on the morphology of the I alveolus of salivary glands of the lone star tick, *Amblyomma americanum*. J. Parasitol. 70, 99-113

Bechara, G.H., Szabó, M.P.J., Ferreira, B.R., Garcia, M.V., 1995. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Brazil. J. Vet. Parasitol. 4, 61-66

Binnington, K.C., 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, *Boophilus microplus*. Int. J.Parasitol. 8, 97-115

Bishop, R., Lambson, B., Wells, C., Pandit, P., Osaso, J., Nkonge, C., Morzaria, S., Musoke, A., Nene, V., 2002. A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory *e* cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. Int. J. Parasitol. 32, 833-842

Coons, L.B., L'amoreaux, W.J., 1986. Developmental changes in the salivary glands of male and female *Dermacentor variabilis* (Say) during feeding. In: Borovsky, D., Spielman, A. (Eds.), Host regulated developmental mechanism in vector arthropods. University of Florida-IFAS, Vero Beach

Fawcett, D.W., Binnington, K., Voigt, W.P., 1986. The cell biology of the ixodid tick salivary gland. In: Sauer, J.R., Hair, A. (Eds.), Morphology, physiology, and behavioral biology of ticks. Ellis Horwood, Chichester

Gill, H.S., Walker, A.R., 1987. The salivary glands of *Hyalomma anatolicum anatolicum*: structural changes during attachment and feeding. Int. J. Parasitol. 17 (8), 1381-1392

Junqueira, L.C., Carneiro, J., 2004. Histologia básica- Texto/Atlas, 10 ed. Guanabara Koogan, Rio de Janeiro

Kaufman, W.R., Sauer, J.R., 1982. Ion and water balance in feeding ticks: mechanism of tick excretion. In: Obenchain, F.D., Galun, R. (Eds.), Physiology of ticks. Pergamon Press, Oxford

Marzouk, A.S., Darwish, Z.E.A., 1994. Changes in the salivary glands of female *Hyalomma (Hyalomma) dromedarii* during and after feeding. J. Egypt. Soc. Parasitol. 24 (1), 39-57

McManus, J.F.A., 1946. Histological demonstration of mucin after periodic acid. Nature 158, 202

McMullen, H.L., Sauer, J.R., Burton, R.L., 1976. Possible role in uptake of water vapour by ixodid ticks salivary glands. J. Insect Physiol. 22, 1281-1285

Needham, G.R., Greenwald, L., Freda, T.J., Coons, L.B., 1983. The ultrastructure of the tick (*Amblyomma americanum*) salivary gland acini responsible for secretion of the higroscopic fluid and localization of Na^{+}/K^{+} -ATPase. Am. Zool. 23, 1013

Olivieri, J.A., Serra-Freire, N.M., 1992. Structure of the salivary glands of the unfed female tick *Amblyomma cajennense* (Fabricius) (Acarina: Ixodidae). Mem. Inst. Oswaldo Cruz 87, suppl. I, 167-174

Ribeiro, A.L., Faccini, J.L.H., Daemon, E., 1996. Estudo das variações morfológicas de *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) no Brasil. Rev. Univ. Rural, Ser. Cienc. Vida 18 (1-2), 25-33

Rudolph, D., Knülle, W., 1974. Site and mechanism of water vapour uptake from the atmosphere in ixodid ticks. Nature 149, 84-85

Rudolph, D., Knülle, W., 1978. Uptake of water vapour from air: process, site, and mechanism in ticks. In: Schmidt-Mielsen, K., Bolio, L., Maddrell, S.H.P. (Eds.), Comparative physiology: *Water, ions and fluid mechanics*. Cambridge University Press, Cambridge

Sonenshine, D.E., 1991. Biology of ticks. Oxford University Press, New York

Till, W.M., 1961. A contribution to the anatomy and histology of the brown ear tick *Rhipicephalus appendiculatus* Neumann. Memoirs of the Entomological Society of Southern Africa, Pretoria 6, 1-124

Walker, A., 1994. Arthropods of humans and domestic animals. A guide to preliminary identification. Chapman & Hall, London

Walker, A.R., Fletcher, J.D., Gill, H., 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. Int. J. Parasitol. 15 (1), 81-100

Wikel, S.K., 1981. The induction of host resistance to tick infestation with a salivary gland antigen. Am. J. Trop. Med. Hyg. 30, 284-288

Capítulo 2

CAPÍTULO 2

TITLE: Salivary glands of females of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae). Degenerative morphological changes detected at the end and after the feeding period.

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RESUMO

Fêmeas de carrapatos *Rhipicephalus sanguineus* ingurgitadas e com três e sete dias pós-ingurgitamento tiveram suas glândulas salivares analisadas morfo-histoquimicamente. Nas ingurgitadas detectou-se diminuição na capacidade secretora, marcada pelo surgimento do processo degenerativo, sendo observados nas glândulas tanto ácinos íntegros (I) como em degeneração (II e III). Naquelas com três dias pós-ingurgitamento o processo degenerativo se intensificou, revelando a presença tanto de ácinos em degeneração quanto em processo de fragmentação e formação de corpos apoptóticos. Nas fêmeas com sete dias pós-ingurgitamento a glândula salivar havia degenerado por completo.

Sendo assim, dos oito tipos de células (**a**, **b**, **c1**, **c2**, **c3**, **c4**, **c5** e **c6**) descritos nos ácinos II de fêmeas de *R. sanguineus* em alimentação (dados não publicados), somente as **a**, **c1** e **c3** foram observadas nas fêmeas ingurgitadas e nos III todos os tipos (**d**, **e** e **f**). No estágio de três dias pós-ingurgitamento, nos ácinos I as células **central** e **periféricas** estavam presentes, nos II somente as **a** e **c3** e os ácinos III não foram mais observados.

Durante o processo degenerativo das glândulas salivares das fêmeas ingurgitadas e com três dias pós-ingurgitamento foram observadas nas células alterações citoplasmáticas, algumas delas caracterizadas pela diminuição dos grânulos de secreção, devido a sua eliminação, outras pela alteração dos mesmos, dando origem a uma massa amorfa, pela retração e vacuolização do citoplasma, além da perda da forma e dos limites celulares. Além das alterações citoplasmáticas foram observadas severas alterações nucleares, como polimorfismo em células e dos núcleos de um mesmo ácino, bem como na disposição e grau de condensação da cromatina.

PALAVRAS-CHAVE: *Rhipicephalus sanguineus*; carrapatos; fêmea; glândula salivar; atividade celular; apoptose; morfologia.

ABSTRACT

The salivary glands of engorged females and females at days three and seven post-engorgement of the tick *Rhipicephalus sanguineus* were morpho-histochemically examined. In engorged females, were detected a decrease in secretory capacity, marked by the onset of a degenerative process, with glands exhibiting intact (type I) as well as degenerating acini (type II and III). In females at day three post-engorgement, the degenerative process progressed, revealing the presence of acini undergoing degeneration as well as a process of fragmentation, and the formation of apoptotic bodies. In females at day seven post-engorgement, the salivary gland was completely degenerated. Thus, of the eight cell types (**a**, **b**, **c1**, **c2**, **c3**, **c4**, **c5** and **c6**) described in type II acini of feeding females of *R. sanguineus* (unpublished data), only types **a**, **c1** and **c3** were observed in engorged females, while in type III acini, all cell types (**d**, **e** and **f**) were present. At day three post-engorgement, in type I acini, **central** and **peripheral** cells were present, in type II acini, only **a** and **c3** cells were observed, while type III acini were no longer observed. During the degenerative process of the glandular tissue of engorged female and females at day three post-engorgement, we observed cytoplasmic changes in cells, some of them characterized by the depletion of secretion granules; others, by changes in secretion granules that originate an amorphous mass due to the condensation and vacuolation of the cytoplasm, and loss of shape and cell boundaries. In addition to cytoplasmic changes, we observed significant nuclear changes, such as polymorphism in cells of the same acinus, variations in size and shape of nuclei, as well as the arrangement and condensation level of chromatin.

KEY WORDS: *Rhipicephalus sanguineus*; ticks; female; salivary gland; cell activity, cell death; apoptosis; morphology.

INTRODUCTION

In general, ticks prefer to feed on mammals, for being endothermic (Oliveira et al., 2005). In the case of the tick *Rhiphicephalus sanguineus*, dogs are the most common host (Rey, 1973; Walker, 1994), although it can also be found in cats, rabbits, buffalos, camels, cattle, goats, horses, sheep, bats, birds, reptiles (Flechtmann, 1973 apud Oliveira et al., 2005) and humans (Rey, 1973).

Ticks are members of one of the most important groups among arthropods, due to the role played by their salivary glands. These organs are responsible for the transmission of infectious agents, making these parasites subjects of great veterinary-medical importance (Balashov, 1983). Salivary glands also allow the feeding of the tick, leading to blood loss for the host. In cattle, both problems result in economic losses, especially in the milk and meat industry.

According to the literature, the activity of glands of feeding ticks can be summarized in three stages: 1) preparatory phase, a 24-35 hour period in which females insert their mouthparts, form a feeding lesion, and secrete cement to attach themselves to the host, 2) slow-feeding phase, a 7-10 day period during which the female gradually increases 10 times its unfed weight, 3) rapid-feeding phase, a period of approximately 24 hours, during which the female rapidly increases in weight (Balashov, 1972 apud Weiss e Kaufman, 2001). The first two stages and the early phase of the third stage are characterized by an intense gland activity (synthesis and secretion of components), while during the final phase of the third stage, the depletion of secretion granules, inactivity, and the early stages of cell degeneration are observed (Marzouk and Darwish, 1994).

The degeneration of salivary glands is hormonally controlled by a steroid (Lomas et al., 1998). The synthesis and release of this hormone begins during early feeding stages and increases until the post-engorgement period (Lomas, 1993, PhD thesis).

During feeding, as soon as females reach their “critical weight”, there is an increase in ecdysteroid synthesis (Weiss and Kaufman, 2001). This increase signals the degeneration of salivary glands (Weiss and Kaufman, 2001), which will be initiated only after engorgement (Kaufman and Lomas, 1996). Another factor probably

stimulating this increase in ecdysteroids in the hemolymph (Lomas e Kaufman, 1992), as well as the rapid engorgement of females, is the male/engorgement factor (Weiss and Kaufman, 2004), which also accelerates gland degeneration (Lomas and Kaufman, 1992; Weiss e Kaufman, 2004).

According to Nunes et al. (2005), the degenerative process of salivary glands of semi-fed females of *R.* (*Boophilus*) *microplus* is a result of a series of morpho-histochemical changes in gland cells, in which secretory cells initially loose all or almost all secretion granules, followed by severe changes in the cytoplasm and nucleus, and form apoptotic bodies.

Once the process of salivary gland degeneration begins, the complete degeneration of this organ will be reached only after oviposition, remaining only the duct system and a mass of connective tissue (Till, 1961). This residue of salivary gland is the final result of cells comprising the gland tissue, due to an event that causes a series of morphological and histochemical changes in gland cells.

Based on these informations and considering that little is known about the morphology and histochemistry of the salivary gland degeneration in ticks, this study aimed at describing the changes undergone by salivary glands at the end of the feeding period (fully engorged females), as well as three and seven days after feeding (females at day three and seven post-engorgement).

MATERIAL AND METHODS

To conduct this study, we utilized engorged females and females at day three and seven post-engorgement of *R. sanguineus*. Unfed individuals were provided by Dr. Gervásio Henrique Bechara of the Department of Veterinary Pathology of UNESP, Jaboticabal campus (São Paulo), from a colony maintained under controlled conditions (29^{o} C, 80% humidity, and 12 hour photoperiod) in BOD incubator. These individuals were placed with some males, in a feeding chamber previously glue with an atoxic and non-lesive preparation (Britannia Adhesives-Unit 4, UK) to the shaved back of the host (rabbit) according to technique described elsewhere (Bechara et al., 1995) for complete feeding. A group of engorged females was utilized for histological procedures while another group was maintained alive in closed containers with breathing holes for three

and seven days to be later analyzed. After the completion of the periods of time examined in this study, salivary glands were removed in saline solution and fixed in 10% buffered neutral formalin and acetone (9:1), during one hour and thirty minutes, at 4^{o} C. Following fixation, the material was dehydrated in increasing concentrations of ethanol (70%, 80%, 90% and 95%), embedded and included in Leica resin, and sectioned at a thickness of 3 μm. Sections were placed in glass slides, stained with Hematoxilyn and Eosin, and PAS (Periodic Acid Schiff), McManus (1946), to demonstrate polysaccharides, and counterstained with Methyl Green for nucleus staining, and mounted in Canada balsam for observation under light microscope.

RESULTS

The identification of the different cell types in acini of salivary glands of *R. sanguineus* is based on the system described and adopted by Binnington (1978). For a visual comparison, results are summarized in tables **1**, **2** and **3**.

In the salivary glands of engorged females, of the three types of acini present in the gland tissue (I, II, and III) only type I acini are intact, exhibiting a regular shape (Fig. 1A). The **central** and **peripheral** cells present a fibrillar PAS positive cytoplasm (Fig. 3A) and their nuclei do not exhibit changes (Figs. 1A, 3A).

The results obtained for degenerating type II and III acini are presented in **Table 1**. Some acini could not be identified, as they have lost their original granular characteristics, they where termed here as **Indeterminate 1** (Fig. 1R). They present an irregular shape and lack cell boundaries (Fig. 1R). Thus, their contents are characterized by a vacuolated cytoplasmic mass with secretion residues (Fig. 1R). This cytoplamic mass surrounds some irregular nuclei (Fig. 1R).

In females at day three post-engorgement, the entire acinar structure is undergoing degeneration and details are described in **Tables 2** and **3**.

In females at day seven post-engorgement, the salivary glands are not longer observed, as the gland tissue is completely degenerated.

All changes observed in acini and gland cells during and after feeding are described in **Figure 4**.

Table 1: Morpho-histochemical results of acini observed undergoing degeneration in salivary glands of engorged females of *Rhipicephalus sanguineus*.

Acini	Cell	Shape		Cytoplasm	Nucleus
		Acinus	Cells		
II	**a**	- regular (Figs. 1B-D);	- cubic (Figs. 1B-D);	- granules PAS – (Fig. 3B);	- irregular (Fig. 1B); - Δ (Fig. 3B);
	b	- ∅;	- ∅;	- ∅;	- ∅;
	c1	- regular (Figs. 1B, C);	- irregular (Figs. 1B, C);	- granules PAS +++ (Fig. 3C);	- !! (Fig. 1C);
	c2	- ∅;	- ∅;	- ∅;	- ∅;
	c3	- regular (Figs. 1D, E);	- irregular (Figs. 1D, E);	- larger granules than those of **c1** PAS +++ (Figs. 3B, C);	- Δ (Fig. 3B); - !! (Fig. 1D);
	c4	- ∅;	- ∅;	- ∅;	- ∅;
	c5	- ∅;	- ∅;	- ∅;	- ∅;
	c6	- ∅;	- ∅;	- ∅;	- ∅;
	indeterminate **1**	- regular (Figs. 1E, F); - irregular (Figs. 1G, H);	- irregular (Figs. 1E-H);	- condensed (Figs. 1E-H);	- ✣ (Fig. 1E); - ★ (Fig. 1F); - ★★ (Fig. 1F); - Δ (Figs. 1G, H); - !! (Figs. 1F, H);
	indeterminate **2**	- regular (Figs. 1F, I); - irregular (Fig. 1J);	- irregular (Figs. 1F, I, J);	- condensed (Figs. 1F I, J);	- ✣ ✣ and irregular (Figs. 1F, I, J); - ∞ (Fig. 1J);
III	**d**	- regular (Fig. 1N); - irregular (Figs. 1K-M, P);	- cubic (Figs. 1K-N, P);	- granules PAS – (Fig. 3D);	- irregular (Figs. 1K-M, P, 3D);
	e	- irregular (Figs. 1K-M, O);	- cubic (Figs. 1K, O); - irregular (Figs. 1K-M);	- ! (Figs. 1K-M, O) PAS – (Figs. 3D, E);	- irregular (Fig. 1K); - irregular and ✣ (Fig. 1M); - ∞ (Figs. 1L, 3D);
	f	- irregular (Fig. 1M);	- irregular (Fig. 1M);	- condensed (Fig. 1M) and fine granulation PAS ++ (Fig. 3E);	- irregular (Fig. 3E); - ∞ (Fig. 1M);
		- regular (Fig. 1N); - irregular (Figs. 1O-Q);	- # (Figs. 1N-Q);	- ? (Figs. 1N, O); - * (Fig. 1P); - * with residues of granules of **d** or **e** (Fig. 1Q);	- ✣ and irregular (Figs. 1N, O); - ∞ (Figs. 1O, Q); - !! (Fig. 1P);

new cell types described in feeding females of *R. sanguineus* (unpublished data); **indeterminate:** cells that lost their original granular characteristics and therefore could not be identified; ∅: cell types not observed; #: loss of cell boundaries; **!**: granule disorganization; **?**: heterogeneous cytoplasmic mass; *: cytoplasmic mass; ✣ : picnotic; ★ : beginning of chromatin margination; ★★ : chromatin margination; Δ: blebs; ∞: undergoing fragmentation; **!!**: fragmented; ✣ ✣ : dilated.

Table 2: Morpho-histochemical results of acini I and II observed undergoing degeneration in salivary glands of *Rhipicephalus sanguineus* females at day three post-engorgement.

Acini	Cell	Shape		Cytoplasm	Nucleus
		Acinus	Cells		
I	**Central and Peripheral**	- regular (Fig. 2A);	** (Fig. 2A);	- ?? PAS +++ (Fig. 3F);	- ✣✣ and irregular (Figs. 2A, 3F);
II	**a**	- irregular (Fig. 2B);	- irregular (Fig. 2B);	- granules PAS - (Figs. 3G-J);	- irregular (Figs. 3G, H) - !! (Figs. 2B, 3G);
	b	- ∅;	- ∅;	- ∅;	- ∅;
	c1	- ∅;	- ∅;	- ∅;	- ∅;
	c2	- ∅;	- ∅;	- ∅;	- ∅;
	c3	- irregular (Fig. 2C);	- irregular (Fig. 2C);	- granules PAS +++ (Fig. 3H);	- irregular (Fig. 2C); - !! (Fig. 3H);
	c4	- ∅;	- ∅;	- ∅;	- ∅;
	c5	- ∅;	- ∅;	- ∅;	- ∅;
	c6	- ∅;	- ∅;	- ∅;	- ∅;
	indeterminate 1	- ∅;	- ∅;	- ∅;	- ∅;
	indeterminate 2	- ∅;	- ∅;	- ∅;	- ∅;
		- irregular (Figs. 2B, C);	- # (Figs. 2B, C);	- ?? PAS +++ (Figs. 3G-J);	- irregular (Figs. 2B, C, 3G, H); - ✣✣ and irregular (Figs. 2B, 3I); - ✣ (Fig. 3G); - ★★ (Fig. 3J); - !! (Fig. 2C);

new cell types described in feeding females of *R. sanguineus* (unpublished data); i**ndeterminate:** cells that lost their original granular characteristics and therefore could not be identified; ******: cell boundaries not evident; ∅: cell types not observed; #: loss of cell boundaries; **??**: granular cytoplasmic mass; ✣✣ : dilated; **!!**: fragmented; ✣ : picnotic; ★★ :chromatin margination.

Table 3: Morpho-histochemical results of acini in advanced stages of salivary gland degeneration of *Rhipicephalus sanguineus* females at day three post-engorgement.

Degeneration Stages		Cell Shape	Cytoplasm	Nucleus
1 (Indeterminate Acini With Irregular Shape)		- # (Figs. 2D-I);	- ∇ (Figs. 2D-I, 3K-M) and ?? PAS +++ (Figs. 3K-M); - secretion residues PAS - (Figs. 3K-M);	- ✣ (Fig. 2D); - ★★ (Figs. 2E, F); - !! (Figs. 2G, 3K); - irregular (Figs. 2D-G, I); - ✣✣ and irregular (Figs. 2H, 3K); - ∞ (Figs. 2H, 3L);
2 (Indeterminate Acini In Early Stages of Fragmentation)	**A**	- # (Fig. 2J);	- ∇ (Figs. 2J, 3N) and ?? PAS +++ (Fig. 3N); - secretion residues (Figs. 2J, 3N);	- !! (Figs. 2J, 3N); - irregular (Fig. 3N);
	B	- # (Fig. 2K);	- residues of cytoplasm (Fig. 2K);	- not observed;
3 (Indeterminate Acini Undergoing Fragmentation)		- # (Figs. 2L, M);	- ∇ (Figs. 2L, M, 3O) and ?? PAS +++ (Fig. 3O); - secretion residues (Fig. 2L);	- irregular (Figs. 2L, 3O); - ★★ (Fig. 2M); - !! (Fig. 3O);
4 (Apoptotic Bodies Formed From The Fragmentation Of Acini)		- cell fragments (Figs. 2N, O);	- residues of cytoplasm with or without granules (Figs. 2N, O, 3P);	- !! (Figs. 2N, O, 3P);

#: loss of cell boundaries; **∇**: vacuolated cytoplasmic mass; **??**:granular cytoplasmic mass; ✣✣: dilated; **!!**: fragmented; ✣: picnotic; ★★:chromatin margination; ∞: undergoing fragmentation.

FIGURES

FIGURE 1:

Histological sections of salivary glands of engorged females of *Rhipicephalus sanguineus* stained with Hematoxylin and Eosin. **A.** Intact type I acini (**I**). **B-D.** Type II acini (**II**) with characteristics of degeneration, exhibiting in: **B**, **a** cell (**a**) filled with secretion granules (**s**) and irregular nucleus (**in**); **C**, irregular **c1** cells (**ic1**) with secretion granules (**s**) and nuclear fragments (**nf**) and **D**, irregular **c3** cell (**ic3**) with secretion granules (**s**) and nuclear fragments (**nf**). **E-J.** Type II acini (**II**) with characteristics of more advanced stages of degeneration and **a** (**a**) and **c3** (**c3**) cells filled with secretion (**s**), and without secretion and irregular shape or **indeterminate 1** (**iin1**) and i**ndeterminate 2** (**iin2**). **E-H.** Irregular **indeterminate 1** cells (**iin1**) with shrinkage of the cytoplasm (**sc**) and gradual nuclear changes, characterized by: **E**, picnotic nucleus (**pn**); **F**, onset of chromatin marginalization (**arrow**) and total chromatin marginalization (**double arrow**); **G** and **H**, blebs (**bb**) and **F** and **H**, fragments of nuclei (**fn**); **F**, **I** and **J.** Irregular **indeterminate 2** cells (**iin2**) with shrinkage of the cytoplasm (**sc**) and gradual nuclear changes, characterized by: **F**, **I** and **J**, dilated and irregular nuclei (**din**); **J**, nucleus undergoing fragmentation (**triple arrow**). **K-M.** Type III acini (**III**) with characteristics of degeneration, exhibiting **d** cells (**d**) filled with secretion granules (**s**) and irregular nuclei (**in**) and irregular **e** cells (**ie**) with secretion resembling an amorphous mass (✳) and gradual nuclear changes, such as: **K**, irregular nuclei (**in**); **L**, nucleus undergoing fragmentation (**triple arrow**) and **M**, irregular and picnotic nucleus (**arrow head**); **M.** Irregular **f** cells (**if**) with shrinkage of the cytoplasm (**sc**) and nuclei undergoing fragmentation (**triple arrow**). **N-Q.** Type III acini (**III**) in more advanced stages of degeneration. **N** and **O.** Cells filled with secretion surrounded by a heterogeneous cytoplasmic mass (**hm**) with nuclei exhibiting various morphological features, characterized by: **N** and **O**, picnotic and irregular nuclei (**arrow head**) and **O**, nucleus undergoing fragmentation (**triple arrow**). **P. d** cells (**d**) filled with secretion granules (**s**), irregular nucleus (**in**), and surrounded by a cytoplasmic mass (**cm**) with nuclear fragments (**nf**). **Q.** Acinus composed of only cytoplasmic mass (**cm**) and nuclei undergoing fragmentation (**triple arrow**). **R. Indeterminate** acinus at stage 1 of degeneration (**Ind1**) consisted of vacuolated cytoplasmic mass (✪) and irregular nucleus (**in**).

c: cytoplasm; **nc**: nucleus of **central** cell; **np:** nucleus of **peripheral** cell, **dt:** duct; **lu:** lumen; **iv:** intraacinar valve; **iac:** irregular-shaped acinus; **d: d** cell; **e: e** cell; **rs:** residue of secretion; **va:** vacuole.

Bars: **A-R**= 20 μm.

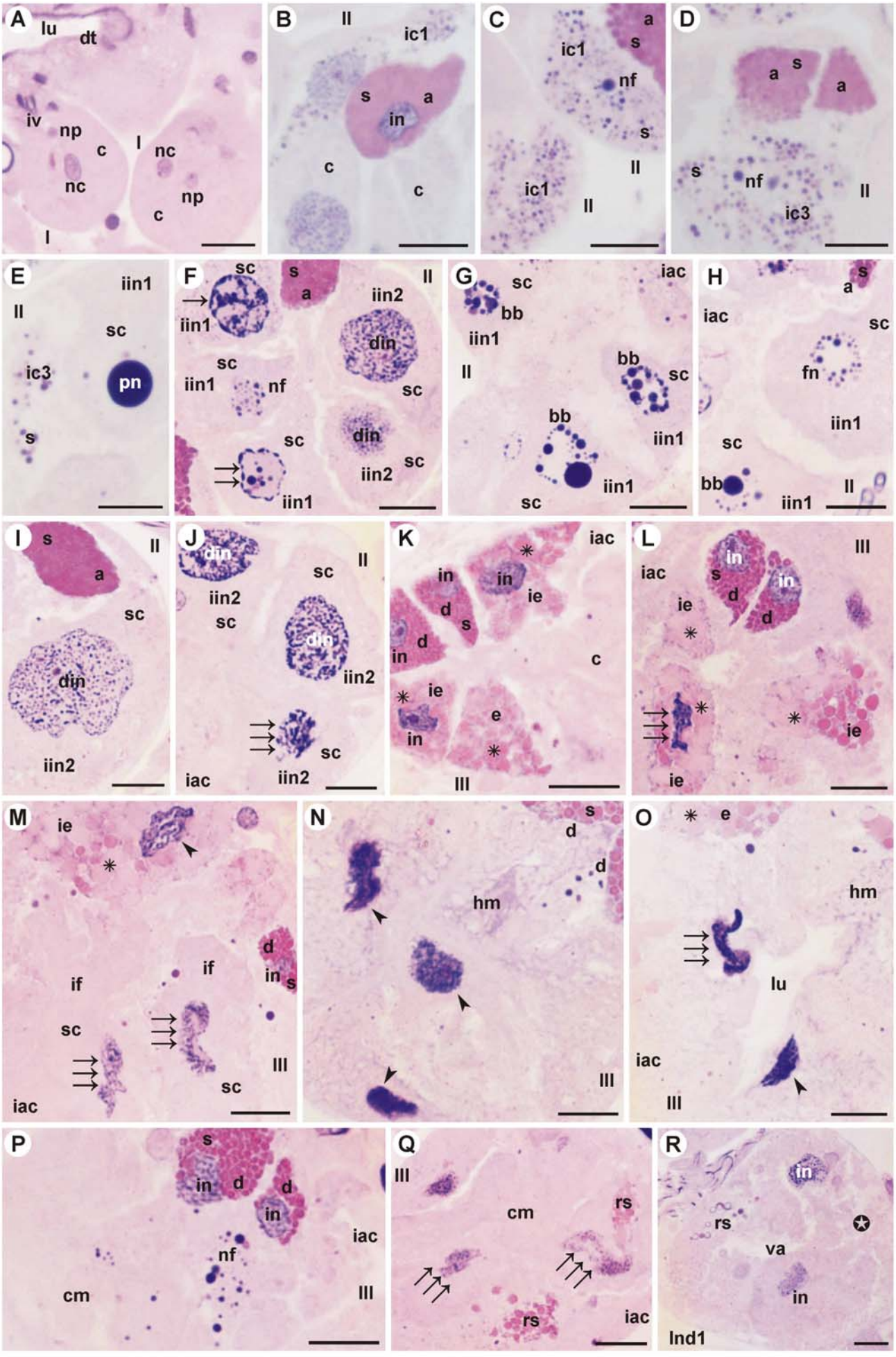
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FIGURE 2:

Histological sections of salivary glands of *Rhipicephalus sanguineus* females at day three post-engorgement stained with Hematoxylin and Eosin. **A.** Type I acini (**I**) with characteristics of degeneration, containing dilated and irregular nuclei of **central** (**dashed arrow**) and **peripheral** (**double dashed arrow**) cells. **B** and **C.** Type II acinus (**II**) with characteristics of more advanced stages of degeneration, exhibiting in: **B**, irregular **a** cell (**ia**) with secretion granules (**s**) and nuclear fragment (**nf**) surrounded by cytoplasmic mass (**cm**) and irregular nuclei (**in**) and dilated and irregular nuclei (**din**); **C**, irregular **c3** cell (**ic3**) with secretion granules (**s**) and irregular nucleus (**in**) surrounded by cytoplasmic mass (**cm**) with irregular nucleus (**in**) and fragments of nuclei (**fn**). **D-M.** Acini in late stages of degeneration, which were not identified and therefore were termed **Indeterminate**. **D-I. Indeterminate** acini at stage 1 of degeneration (**Ind1**) with irregular shape, consisted of vacuolated cytoplasmic mass (✪) and in: **D**, picnotic nucleus (**pn**) and irregular nucleus (**in**); **E** and **F**, irregular nuclei (**in**) and nuclei with chromatin margination (**double arrow**); **G**, nuclear fragments (**nf**) and irregular nucleus (**in**); **H**, fragmenting nucleus (**triple arrow**) and irregular and dilated nuclei (**din**) and **I**, irregular nuclei (**in**). **J** and **K. Indeterminate** acini at stage 2 of degeneration divided into sub-types, **Indeterminate** acinus 2 A (**Ind2A**) and 2 B (**Ind2B**), which are in early stages of fragmentation. **J. Indeterminate** acinus in stage 2A (**Ind2A**) exhibits vacuolated cytoplasmic mass (✪) and nuclear fragments (**nf**). **K. Indeterminate** acinus in stage 2B (**Ind2B**) with only cytoplasmic residues (**cr**). **L** and **M. Indeterminate** acini in stage 3 of degeneration (**Ind3**) undergoing fragmentation and consisted of vacuolated cytoplasmic mass (✪), presenting: **L**, irregular nuclei (**in**) and **M**, nucleus with chromatin marginalization (**double arrow**). **N** and **O**. Apoptotic bodies (**ab**) containing cytoplasm (**c**) or secretion (**s**) and nuclear fragments (**nf**), note in: **O**, apoptotic bodies (**ab**) containing secretion granules (**s**) of **c3** cells or only cytoplasm (**c**).

dt: ducto; **rs:** residue of secretion; **va:** vacuole.

Bars: **A-O**= 20 μm.

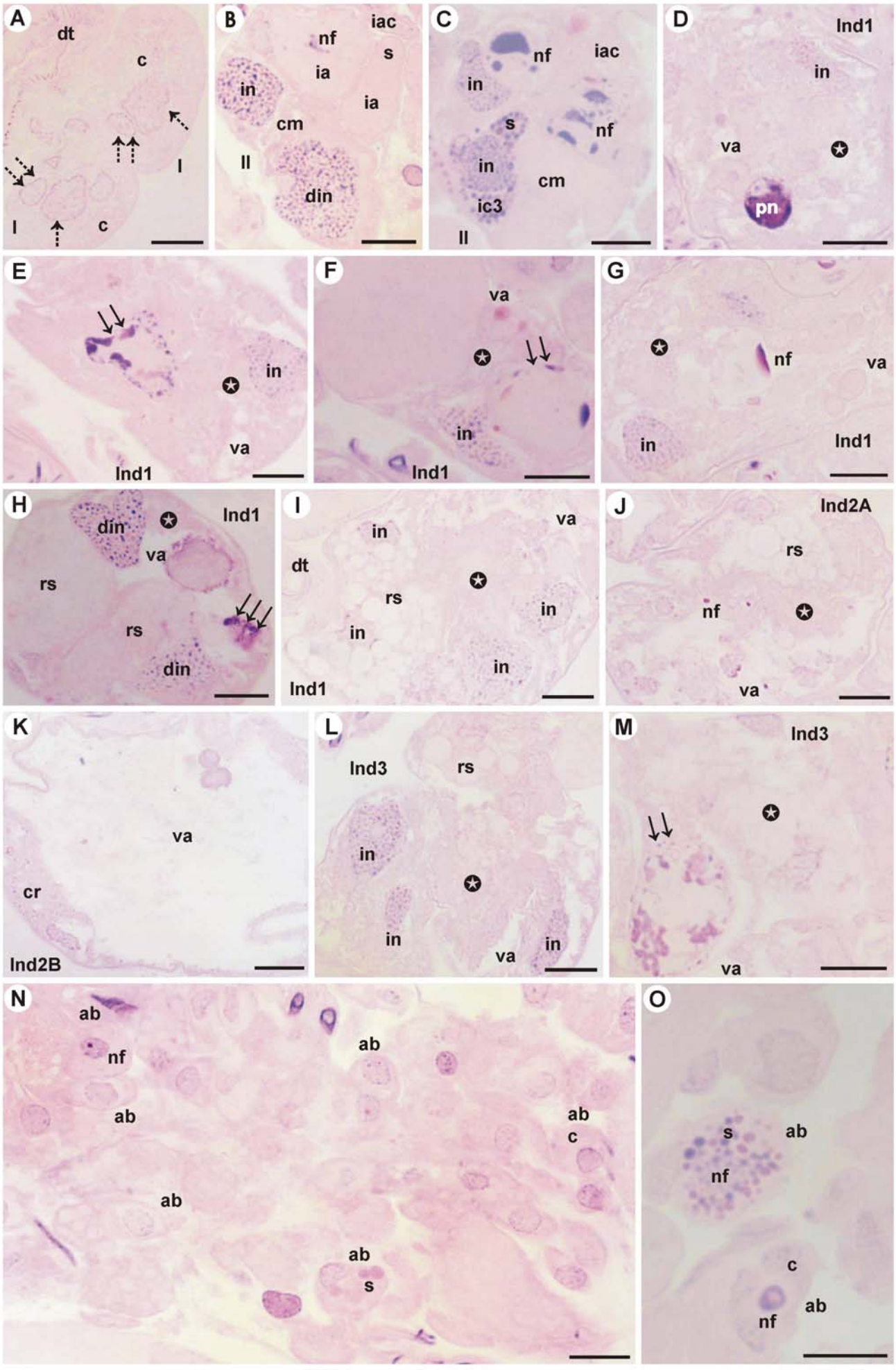
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FIGURE 3:

Histological sections of salivary glands of females of *Rhipicephalus sanguineus* stained with PAS and counterstained with Methyl Green. **A-E.** Engorged females. **A.** Intact type I acinus (**I**). **B** and **C.** Type II acini (**II**) undergoing degeneration, exhibiting in: **B**, **a** cells (**a**) and irregular **c3** cells (**ic3**), filled with secretion (**s**) and blebbed nuclei (**bb**) and **C**, **c1** (**ic1**) and **c3** (**ic3**) cells exhibiting irregular shape and filled with secretion (**s**). **D** and **E.** Type III aciniI (**III**) undergoing degeneration, with: **D**, **d** cells (**d**) and irregular **e** cell (**ie**) filled with secretion (**s**), **d** cells (**d**) with irregular nuclei (**in**) and **e** (**e**) undergoing fragmentation (**triple arrow**) and **E**, irregular shaped **f** cells (**if**), cytoplasm with fine granulation (⁂) and irregular nuclei (**in**). **F-P.** Females at day three post-engorgement. **F.** Type I acinus (**I**) undergoing degeneration, containing granular cytoplasmic mass (✦) and **central** (**dashed arrow**) and **peripheral** (**double dashed arrow**) nuclei, both dilated and irregular. **G-J.** Type II acini (**II**) with more prominent characteristics of degeneration, exhibiting irregular shaped **a** cells (**ia**) filled with secretion granules (**s**), irregular nuclei (**in**) and nuclear fragment (**nf**), irregular **c3** cells (**ic3**) with secretion granules (**s**) and nuclear fragment (**nf**), a granular cytoplasmic mass is also observed in these acini (✦) with nuclei with various aspects, in: **G**, irregular (**in**) and picnotic (**pn**); **H**, irregular (**in**); **I**, dilated and irregular (**din**) and **J**, with chromatin marginalization (**double arrow**). **K-O.** Acini in late stages of degeneration termed **Indeterminate**. **K-M. Indeterminate** acini in stage 1 of degeneration (**Ind1**) with irregular shape, composed of a cytoplasmic mass with a granular and vacuolated aspect (**cgv**) and nuclei with different characteristics, in: **K**, dilated and irregular (**din**) and nuclear fragments (**nf**) and **L**, undergoing fragmentation (**triple arrow**). **N. Indeterminate** acinus in stage 2A of degeneration (**Ind2A**) in early stages of fragmentation and composed of a cytoplasmic mass with a granular and vacuolated aspect (**cgv**), irregular nucleus (**in**) and nuclear fragments (**nf**); **O. Indeterminate** acinus in stage 3 of degeneration (**Ind3**) undergoing fragmentation and composed of a cytoplasmic mass with a granular and vacuolated aspect (**cgv**), irregular nucleus (**in**) and nuclear fragments (**nf**). **O** and **P.** Apoptotic bodies (**ab**) containing nuclear fragments (**nf**) and cytoplasm (**c**) or secretion (**s**).

fc: fibrillar cytoplasm ;**nc:** nucleus of **central** cell; **np:** nucleus of **peripheral** cell; **dt:** duct; **lu:** lumen; **rs:** residue of secretion; **va:** vacuole.

Bars: **A-P**= 20μm.

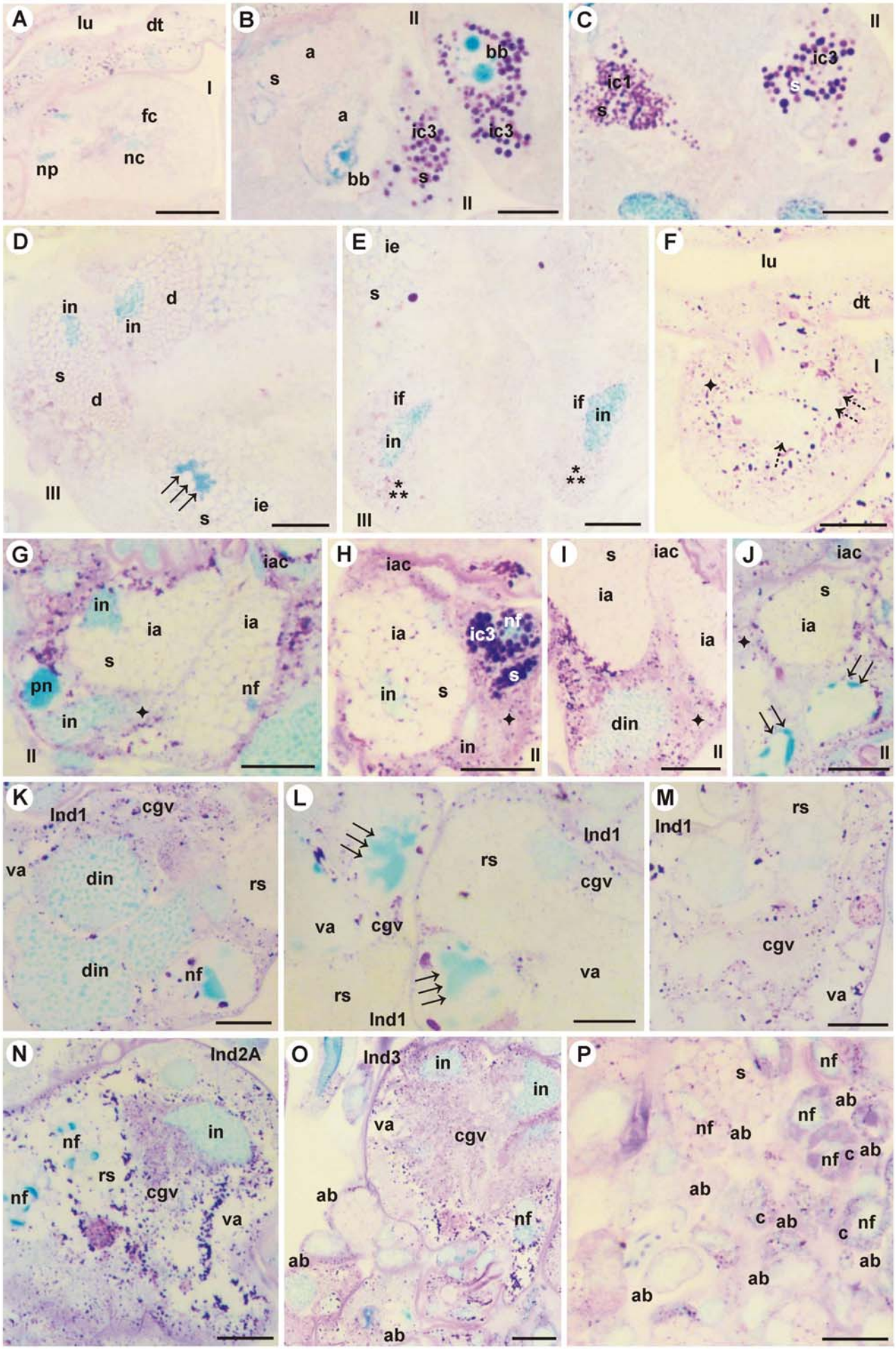
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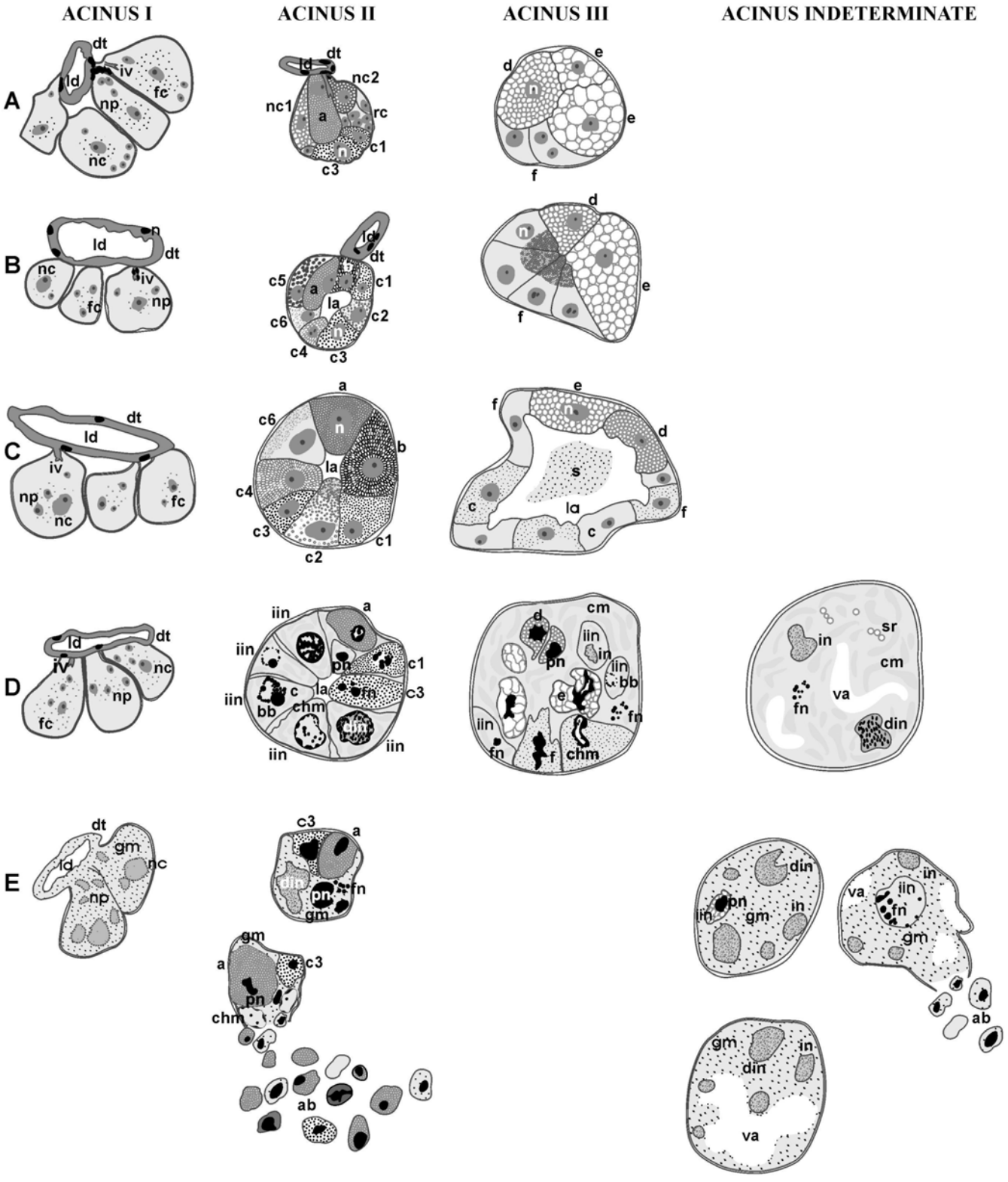
FIGURE 4:

Schematic representation of changes undergone by type I, II, and III acini of the salivary glands of *Rhpicephalus sanguineus* females during and after feeding.

A. Unfed females;
B. Two-days fed females;
C. Four-days fed females;
D. Engorged females;
E. Females at day three post-engorgement;

rc: undifferentiated cell; **nc1: undefined** cell **1**; **nc2: undefined** cell **2**; **a: a** cell; **b: b** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **c6: c6** cell; **d: d** cell; **e: e** cell; **f: f** cell; **iin: indeterminate** cell; **dt:** duct; **ld:** lumen of the duct; **iv:** intraacinar valve; **fc:** fibrillar cytoplasm; **cn: central** cell nucleus; **pn: peripheral** cell nucleus; **gm:** granular cytoplasmic mass; **n:** nucleus; **gm:** granular cytoplasmic mass; **c:** cytoplasm; **din:** dilated and irregular nucleus; **pn:** picnotic nucleus; **chm:** chromatin margination; **bb:** blebs; **fn:** fragmented nucleus; **ab:** apoptotic bodies; **s:** secretion; **cm:** cytoplasmic mass; **in:** irregular nucleus; **va:** vacuole; **rs:** residue of secretion.


ACINUS I
ACINUS II
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ACINUS INDETERMINATE
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DISCUSSION

The present study demonstrated morphological changes in the salivary glands of *Rhipicephalus sanguineus* females as a result of the degenerative process at the end and after the feeding period, confirming and complementing the information available in the literature.

The salivary glands of engorged females of *R. sanguineus* exhibited a prominent decrease in secretory capacity, marked by the loss of cytoplasmic granules and the appearance of characteristics of degeneration. In these females, these changes make their feeding process unfeasible, forcing them to stop feeding, as observed by Harris and Kaufman (1984). These authors reported that the gland degeneration of *Amblyomma hebraeum* is characterized by a reduction of over 90% in the maximum secretory capacity of this tissue.

We observed in engorged females, intact and therefore functional type I acini; type II and III acini in different stages of degeneration; and acini that could not be identified due to their advanced stages of degeneration. The latter were here termed **Indeterminate**. In type II and III acini, among acini of the same type, we observed variations in the intensity of the degenerative process, with some acini exhibiting cells with granules while others, cells with few or no granules. Among cells of the same acinus, in type II and III acini, a variation in the intensity of the degenerative process was also observed, with cells still with granules, although already exhibiting degenerative characteristics, coexisting with cells without granules, therefore in an advanced degenerative stage.

In *R. sanguineus* females at day three post-engorgement, we observed a progression of the degenerative process compared to the observed in engorged females, exhibiting type I and II acini undergoing degeneration, **Indeterminate** acini, as well as several apoptotic bodies. These results support Till's observations (1961) in *R. appendiculatus* that degeneration is intensified after the end of feeding, especially two days after engorgement, with the depletion of most secretion granules.

Our data, however, on the characteristics of degeneration in the glands of engorged females contradict those obtained by Lomas et al. (1998), L'Amoreaux et al. (2003) and Nunes et al. (2005), in females of *A. hebraeum*, *Dermacentor variabilis*, and

R. (Boophilus) microplus, respectively. According to these authors, salivary gland degeneration begins 24 hours following engorgement in *A. hebraeum*, five days after engorgement in *D. variabilis*, and already in semi-engorged females of *R. (Boophilus) microplus*. This process is complete within 4 days following engorgement in *A. hebraeum* (Lomas et al. (1998) and in approximately 33 days after engorgement in *D. variabilis* (L'Amoreaux et al., 2003). Although the salivary glands of *R. sanguineus* females at day three post-engorgement present characteristics of advanced stages of degeneration, this process is probably not complete in four days, as reported for *A. hebraeum* (Lomas et al., 1998), but it would not last as long as the observed for *D. variabilis* (L'Amoreaux et al., 2003).

Thus, these data suggest that the onset of degeneration observed in glands of engorged females of *R. sanguineus* occurs earlier than in *A. hebraeum* (Lomas et al., 1998) and *D. variabilis* (L'Amoreaux et al., 2003). On the other hand, it occurs later than that of semi-engorged females of *R. (B.) microplus* (Nunes et al., 2005), and ends within about seven days post-engorgement, later than in *A. hebraeum* (Lomas et al., 1998).

Changes in cells of degenerating acini of salivary glands detected in the two situations examined in our study clearly indicate that salivary gland degeneration in *R. sanguineus* is asynchronous among different acini, as well as among cells of the same acini. This supports the results obtained by Till (1961), which described the asynchronous degeneration of the gland tissue of *R. appendiculatus* females, and that type II and III acini are the first ones to degenerate; by L'Amoreaux et al. (2003) that demonstrated that type II and III acini of *D. variabilis* females degenerate earlier than type I acini; and by Nunes et al. (2006), which described an asynchronous degeneration of acini and salivary gland cells of semi-engorged females of *R. (Boophilus) microplus*.

Thus, based on the different stages of degeneration among acini of the same type and the location of acini in the glands of Ixodidae in general, our results suggest that the degeneration process in *R. sanguineus* females begins in the posterior region of the gland, where the first signs of degeneration were detected, while intact acini were observed in the anterior region. This indicates that the degeneration of salivary glands of *R. sanguineus* females follows the same pattern described by most insects studied (Silva de Moraes, 1998, PhD thesis; Abreu, 2004).

Regarding size, type I acini of engorged females of *R. sanguineus* did not exhibit differences compared to those of females during the period of large blood consumption (unpublished data). Also, **central** and **peripheral** cells were intact, as observed by Binnington (1978) and Nunes et al. (2006).

In glands of *R. sanguineus* females at day three post-engorgement, characteristics of degeneration were detected in **central** as well as **peripheral** cells, unlike the observed by Bowman and Sauer (2004), which reported that in *Ixodes ricinus* at day 12 post-engorgement, these acini do not exhibit signs of cell death, while two other types are completely degenerated; and L'Amoreaux et al. (2003) that observed the degeneration of these structures only after day 13 post-engorgement.

The late degeneration of type I acini might be associated with their role in glands of females that completed feeding. According to Bowman and Sauer (2004), in *I. ricinus*, these acini remain functional to maintain the hydration of the female during egg laying. L'amoreaux et al. (2003) agree with this role, however, they report that this occurs during the period of pre-oviposition, since these structures undergo cells death after the first week of oviposition. This supports the data obtained in our study, since we observed the first signs of degenerative characteristics in type I acini only in the beginning of the oviposition period.

Type II acini of engorged females of *R. sanguineus* slightly decreased in size compared to those of females during the period of large blood consumption (unpublished data). This was observed by Binnington (1978), which reported that in females of *B. microplus*, at the end of engorgement, acini decrease in size. However, our results contradict those obtained by Marzouk and Darwish (1994), which reported an increase in the size of these structures in engorged females of *Hyalomma (Hyalomma) dromedarii*.

In engorged females of *R. sanguineus*, these acini exhibited characteristics of degeneration, and in the same acinus, degenerating cells were observed with secretion granules, coexisting with degenerating cells with few granules. The different levels of degeneration might be the result of a functional asynchronism of cells, in which they may be active in different moments during the feeding process (Binnington, 1978; Walker et al., 1985; Gil and Walker, 1987; Marzouk and Darwish, 1994) as well as inactive in different moments. The data are in agreement with those obtained by Nunes

et al. (2006), which proposed that these are typical characteristics of the apoptotic process, with cells in different stages coexisting in the same tissue.

In this study, **a**, **c1** and **c3** cells still containing secretion granules but already exhibiting nuclear changes were observed in type II acini. Additionally, were also found in the same acinus, cells with few or no secretion granules, reduced size, and severe signs of degeneration, especially in the nucleus. These cells could not be identified and were termed **indeterminate**. The depletion of most secretion granules in the different cells types and consequently the presence of cells with atypical characteristics (**indeterminate** cells) are in agreement with the results obtained by Binnington (1978). This author reported that at the end of engorgement, type II acini of *B. microplus* females undergo drastic changes, such as the decrease in size and loss of granules, making their identification difficult.

The presence of cells with granules in *R. sanguineus*, however, contradicts the data obtained by Binnington (1978), Nunes et al. (2006), Walker et al. (1985) and Marzouk and Darwish (1994). In *B. microplus* at the end of engorgement, the only cells observed containing small amounts of granules were **a**, **b**, **c1** and **c3**; in *R. (B) microplus*, **a**, **b**, **c2** and **c3**; and in *R. appendiculatus*, at the final feeding stage, only **a**, **b**, **c1** and **c2** remained, while during this period, **c1** hypertrophied. In engorged females of *H. (Hyalomma) dromedarii*, **a**, **b** and **c** cells contained fewer secretion granules when compared to feeding females, and **c** cells exhibited large cytoplasmic vacuoles.

This clearly indicates that for each tick species, or depending on their requirements, the active period of each cell type may vary and consequently each cell type does not play exactly the same role in the secretory cycle of salivary glands.

The present study also showed that in *R. sanguineus,* **a**, **c1** and **c3** cells of engorged females were the last ones to degenerate, due to their role in the feeding process. In addition, they were the first ones to become active, indicated by the presence of secretion granules (unpublished data) and the last ones to lose their function.

The cells termed here as **indeterminate** exhibited characteristics that clearly indicated cytoplasmic and nuclear changes, as a consequence of the advanced stage of degeneration. Since these cells exhibit different cytoplasmic and nuclear aspects, they were termed **indeterminate 1** and **2**.

In type II acini of females at day three post-engorgement, the degenerative process was more prominent when compared to those of engorged females. In the former, few type II acini were observed, indicating that many of them presented cells in advanced stages of the degeneration. The few type II acini observed did not seem to exhibit changes in size compared to those of engorged females. Marzouk and Darwish (1994), on the contrary, showed that in *H. (Hyalomma) dromedarii* type II acini in females after engorgement increase in size. Some type II acini do not contain granules, thus the increase in size might be associated with the hypertrophy of these acinar structures, as a result of the degenerative process.

Type II acini of *R. sanguineus* also exhibited some **a** and **c3** cells, being the former the most abundant. These two cell types contained secretion, although they already exhibited signs of degeneration. In addition to **a** and **c3** cells**,** were also observed a cytoplasmic mass without cell boundaries containing changed nuclei, characterizing a very advanced stage of degeneration of cells of these acini.

Compared to other species, in *R. sanguineus* **a** and **c3** cells were the last to degenerate; while in *B. microplus*, **b** and **c3** cells (Binnington, 1983); in *R. (Boophilus) microplus*, **a**, **b**, **c2** and **c3** (Nunes et al., 2006); and in *R. appendiculatus*, **a** cells remained, although reduced in size and with few granules, until the tick detachment the (Walker et al., 1985).

Type III acini of engorged females of *R. sanguineus* did not exhibit changes in size when compared to females during the period of large blood consumption (unpublished data), unlike the observed by Marzouk and Darwish (1994) that demonstrated a significant increase in engorged females of *H. (Hyalomma) dromedarii*.

These acini in engorged females of *R. sanguineus* exhibited a decrease in the diameter of the lumen, and cells, previously squamous, became cubic again in the beginning of the period of large blood consumption (unpublished data). These data support Till's observations (1961) that in females of *R. appendiculatus*, the lumen of these acini greatly increase during the period of large blood consumption. This, however, occurs in the end of the feeding cycle, during which consumption was probably lower, the diameter of lumen decreased and acini returned to their original shape (small lumen and cubic cells). On the other hand, Binnington (1978) and Gill and Walker (1987) reported in females of *B. microplus* and *H. anatolicum anatolicum*,

respectively, the presence of type III acini with squamous cells and enlarged lumen at the end of the feeding period.

In addition to the morphological changes described here for type III acini, changes in **d**, **e** and **f** cells were also observed as a result of the degenerative process, which became more prominent in some cell types of the same acinus, characterizing an asynchronous degeneration. These data support those obtained by Nunes et al. (2006) that also observed an asynchronous degeneration of **d**, **e** and **f** cells of type III acini of semi-engorged females of *R. (Boophilus) microplus*. In these acini, additionally to **d**, **e** and **f** cells, a cytoplasmic mass with no cell boundaries was also observed containing fragments of nuclei.

Although cells **d** and **e** contained secretion, in some cases, not in the form of granules, but as an amorphous content, these cells exhibited other cytoplasmic and nuclear changes that characterized cell death. These results support Binnington's observations (1978) that **d** and **e** cells of *B. microplus* females at the end of engorgement exhibit few granules and picnotic nuclei; and Marzouk and Darwish's (1994) findings that **d** and **e** cells contain disperse granules due to the increase in size of the acinus.

Our results obtained for **d** and **e** cells of *R. sanguineus* are partially in agreement with those reported by Nunes et al. (2006) These authors observed **d** cells with reduced size, residual secretion and picnotic nuclei in semi-engorged females of *R. (Boophilus) microplus*, as observed in our study. However **e** cells were not observed by these authors. This might be due to the complete or advanced stages of degeneration, as also reported by Gill and Walker (1987) in *H. anatolicum anatolicum* females in the final phase of feeding, when these cells are extremely compressed between well developed interstitial cells.

In engorged females examined in this study, **f** cells did not contain secretion granules, but exhibited a cytoplasm with a fine granular material, as well as nuclear changes suggestive of degeneration. Nunes et al. (2006) also observed **f** cells with cytoplasm with a fine granular material, but apparently preserved nuclei. On the other hand, **f** cells of *D. variabilis* females during the rapid feeding phase already show signs of degeneration (Coons and L'Amoreaux, 1986).

Cell changes observed in type III acini of engorged females of *R. sanguineus* indicate that **f** cells might be the first to degenerate, since they loose their function due to a decrease in the need to eliminate water and ions present in the hemolymph from the blood meal. Thus, the commonly observed cytoplasmic mass, containing changed nuclei and surrounded by other cells (**d** or **e**) in III type acinus, probably indicate an advanced stage of degeneration of **f** cells. Cells **d** seem to be the last ones to degenerate, since: 1) they still contained secretion granules and coexisted with **e** cells, whose granules were disorganized caused by cell degeneration, 2) they were the only cells present in the same acinus in advanced stages of degeneration, and were surrounded by a mass of cytoplasmic cells with nuclear fragments.

The results obtained in the present study indicate that type III acini were the first to degenerate and in females at day three post-engorgement, the degenerative process progressed. Thus, in these females, type III acini completely lost their morphological characteristics and histochemical properties and could not be identified and/or were completely degenerated, and their fragments (apoptotic bodies) became a residual mass commonly observed in the gland tissue. These data support the results obtained by L'Amoreaux et al. (2003), which reported that in *D. variabilis* females in different post-engorgement stages, type III acini are the most affected by the degenerative process and the first to be eliminated from the gland tissue.

Since the degenerative process of salivary glands of females of the tick *R. sanguineus* was asynchronous along the gland, in engorged females, intact acini were observed at the same time as acini exhibiting characteristics of advanced stages of degeneration. Some could be identified while others could not. The latter were classified as **Indeterminate**.

The presence of **Indeterminate** acini already in engorged females of *R. sanguineus* supports the findings by Nunes et al. (2006) that also reported the presence of residues of acini, the result of advanced stages of degeneration in salivary glands of semi-engorged females of *R. (Boophilus) microplus*. In the present study were observed an increase of these acini in females at day three post-engorgement, due to the progression of the degenerative process in this situation.

Thus, salivary glands of *R. sanguineus* underwent changes throughout the degenerative process that caused changes in acini (**Indeterminate**). In females at day

three post-engorgement, acini were extremely irregular and fragmented, resulting in the formation of apoptotic bodies. These data support those obtained by Nunes et al. (2005) that also showed the presence of apoptotic bodies in the final phase of the degenerative process of salivary glands of semi-engorged females of *R. (Boophilus) microplus*. According to Till (1961) in *R. appendiculatus*, the final result of the degeneration process is a mass of connective tissue and the duct system.

During the process of salivary gland degeneration of engorged females and females at day three post-engorgement of *R. sanguineus*, changes were not the same in different cells. In some of them, we observed a decrease in secretion granules, which were eliminated, while in others, granules were disorganized and became a mass. Both cytoplasmic changes were followed by severe nuclear changes, which varied in size, shape, condensation level, and chromatin arrangement, even in cells of the same acinus. Thus the degenerative process intensified resulting in other changes, such as condensation of cytoplasm, loss of cell boundaries, loss of shape of cell and cytoplasmic vacuolation. All these cell changes caused a disorganization of acini and consequently the formation of apoptotic bodies. Nunes et al. (2005, 2006) also reported significant changes in the cytoplasm and nucleus, in addition to the formation of apoptotic bodies in *R. (Boophilus) microplus*.

The results obtained in this study and the information available in the literature (L'Amoreaux et al., 2003; Bowman and Sauer, 2004; Nunes et al., 2005, 2006a, b) suggest that the salivary gland degeneration of *R. sanguineus* females occurs by apoptosis.

Nunes et al. (2006) concluded that in *R. (Boophilus) microplus* the gland tissue degenerates due to the ontogenetic development of individuals, in which cells are programmed to be deactivated in certain moments. In the present study, we observed that salivary glands not only degenerate to complete the feeding process of ticks, but also to avoid an investment and waste of energy in the maintenance of an organ that has lost all or most of its functions.

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REFERENCES

Abreu, R.M.M., Silva de Moraes, R.L.M., Taboga, S.R., Camargo-Mathias, M.I., 2004. Cytochemical studies of the nuclei of the venom glands' cells of *Apis mellifera* (Hymenoptera, Apidae). Acta Histochem. Cytochem. 37 (5), 195-299

Balashov, Y.S., 1983. Salivary glands. In: Raikhel, A.S., Hoogstraal, I.I. (Eds.), An atlas of ixodid tick ultrastructure. Entomological Society of America, Lanham

Balashov, Y.S., 1972. Bloodsucking ticks (Ixodoidea)- vectors of diseases of man and animals. Misc. Publ. Entomol. Soc Am. 8, 161-376

Bechara, G.H., Szabó, M.P.J., Ferreira, B.R., Garcia, M.V., 1995. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Brazil. J. Vet. Parasitol. 4, 61-66

Binnington, K.C., 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, *Boophilus microplus*. Int. J. Parasitol. 8, 97-115

Bowman, A.S., Sauer, J.R., 2004. Tick salivary glands: function, physiology and future. Parasitology 129, S67-S81

Coons, L.B., L'amoreaux, W.J., 1986. Developmental changes in the salivary glands of male and female *Dermacentor variabilis* (Say) during feeding. In: Borovsky, D., Spielman, A. (Eds.), Host regulated developmental mechanism in vector arthropods. University of Florida-IFAS, Vero Beach

Flechtmann, C.H.W., 1973. Ácaros de importância médico-veterinária. Editora Livraria Nobel S.A., São Paulo

Gill, H.S., Walker, A.R., 1987. The salivary glands of *Hyalomma anatolicum anatolicum*: structural changes during attachment and feeding. Int. J. Parasitol. 17 (8), 1381-1392

Harris, R.A. Kaufman, W.R., 1984. Neural involvement in the control of salivary gland degeneration in the ixodid tick *Amblyomma Hebraeum*. J. Exp. Biol. 109, 281-290

Kaufman, W.R., Lomas, L.O., 1996. "Male factor" in ticks: their role in feeding and egg development. Invertebr. Reprod. Dev. 30, 191-198

L'Amoreaux, W.J., Junaid, L., Trevidi, S., 2003. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. Tissue Cell 35, 95-99

Lomas, O.L., Gelman, D., Kaufman, W. R., 1998. Ecdysteroid regulation of salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*: a reconciliation of *in vivo* and *in vitro* observations. Gen. Comp. Endocrionol. 109, 200-211

Lomas, L.O., Kaufman, W.R., 1992. An indirect mechanism by which a protein from the male gonad hastens salivary gland degeneration in the female ixodid tick, *Amblyomma hebraeum*. Arch Insect Biochem. Physiol. 21, 169-178

Marzouk, A.S., Darwish, Z.E.A., 1994. Changes in the salivary glands of female *Hyalomma (Hyalomma) dromedarii* during and after feeding. J. Egypt. Soc. Parasitol. 24 (1), 39-57

McManus, J.F.A., 1946. Histological demonstration of mucin after periodic acid. Nature 158, 202

Nunes, E.T., Bechara, G.H., Saito, K.C., Denardi, S.E., O liveira, P.R., Camargo-Mathias, M.I., 2005. Morphological, histological, and ultrastructural characterization of degeneration salivary glands in females of the cattle-tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae). Micron 36, 437-447

Nunes, E.T., Camargo-Mathias, M.I., Bechara, G.H., 2006 a. *Rhipicephalus (Boophilus) microplus* (Canestrine, 1887) (Acari: Ixodidae): acid phosphatase and ATPase activities localization in salivary glands of females during the feeding period. Exp. Parasitol. 114, 109-117

Nunes, E.T., Camargo-Mathias, M.I., Bechara, G.H., 2006b. Structural and cytochemical changes in the salivary glands of the *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae) tick female during feeding. Vet. Parasitol. 140, 114-123

Oliveira, P.R., Bechara, G.H., Denardi, S.E., Saito, K.C., Nunes, E.T., Szabó, M.P.J., Camargo-Mathias, M.I., 2005. Comparison of the external morphology of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) ticks from Brazil and Argentina. Vet. Parasitol. 129, 139-147

Rey, L., 1973. Parasitologia. Guanabara Koogan, Rio de Janeiro

Till, W.M., 1961. A contribution to the anatomy and histology of the brown ear tick *Rhipicephalus appendiculatus* Neumann. Memoirs of the Entomological Society of Southern Africa, Pretoria, 6, 1-124

Walker, A., 1994. Arthropods of humans and domestic animals. A guide to preliminary identification. Chapman & Hall, London

Walker, A.R., Fletcher, J.D., Gill, H., 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. Int. J. Parasitol. 15 (1), 81-100

Weiss, L.B., Kaufman, W.R., 2001. The relationship between 'critical weight' and 20-hydroxiecdysone in the female ixodid tick, *Amblyomma Hebraeum*. J. Insect Physiol. 47, 1261-1267

Weiss, L.B., Kaufman, W.R., 2004. Two feeding-induced proteins from the male gonad trigger engorgement of the female tick *Amblyomma hebraeum*. Proc. Natl. Acad. Sci. S.A. 101 (16), 5874-5879

Capítulo 3

CAPÍTULO 3

TITLE: Apoptosis in salivary glands of females of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae)

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RESUMO

As glândulas salivares de fêmeas de carrapatos *Rhipicephalus sanguineus* nos estados de jejum, ingurgitado e com três dias pós-ingurgitamento foram submetidas a testes citoquímicos para análises enzimática e da viabilidade celular. No estudo comparativo do tecido glandular nestas três situações observou-se que a marcação nas células dos vários tipos de ácinos foi diferente, em especial para os ácinos III, II e I nas situações onde estava havendo degeneração, os quais foram nesta seqüência afetados pela morte celular. Este estudo, também revelou: alterações nos núcleos, na intensidade da marcação da fosfatase ácida e da ATPase e na permeabilidade da membrana plasmática, sendo que a positividade para fosfatase foi inversamente proporcional à da ATPase e a positividade da ATPase foi sempre proporcional à integridade da membrana. O tecido glandular das fêmeas em jejum apresentou alta atividade metabólica com células com núcleo e membrana plasmática íntegros. Conclui-se que a presença de fosfatase ácida nestes indivíduos faz parte da fisiologia de alguns ácinos, visto que este tecido não se encontra em degeneração. Nas fêmeas ingurgitadas foi observada membrana íntegra nas células dos ácinos I e II, bem como alterações nucleares, queda na atividade da ATPase e maior marcação para fosfatase ácida, alterações estas que fazem parte do processo degenerativo. Naquelas com três dias pós-ingurgitamento o processo degenerativo já em estágio mais avançado revelou perda da integridade da membrana na maioria das células (de alguns ácinos I, da maioria dos II e de todos os III), intensa alteração nuclear, redução da atividade ATPásica, forte marcação para fosfatase ácida, culminando com a formação de corpos apoptóticos. Na morte celular destas glândulas as alterações nucleares precederam as citoplasmáticas na seguinte seqüência: alteração do núcleo, perda da atividade da ATPase, perda da integridade da membrana plasmática, maior atividade da fosfatase ácida e finalmente formação de corpos apoptóticos. A presença de fosfatase ácida com papel secundário (tardio) na morte celular, degradando os resíduos celulares finais, caracterizaram o processo de morte celular em glândulas de fêmeas de *R. sanguineus* como sendo apoptose atípica ou não clássica.

PALAVRAS-CHAVE: *Rhipicephalus sanguineus;* glândula salivar; carrapatos; fêmea; estágio de alimentação; atividade enzimática; morte celular.

ABSTRACT

The salivary glands of females of the tick *Rhipicephalus sanguineus* at three feeding stages: unfed, engorged, and at day three post-engorgement, were subjected to cytochemical methods of enzymatic analysis and cell viability. Comparing gland tissues at these stages, we observed distinct staining patterns in cells of different types of acini, specially in degenerating types III, II, I acini, which were affected in this sequence by cell death. This study also revealed changes in: nuclei, staining intensity for acid phosphatase and ATPase activities, and permeability of the plasmic membrane. Acid phosphatase activity was inversely proportional to that of ATPase, while ATPase activity was always proportional to membrane integrity. The gland tissue of unfed females exhibited high metabolic activity and cells with intact nucleus and plasmic membrane, suggesting that the presence of acid phosphatase detected in these individuals may participate in the normal physiology of some acini, as they were not undergoing degeneration. In cells of types I and II acini of engorged females, we observed intact membranes, as well as changes associated with the degenerative process, characterized by nuclear changes, decrease in ATPase activity, and stronger acid phosphatase activity. At day three post-engorgement, degeneration progressed to more advanced stages, loss of membrane integrity was observed in most cells (of some type I acini, most type II acini, and all type III acini), as well as prominent nuclear changes, decrease in ATPase activity, and intense acid phosphatase activity, resulting in the formation of apoptotic bodies. During the death of cells in these glands, nuclear changes preceded cytoplasmic ones in the following sequence: nuclear changes, loss of ATPase activity, loss of integrity of the plasmic membrane, increase in acid phosphatase activity, and finally formation of apoptotic bodies. The presence of acid phosphatase with a secondary role (late) during cell death, degrading final cell remnants, characterized this process in the glands of *R. sanguineus* females as atypical or non-classic apoptosis.

KEY WORDS: *Rhipicephalus sanguineus;* salivary gland; ticks; female; feeding stage; enzymatic activity; cell death.

INTRODUCTION

The onset of characteristics associated with salivary gland degeneration of females of the tick *Rhipicephalus sanguineus* leads to the completion of the feeding process of these individuals (Furquim, 2005). This degenerative process is triggered when the gland tissue has already fulfilled its role in allowing the female to feed and reproduce (Oliver, 1986). Feeding then ends and a new activity of the female organism, egg laying, begins (Bowman and Sauer, 2004).

According to some authors, salivary gland degeneration in ticks does not occur by necrotic or pathological death, but rather a programmed and regulated physiological process (programmed cell death) (Bowman and Sauer, 2004). In ticks, gland degeneration is regulated by an ecdysteroid hormone (Lomas et al., 1998), as also observed in different insect organs (Jiang et al., 1997; Pelt-Verkuil, 1979).

Studies have also demonstrated that the binding of this ecdysteroid to its corresponding receptors located on the surface of cells of salivary glands signals the degenerative process in female ticks (Mao et al., 1995; Mao and Kaufman, 1999). This binding then triggers a cascade of chemical reactions in the cytoplasm and nucleus of gland cells, resulting in their death.

In the literature, two main types of genetically programmed cell death have been described: apoptotic and autophagic one (Clarke, 1990; Bowen, 1993; Zakeri and Ahuja, 1997; Jiang et al., 1997). The latter is commonly observed in insect tissues, especially during metamorphosis with the participation of acid hydrolases (acid phosphatase) playing an essential role (Pipan and Rakovec, 1980; Armbruster et al., 1986; Cummings and Bowen, 1992; Lockshin and Zakeri, 1996; Jochová et al., 1997; Gregorc et al., 1998).

L'Amoreaux et al. (2003), Nunes et al. (2005, 2006a, b), however, studying salivary gland degeneration in female ticks of *Dermacentor variabilis* and *R. (Boophilus) microplus*, respectively, suggested that the degeneration of acini may occur by apoptotic cell death. Also, Bior et al. (2002) reported in the gland tissue of unfed and feeding *Amblyomma americanum* males, the presence of a gene expressing a protein that inhibits the apoptotic process, demonstrating the possibility of apoptosis in gland cells.

Zakeri et al. (1995) and Clarke (1990) reported that apoptotic cell death does not always exhibit a classic apoptosis profile, characterized by nuclear and cytoplasmic breakdown, absence of hydrolases and, therefore, of autophagy, resulting in the formation of apoptotic bodies that are later phagocytized by macrophages or other neighboring cells (heterophagy) (Bowen and Bowen, 1990; Kerr et al., 1995; Lockshin and Zakeri, 1996; Zakeri and Ahuja, 1997; Häcker, 2000). According to Clarke (1990) and Zakeri et al. (1995), hydrolases (autophagy) may influence apoptosis in some cells of certain tissues, but much controversy still remains regarding its exact role during the process of apoptotic cell death (Bowen and Bowen, 1990; Bowen, 1993).

According to Bowen and Bowen (1990), apoptosis is an ATP-dependent process. However, at the final stages a decrease in ATP levels would be observed and consequently a decrease or even absence of ATPase activity. This could be used as an indication of cells in late stages of apoptosis (Mullarkey, 1987 apud Bowen and Bowen, 1990; Bowen et al., 1988 apud Bowen and Bowen, 1990).

Another indication of cells in these stages is loss of integrity (of functionality) of the plasmic membrane due to changes in ion pumps (McGahon et al., 1995), which would occur only when energy production systems in the cell fail (Walker and Lucas, 1972 apud Silva de Moraes 1998; Rosenau, 1973 apud Silva de Moraes 1998). Thus, according to McGahon et al. (1995), cells with intact membranes may be differentiated from those with changed membranes by utilizing permeable and non-permeable stains. The latter only penetrates cells in advanced stages of apoptosis or dead cells, in which ion pumps are deficient or inactive.

Based on the presented information, the purpose of this study was to describe the cell changes due the salivary gland degeneration of engorged females and females at day three post-engorgement of *R. sanguineus*, comparing with healthy gland tissues of unfed females, and identifing the type of cell death in these organs.

MATERIAL AND METHODS

In this study, we utilized unfed, engorged females and females at day three post-engorgement of the tick *R. sanguineus*. Unfed individuals were provided by Dr. Gervásio Henrique Bechara of the Department of Veterinary Pathology of UNESP, Jaboticabal campus (São Paulo), from a colony maintained under controlled conditions (29^{o} C, 80% humidity, and 12 hour photoperiod) in BOD incubator. These individuals were placed with some males, in a feeding chamber previously glue with an atoxic and non-lesive preparation (Britannia Adhesives-Unit 4, UK) to the shaved back of the host (rabbit) according to technique described elsewhere (Bechara et al., 1995) for complete feeding.

A group of unfed individuals was assigned to cytochemical methods, while another group was placed with males in a feeding chamber attached to the host (rabbit) to complete feeding. A group of engorged females was assigned to cytochemical procedures, and another group was maintained alive in closed containers with small breathing holes for three days to be later analyzed.

After the completion of the time periods examined in this study, salivary glands were removed in saline solution and processed according to the following methods described below for observation under light and fluorescence microscopy.

For light microscopy, the material was fixed in 10% buffered neutral formalin and acetone (9:1) for one hour and thirty minutes at 4^{o} C, then processed according to the methods described by Hussein et al. (1990) for detection of acid phosphatase and ATPase activities. The material was then dehydrated in increasing concentrations of ethanol (70%, 80%, 90% and 95%), embedded in Leica resin, and sectioned at a thickness of 7 μm. Sections were placed on glass slides, counterstained with Hematoxylin for 2 minutes, and mounted in Canada balsam for later examination under light microscope. For the demonstration of acid phosphatase activity, whole mount preparations of some glands were also examined.

In both enzymatic experiments, control samples were incubated without substrate.

For fluorescence microscopy, after dissection, glands were placed on glass slides and received two drops of Acridine Orange (100 μg/mL) and Ethidium Bromide (100

μg/mL) both in PBS, according to MacGahon et al. (1995), to demonstrate cell viability and detection of apoptotic and/or necrotic cells. Slides were then covered with cover glasses and immediately examined under fluorescence microscope with a 488 nm excitation filter. Healthy cells are homogeneously green (cytoplasm and nucleus) or the nucleus is homogeneously green and the cytoplasm orange/red. Cells in early stages of apoptosis exhibit green or red-orange cytoplasm and green nucleus with clusters of bright green condensed chromatin. Cells in late stages of apoptosis present red cytoplasm and nucleus with bright orange condensed chromatin. Necrotic cells exhibit red cytoplasm and homogeneously orange nucleus.

RESULTS

Unfed females

The salivary glands of unfed females of *R. sanguineus* exhibit healthy cells strongly stained for RNA (Figs. $2A_1$–A_3, A_5) and ATPase (Figs. $1A_1$-A_5), and weakly stained for acid phosphatase (Figs. $3A_1$-A_6). The plasmic membrane and nuclei are intact (Figs. $2A_1$-A_5).

Type I acinus

The basal membrane is strongly stained for ATPase (Fig. $1A_1$) and the plasmic membrane of cells is intact (Fig. $2A_1$). The cytoplasm is strongly positive for RNA (Fig. $2A_1$), moderately positive for ATPase (Fig. $1A_1$) and acid phosphatase (Fig. $3A_4$).

Type II acinus

The basal membrane of the acinus and the plasmic membrane of cells are strongly positive for ATPase (Figs. $1A_2$, A_3), the cytoplasm is strongly positive for RNA (**undifferentiated** cells) (Figs. $2A_2$, A_3), weakly positive for acid phosphatase (Fig. $3A_5$) and, in most cells, strongly positive for ATPase (Fig. $1A_2$).

Type III acinus

The basal membrane of the acinus and the plasmic membrane of cells are strongly positive for ATPase (Figs. $1A_4$, A_5). The cytoplasm is moderately positive for

RNA (Fig. $2A_4$, A_5), weakly positive for acid phosphatase (Fig. $3A_6$), and in most cells, strongly positive for ATPase (Fig. $1A_4$).

Engorged females

Here, degenerative characteristics are already observed (Figs. $1B_4$-C_2, C_4, D_1, D_3, $2B_2$-C_2, $3B_3$-B_5, C_2-D_1). Only type I acini are intact (Figs. $1B_2$, B_3, $2B_1$, $3B_2$, $3C_1$). Degenerative characteristics are most frequently observed in type III (Figs. $1C_4$, D_1, $2B_4$, C_1, $3B_4$, C_3) and **Indeterminate** acini (Figs. $1D_3$, $2C_2$, $3D_1$). In general, acini exhibit weaker staining for ATPase (Figs. $1B_3$-C_2, C_4, D_1, D_3) and stronger staining for acid phosphatase (Figs. $3B_2$-D_1).

Type I acinus

The basal membrane of most acini is strongly positive for ATPase (Fig. $1B_2$) and the plasmic membrane of all cells is intact (Fig. $2B_1$). The cytoplasm is strongly positive for RNA (Fig. $2B_1$), moderately positive for acid phosphatase (Fig. $3C_1$), and in most cells, moderately positive for ATPase (Fig. 1B3). Nuclei are intact (Fig. 2B1).

Type II acinus

The basal membrane of most acini is strongly positive for ATPase (Figs. $1B_4$-C_1) while the plasmic membrane of most cells is negative (Figs. $1B_5$, C_1). All cell membranes are intact (Figs. $2B_2$, B_3). The cytoplasm of cells is moderately positive for acid phosphatase (Fig. $3C_2$), and in most cells, moderately positive for ATPase (Figs. $1B_5$-C_2). Nuclei exhibit changes (Figs. $2B_2$, B_3).

Type III acinus (reduced lumen)

In most type III acini, the lumen is reduced (Figs. $1C_3$-D_1) and they basal membrane is strongly positive (Fig. $1C_3$) or negative for ATPase in some regions, exhibiting an irregular staining pattern (Fig. $1D_1$). The plasmic membrane of all cells is negative for ATPase (Figs. $1C_3$-D_1) and has lost its integrity (Figs. $2B_4$, C_1). The cytoplasm of cells is strongly positive for acid phosphatase (Fig. $3C_3$), and in most cells, strongly positive for ATPase (Fig. $1C_3$). Nuclei exhibit changes (Figs. $2B_4$, C_1).

Type III acinus (dilated lumen)

The basal membrane of acini is strongly positive for ATPase while the plasmic membrane of all cells is negative for ATPase (Fig. $1D_2$) and non-functional (Figs. $2B_4$, C_1). The cytoplasm of cells is strongly positive for ATPase (Fig. $1D_2$) and acid phosphatase (Fig. $3C_4$). Nuclei exhibit changes (Figs. $2B_4$, C_1).

Indeterminate acinus

The basal membrane of acini is moderately positive for ATPase (Fig. $1D_3$). The plasmic membrane of all cells is negative for ATPase (Fig. $1D_3$) and has already lost its integrity (Fig. $2C_2$). The cytoplasm is strongly positive for acid phosphatase (Fig. $3D_1$), and in most cells, negative for ATPase (Fig. $1D_3$). Nuclei exhibit changes (Fig. $2C_2$).

Females at day three post-engorgement

In these ticks, degeneration has progressed into more advanced stages. Types II (Figs. $1E_6$, F_1, $2D_3$, $3D_4$, E_3, E_4), III (Figs. $1F_2$, F_3, $2E_1$, $3E_1$, E_5, F_1), and **Indeterminate** (Figs. $1G_1$-G_3, $2E_2$, $3F_2$, F_3) acini exhibit more prominent changes. Apoptotic bodies (Figs. $1G_3$, $2E_3$, $3F_4$) are present. Stronger staining for acid phosphatase (Figs. $3D_3$-F_3) and weaker staining for ATPase (Figs. $1E_1$-G_3) are observed. Apoptotic bodies exhibit only strong staining for acid phosphatase (Figs. $1G_3$, $3F_4$) and the plasmic membrane is not intact (Fig. $2E_3$).

Type I acinus

The basal membrane of most acini is moderately positive for ATPase (Fig. $1E_1$) and the plasmic membrane of most cells is intact (Fig. $1C_3$). The cytoplasm of cells is strongly positive for RNA (Fig. $1C_3$), weakly positive (Fig. $1E_1$) or negative (Figs. $1E_2$, E_3) for ATPase, and strongly positive for acid phosphatase (Fig. $3E_2$). Nuclei exhibit changes (Fig. 1D1).

Type II acinus

The basal membrane of most acini is moderately positive for ATPase (Figs. $1E_4$, E_5), and in most cells, the plasmic membrane is negative for ATPase (Figs. $1E_5$-F_1) and has lost its integrity (Fig. $2D_3$). The cytoplasm of cells is strongly (Fig. $3E_4$) or extremely positive (Fig. $3E_3$) for acid phosphatase, and in most cells, is negative for ATPase (Figs. $1E_4$-F_1). Nuclei exhibit changes (Figs. $2D_2$, D_3).

Type III acinus

The basal membrane is weakly positive (Fig. $1F_2$) or negative (Fig. $1F_3$) for ATPase. The plasmic membrane is negative for ATPase (Figs. $1F_2$, F_3) and has lost its integrity (Fig. $2E_1$). In the cytoplasm of most cells, ATPase activity is absent (Figs. $1F_2$, F_3) while acid phosphatase activity is intense (Fig. $3E_5$, F_1). Nuclei exhibit changes (Fig. $2E_1$).

Indeterminate acinus

The basal membrane of most acini is negative for ATPase (Figs. $1G_2$, G_3). The plasmic membrane of all cells is negative for ATPase (Figs. $1F_4$-G_3) and has lost its integrity (Fig. $2E_2$). The cytoplasm of most cells is negative for ATPase (Figs. $1F_4$-G_3) and strongly positive for acid phosphatase (Fig. $3F_3$). Nuclei exhibit changes (Fig. $2E_2$).

To better compare the results, the data are summarized in **Table 1**.

Table 1: Enzymatic activity and viability of cells of salivary glands of unfed, engorged, and at day three post-engorgement females of *Rhipicephalus sanguineus*.

		Type I Acini	Type II Acini	Type III Acini	Indeterminate Acini
UNFED	**Basal Membrane of Acinus**	- ATPase +++ (Fig. $1A_1$);	- ATPase +++ (Figs. $1A_2$, A_3);	- ATPase +++ (Figs. $1A_4$, A_5);	
	Plasmic Membrane	- intact (Fig. $2A_1$);	- ATPase +++ (Figs. $1A_2$, A_3); - intact (Figs. $2A_2$, A_3);	- ATPase +++ (Figs. $1A_4$, A_5); - intact (Figs. $2A_4$, A_5);	
	Cytoplasm	- ATPase ++ (Fig. $1A_1$); - fa ++ (Fig. $3A_4$);	- ATPase +++ (Fig. $1A_2$) and – (Figs. $1A_2$, A_3); - fa + (Fig. $3A_5$);	- ATPase +++ (Fig. $1A_4$) and – (Figs. $1A_4$, A_5); - fa + (Fig. $3A_6$);	
	Nucleus	- intact (Fig. $2A_1$);	- intact (Figs. $2A_2$, A_3);	intact (Figs. $2A_4$, A_5);	
ENGORGED	**Basal Membrane of Acinus**	- ATPase +++ (Fig. $1B_2$) and ++ (Fig. $1B_3$);	- ATPase +++ (Figs. $1B_4$-C_1) and ++ (Fig. $1C_2$);	**reduced lumen:** - ATPase +++ (Fig. $1C_3$), ++ (Fig. $1C_4$) and +/– (Fig. $1D_1$); **dilated lumen:** - ATPase +++ (Fig. $1D_2$);	- ATPase ++ (Fig. $1D_3$);
	Plasmic Membrane	- intact (Fig. $2B_1$);	- ATPase ++ (Figs. $1B_4$, C_2) and – (Figs. $1B_5$, C_1); - intact (Figs. $2B_2$, B_3);	- ATPase – (Figs. $1C_3$-D_2); - not intact (Figs. $2B_4$, C_1);	- ATPase – (Fig. $1D_3$); - not intact (Fig. $2C_2$);
	Cytoplasm	- ATPase ++ (Fig. $1B_3$) and – (Fig. $1B_2$); - fa ++ (Fig. $3C_1$);	- ATPase + + (Fig. $1B_5$-C_2) and – (Fig. $1B_4$, C_2); - fa ++ (Fig. $3C_2$);	**reduced lumen:** - ATPase +++ (Fig. $1C_3$), ++ (Fig. $1C_4$) and + (Fig. $1D_1$); - fa +++ (Fig. $3C_3$); **dilated lumen:** - ATPase +++ (Fig. $1D_2$); - fa +++ (Fig. $3C_4$);	- ATPase + (Fig. $1D_3$) and – (Fig. $1D_3$); - fa +++ (Fig. $3D_1$);
	Nucleus	- intact (Fig. $2B_1$);	- # (Figs. $2B_2$, B_3);	- ? (Figs. $2B_4$, C_1) and ! (Fig. $2B_4$);	- ? (Fig. $2C_2$);
3 DAYS AFTER	**Basal Membrane of Acinus**	- ATPase ++ (Fig. $1E_1$), + (Fig. $1E_2$) and +/– (Fig. $1E_3$);	- ATPase ++ (Figs. $1E_4$, E_5), +/– (Fig. $1E_6$) and – (Fig. $1F_1$);	- ATPase + (Fig. $1F_2$) and – (Fig. $1F_3$);	- ATPase ++ (Fig. $1F_4$), +/– (Fig. $1G_1$) and – (Figs. $1G_2$, G_3);
	Plasmic Membrane	- > intact (Fig. $2C_3$); - < not intact (Fig. $2D_1$);	- ATPase ++ (Fig. $1E_4$) and – (Figs. $1E_5$-F_1); - > not intact (Fig. $2D_3$); - < intact (Fig. $2D_2$);	- ATPase – (Figs. $1F_2$, F_3); - not intact (Fig. $2E_1$);	- ATPase – (Figs. $1F_4$-G_3) - not intact (Fig. $2E_2$);
	Cytoplasm	- ATPase + (Fig. $1E_1$) and – (Figs. $1E_2$, E_3); - fa +++ (Fig. $3E_2$);	- ATPase ++ (Fig. $1E_4$), + (Figs. $1E_6$, F_1) and – (Fig. $1E_5$ - fa ++++ (Fig. $3E_3$) and +++ (Fig. $3E_4$);	- ATPase + (Figs. $1F_2$, F_3) and – (Figs. $1F_2$, F_3); - fa ++++ (Figs. $3E_5$, F_1) and +++ (Fig. $3E_5$);	- ATPase ++ (Fig. $1G_1$), + (Fig. $1F_4$) and – (Figs. $1G_2$, G_3); - fa ++++ (Fig. $3F_2$) and +++ (Fig. $3F_3$);
	Nucleus	- # (Fig. $2D_1$);	- # (Fig. $2D_2$), ? (Fig. $2D_3$), ! (Fig. $2D_3$) and ** (Fig. $2D_2$);	- #, ? and ! (Fig. $2E_1$);	- ? and * (Fig. $2E_2$);

(++++) extremely positive; (+++) strongly positive; (++) moderately positive; (+) weakly positive; (–) negative; (**af**) acid phosphatase; (**>**) most cells; (**<**): some cells; (**+/-**) irregular staining; (#) nucleus with clusters of condensed chromatin; (**?**) picnotic nucleus; (**!**) blebs; (**) fragmenting nucleus; (*) fragmented nucleus.

FIGURES

FIGURE 1:

ATPase activity in the female salivary glands of *Rhipicephalus sanguineus*. **A_1-B_1.** Unfeds: observe that types I (**I**), II (**II**), III (**III**), and **Indeterminate** (**Ind**) acini are strongly stained for ATPase (**arrow**). **B_1.** Negative control. **B_2-D_4.** Engorged: observe weaker staining for ATPase (**arrow**) in types I (**I**), II (**II**) and III (**III**) acini. Note in: **B_5** and **C_2**, weaker staining for ATPase (**arrow**) in the basal membrane of type II acini (**II**); **B_4-C_2**, weaker staining for ATPase (**arrow**) in the plasmic membrane and cytoplasm of cells of type II acini (**II**); **B_5** and **C_1**, plasmic membrane of some cells of type II acini (**II**) with no ATPase activity. Observe in: **C_4**, lower ATPase activity (**arrow**) in the basal membrane and in the cytoplasm of cells of type III acini (**III**) and **D_1**, irregular staining for ATPase (**arrow**) in the basal membrane of type III acinus (**III**). **D_4.** Negative control. **E_1-G_4.** Day three post-engorgement: note even weaker staining for ATPase activity in types I (**I**), II (**II**), III (**III**), and **Indeterminate** (**Ind**) acini. Observe in **E_3**, type I acinus (**I**) with irregular staining for ATPase along the basal membrane and no staining in the cytoplasm of cells. Note in: **E_6**, irregular staining for ATPase in the basal membrane of type II acinus (**II**); **F_1**, absence of ATPase activity in the basal membrane of type II acinus (**II**) and **E_5-F_1**, absence of staining for ATPase in the plasmic membrane of cells of type II acini (**II**). Note in: **F_3**, absence of ATPase activity in the basal membrane of type III acinus (**III**) and **F_2** and **F_3**, no staining for ATPase in the plasmic membrane of cells of type III acinus (**III**). In **G_1**, irregular ATPase activity in the basal membrane of **Indeterminate** acinus (**Ind**); **G_2** and **G_3**, no staining for ATPse in the basal membrane of **Indeterminate** acinus (**Ind**); **G_1** and **G_3**, absence of ATPase activity in the membrane of cells of **Indeterminate** acinus (**Ind**). Note in **F_4-G_3**, no ATPase staining in the membrane of apoptotic body (**ab**). **G_4.** Negative control.

I-III: types acini; **Ind: Indeterminate** acinus; **arrow:** staining for ATPase; **n:** staining for ATPase in the nucleus; **dt:** duct.

Bars: E_1 and **E_2**= 12, 5 μm; **A_5-B_3**, **C_3-E_6** and **F_4**= 25 μm; **A_1-A_4**, **B_4-D_4**, **F_1-F_3** and **G_1-G_4**= 50 μm.

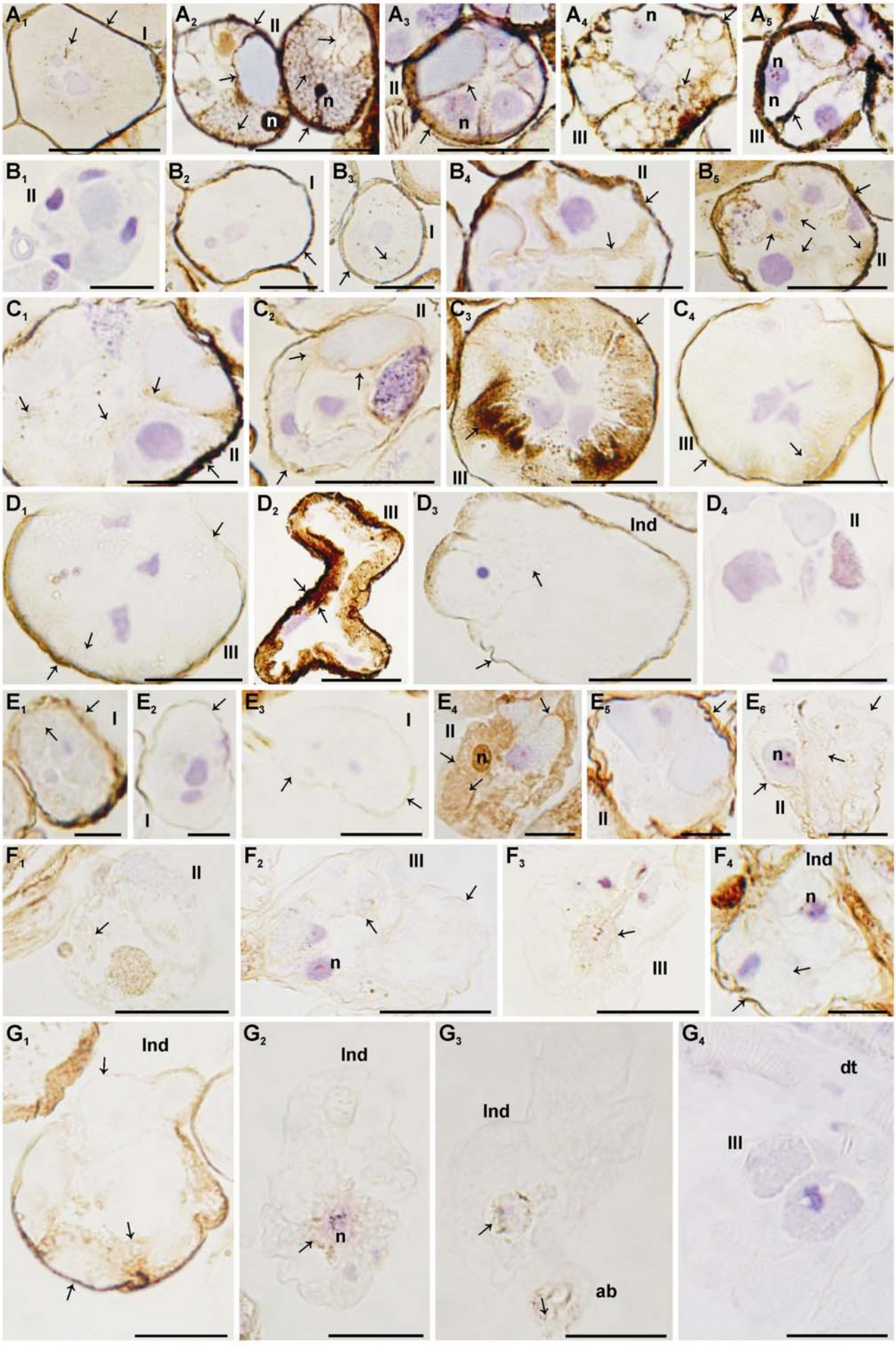
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FIGURE 2:

Total preparation of female salivary glands of *Rhipicephalus sanguineus* stained with Ethidium Bromide and Acridine Orange. **A_1-A_5.** Unfeds: Observe healthy cells of types I (**I**), II (**II**), and III (**III**) acini with intact plasmic membrane and nuclei (**tn**). **B_1-C_2.** Engorged: in **B_1**, intact type I acinus (**I**). Note in: **B_2** and **B_3**, type II acini (**II**) undergoing degeneration with cells exhibiting nuclear changes and membrane still intact; **B_4** and **C_1**, type III acini (**III**) undergoing degeneration with nuclear changes and loss of plasmic membrane integrity, and **C_2**, **Indeterminate** acinus (**Ind**) with cells exhibiting nuclear changes and loss of plasmic membrane integrity. **C_3-E_3.** Day three post-engorgement: in **C_3**, type I acinus (**I**) undergoing degeneration and exhibiting plasmic membrane still intact. Observe in: **D_1**, type I acinus (**I**) undergoing degeneration with cells exhibiting nuclear changes and loss of membrane integrity; **D_3**, cells of type II acinus (**II**) exhibiting nuclear changes and loss of plasmic membrane integrity; **E_1**, Type III acinus (**III**) undergoing degeneration with cells exhibiting nuclear and membrane changes; **E_2**, **Indeterminate** acinus (**Ind**) exhibiting degenerating cells with nuclei and membrane changed, and **E_3**, apoptotic bodies (**ab**) with loss membranes integrity.

I-III: types acini; **Ind: Indeterminate** acinus; **arrow:** cytoplasmic RNA; **tn:** intact nucleus; **dashed arrow:** nucleus with clusters of condensed chromatin; **pn:** picnotic nucleus; **bb:** blebs; **∗:** fragmenting nucleus; **fn:** fragmented nucleus.

Bars: A1-A4 and **B1**= 12, 5 μm; **A5** and **B2-E3**= 25 μm.

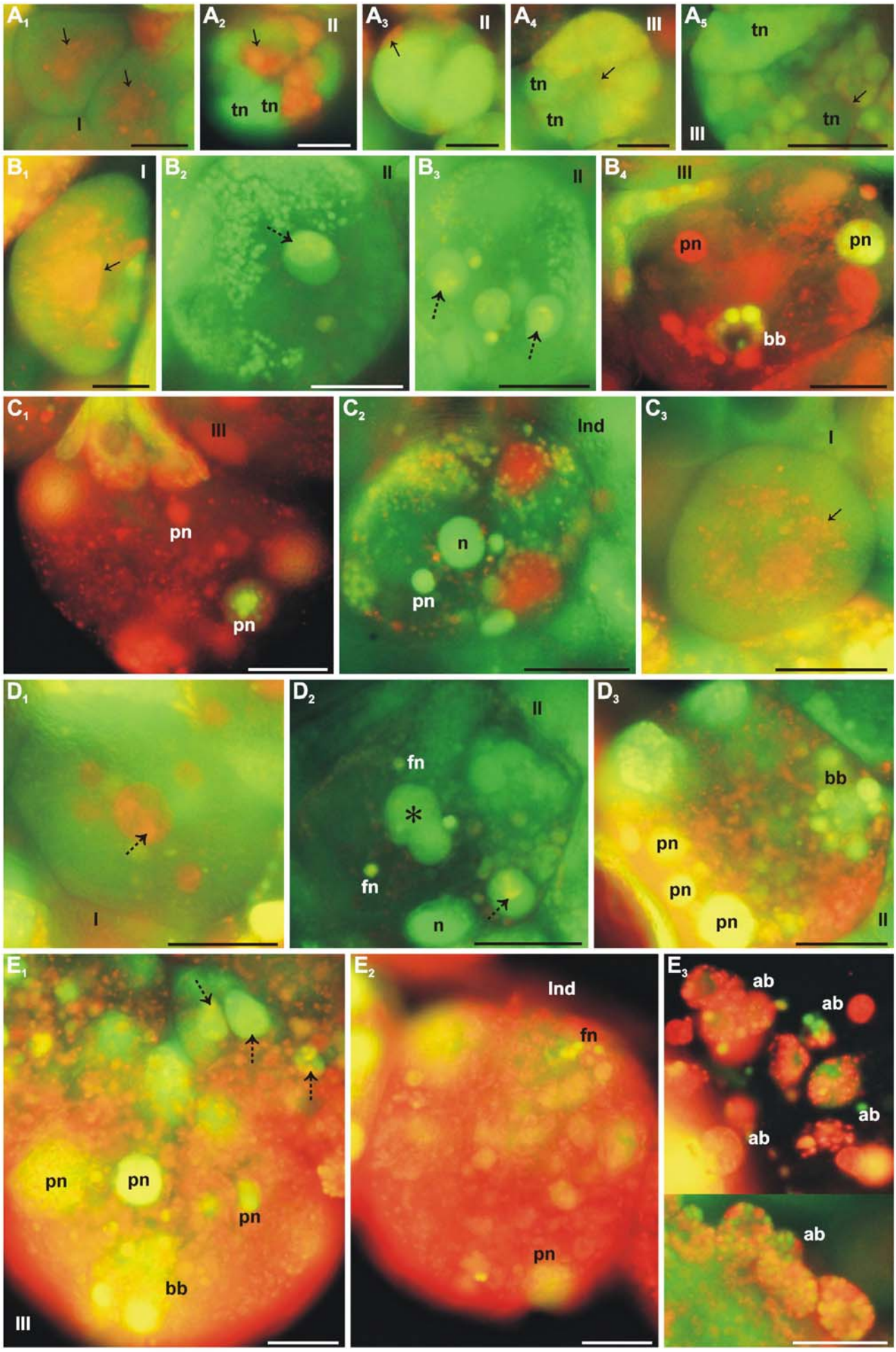
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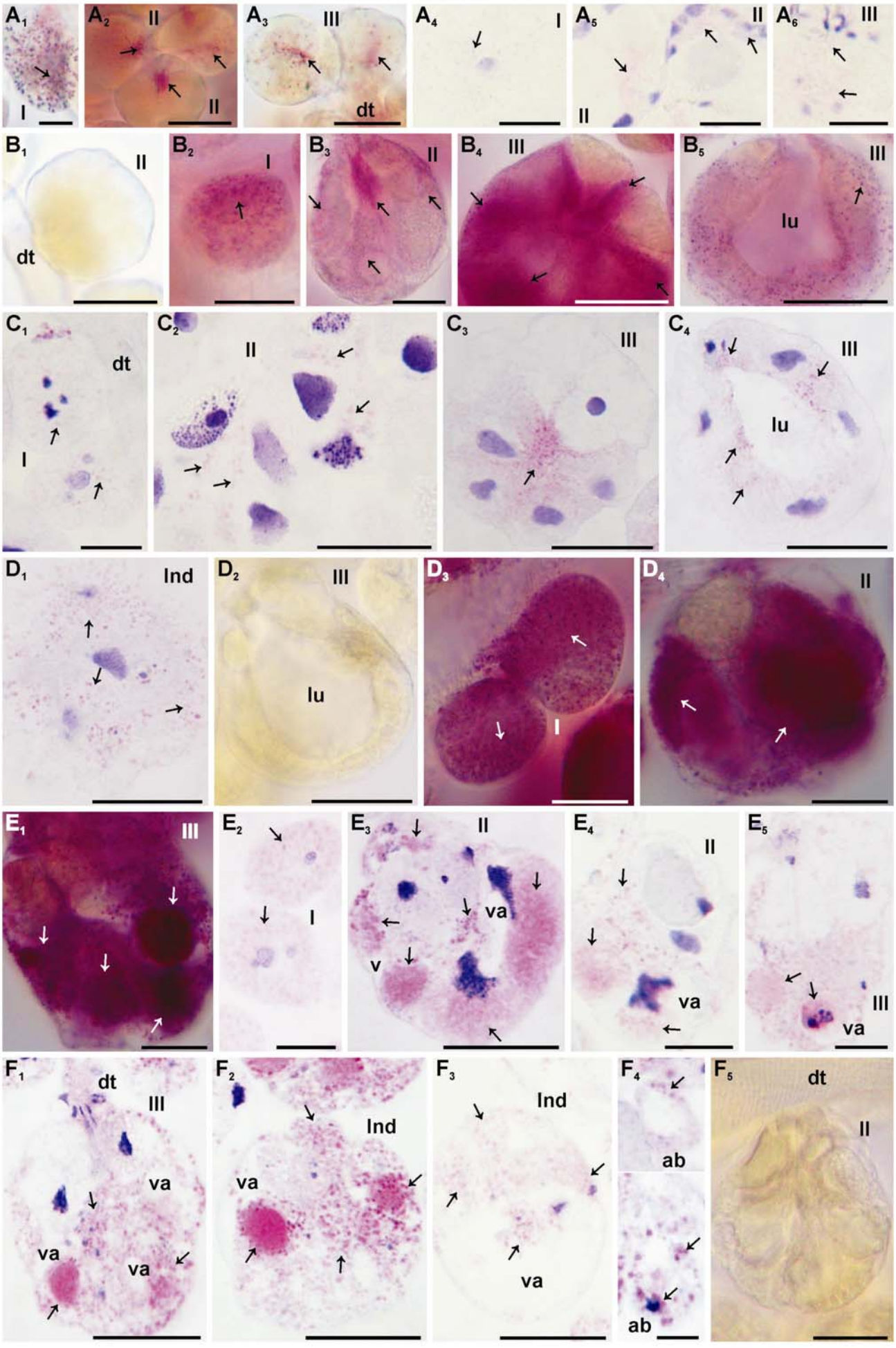
FIGURE 3

Acid phosphatase activity in the females salivary glands of *Rhipicephalus sanguineus*. **A_1-B_1.** Unfeds: Observe in: **A_1-A_3**, total preparation of types I (**I**), II (**II**), and III (**III**) acini showing low acid phosphatase activity (**arrow**) and **A_4-A_6**, histological sections of types I (**I**), II (**II**), and III (**III**) acini exhibiting weak staining for acid phosphatase (**arrow**) in the cytoplasm of cells. **B_1.** Negative control. **B_2-D_2.** Engorged: in **B_2-B_5**, total preparation of types I (**I**), II (**II**), and III (**III**) acini showing stronger staining for acid phosphatase (**arrow**). Note in **B_4** and **B_5**, prominent staining for acid phosphatase (**arrow**) in the cytoplasm of cells of type III acinus (**III**). **C_1-D_1.** Histological sections of types I (**I**), II (**II**), III (**III**), and **Indeterminate** (**Ind**) acini. Observe in **C_3**, **C_4** and **D_1**, cytoplasm of cells of types III (**III**) and **Indeterminate** (**Ind**) acini showing stronger staining for acid phosphatase (**arrow**). **D_2.** Negative control. **D_3-F_5.** Day three post-engorgement: in **D_3-E_1**, total preparation of types I (**I**), II (**II**), and III (**III**) acini, note stronger staining for acid phosphatase (**arrow**) in the cytoplasm of cells of the three types of acini (**I**, **II,** and **III**). Observe in **D_4** and **E_1**, the most prominent staining for acid phosphatase (**arrow**). In **E_2-F_4.** Histological sections of types I (**I**), II (**II**), III (**III**), and **Indeterminate** (**Ind**) acini, as well as apoptotic bodies (**ab**). Note in: **E_3**, **E_5-F_2**, extremely strong staining for acid phosphatase activity (**arrow**) in cells of types II (**II**), III (**III**), and **Indeterminate** (**Ind**) acini, and **E_3-F_3**, presence of vacuoles (**va**) in the cytoplasm of cells of types II (**II**), III (**III**), and **Indeterminate** (**Ind**) acini. **F_5.** Negative control.

arrow: staining for acid phosphatase; **dt:** duct; **lu:** lumen.

Bars: **A_1**= 10 μm; **F_4**= 12, 5 μm; **A_2**, **A_3**, **B_1-B_3**, **D_3-E_1** and **F_5**= 20 μm; **A_4-A_6**, **C_1**, **E_2**, **E_4** and **E_5**= 25 μm; **B_4**, **B_5** and **D_2**= 40 μm; **C_2-D_1**, **E_3** and **F_1-F_3**= 50 μm.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DISCUSSION

The present study revealed structural and enzymatic aspects of the salivary glands of unfed, and engorged, and day three post-engorgement females of *Rhipicephalus sanguineus*. In the latter two stages, changes undergone by cells characterized the degeneration of this organ.

In unfed females of *R. sanguineus*, the presence of ATPase in different cell compartments confirmed the need of a large consumption of energy for metabolic processes in these areas, in addition to contributing to the maintenance of the intracellular environment, since the ion pump of the membrane is functional. The ATPase staining observed in the nucleus might be associated with transcription activity, supporting the results obtained by Azeredo-Oliveira and Mello (1986), which reported the presence of ATPase in the euchromatin and nucleolus of cells of Malphigian tubules of *Triatoma infestans*.

In this study, acid phosphatase staining was also observed in unfed females, especially in cells of types I and II acini. In these cells, this enzyme might be involved in the normal cell metabolism. According to the literature, the activity of this enzyme is not always associated with cell destruction. Costa and Cruz-Landim (2001) and Britto and Caetano (2006) suggested that it may participate in the metabolism of different stages of the secretory cycle of some glands.

Binnington (1978), Walker et al. (1985), and Nunes et al. (2006) also reported acid phosphatase in active gland cells of feeding females of *Boophilus microplus*, *R. appendiculatus*, and *R. (Boophilus) microplus*, respectively. The latter authors demonstrated the participation of these enzymes in the cell metabolism during the production and discharge of secretion (Nunes et al., 2006). In unfed females examined in this study, the presence of acid phosphatase in type I acini might be associated with protein synthesis, since large quantities of RNA were observed, as reported by Costa and Cruz-Landim (2001) in hypopharyngeal glands of *Scaptotrigona postica*. In type II and III acini, where cells are in pre-secretory phase, the participation of acid phosphatase might be associated with the production of secretion, as observed in other studies (Costa and Cruz-Landim, 2001).

The salivary gland cells of *R. sanguineus* females, engorged and at day three post-engorgement, underwent structural (nucleus and plasmic membrane) and

enzymatic changes (ATPase and acid phosphatase) that resulted in their death, confirming an asynchrony in the process of cellular and acinar degeneration. Types II and III acini seem the first to be affected and the degenerative process intensifies in females at day three post-engorgement.

In engorged females, we observed a decrease in ATPase and increase of acid phosphatase activities in type I acinus, the only intact, and types II, III, and **Indeterminate** acini. In types III and **Indeterminate** acini, the plasmic membrane lost its integrity and did not exhibit ATPase activity, confirming the observed by Nunes et al. (2006). These authors detected a decrease in enzymatic activity (ATPase and acid phosphatase) in semi-engorged females of *R. (Boophilus) microplus*, as well as a heterogeneous staining pattern for ATPase throughout the gland tissue.

The intense ATPase and acid phosphatase activities observed in the cytoplasm of cells of type III acinus (with dilated lumen) of glands of engorged females of *R. sanguineus* are probably not associated with cell death, but rather with a re-structuring of cells and shape of acini, returning from squamous to cubic (Till, 1961). This process requires a lot of energy, as indicated by the intense staining for ATPase. These data support those obtained by Walker et al. (1985) that correlated the presence of acid phosphatase with changes in shape of cells during the phase of large blood consumption of the tick.

At day three post-engorgement, gland cells of *R. sanguineus* females exhibited more prominent enzymatic changes, as the degeneration process progressed.

In engorged females as well as those at day three post-engorgement, we observed ATPase staining in the basal membrane of different acini, as reported by Nunes et al. (2006). In some acini, ATPase activity was irregular, characterized by stained and non-stained areas. In the latter, loss of membrane integrity was observed.

Type II acinus of engorged females and females at day three post-engorgement exhibited a characteristic not observed in other types of acini. The cells of some of type II acini presented an intact plasmic membrane, but absence of ATPase activity. One possible explanation might be the presence of a functional plasmic membrane only in the basal portion that could not be distinguished from the basal membrane of the acinus. Thus, only an ATPase positive halo was observed around it.

Our findings regarding acid phosphatase activity were inversely proportional to those of ATPase in engorged females and those at day three post-engorgement. This was also observed by Weber (1969) that reported a simultaneous increase in acid and basic phosphatase activities and a decrease in ATPase activity during degeneration.

The decrease in ATPase activity and increase in acid phosphatase activity observed in our study during gland degeneration were accompanied by the following morphological changes reported by Furquim (2005): a) decrease in the amount of secretion in cells, b) nuclear breakdown, c) cytoplasmic shrinkage, d) loss of shape and cell boundaries (cell individuality), e) vacuolation, and f) cell fragmentation. The decrease in the amount of secretion and nuclear breakdown precede all other events. These changes, in addition to bodies containing cytoplasmic and nuclear remnants, resulted from cytoplasm shrinkage and cell fragmentation and enclosed by membranes, characterize an apoptotic cell death (Clarke, 1990; Bowen, 1993; Kerr et al., 1995; Lockshin and Zakeri, 1996; Häcker, 2000). In this study, the decrease and/or absence of ATPase activity in the plasmic membrane, as well as the loss of integrity observed in engorged females and females at day three post-engorgement contradict the observed by Bowen and Bowen (1990). These authors suggested that apoptosis is an ATP-dependent process and its decrease and that of ATPase occur only in advanced stages of the process, with a functional plasmic membrane present until cell fragmentation (Bowen and Bowen, 1990; Bowen, 1993; Kerr et al., 1995; Lockshin and Zakeri, 1996).

In *R. sanguineus* salivary glands, the absence of ATPase activity and loss of integrity of the plasmic membrane occurred mainly in females at day three post-engorgement. At this stage, unlike the described for apoptotic death, we observed intact and fragmenting acini, in addition to ATPase-negative apoptotic bodies lacking intact membranes. Although these changes occurred after nuclear changes, cytoplasmic shrinkage, and loss of cell shape, they were observed before than the normally reported for apoptosis, which is before the formation of apoptotic bodies, since the latter still exhibit a functional membrane soon after they are formed (Bowen and Bowen; 1990; Kerr et al., 1995).

In *R. sanguineus*, acid phosphatase activity was more intense in engorged females than in unfed ones, and was intensified in those at day three post-engorgement. According to the literature, a process of cell death associated with acid phosphatase

(acid hydrolases) is characterized as "autophagic death" (Zakeri et al., 1995; Lockshin and Zakeri, 1996; Cummings and Bowen, 1992; Gregorc et al., 1998; Clarke, 1990; Bowen, 1993; Pipan and Ravoc, 1980; Jochová et al., 1997). Several authors, however, disagree on the real role of acid hydrolases in apoptotic death (Bowen and Bowen, 1990; Clarke, 1990; Bowen, 1993).

In degenerating salivary glands of *R. sanguineus* females, acid phosphatase occurred after other changes. This enzyme was detected mainly in acini in late stages of degeneration, in which several cytoplasmic vacuoles were also observed. Furquim (2005) also reported the presence of these structures. Cytoplasmic vacuolation, in addition to a high acid phosphatase activity in females at day three post-engorgement, would strongly suggest an autophagic death, as commonly observed during insect metamorphosis (Zakeri et al., 1995; Lockshin and Zakeri, 1996). According to Cummings and Bowen (1992) and Gregorc et al. (1998), the presence of this enzyme might be considered a prelude to autophagic cell death, with early destruction of the cytoplasm (extensive vacuolation) and later nuclear breakdown (Clarke, 1990; Bowen, 1993; Lockshin and Zakeri, 1996; Zakeri et al., 1995; Pipan and Ravoc, 1980; Jochová et al., 1997). However, it is important to point out that in the present study the nuclear breakdown was observed before an intense acid phosphatase activity, and therefore, before cytoplasmic vacuolation. This suggests that acid phosphatase might have a role in later stages, degrading part of the cytoplasm, and therefore not characterizing the cell death in the glands of *R. sanguineus* females as autophagic. One possible hypothesis might be an apoptotic death combined with autophagy, corroborating Levy and Bautz (1985), Clarke (1990), Zakeri et al. (1995), Bowen et al. (1996), Dai and Gilbert (1997), Lockshin and Zakeri (1996), Gregorc and Bowen (1997), Jochová et al. (1997) and Yamamoto et al. (2000). These authors have suggested that the process of cell death may exhibit characteristics of more than one type.

The early decrease of ATPase activity observed in this study as well as loss of membrane integrity, and intense acid phosphatase activity did not characterize a classic apoptotic death. Bowen et al. (1993) reported that during cell death of salivary gland cells of *Calliphora vomitoria*, ATPase activity ceased at some point, and massive cytoplasmic vacuolation was observed, indicating that in programmed cell death,

ATPase and preservation of membrane integrity until late stages of the process are not always observed, as suggested by Bowen and Bowen (1990) and Kerr et al. (1995).

Regarding whether or not autophagy is involved in apoptotic death in invertebrates, some studies have demonstrated that programmed death exhibit characteristics distinct from those of classic apoptosis in insects, as vacuolation in cells occurs due to autophagy (Zakeri et al., 1995; Dai and Gilbert 1997; Bowen et al., 1996; Gregorc and Bowen, 1997; Levy and Bautz, 1985). In addition, cells increase in size instead of shrinking (Bowen et al., 1996). Studying cell death in prothoracic glands of *Manduca sexta* during metamorphosis, Dai and Gilbert (1997) reported changes similar to those observed in the present study and classified it as apoptotic death. According to Hurle and Hinchliffe (1978) apud Bowen and Bowen (1990) and Levy and Bautz (1985), the participation of acid hydrolases in the apoptotic process might be associated with cell fragmentation. This could also be the case in our study, as strong acid phosphatase activity was observed in fragmenting acini, as well as in apoptotic bodies. Zakeri et al. (1995) also reported that apoptosis and autophagic death might overlap in some aspects, including regarding acid hydrolase activity.

Thus, the cell death process leading to the degeneration of the salivary gland tissue of *R. sanguineus* females exhibits features of classic apoptosis combined with autophagy, characterizing an atypical apoptosis, with acid phosphatase participating in the removal of cytoplasmic remnants and cell fragmentation. In *R. sanguineus* females, this enzyme may have an endogenous origin through *de novo* synthesis commonly observed in autophagic death, although it may and also occur during apoptotic death (Zakeri et al., 1995), or an exogenous origin, from the hemolymph. Levy and Bautz (1985), Armbruster et al. (1986), Jones and Bowen (1993) reported the presence of hemocytes containing large amounts of acid phosphatase adhered to degenerating tissues. Nunes et al. (2006) suggested that acid phosphatase detected in the gland tissue of *R. (Boophilus) microplus* females may come from the hemolymph. Therefore, these findings suggest that a) the gradual increase in acid phosphatase in cells of salivary glands of females, from unfed to three day post-engorgement, indicates that the synthesis of this enzyme begins in engorged females, and b) the absence of hemocytes around or in the gland tissue suggests that the origin of acid phosphatase in *R. sanguineus* is exclusively endogenous.

The results presented in this study, in addition to those reported by Furquim (2005) in females of this species, clearly indicate that the salivary gland tissue of *R. sanguineus* females dies by atypical apoptosis, characterized by events that occur in the following order: a) early nuclear breakdown, chromatin condensation and margination, bleb formation in the nuclear envelope, and finally nuclear fragmentation, b) cytoplasmic shrinkage and loss of cell shape, c) decrease and/or absence of ATPase activity, loss of membrane integrity, and loss of cell boundaries, d) intense acid phosphatase activity, resulting in cytoplasmic vacuolation and cell fragmentation, and e) fragmentation of acini with release of apoptotic bodies. The latter are not removed by heterophagy from the hemolymph, unlike the observed by Till (1961), Kerr et al. (1995), Bowen et al. (1993), Bowen and Bowen (1990), and Häcker (2000), but rather through the participation of the acid phosphatase synthesized by salivary gland cells, characterizing an apoptotic process involving autophagy.

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REFERENCES

Armbruster, L., Levy, M., Mathieu, N., Bautz, A.M., 1986. Acid phosphatase activity in the hemolynph, hemocytes, fat body and salivary glands during larval and prepupal development in *Calliphora erythrocephala* (Diptera: Calliphoridae). Comparative Biochemistry and Physiology 84B (3), 349-354.

Azeredo-Oliveira, M.T.V., Mello, M.L.S., 1986. Mg^{2+}-dependent ATPase activity investigated in heterochromatin, euchromatin and nucleoli of *Triatoma infestans*. Revista Brasileira de Genética IX (2), 223-229.

Bechara, G.H., Szabó, M.P.J., Ferreira, B.R. Garcia, M.V., 1995. *Rhipicephalulus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions, Brazilian Journal of Veterinary Parasitology 4, 61-66.

Binnington, K.C., 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, *Boophilus microplus*. International Journal for Parasitology 8, 97-115.

Bior, A.D., Essemberg, R.C., Sauer, J.R., 2002. Comparison of differentially expressed genes in the salivary glands of male ticks, *Amblyomma americanum* and *Dermacentor andersoni*. Insect Biochemistry and Molecular Biology 32, 645-655.

Bowen, I.D., 1993. Apoptosis or programmed cell death? Cell Biology International 17 (4), 365-380.

Bowen, I.D., Bowen, S.M., 1990. Programmed Cell Death in Tumours and Tissues. Chapman and Hall, London, pp. 15-66.

Bowen, I.D., Morgan, S.M., Mullarkey, K., 1993. Cell death in the salivary glands of metamorphosing *Calliphora vomitoria*. Cell Biology International 17 (1), 13-34.

Bowen, I.D., Mullarkey, K., Morgan, S.M., 1996. Programmed cell death during metamorphosis in the blow-fly *Calliphora vomitoria*. Microscopy Research and Technique 34, 202-217.

Bowen, I.D., Worrill, N.A., Winters, C.A., Mullarkey, K., 1988. The use of backscattered electron imaging, X-ray microscopy in demonstrating physiological cell death. Scanning Microscopy 2, 1453-1462.

Bowman, A.S., Sauer, J.R., 2004. Tick salivary glands: function, physiology and future. Parasitology 129, S67-S81.

Britto, F.B., Caetano, F.H., 2006. Morphological features and occurrence of degenerative characteristics in the hypopharyngeal glands of the paper wasp *Polistes versicolor* (Olivier) (Hymenoptera: Vespedae). Micron 37, 742-747.

Clarke, P.G.H., 1990. Developmental cell death: morphological diversity and multiple mechanisms. Anatomy and Embryology 181, 195-213.

Costa, R.A.C., Cruz-Landim, C., 2002. Distribution of acid phosphatases in the hypopharyngeal glands from workers, queens and males of a Brazilian stingless bee *Scaptotrigona postica* Latreille: an ultrastructural cytochemical study. Histochemical Journal 33, 653-662.

Cummings, L.A., Bowen, I.D., 1992. The localization of acid phosphatase as a prelude to cell death in the blow-fly *Calliphora erithrocephala*. Micron and Microscopica Acta 23 (3), 357-358.

Dai, J., Gilbert, L.I., 1997. Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. Insect Biochemistry and Molecular Biology 27 (1), 69-78.

Furquim, K.C.S., 2005. Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados, 2º Relatório Científico CAPES, Instituto de Biociências-UNESP, Rio Claro (S.P.), Brasil, 40 p.

Gregorc, A., Bowen, I.D., 1997. Programmed cell death in the honey-bee (*Apis mellifera* L.) larvae midgut. Cell Biology International 21 (3), 151-158.

Gregorc, A., Bowen, I.D., Pogacnik, A., 1998. Acid phosphatase activity in the midgut of honeybee (*Apis melliphera* L.) larvae. Apidologie 29, 579-584.

Häcker, G., 2000. The morphology of apoptosis. Cell and Tissue Research 301, 5-17.

Hurle, J., Hinchliffe, J.R., 1978. Cell death in the posterior necrotic zone (PNZ) of the chick wing-bud: a stereoscan and ultrastructural survey of autolysis and cell fragmentation. Journal of Embryology and Experimental Morphology 43, 123-136.

Hussein, M.A., Bowen, I.D., Lewis, G.H., 1990. The histochemical localization of ATPase, cholinesterase and acid phosphatase activity in *Culex pipiens* (Diptera, Culicidae) larvae using a methacrylate embedding technique. Cell Biology International Reports 14 (9), 775-781.

Jochová, J., Zakeri, Z., Lockshin, R.A., 1997. Rearrangement of the tubulin and actin cytoskeleton during programmed cell death in *Drosophila* salivary glands. Cell Death and Differentiation 4, 140-149.

Jiang, C., Baehrecke, E.B., Thummel, C.S., 1997. Steroid regulated programmed cell death during *Drosophila* metamorphosis. Development 124, 4673-4683.

Jones, H.E., Bowen, I.D., 1993. Acid phosphatase activity in the larval salivary glands of developing *Drosophila melanogaster*. Cell Biology International 17 (3), 305-315.

Kerr, J.F.R., Gobé, G.C., Winterford, C.M., Harmon, B., 1995. Anatomical Methods in Cell Death. In: Schwartz, L.M., Osborne, B.A. (Eds.), Cell Death. Academic Press, San Diego, pp. 1-27.

L'Amoreuax, W.J., Junaid, L., Trevidi, S., 2003. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. Tissue & Cell 35, 95-99.

Levy, M., Bautz, A.M., 1985. Degeneration of larval salivary glands during metamorphosis of the blowfly, *Calliphora erythrocephala* Meigen (Diptera: Calliphoridae). International Journal of Insect Morphology & Embryology 14 (5), 281-290.

Lockshin, A.R., Zakeri, Z., 1996. The Biology of Cell Death and its Relationship to Aging. In: Holbrook, N., Martin, G.R., Lockshin, R.A. (Eds.), Cellular Aging and Cell Death. Wiley-Liss, New York, pp. 167-180.

Lomas, O.L., Gelman, D., Kaufman, W. R., 1998. Ecdysteroid regulation of salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*: a reconciliation of *in vivo* and *in vitro* observations. General and Comparative Endocrionology 109, 200-211.

Mao, H., MacBlain, W.A., Kaufman, W.R., 1995. Some properties of the ecdysteroid receptor in the salivary gland of the ixodid tick, *Amblyomma hebraeum*. General and Comparative Endocrinology 99, 340-348.

Mao, H., Kaufman, W.R., 1999. Profile of the ecdysteroid hormone and its receptor in the salivary gland of the adult female tick, *Amblyomma hebraeum*. Insect Biochemistry and Molecular Biology 29, 33-42.

MacGahon, A.J., Martin, S.J., Bissonnette, R.P., Mahboubi, A., Shi, Y., Mogil, R.J., 1995. The End of the (Cell) Line: Methods for the Study of Apoptosis in Vitro. In:

Schwartz, L.M., Osborne, B.A. (Eds.), Cell Death. Academic Press, San Diego, pp. 153-185.

Mullarkey, K., 1987. Gene expression in the salivary gland of the blowfly (*Calliphora vomitoria*) larva. PhD Thesis, University of Exerter, Exerter, UK.

Nunes, E.T., Bechara, G.H., Saito, K.C., Denardi, S.E., Oliveira, P.R., Camargo-Mathias, M.I., 2005. Morphological, histological and ultrastructural characterization of degenerating salivary glands in females of the cattle-tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae). Micron 36, 437-447.

Nunes, E.T., Camargo-Mathias, M.I., Bechara, G.H., 2006 a. *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae): acid phosphatase and ATPase activities localization in salivary glands of females during the feeding period. Experimental Parasitology 114, 109-117.

Nunes, E.T., Camargo-Mathias, M.I., Bechara, G.H., 2006 b. Structural and cytochemical changes in the salivary glands of the *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae) tick female during feeding. Veterinary Parasitology 140, 114-123.

Oliver, J.H., Jr., 1986. Relationship among feeding, gametogenesis, mating and syngamy in ticks. In: Borovsky, D., Spielman, A. (Eds.), Host Regulated Developmental Mechanism in Vector Arthropods. University of Florida-IFAS, Vero Beach, pp. 93-99.

Pelt-Verkuil, E.V., 1979. Hormone mediated induction of acid phosphatase activity in the fat body of *Calliphora erythrocephala* prior to metamorphosis. Journal of Insect Physiology 25, 965-973.

Pipan, N., Rakovec, V., 1980. Cell death in the midgut epithelium of the worker honey bee (*Apis mellifera carnica*) during metamorphosis. Zoomorphologie 97, 217-224.

Rosenau, W., Goldberg, M.L., Burke, G.C., 1973. Early biochemical alterations induced by lymphotoxin in target cells. The Journal of Immunology 111, 1128-1135.

Silva de Moraes, R.L.M., 1998. Morte celular na glândulas hipofaríngeas de *Apis mellifera* (Hymenoptera, Apidae). Tese de Livre Docência, Instituto de Biociências-Unesp, Rio Claro, 97 p.

Till, W.M., 1961. A Contribution to the Anatomy and Histology of the Brown Ear Tick *Rhipicephalus appendiculatus*. Memoirs of the Entomological Society of Southern Africa, Pretoria, 6, 1-124.

Walker, A.R., Fletcher, J.D., Gill, H., 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. International Journal for Parasitology 15 (1), 81-100.

Walker, S.M., Lucas, Z.J., 1972. Cytochemical activity of lymphocytes: II. Studies on mechanism of lymphotoxin-mediated cytotoxicity. The Journal of Immunology 109, 1233-1244.

Weber, R., 1969. Tissue Involution and Lysosomal Enzimes During Anuran Metamorphosis. In: Dingle, J.T., Fell, H.B. (Eds.), Lysosomes in Biology and Pathology, vol. 2. North Holland Publishing Co., Amsterdam and London, pp. 437-461.

Yamamoto, S., Sawada, K., Shimomura, H., Kawamura, K., James, T.N., 2000. On the nature of cell death during remodeling of hypertrophied human myocardium. Journal of Molecular and Cellular Cardiology 32, 161-175.

Zakeri, Z.F., Ahuja, H.S., 1997. Cell death/apoptosis: normal, chemically induced, and teratogenic effect. Mutation Research 396, 149-161.

Zakeri, Z., Bursch, W., Tenniswood, M., Lockshin, R.A., 1995. Cell death: programmed, apoptosis, necrosis, or other? Cell death and Differentiation 2, 87-96.

Capítulo 4

CAPÍTULO 4

TITLE: Morpho-histochemical characterization of salivary gland cells of males of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, ixodidae) at different feeding stages. Description of new cell types.

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RESUMO

O estudo morfo-histoquímico das células das glândulas salivares de machos de *R. sanguineus* em jejum e em alimentação (dois e quatro dias de infestação) mostrou as alterações que nelas ocorreram durante a alimentação destes carrapatos, bem como auxiliou na descrição de novos tipos celulares.

As glândulas nos machos em jejum encontram-se pouco desenvolvidas, sendo observados os ácinos I, os II contendo células "i**ndiferenciadas**", i**ndefinidas 1** e **2** (estas últimas com grânulos atípicos), **a**, **c1** e **c3**, nos ácinos III as células **d** e **e**, e nos IV as **g**.

Nos machos com dois dias de infestação, portanto em início do ciclo secretor, os ácinos I possuem a mesma morfologia encontrada nos indivíduos em jejum. Nos ácinos II, III e IV existe aumento no tamanho, devido às células estarem repletas de grânulos de secreção, que em alguns casos ainda estão em processo de maturação. Nos ácinos II observam-se as células **a**, **b** e **c1-c8**. As **c7** e **c8** são aqui descritas pela primeira vez. As **c7** foram assim chamadas devido à adição de polissacarídeos na constituição da secreção (nos indivíduos em jejum eram chamadas de i**ndefinidas 1**). Os ácinos III apresentaram as células **d** e **e** repletas de grânulos e os IV as **g** com grânulos em vários estágios de maturação.

Nos machos com quatro dias de infestação, onde as glândulas salivares encontravam-se no final do ciclo secretor, os ácinos I não sofreram alterações. Os granulares continham células com menos grânulos de secreção e já maduros. Nos ácinos II estavam presentes as células **a**, **b**, **c1-c5**, nos III as **d** e **e** e nos IV as **g** com pouca ou nenhuma secreção.

O presente estudo mostrou que nas glândulas salivares de machos de *R. sanguineus*: as células **a**, **c1** e **c3** do ácino II e as **d** e **e** do ácino III não apresentaram diminuição da secreção, permanecendo continuamente ativas durante todo o período de alimentação, indicando que no intervalo entre os estágios de alimentação as células glandulares readquiriram as mesmas características encontradas nos indivíduos em jejum, sugerindo que as mesmas sofrem reprogramação para serem reativadas no próximo ciclo.

PALAVRAS-CHAVE: *Rhipicephalus sanguineus*; carrapato; glândula salivar; morfologia; ciclo celular; reprogramação celular.

ABSTRACT

This morpho-histochemical study describes the changes undergone by cells of the salivary glands of unfed and feeding (at day two and four post-attachment) *R. sanguineus* males, as well as new cell types.

The glands of unfed males are little developed. Types I and II acini are observed with cells "**undifferentiated**", **undefined 1** and **2** (the latter, with atypical granules), **a**, **c1** and **c3**; type III acini are composed of cells **d** and **e**; and type IV acini present cells **g**.

In males at day two post-attachment, in which the secretory cycle is beginning, type I acini exhibit the same morphology of unfed individuals. An increase in size is observed in types II, III, and IV acini, as cells are filled with secretion granules. Some granules are still undergoing maturation. In type II acinus, cells **a**, **b** and **c1–c8** are observed. Cells **c7** and **c8** are described for the first time. Cells **c7** are termed as such due to the addition of polysaccharides in the composition of the secretion granules (in unfed individuals, they are termed **undefined 1).** Type III acini exhibit cells **d** and **e** completely filled with granules, and in type IV acini, cells **g** contain granules in several stages of maturation.

In males at day four post-attachment, salivary glands are in the late stages of the secretory cycle, and type I acini do not exhibit changes. Granular acini exhibit cells with fewer secretion granules, which are already mature. In type II acini, cells **a**, **b**, **c1–c5** are present, type III acini exhibit cells **d** and **e**, and type IV contain cells **g** with little or no secretion.

The present study shows that in the salivary glands of *R. sanguineus* males: cells **a**, **c1,** and **c3** of type II acinus, and cells **d** and **e** of type III acinus do not exhibit changes in granular content, remaining continuously active during the entire feeding period. This indicates that during the intervals among feeding stages, gland cells reacquire the same characteristics found in unfed individuals, suggesting that they undergo reprogramming to be active in the next cycle.

KEY WORDS*:* *Rhipicephalus sanguineus*; tick; salivary gland; morphology; cell cycle; cell reprogramming.

INTRODUCTION

The morphological structure of the salivary gland of male ticks is, in general, similar to those of females (Till, 1961), differing only in the presence of one additional type of acinus, termed type IV acinus (Binnington, 1978; Walker et al., 1985; Gill and Walker, 1987).

In Ixodidae, this gland is essential for the maintenance of life as well as the reproductive capacity of individuals (Sanders et al., 1996), as it is a multifunctional organ that allows these parasites to feed.

The different types of acini found in the glands of ticks are associated with the several roles played by these organs: type I acini maintain hydration levels and osmoregulation in these parasites (Walker et al., 1985; Binnington, 1978; Serra-Freire and Olivieri, 1993; Gill and Walker, 1987; McMullen et al., 1976 apud Fawcett et al., 1986; Gaede and Knulle, 1997, apud Bowman and Sauer, 2004); types II and III secrete compounds necessary for the attachment of the tick to the host (formation of cement cone) and to manipulate the host, allowing the parasite to feed on blood (Binnington, 1978; Walker et al., 1985; Gill and Walker, 1987); the function of type IV acinus is not completely understood. Some authors suggest a role in mating (Feldman-Meshsam et al., 1970) while according to other, these acini might also be involved in the formation of the cement cone (Fawcett et al., 1986).

In the salivary glands of ticks, the structure of cells is unique for each type of acinus. Type I acini are agranular and consist of a **central** cells surrounded by several **peripheral** ones. Type II, III, and IV acini are granular; type II acini are composed of cells **a**, **b**, **c1**–**c4**; type III acini exhibit cells **d**, **e** and **f**; and type IV acini consist of only cells **g** (Binnington, 1978; Walker et al., 1985; Gill and Walker, 1987). A recent study on the salivary glands of *Rhipicephalus sanguineus* females in different feeding stages reanalyzed the cell types and showed that in unfed females, type II acini are also comprised of cells **undefined 1** and **2**, and cells **c5** and **c6** are observed in feeding females (Furquim, 2004).

Secretory cells of salivary glands of ticks are characterized by specific granules of varied composition, such as proteins, glycoproteins, lipoproteins, and enzymes,

among others (Binnington, 1978; Walker et al., 1985; Marzouk and Darwish, 1994). The secretion produced by all cells comprises the tick saliva.

Regarding its release, secretion production in the salivary glands of male ticks is not continuous as in females, since males do not feed continuously and even when they cease feeding and detach from the host, salivary gland cells remain functionally ready for the next reattachments, maintenance in the host, and blood consumption.

Sanders et al. (1996), studying feeding males of *Amblyomma americanum*, suggested that the stimulus for the development of salivary glands is the contact with the host and during attachment and feeding, there is an increase in the amount and types of protein synthesized by the gland, resulting in an increase in size of the organ.

Thus, the present study aimed at identifying the different cell types present in the salivary glands of *R. sanguineus* males, as well as defining the periods when these cells are active during feeding.

MATERIAL AND METHODS

For this study, were utilized male ticks of *Rhipicephalus sanguineus* in the following feeding stages: unfed, and at day two and four post-attachment to the host. Unfed individuals were provided by Dr. Gervásio Henrique Bechara of the Department of Veterinary Pathology of UNESP, Jaboticabal campus (São Paulo), from a colony maintained under controlled conditions (29^{o} C, 80% humidity, and 12 hour photoperiod) in BOD incubator. A group of unfed individuals was assigned to histological methods while another group was placed with some females in a feeding chamber previously glue with an atoxic and non-lesive preparation (Britannia Adhesives-Unit 4, UK) to the shaved back of the host (rabbit) according to technique described elsewhere (Bechara et al., 1995) to monitor the feeding process.

Upon completion of the feeding periods, males were collected and salivary glands were removed in saline solution and fixed in a 10 % neutral buffered formalin and acetone solution (9:1) for one hour and thirty minutes at 4^{o} C. After fixation, the material was dehydrated in a series of increasing concentrations of alcohol (70%, 80%, 90% and 95%), embedded in resin (Leica), and sectioned at 3 μm. Sections were mounted on glass slides and stained with Hematoxylin-Eosin and PAS (Periodic Acid

Schiff), McManus (1946), for detection of polysaccharides, and counterstained with Methyl Green. Slides were mounted with Canada balsam and examined under light microscope.

RESULTS

For the identification of the different cell types of type II acinus of *R. sanguineus* salivary gland, were utilized the system described and adopted for females of the same species (Furquim, 2004). Additionally, we characterize new cell types not previously observed and described in *R. sanguineus* females.

For comparison of results, the data obtained are summarized in Tables **1**, **2**, and **3**.

Table 1: Morpho-histochemical results of the salivary glands of unfed males of *Rhipicephalus sanguineus*, including the description of new cell types.

Acini	Cells	Cell Characteristics		
		Localization	**Shape**	**Cytoplasm**
I	**central and peripheral**	- one **central** cell (Fig. $1A_1$); - several **peripheral** cells (Fig. $1A_1$);	- ∅ (Fig. $1A_1$);	- fibrillar PAS ++ (Fig. $2A_1$);
II	**undifferentiated**	- ? (Fig. $1A_2$);	- cubic (Fig. $1A_2$);	- scarce, no granules (Fig. $2A_2$);
	undefined 1	- beside **a** cells (Fig. $1A_3$);	- cubic (Fig. $1A_3$);	- larger granules PAS – (Fig. $2A_3$);
	2	- near to **a** cells (Fig. $1A_4$);	- cubic (Fig. $1A_4$);	- smaller granules (Fig. $1A_4$);
	a	- hilus of the acinus (Fig. $1A_2$);	- cubic (Fig. $1A_2$);	- granules PAS – (Fig. $2A_4$);
	c1	- near to **a** cells (Fig. $1A_4$);	- cubic (Fig. $1A_4$);	- granules PAS +++ (Fig. $2A_5$);
	c3	- fundus of the acinus (Fig. $1A_3$);	- cubic (Fig. $1A_3$);	- larger granules than those of **c1** cells PAS +++ (Fig. $2A_4$);
III	**d**	- hilus of the acinus (Fig. $1B_1$);	- cubic (Fig. $1B_1$);	- granules PAS – (Fig. $2B_1$);
	e	- beside **d** cells (Fig. $1B_1$);	- cubic (Fig. $1B_1$);	- larger granules than those of **d** cells PAS – (Fig. $2B_1$);
	f	- fundus of the acinus (Fig. $1B_2$);	- cubic (Fig. $1B_2$);	- scarce, no granules (Fig. $1B_2$);
IV	**g**	- throughout acinus (Fig. $1B_3$);	- cubic (Fig. $1B_3$);	- scarce, no granules (Fig. $2B_2$);

∅: cell boundary not evident; ☐ cell types already described for type II acini of *R. sanguineus* females (Furquim, 2004); **?**: location not defined; **Undifferentiated**: undifferentiated cells still without secretion granules; **Undefined:** cells exhibiting immature secretion granules (atypical);

Cells **b**, **c2**, **c4**, **c5**, **c6**, **c7,** and **c8** were not included in the table, as they were not observed in the gland tissue of unfed *R. sanguineus* males.

Table 2: Morpho-histochemical results of the salivary glands of *Rhipicephalus sanguineus* males at day two post-attachment, including the description of new cell types.

Acini	Cells	Cell Characteristics		
		Localization	**Shape**	**Cytoplasm**
I	**central and peripheral**	- one **central** cell (Fig. $1B_4$); - several **peripheral** cells (Fig. $1B_4$);	- ∅ (Fig. $1B_4$);	- fibrillar PAS ++ (Fig. $2B_3$);
II	**a**	- hilus of the acinus (Fig. $1B_5$);	- cubic (Fig. $1B_5$);	- granules PAS – (Fig. $2B_4$);
	b	- beside **a** cells (Fig. $1C_1$);	- cubic (Fig. $1C_1$);	- elliptic and heterogeneous granules PAS+ and PAS ++ (Fig. $2C_1$);
	c1	- near to **a** cells (Fig. $1C_2$);	- cubic (Fig. $1C_2$);	- granules PAS +++ (Fig. $2C_2$);
	c2	- near to **a** cells (Fig. $1C_2$);	- cubic (Fig. $1C_2$);	- granules PAS + (Fig. $2C_3$);
	c3	- fundus of the acinus (Fig. $1C_3$);	- cubic (Fig. $1C_3$);	- larger granules than those of **c1** cells PAS +++ (Fig. $2C_3$);
	c4	- fundus of the acinus (Fig. $1C_4$);	- cubic (Fig. $1C_4$);	- elliptic granules PAS – (Fig. $2C_4$);
	c5	- near **a** cells (Fig. $1C_5$);	- cubic (Fig. $1C_5$);	- smaller granules than those of **a** cells and larger than those of **c3** cells PAS ++ (Fig. $2C_5$);
	c6	- near **a** cells (Fig. $2D_1$);	- cubic (Fig.$2D_1$);	- fine granules PAS ++ (Fig. $2D_1$);
	c7	- near **a** cells (Fig. $1C_6$);	- cubic (Fig. $1C_6$);	- larger granules than those of **a** cells PAS ++ (Fig. $2D_2$);
	c8	- near **a** cells (Fig. $1D_1$);	- cubic (Fig. $1D_1$);	- larger granules than those of **c3** cells PAS + (Fig. $2D_3$);
III	**d**	- hilus of the acinus (Fig. $1D_2$);	- cubic (Fig. $1D_2$);	- granules PAS – (Fig. $2D_4$);
	e	- beside **d** cells (Fig. $1D_2$);	- cubic (Fig. $1D_2$);	- larger granules than those of **d** cells PAS – (Fig. $2D_4$);
IV	**g**	- throughout acinus (Fig. $1D_3$);	- cubic (Fig. $1D_3$);	- homogeneous granules PAS ++ (fig. 2E1) and heterogeneous granules PAS + and PAS ++ (Fig. $2E_2$);

∅: cell boundary not evident; ☐ cell types already described for type II acini of *R. sanguineus* females (Furquim, 2004); ■ new cell types described in the present study; Cells **undifferentiated**, **undefined 1** and **2**, and **f** were not included in the table, as they were not observed in the gland tissue of *R. sanguineus* males at day two post-attachment.

Table 3: Morpho-histochemical results of the salivary glands of *Rhipicephalus sanguineus* males at day four post-attachment, including the description of new cell types.

Acini	Cells	Cell Characteristics		
		Localization	**Shape**	**Cytoplasm**
I	**central and peripheral**	- one **central** cell (Fig. $1D_4$); - several **peripheral** cells (Fig. $1D_4$);	- ∅ (Fig. $1D_4$);	- fibrillar PAS – (Fig. $2E_3$);
II	**a**	- hilus of the acinus (Fig. $1D_5$);	- cubic (Fig. $1D_5$);	- granules PAS – (Fig. $2E_4$);
	b	- beside **a** cells (Fig. $1D_5$);	- cubic (Fig. $1D_5$);	- elliptic and heterogeneous granules PAS++ and PAS +++ (Fig. $2E_4$);
	c1	- near to **a** cells (Fig. $1E_1$);	- cubic (Fig. $1E_1$);	- granules PAS +++ (Fig. $2F_1$);
	c2	- near to **a** cells (Fig. $1E_2$);	- cubic (Fig. $1E_2$);	- granules PAS + (Fig. $2F_2$);
	c3	- fundus of the acinus (Fig. $1E_2$);	- cubic (Fig. $1E_2$);	- larger granules than those of **c1** cells PAS +++ (Fig. $2F_3$);
	c4	- fundus of the acinus (Fig. $1E_3$);	- cubic (Fig. $1E_3$);	- elliptic granules PAS – (Fig. $2F_4$);
	c5	- near **a** cells (Fig. $1E_4$);	- cubic (Fig. $1E_4$);	- smaller granules than those of **a** cells and larger than those of **c3** cells PAS ++ (Fig. $2F_5$);
III	**d**	- hilus of the acinus (Fig. $1F_1$);	- cubic (Fig. $1F_1$);	- granules PAS – (Fig. $2G_1$);
	e	- beside **d** cells (Fig. $1F_1$);	- cubic (Fig. $1F_1$);	- larger granules than those of **d** cells PAS – (Fig. $2G_1$);
IV	**g**	- throughout acinus (Fig. $1F_2$, F_3);	- cubic (Fig. $1F_2$, F_3);	- granules PAS +++ (fig. $2G_1$, G_2);

∅: cell boundary not evident; ☐ cell types already described for type II acini of *R. sanguineus* females (Furquim, 2004);

Cells **undifferentiated**, **undefined 1** and **2**, **c6**, **c7**, **c8**, and **f** were not included in the table, as they were not observed in the gland tissue of *R. sanguineus* males at day four post-attachment.

FIGURES

FIGURE 1:

Histological sections of types I, II, III, and IV acini of males salivary glands of unfed and at days two and four post-attachment of *Rhipicephalus sanguineus* stained with Hematoxilin and Eosin.

A_1-B_3. Unfed males.
B_4-D_3. Males at day two post-attachment.
D_4-F_3. Males at day four post-attachment.

I-IV: types acini; **rc: undifferentiated** cell; **nc1: undefined** cell **1**; **nc2: undefined** cell **2**; **a: a** cell; **b: b** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **c7: c7** cell; **c8: c8** cell; **d: d** cell; **e: e** cell; **f: f** cell; **g: g** cell; **c:** cytoplasm; **cn: central** cell nucleus; **pn: peripheral** cell nucleus; **n:** nucleus; **dt:** ducto; **iv:** intraacinar valve; **s:** secretion; **lu:** lumen.

Bars: A_1-F_3= 20 μm.

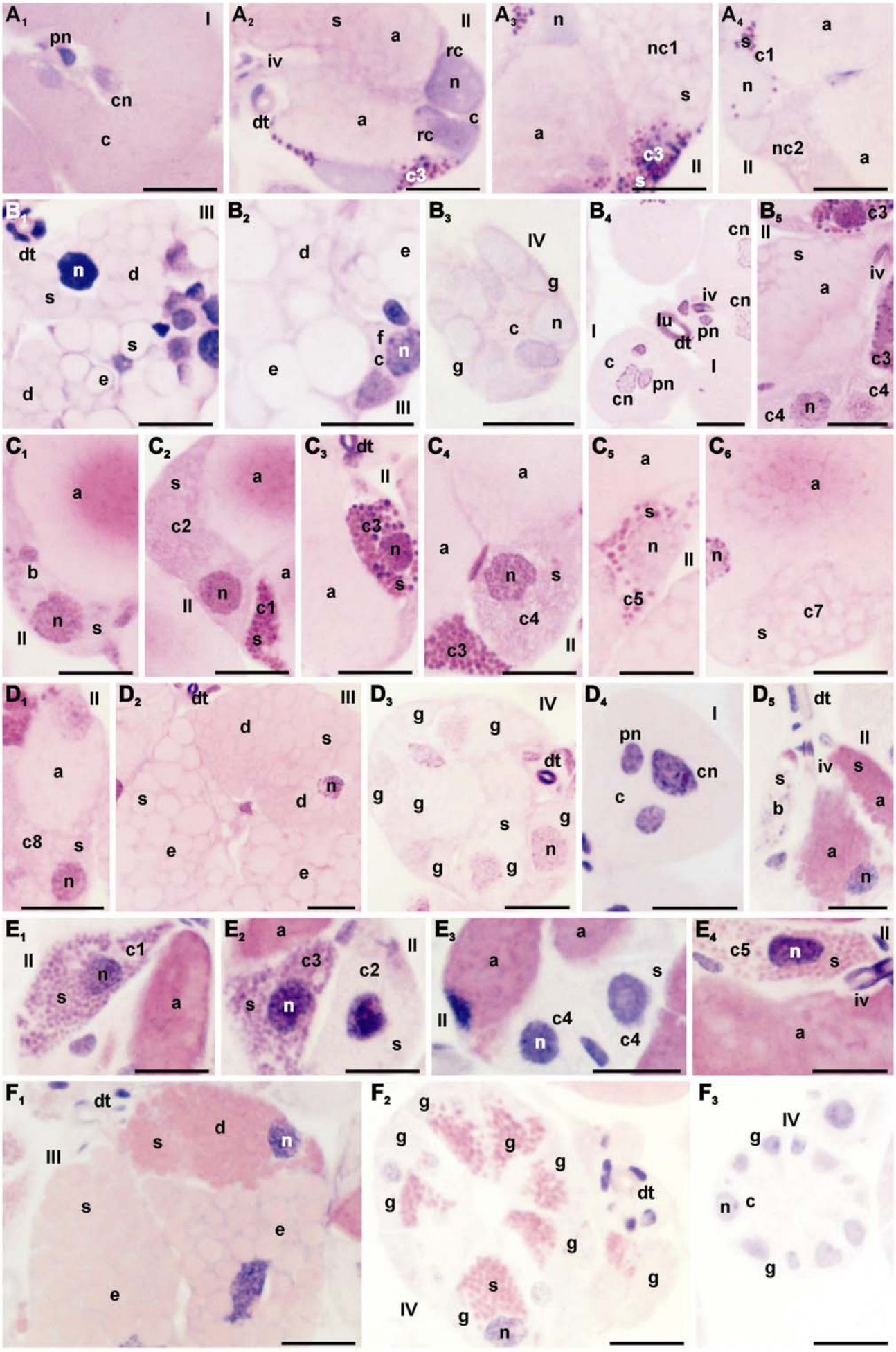
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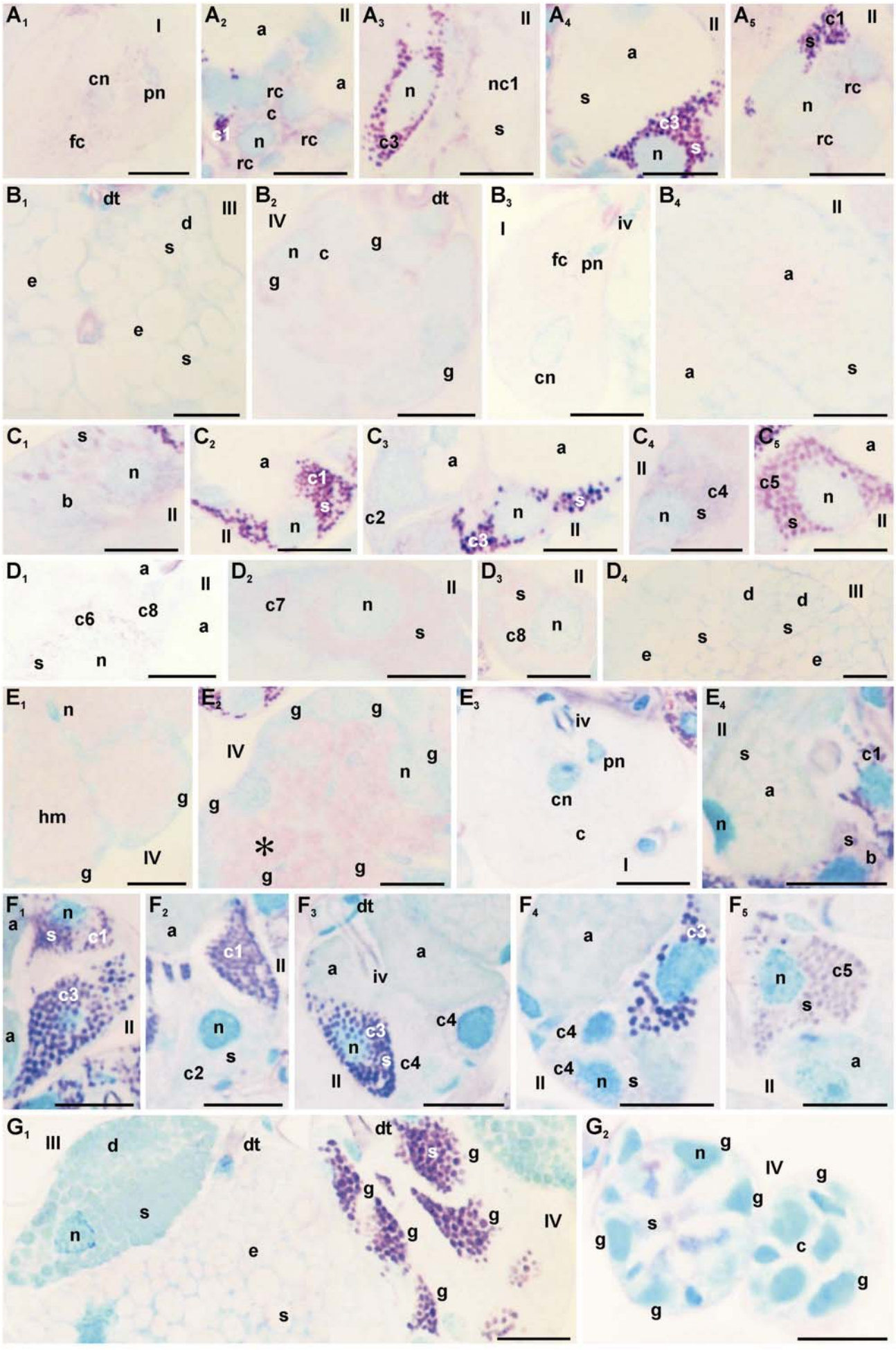
FIGURE 2:

Histological sections of types I, II, III, and IV acini of males salivary glands of unfed and at days two and four post- attachment of *Rhipicephalus sanguineus* stained with PAS and counterstained with Methyl Green.

A_1-B_2. Unfed males.
B_3-E_2. Males at day two post-attachment.
E_3-G_2. Males at day four post-attachment.

I-IV: types acini; **rc: undifferentiated** cell; **nc1: undefined** cell **1**; **a**: a cell; **b: b** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5**: c5 cell; **c6: c6** cell; **c7: c7** cell; **c8: c8** cell; **d: d** cell; **e: e** cell; **g: g** cell; **fc:** fibrillar cytoplasm; **cn: central** cell nucleus; **pn: peripheral** cell nucleus;**c:** cytoplasm; **n:** nucleus; **s:** secretion; **dt:** duct; **iv:** intraacinar valve; **hm:** homogeneous granules; ***:** heterogeneous granules.

Bars: B_2= 10 μm**; A_1-B_1** and **B_3-G_2**= 20 μm.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DISCUSSION

The histology of the salivary glands of ticks has been described by several authors especially in females (Till, 1961; Binnington, 1978; Walker et al., 1985; Sonenshine, 1991; Serra-Freire and Olivieri, 1993), as they, unlike males, feed continuously until engorgement, ingesting larger quantities of blood and causing more damages to the host.

For this study, we utilized the classification system developed by Binnington (1978), Walker et al. (1985), Gill and Walker (1987) for types I, III, and IV acini. For type II acinus, we adopted the system proposed by Furquim (2004) described for *Rhipicephalus sanguineus* females that includes four new cell types: **undefined 1** and **2**, **c5** and **c6**. In addition to these, we described in males, for the first time, two additional cell types, **c7** and **c8**. These might be present only in males of this species, or if present in females, they may be active only during the first day of attachment to the host (Furquim, 2004).

In this study, we observed that gland cells of males were activated with the onset of feeding, supporting Sanders et al. (1996) that in *Amblyomma americanum* reported that the beginning of feeding promoted the development and increase of production of different proteins in the salivary glands.

In feeding males, the authors observed that as soon as the secretory cycle ended, the aspect of these organs returned to the observed in unfed individuals. This was also reported by Wang et al. (1999), which described a reprogramming in the expression of the gland tissue of *R. appendiculatus* when feeding ceased and that was maintained until the next attachment. In *R. sanguineus*, only cells **a**, **c1**, **c3**, **d**, and **e** were continuously active and therefore, exhibited mature granules in the cytoplasm since the first secretory cycle. In the remaining cell types, granules were released at the end of each cycle, restarting the secretory activity when reattachment occurred.

Our findings indicate that the glands of males at day two post-attachment were in early stages of the secretory cycle while at day four post-attachment, glands were in late stages of one of the several cycles of this organ. Wang et al. (1999), studying *R. appendiculatus* females and males, showed that the salivary gland cycle in male ticks is based on an inversely proportional relationship between the amount of blood consumed

and the difficulty of the gland to reprogram its expression. Since males do not consume large volumes of blood due to the physical resistance caused by the presence of the dorsal scutum, they do not reach the "critical weight" (Weiss and Kaufman, 2001) and glands still remain active during intervals between one cycle and the next, allowing feeding in the next attachment to the host.

The histological data presented in our study showed that type I acinus did not undergo structural or size changes during feeding, as reported by Till (1961), Walker et al. (1985), Binnington (1978), and Furquim (2004), in males of *R. appendiculatus* and *B. microplus*, and females of *R. sanguineus*, respectively.

In *R. sanguineus*, the most developed type II acinus and with more cell types were observed in males at day two post-attachment. Ten types of cells were observed, unlike Walker et al. (1985) and Gill and Walker (1987) that reported five types (**a**, **b**, **c1**–**c3**), and Binnington (1978) that described six types (**a**, **b**, **c1**–**c4**).

In unfed males, cells **a** were already filled with immature secretion granules; at day two post-attachment, cells became larger and secretion granules was maturing; and at day four, cells were smaller and with mature granules. These results confirm those obtained by Binnington (1978), Walker et al. (1985), and Gill and Walker (1987).

In *R. sanguineus*, the secretion present in cells **a** does not contain polysaccharides, unlike the observed by Serra-Freire and Olivieri (1993) that reported this compound in *A. cajennense*. According to Binnington (1978), Walker et al. (1985), Fawcett et al. (1986), Gill and Walker (1987), and Sonenshine (1991), these cells might be involved in the secretion of cement precursors, a likely role played by this type of cell in the individuals examined in this study.

Cells **b** were not observed in unfed males, contrary to Binnington (1978) and Gill and Walker (1987), which reported in *B. microplus* and *H. anatolicum anatolicum*, respectively, the presence of secretion in the cytoplasm of these cells in unfed males. In males at day two post-attachment, cells **b** were filled with heterogeneous granules, including the presence of polysaccharides (PAS staining was observed in one area of the granule, while staining was absent in another). At day four post-attachment, these granules did not undergo histochemical changes, but were present in fewer numbers, as also reported by Walker et al. (1985). These authors detected activity in cells **b** of *R. appendiculatus* during early stages, which decreased throughout feeding. In addition,

Walker et al. (1985) suggested that the secretion synthesized by cells **b** may be involved in the manipulation of the host response, and this could also be the case in *R. sanguineus* males.

Cells **c1** were active in unfed males; in those at day two post-attachment, they were more developed and remained unchanged in those at day four post-attachment. According to Gill and Walker (1987), the secretory activity of these cells increases during feeding and most granules are released around 72 hours of feeding, while Walker et al. (1985) reported a hypertrophy of these cells during feeding.

Previous studies have shown that the secretion produced by cells **c1** in *R. sanguineus* females might be fully active in individuals after four days of feeding, the beginning of a phase characterized by the largest consumption of blood (Furquim, 2004). Thus, cells **c1** might also play a similar role in males, participating in the consumption of blood.

The present study showed that cells **c2** of unfed males exhibited secretion granules, unlike the observed by Gill and Walker (1987). At day two post-attachment, cells were filled with granules and still active at day four post-attachment, confirming the obtained by Binnington (1978), Walker et al. (1985), and Gill and Walker (1987).

The secretion of cells **c2** analyzed in our study might play a role in the consumption of blood by the tick, as also described in *B. microplus* (Binnington, 1978).

In unfed males, cells **c3** already contained secretion, contrary to the reported by Walker et al. (1985), which demonstrated that in *R. appendiculatus*, these cells are inactive in individuals at this stage. In the individuals here examined, at day two post-attachment, cells **c3** were more developed and remained active at day four post-attachment. These results also contradict those obtained by Walker et al. (1985) that observed hypertrophy in these cells in more advanced stages of feeding.

Cells **c4** of unfed *R. sanguineus* males did not exhibit secretion granules in the cytoplasm, unlike the observed by Binnington (1978) in unfed *B. microplus* males. In *R. sanguineus* at day two post-attachment, these cells were developed; and at day four post-attachment, they were less active. The morphological and histochemical characteristics of the granules were similar to those found in *B. microplus* (Binnington, 1978), suggesting that in *R. sanguineus*, cells **c4** also produce and secrete enzymes assisting in feeding, promoting the consumption of blood by the tick.

The cells **c5** and **c6** were not observed on unfed *R. sanguineus* males, confirming the observed in females of the same species (Furquim, 2004). At day two post-attachment, these cells were developed and at day four post-attachment, cells **c5** remained active while **c6** were no longer observed, contrary to the observed in females after four days of feeding, in which **c6** were still active (Furquim, 2004).

Based on the results reported in a previous study on *R. sanguineus* females, the secretion of cells **c5** and **c6** may play a role in the initial phase of the secretory cycle, inhibiting the host response in order to overcome any specific resistance to the attachment of the parasite (Furquim, 2004).

In *R. sanguineus* males, cell types **c7** and **c8** were not observed in unfed individuals, but were present in those at day two post-attachment, and at day four post-attachment, these cells were no longer observed, suggesting the end of their activities. As the secretory cycle progressed, polysaccharides were demonstrated in the granules of cells **undefined 1**, previously observed only in unfed males. This histochemical change in composition characterized these cells as a new cell type: **c7**. If cells **c7** and **c8** were only present in *R. sanguineus* males, they probably would play specific roles in these individuals, such as during mating. If they were also present in females, they might be involved in the formation of the feeding lesion that occurs during the first two days of attachment (Balashov, 1972 apud Kaufman and Lomas, 1996).

The type III acinus of males examined here, unlike the observed in females (Furquim, 2004), did not undergo prominent morphologic changes during feeding, since males consumed small amounts of blood, and thus the participation of these acini was not needed in the excretion of water and ions. In *R. sanguineus* males, we only observed changes in size of these acini. In males at day two post-attachment, type III acini were larger than those of males at day four post-attachment.

Cells **d** and **e** of type III acinus of unfed *R. sanguineus* males already contained secretion, confirming the reported by Binnington (1978), Walker et al. (1985), Gill and Walker (1987), and Serra-Freire and Olivieri (1993). These cells became even more developed at day two post-attachment, but a decrease in activity was observed at day four post-attachment. Binnington (1978), Walker et al. (1985), and Gill and Walker (1987), however, did not observe changes in secretory activity during feeding. The histochemical results on the secretion of these cells confirm those obtained by

Binnington (1978) and Walker et al. (1985). These authors demonstrated the presence of: lipoproteins, as cement precursors (Binnington,1978; Walker et al., 1985; Bishop et al., 2002, Gill and Walker, 1987); aminopeptidases participating in the infiltration of cement in the host's skin (Walker et al., 1985); and phenol and phenoloxidase, associated with the process of hardening of the cement cone (Binnington, 1978). These functions might also be performed by these cell types in the males here examined.

Cells **f** of *R. sanguineus* males in the three conditions examined were inactive, as reported by Binnington (1978) and Gill and Walker (1987). This was probably due to the low ingestion of blood, since there was no need for the cells to secrete compounds exclusively active in the early phase of blood consumption (Furquim, 2004), or in osmoregulation (Binnington, 1978; Walker et al., 1985; Coons and Lamureaux, 1986; Gill and Walker, 1987), unlike the observed by Walker et al. (1985).

Type IV acini of unfed *R. sanguineus* males were "undifferentiated", confirming the results obtained by Binnington (1978), Walker et al. (1985), Gill and Walker (1987), and Serra-Freire and Olivieri (1993). At day two post-attachment, these acini became larger, as described by Walker et al. (1985) and Gill and Walker (1987), while at day four post-attachment, the secretory activity decreased, contrary to the reported by Binnington (1978), which observed an increase in the secretory activity during feeding and cells filled with secretion in males after approximately 96 hours of feeding.

According to the literature, the role of type IV acinus is to produce cement in some Ixodidae (Fawcett et al., 1986), as well as to facilitate the transfer of the spermatophore to the females (Feldman-Meshsam et al., 1970 apud Fawcett et al., 1986). Our findings, however, do not suggest that these acini are involved in the formation of the cone, since males exhibit all cell types present in females (Furquim, 2004). Consequently, a specific structure for the construction of the cone is not necessary. Thus, type IV acini of *R. sanguineus* might participate in the lubrication of the spermatophore as well as in physiological and behavioral changes in females after mating. Weiss and Kaufman (2004) have demonstrated that the proteins produced in the gonads of *A. hebraeum* males and that are transferred to females through mating are not capable of trigger all physiological and behavioral responses, such as the development of the ovary.

The results obtained in the present study suggest that the salivary glands of *R. sanguineus* males do not exhibit histological differences compared to females, except by the presence of type IV acinus and cells **c7** and **c8**. If they in fact are only present in males, these cells might provide lubrication of the spermatophore and trigger responses in females that allow them to reproduce.

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REFERENCES

Balashov, Y.S., 1972. Bloodsucking ticks (Ixodidae)- vectors of disease of man and animals. Hoogstraal, H., Tachell, R.J. (Eds.), Misc. Publ. Entomol. Soc. Am. 8, 161-376.

Bechara, G.H., Szabó, M.P.J., Ferreira, B.R., Garcia, M.V., 1995. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Brazil. J. Vet. Parasitol. 4, 61-66.

Binnington, K.C., 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, *Boophilus microplus*. Int. J. Parasitol. 8, 97-115.

Bishop, R., Lambson, B., Wells, C., Pandit, P., Osaso, J., Nkonge, C., Morzaria, S., Musoke, A., Nene, V., 2002. A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory *e* cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. Int. J. Parasitol. 32, 833-842.

Bowman, A.S., Sauer, J.R., 2004. Tick salivary glands: function, physiology and future. Parasitology 129, S67-S81.

Coons, L.B., L'amoreaux, W.J., 1986. Developmental changes in the salivary glands of male and female *Dermacentor variabilis* (Say) during feeding. In: Borovsky, D., Spielman, A. (Eds.), Host Regulated Developmental Mechanism in Vector Arthropods. University of Florida-IFAS, Vero Beach, pp. 86-92.

Fawcett, D.W., Binnington, K., Voigt, W.P., 1986. The cell biology of the ixodid tick salivary gland. In: Sauer, J.R., Hair, A. (Eds.), Morphology, Physiology, and Behavioral Biology of Ticks. Ellis Horwood, Chichester, pp. 22-45.

Feldman-Muhsam, B., Borut, S., Saliternik-Givant, S., 1970. Salivary secretion of the male tick during copulation. J. Insect Physiol. 16, 1945-1949.

Furquim, K.C.S., 2004. Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados. 1º Relatório Científico CAPES, Instituto de Biociências, UNESP, Rio Claro (S.P.), Brasil, 37 pp.

Gaede, H., Knulle, W., 1997. On the mechanism of water vapour sorption from unsaturated atmospheres by ticks. J. Exp. Biol. 200, 1491-1498.

Gill, H.S., Walker, A.R., 1987. The salivary glands of *Hyalomma anatolicum anatolicum*: structural changes during attachment and feeding. Int. J. Parasitol. 17 (8), 1381-1392.

Kaufman, W.R., Lomas, L.O., 1996. "Male factors" in ticks: their role in feeding and egg development. Invertebr. Reprod. Develop. 30 (1-3), 191-198.

Marzouk, A.S., Darwish, Z.E.A., 1994. Changes in the salivary glands of female *Hyalomma (Hyalomma) dromedarii* during and after feeding. J. Egypt. Soc. Parasitol. 24 (1), 39-57.

McMullen, H.L., Sauer, J.R., Burton, R.L., 1976. Possible role in uptake of water vapour by ixodid tick salivary glands. J. Insect Physiol. 22, 1281-1285.

McManus, J.F.A., 1946. Histological demonstration of mucin after periodic acid. Nature 158, 202.

Sanders, M.L., Scott, A.L., Glass, G.E., Schwartz, B.S., 1996. Salivary gland changes and host antibody responses associated with feeding of male lone star ticks (Acari: Ixodidae). J. Med. Entomol. 33 (4), 628-634.

Serra-Freire, N.M., Olivieri, J.A., 1993. Structure of the salivary glands of the unfed male tick *Amblyomma cajennense* (Fabricius) (Acari: Ixodidae). Mem. Inst. Oswaldo Cruz 88 (2), 207-213.

Sonenshine, D.E., 1991. Biology of Ticks. Oxford University Press, New York, pp 141-158.

Till, W.M., 1961. A Contribution to the Anatomy and Histology of the Brown Ear Tick *Rhipicephalus appendiculatus* Neumann. Memoirs of the Entomological Society of Southern Africa, Pretoria, 6, 1-124.

Walker, A.R., Fletcher, J.D., Gill, H., 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. Int. J. Parasitol. 15 (1), 81-100.

Wang, H., Henbest, P.J., Nuttall, P.A., 1999. Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by re-programming of salivary gland protein expression. Parasitology 119, 143-149.

Weiss, B.L., Kaufman, W.R., 2001. The relationship between "critical weight" and 20-hydroxyecdysone in the female ixodid tick, *Amblyomma hebraeum*. J. Insect Physiol. 47, 1261-1267.

Weiss, B.L., Kaufman, W.R., 2004. Two feeding-induced proteins from the male gonad trigger engorgement of the female tick *Amblyomma hebraeum*. Proc. Natl. Acad. Sci. S.A. 101 (16), 5874-5879.

Capítulo 5

CAPÍTULO 5

TITLE: Degeneration of salivary glands of males of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae).

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RESUMO

O presente estudo analisou morfo-histoquímicamente as glândulas salivares de machos de *Rhipicephalus sanguineus* com sete dias de infestação, três e sete dias pós-remoção do hospedeiro. Houve degeneração do órgão nas três situações analisadas, que se intensificou com o decorrer do tempo fora do hospedeiro, sendo maior nos machos com sete dias pós-remoção que naqueles com sete dias de infestação. Somente nestes últimos os ácinos I apresentaram-se íntegros e os outros com características de degeneração. Nos indivíduos com sete dias de infestação os ácinos II tiveram identificadas as células **a**, **c1-c5**, **c8** e **indeterminadas**, onde apenas as **c1** e **c8** eventualmente estavam íntegras. As outras estavam em degeneração assim como nos ácinos III todas as células **d**, **e** e **f** e nos IV todas as **g**.

Todas as células (**a**, **c1-c5**, **c8** e **indeterminadas**) observadas nos ácinos II de machos com três dias pós-remoção do hospedeiro, as **d** e **e** nos III e as **g** nos IV estavam degenerando. Em alguns dos ácinos **Indeterminados** ainda pode-se observar o limite de algumas células e em outros somente uma massa citoplasmática. Nos indivíduos com três dias pós-remoção foram observados corpos apoptóticos.

Nos machos com sete dias pós-remoção do hospedeiro o processo degenerativo se intensificou, foram observadas as células **a**, **c1**, **c3-c5**, **c8** e **indeterminadas** nos ácinos II e as **d** e **e** nos III, todas em degeneração. Nos IV, ainda havia resíduo de secreção, e nos **Indeterminados** somente uma massa citoplasmática. Nos indivíduos com sete dias pós-remoção também se observou a presença de corpos apoptóticos.

O presente estudo mostrou ainda que as células das glândulas salivares de machos de *R. sanguineus* quando em degeneração sofrem as seguintes alterações: a) diminuição da produção da secreção com ou sem rompimento dos grânulos, b) mudança da morfologia nuclear, c) retração citoplasmática, d) perda da forma celular, e) perda dos limites celulares e f) vacuolização citoplasmática. Todas elas somadas resultam na fragmentação celular com liberação de corpos apoptóticos.

PALAVRAS-CHAVE: *Rhipicephalus sanguineus*, glândula salivar, carrapato, degeneração, estágio de alimentação, morfologia.

ABSTRACT

The present morpho-histochemical study examined the salivary glands of *Rhipicephalus sanguineus* males at day seven post-attachment, and at days three and seven post-detachment from the host. Degeneration of this organ occurred in the three stages analyzed and it advanced as time away from the host progressed. Thus, characteristics of degeneration were more prominent in males at day seven post-detachment than in males at day seven post-attachment. In the latter, type I acini were intact; while in other stages these acini exhibited signs of degeneration. In type II acini of individuals at day seven post-attachment, cells **a**, **c1**–**c5**, **c8**, and **indeterminate** were identified. Only **c1** and **c8** were intact. The remaining cell types were undergoing degeneration, as well as all cells **d**, **e** and **f** in type III acini, and all cells **g** in type IV acini.

In males at day three post-detachment from the host, all cells (**a**, **c1**–**c5**, **c8** and **indeterminate**) observed in type II acini, cells **d** and **e** in type III acini, and **g** in type IV acini were undergoing degeneration. In some **Indeterminate** acini, the boundaries of cells still could be distinguished, while in others, only a cytoplasmic mass was observed. At day three post-detachment, apoptotic bodies were observed.

In males at day seven post-detachment from the host, the degeneration process progressed. All cells **a**, **c1**, **c3**–**c5**, **c8** and **indeterminate** in type II acini, and **d** and **e** in type III acini were undergoing degeneration. Type IV acini still contained remnants of secretion and in **Indeterminate** acini, only a cytoplasmic mass could be observed. At this stage, apoptotic bodies were also present.

In addition, the present study revealed that cells of salivary glands of *R. sanguineus* males when degenerating the following changes: a) decrease in secretion production with or without granule breakage, b) changes in nuclear morphology, c) cytoplasm shrinkage, d) loss of cell shape, e) loss of cell boundaries, and e) cytoplasmic vacuolation. Together, these changes result in cell fragmentation with release of apoptotic bodies.

KEY WORDS: *Rhipicephalus sanguineus*, salivary gland, tick, degeneration, feeding stage, morphology.

INTRODUCTION

Male ticks present salivary glands morphologically similar to those of females (Till, 1961), and as the latter, require compounds in the saliva to assist in the manipulation of the host (Sanders et al., 1996).

The salivary glands of these individuals consist of types I, II, III and IV acini (Binnington, 1978; Walker et al., 1985; Gill and Walker, 1987). In *R. sanguineus*, type I acini are composed of one **central** cell and several **peripheral** ones; while type II acini contain cells **a**, **b**, **c1**–**c8**; type III acini, cells **d**, **e** and **f**; and type IV acini, cells **g** (Furquim, 2006).

Since males exhibit several feeding stages (attach and detach several times from the host), salivary glands undergo many secretory cycles during infestation (Wang et al., 1999). Previous studies conducted with unfed and feeding *R. sanguineus* males have also demonstrated that in each secretory cycle, the gland undergo the following stages: 1) intense gland activity with synthesis and storage of secretion, 2) secretion maturation, 3) secretion discharge, and 4) acquisition of characteristics observed in unfed males, allowing the gland to restart a new secretory cycle through cell reprogramming (Furquim, 2006).

Walker et al. (1985), studying the salivary glands of *R. appendiculatus* males, reported that the gland tissue of these individuals do not undergo degeneration and remain functional after the end of each feeding period.

In female ticks, gland degeneration is signaled by the ecdysteroid hormone (Lomas et al., 1998). In *Amblyomma hebraeum* males exposed to 20-hydroxyecdysone (ecdysteroid hormone) *in vitro* for four days, the secretory capacity of the gland decreased without inducing the formation of autophagic vacuoles in the cytoplasm, not increasing the synthesis of this ecdysteroid when comparing feeding males (seven and fourteen days) to those after detachment from the host (Kaufman, 1990). Clearly, these studies are not conclusive to confirm whether the ecdysteroid hormone signals gland degeneration in *A. hebraeum* males, as observed in females of the same species (Lomas et al., 1998).

Due to the scarce information available in the literature regarding gland degeneration in ticks, this study aimed at describing morphologically and

histochemically the degenerating salivary glands of *R. sanguineus* males at day seven post-attachment, and at day three and seven post-detachment.

MATERIAL AND METHODS

For this study, were utilized males of the tick *Rhipicephalus sanguineus* at day seven post-attachment to the host, and at day three and seven post-detachment from the host (rabbit).

Unfed individuals were provided by Dr. Gervásio Henrique Bechara of the Department of Veterinary Pathology of UNESP, Jaboticabal campus (São Paulo, Brazil) from a colony maintained under controlled conditions (29^{o} C, 80% humidity, and 12 hour photoperiod) in BOD incubator.

Males were placed with some females inside a feeding chamber previously glue with an atoxic and non-lesive preparation (Britannia Adhesive-Unit 4, UK) to the shave back of the host (rabbit) according to technique described elsewhere (Bechara et al., 1995). After seven days post-attachment, males were collected and a group was assigned to histological procedures. Another group was maintained alive for three and seven days in closed containers with breathing holes to be later analyzed morphologically and histochemically.

Upon completion of the feeding periods, males were collected and salivary glands were removed in saline solution and fixed in a 10 % neutral buffered formalin and acetone solution (9:1) for one hour and thirty minutes at 4^{o} C. After fixation, the material was dehydrated in a series of increasing concentrations of alcohol (70%, 80%, 90% and 95%), embedded in resin (Leica), and sectioned at 3 μm thickness. Sections were mounted on glass slides and stained with Hematoxylin-Eosin and PAS (Periodic Acid Schiff) McManus (1946) for detection of polysaccharides. Samples were counterstained with Methyl Green to demonstrate chromatin, later mounted in Canada balsam, and observed under light microscope.

RESULTS

Males at day seven post-attachment

The salivary glands of these individuals exhibit intact type I acinus (Figs. 1, 53), while types II (Figs. 2-10, 54-59), III (Figs. 11-16, 60) and IV (Figs. 17-19, 61) acini are undergoing degeneration.

Intact acini present regular shape (Figs. 1, 53); cells containing fibrillar structures in their cytoplasm (Fig. 53) and nuclei of **central** cells as well as **peripheral** ones do not exhibit changes (Figs. 1, 53).

Type II acini may be regular (Figs. 3, 8, 10, 54, 55, 57, 59) or irregular (Fig. 2, 4-7, 9, 56, 58), some cells **c1** and **c8** are intact (Figs. 55, 57) while others are undergoing degeneration and exhibit nuclear changes (Figs. 54, 3). Degenerating cells are, **a:** cubic (Figs. 2, 3, 54-56, 58, 59) or irregular (Fig. 4) cells exhibiting intact (Figs. 2, 54-56, 58) or broken (Figs. 3, 4) granules and nuclear changes (Figs. 3); **c1:** cubic cells exhibiting intact granules (Figs. 54, 55) and intact (Fig. 55) or changed (Fig. 54) nuclei; **c2:** cubic (Figs. 5, 55) or irregular (Fig. 6) cells exhibiting intact granules (Figs. 5, 6, 55) and nuclear changes (Figs. 5); **c3:** cubic cells exhibiting broken granules and nuclear changes (Figs. 7, 54, 55); **c4:** cubic (Figs. 8, 56) or irregular (Fig. 9) cells exhibiting intact granules (Figs. 8, 9, 56) and nuclear changes (Figs. 9 and 56); **c5:** cubic cells exhibiting broken granules (Figs. 2, 6, 56-58) and nuclear changes (Figs. 2, 57, 58) and **c8:** cubic cells exhibiting intact granules (Figs. 3, 57, 59) and intact (Fig. 57) or changed (Figs. 3) nuclei. Were also observed some cells that could not be identified due to advanced stages of degeneration. These cells were termed **indeterminate:** cubic (Figs. 4, 59) or irregular (Figs. 10, 58) cells exhibiting remnants of secretion (Figs. 4, 10, 58, 59) and nuclear changes (Figs. 10, 58, 59). Included in this group are cells **b**, **c6**, and **c7** that either already degenerated or are in late stages of degeneration, and are no longer observed.

Type III acini may be irregular (Figs. 12, 15, 16) and some, fragmenting (Fig. 13). Degenerating cells are all cells **d:** cubic (Figs. 11, 14, 60), irregular (Fig. 12) or fragmenting (Fig. 13) cells exhibiting broken granules (Figs. 11-14, 60) and nuclear changes (Figs. 11-13); **e:** cubic (Figs. 14, 60), irregular (Fig. 15) or fragmenting (Fig.

13) cells exhibiting broken granules (Fig. 13-15, 60) and nuclear changes (Figs. 15 and 60), and **f:** irregular cells exhibiting nuclear changes (Figs. 16).

In type IV acini, cells **g** are cubic (Figs. 17, 61), irregular (Fig. 18) or fragmenting (Fig. 19) and present broken granules (Figs. 17-19, 61), cytoplasm shrinkage (Fig. 18) and nuclear changes (Figs. 17, 18, 61).

Males at day three post-detachment

In these individuals, all acini exhibit more prominent degenerative characteristics (Figs. 20-35, 62-67). Acini with cells that have lost their original granular characteristics are also observed, and could not be identified: these were termed **Indeterminate** (Figs. 33, 34). Apoptotic bodies are formed (Fig. 35).

Type I acini are regular (Figs. 20, 62) with cells exhibiting fibrillar structures in the cytoplasm (Fig. 62), in addition to nuclear changes (Figs. 20, 62).

Type II acini may be irregular (Figs. 22-24, 63-65). Cells **a:** cubic (Figs. 21, 22, 24, 64, 65) or irregular (Figs. 23, 63) cells exhibiting intact (Figs. 21, 63-65) or broken (Figs. 22-24) granules and nuclear changes (Figs. 21, 63); **c1:** cubic cells exhibiting broken granules (Figs. 24, 64) and nuclear changes (Figs. 64); **c2:** cubic (Fig. 24) or irregular cells (Fig. 25) exhibiting intact granules and nuclear changes (Figs. 24, 25); **c3:** cubic (Figs. 21, 24, 64) or irregular (Figs. 22, 25) cells exhibiting broken granules (Fig. 21, 22, 24, 25, 64) and nuclear changes (Figs. 21, 22, 24); **c4:** irregular cells exhibiting intact granules, and nuclear changes (Figs. 23); **c5:** cubic cells exhibiting broken granules (Figs. 23, 26, 63-65), and **c8:** irregular cells exhibiting broken granules (Figs. 23, 64, 65) and intact (Fig. 64) or changed (Fig. 65) nuclei are undergoing degeneration. **indeterminate** cells: irregular (Figs. 27, 64, 65) cells exhibiting remnants of secretion (Figs. 64, 65) are observed in higher numbers than in the previous feeding stage (Figs. 4, 10, 58, 59).

Type III acini are regular (Figs. 28, 66), irregular (Fig. 29) or fragmenting (Fig. 30). Degenerating cells are, **d:** cubic (Figs. 28, 66), irregular (Fig. 29) or fragmenting (Fig. 30) cells exhibiting broken granules (Figs. 28-30, 66) and nuclear changes (Figs. 28, 30) and **e:** cubic (Figs. 28, 66), irregular (Fig. 29) or fragmenting (Fig. 30) cells

exhibiting broken granules (Figs. 28-30, 66) and nuclear changes (Fig. 66). Cells **f** are no longer observed.

In type IV acini, cells **g** are irregular (Figs. 31, 67) or fragmenting (Fig. 32) and exhibit broken granules (Figs. 31, 32, 67), cytoplasmic shrinkage (Fig. 31), and nuclear changes (Figs. 31, 67).

Indeterminate acini are irregular (Figs. 33, 34), showing cytoplasmic shrinkage (Fig. 33), and nuclear changes (Fig. 33). Some acini still contain a vacuolated cytoplasmic mass and some changed nuclei (Fig. 34).

Males at day seven post-detachment from the host

At this stage (Figs. 36-52, 68-75), more prominent degenerative characteristics are observed than at day three post-detachment from the host (Figs. 20-35, 62-67), such as **indeterminate** cells (Figs. 39, 42-44, 69, 70, 72), **Indeterminate** acini (Fig. 51), and more abundant apoptotic bodies (Fig. 52).

Type I acini are regular (Figs. 36, 68) and the cytoplasm of cells contain fibrillar structures (Fig. 68) and changed nuclei (Figs. 36, 68).

Type II acini may be irregular (Figs. 37, 41-45, 70, 72). Cells exhibit the following degenerative changes, **a:** cubic (Figs. 37, 38, 41, 42, 44, 69, 70, 72) or irregular (Figs. 39, 43) cells exhibiting broken granules (Figs. 37-39, 41-44, 69, 70, 72) and nuclear changes (Figs. 38, 69, 70); **c1:** cubic cells exhibiting broken granules (Fig. 38, 39, 70) and nuclear changes (Figs. 39, 70); **c3:** cubic (Figs. 39, 40, 43, 70, 71) or irregular (Fig. 41) cells exhibiting broken granules (Figs. 39-41, 43, 44, 70, 71) and nuclear changes (Figs. 41, 71); **c4:** irregular cells exhibiting intact granules and nuclear changes (Figs. 38, 71); **c5:** cubic cells exhibiting broken granules (Figs. 42, 69, 72) and nuclear changes (Figs. 42, 69); **c8:** irregular cells exhibiting broken granules (Figs. 38, 69, 71, 72) and nuclear changes (Fig. 69), and **indeterminate:** cubic (Figs. 39, 42, 43, 69, 70, 72) or irregular (Fig. 44) cells exhibiting remnants of secretion (Figs. 44, 69, 70, 72) and nuclear changes (Figs. 39, 42, 43, 44, 69). Some type II acini appear as a cytoplasmic mass (Figs. 41, 45) containing remnants of secretion (Fig. 45) and changed nuclei (Figs. 41, 45).

Type III acini are regular (Fig. 46), irregular (Figs. 47, 73, 74) or fragmenting (Fig. 48). Cells **d** and **e** are cubic (Fig. 46), irregular (Figs. 47, 73, 74) or fragmenting

(Fig. 48) and exhibit broken granules (Figs. 46-48, 73, 74) and nuclear changes (Figs. 46-48, 73).

In irregular (Figs. 49, 75) or fragmenting (Fig. 50) type IV acini, only a cytoplasmic mass with secretion is observed (Figs. 49, 50, 75). Nuclei exhibit changes (Figs. 49, 50, 75).

Indeterminate acini are irregular, vacuolated, with remnants of secretion in the cytoplasmic mass (Fig. 51). Nuclei exhibit changes (Fig. 51).

To better compare the results, the data are summarized in Tables **1**, **2**, and **3**.

Table 1: Morpho-histochemical aspects of cells and acini of salivary glands of *Rhipicephalus sanguineus* males at day seven post-attachment.

Acini	Cells	Cell Characteristics		
		Shape	**Cytoplasm**	**Nucleus**
I	**central and peripheral**	- ∅ (Figs. 1, 53);	- fibrillar structures PAS ++ (Fig. 53);	- no changes (Figs. 1, 53);
II	**a**	- cubic (Figs. 2, 3, 54-56, 58, 59); - irregular (Fig. 4);	- granules PAS – (Figs. 2, 54-56, 58) - * (Figs. 3, 4);	- picnotic (Fig. 3);
	c1	- cubic (Figs. 54, 55);	- granules PAS +++ (Figs. 54, 55);	- intact (Fig. 55); - enlarged (Fig. 54);
	c2	- cubic (Figs. 5, 55); - irregular (Fig. 6);	- granules PAS + (Figs. 5, 6, 55);	- enlarged and irregular (Fig. 5);
	c3	- cubic (Figs. 7, 54, 55);	- * (Figs. 7, 54, 55);	- picnotic (Fig. 7); - enlarged (Figs. 54, 55);
	c4	- cubic (Figs. 8, 56) - irregular (Fig. 9);	- elliptic granules PAS – (Figs. 8, 9, 56);	- enlarged (Fig. 9); - irregular (Fig. 56);
	c5	- cubic (Figs. 2, 6, 56-58);	- * (Figs. 2, 6, 56-58);	- enlarged and irregular (Fig. 2); - enlarged (Figs. 57, 58);
	c8	- cubic (Figs. 3, 57, 59);	- larger granules than those of **c3** cells PAS + (Figs. 3, 57, 59);	- irregular (Fig. 3); - intact (Fig. 57);
	indeterminate	- cubic (Figs. 4, 59); - irregular (Figs. 10, 58);	- remnants of secretion (Fig. 4, 10, 58, 59);	- picnotic (Fig. 10); - enlarged (Figs. 58, 59);
III	**d**	- cubic (Figs. 11, 14, 60); - irregular (Fig. 12); - fragmenting (Fig. 13);	- * (Figs. 11, 12-14, 60);	- picnotic (Figs. 11, 12); - irregular (Fig. 13);
	e	- cubic (Figs. 14, 60); - irregular (Fig. 15); - fragmenting (Fig. 13);	- * (Figs. 13, 14, 15, 60);	- enlarged and irregular (Fig. 15); - picnotic (Fig. 60);
	f	- irregular (Fig. 16);	- scarce (Fig. 16);	- irregular (Fig. 16);
IV	**g**	- cubic (Figs. 17, 61); - irregular (Fig. 18); - fragmenting (Fig. 19);	- * (Figs. 17-19, 61); - cytoplasmic shrinkage (Fig. 18);	- picnotic (Figs. 17, 61); - enlarged (Fig. 18); - fragmenting (Fig. 18);

∅: cell boundaries not evident; *: broken secretion granules;

Table 2: Morpho-histochemical aspects of cells and acini of salivary glands of *Rhipicephalus sanguineus* males at day three post-detachment.

Acini	Cells	Cell Characteristics		
		Shape	**Cytoplasm**	**Nucleus**
I	**central and peripheral**	- ∅ (Figs. 20, 62);	- fibrillar structures PAS +++ (Fig. 62);	- chromatin margination (Figs. 20, 62);
II	**a**	- cubic (Figs. 21, 22, 24, 64, 65); - irregular (Fig. 23, 63);	- granules PAS – (Figs. 21, 63-65); - * (Figs. 22-24);	- fragmented (Fig. 21); - picnotic (Fig. 63);
	c1	- cubic (Figs. 24, 64);	- * (Figs. 24, 64);	- enlarged (Fig. 64);
	c2	- cubic (Fig. 24); - irregular (Fig. 25);	- granules (Figs. 24, 25);	- enlarged (Figs. 24, 25);
	c3	- cubic (Figs. 21, 24, 64); - irregular (Figs. 22, 25);	- * (Figs. 21, 22, 24, 25, 64);	- picnotic (Fig. 21); - enlarged (Figs. 22, 24);
	c4	- irregular (Fig. 23);	- elliptic granules PAS – (Fig. 23);	- irregular (Fig. 23); - enlarged and irregular (Fig. 23);
	c5	- cubic (Figs. 23, 26, 63-65);	- * (Figs. 23, 26, 63-65);	- not observed;
	c8	- irregular (Figs. 23, 64, 65);	- * (Figs. 23, 64, 65);	- intact (Fig. 64); - irregular (Fig. 65);
	indeterminate	- irregular (Figs. 27, 64, 65);	- remnants of secretion (Figs. 64, 65);	- not observed;
III	**d**	- cubic (Figs. 28, 60); - irregular (Fig. 29); - fragmenting (Fig. 30);	- * (Figs. 28-30, 66);	- irregular (Fig. 28); - picnotic (Fig. 30);
	e	- cubic (Figs. 28, 66); - irregular (Fig. 29); - fragmenting (Fig. 30);	- * (Figs. 28-30, 66);	- picnotic (Fig. 66);
IV	**g**	- irregular (Figs. 31, 67); - fragmenting (Fig. 32);	- * (Figs. 31, 32, 67); - cytoplasmic shrinkage (Fig. 31);	- irregular (Fig. 31); - picnotic (Fig. 67);
Indeterminate		- irregular (Fig. 33);	- cytoplasmic shrinkage (Fig. 33);	- fragmented (Fig. 33);
		- # (Fig. 34);	- ! vacuolated (Fig. 34);	- enlarged (Fig. 34);

∅: cell boundaries not evident; *: broken secretion granules; #: loss of cell boundaries; **!**: cytoplasmic mass;

Table 3: Morpho-histochemical aspects of cells and acini of salivary glands of *Rhipicephalus sanguineus* males at day seven post-detachment.

Acini	Cells	Cell Characteristics		
		Shape	**Cytoplasm**	**Nucleus**
I	**central and peripheral**	- ∅ (Figs. 36, 68);	- fibrillar structures PAS +++ (Fig. 68);	- enlarged and with chromatin margination (Figs. 36, 68);
II	**a**	- cubic (Figs. 37, 38, 41, 42, 44, 69, 70, 72); - irregular (Figs. 39, 43);	- * (Figs. 37-39, 41-44, 69, 70, 72);	- picnotic (Figs. 38, 69, 70);
	c1	- cubic (Figs. 38, 39, 70);	- * (Figs. 38, 39, 70);	- enlarged (Fig. 39); - picnotic (Fig. 70);
	c3	- cubic (Figs. 39, 40, 43, 70, 71); - irregular (Fig. 41);	- * (Figs. 39-41, 43, 44, 71);	- enlarged and irregular (Fig. 41); - enlarged (Fig. 71);
	c4	- irregular (Figs. 38, 71);	- elliptic granules PAS – (Figs. 38, 71);	- irregular (Fig. 38); - picnotic (Fig. 38); - enlarged (Fig. 71);
	c5	- cubic (Figs. 42, 69, 72);	- * (Figs. 42, 69, 72);	- enlarged and irregular (Fig. 42); - enlarged (Fig. 69);
	c8	- irregular (Figs. 38, 69, 71, 72);	- * (Figs. 38, 69, 71, 72);	- picnotic (Figs. 69);
	indeterminate	- cubic (Figs. 39, 42, 43, 69, 70, 72); - irregular (Fig. 44);	- remnants of secretion (Figs. 44, 69, 70, 72);	- enlarged (Figs. 39, 43, 69); - enlarged and irregular (Figs. 42, 44);
		- # (Figs. 41, 45);	- ! with (Fig. 45) or without (Fig. 41) remnants of secretion;	- irregular (Fig. 41); - enlarged (Fig. 41); - enlarged and irregular (Fig. 45);
III	**d**	- cubic (Fig. 46); - irregular (Figs. 47, 73, 74); - fragmenting (Fig. 48);	- * (Figs. 46-48, 73);	- picnotic (Figs. 47, 48); - enlarged and irregular (Fig. 73);
	e	- cubic (Fig. 46); - irregular (Figs. 47, 73, 74); - fragmenting (Fig. 48);	- * (Figs. 46-48, 74);	- enlarged and irregular (Fig. 46); - irregular (Fig. 47); - picnotic (Figs. 46, 47, 48);
IV		- # (Figs. 49, 50, 75);	- cytoplasmic mass with * (Figs. 49, 50, 75);	- fragmenting (Figs. 49, 50); - enlarged and irregular (Fig. 75); - picnotic (Fig. 75);
Indeterminate		- # (Fig. 51);	- ! vacuolated and with remnants of secretion (Fig. 51);	- irregular (Fig. 51); - enlarged (Fig. 51); - enlarged and irregular (Fig. 51)

∅: cell boundaries not evident; *: broken secretion granules; #: loss of cell boundaries; !: cytoplasmic mass;

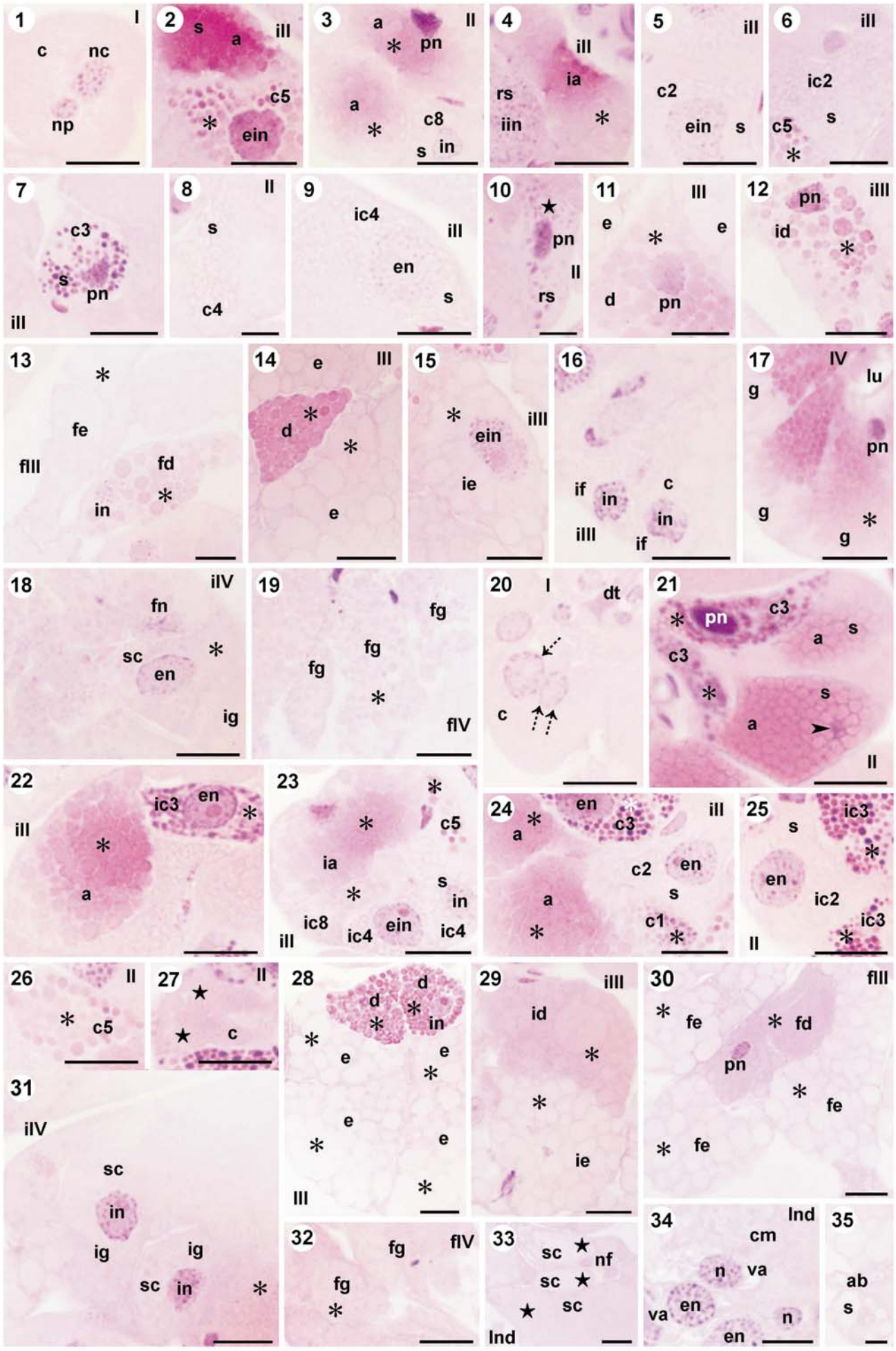
FIGURES

FIGURES 1-35:

Histological sections of salivary glands of males of *Rhipicephalus sanguineus* stained with Hematoxylin-Eosin. **1-19**. Males at day seven post-attachment. **1.** Intact type I acinus; **2-10.** Type II acini undergoing degeneration, note in **2**, **4-7** and **9**, irregular shaped type II acini (**iII**). **11-16.** Type III acini undergoing degeneration, observe in **12**, **15**, and **16**, irregular shaped type III (**iIII**) and **13**, type III acinus undergoing fragmentation (**fIII**). **17-19.** Type IV acini undergoing degeneration, note in **18**, irregular shaped type IV acinus (**iIV**) and **19**, type IV acinus undergoing fragmentation (**fIV**). **20-35.** Males at day three post-detachment. **20.** Type I acinus with characteristics of degeneration. **21-27.** Type II acini undergoing degeneration, note in **22-24**, irregular shaped type II acini (**iII**). **28-30.** Type III acini undergoing degeneration, observe in **29**, irregular shaped type III acinus (**iIII**) and **30**, type III acinus undergoing fragmentation (**fIII**). **31** and **32**. Type IV acini undergoing degeneration, in **31**, irregular shaped type IV acinus (**iIV**) and **32**, type IV acinus undergoing fragmentation (**fIV**). **33** and **34**. **Indeterminate** acini (**Ind**). **35.** Apoptotic body (**ab**).

I-IV: acini with regular shape; **a**, **c1**-**c5**, **c8**, **d**, **e** and **g:** cells with cuboidal shape; **iin: indeterminate** cuboidal cell; **ia**, **ic2**-**ic4**, **ic8**, **id**, **ie**, **if** and **ig:** cells with irregular shape; ⋆ **:** irregular shaped **indeterminate** cell; **fd**, **fe** and **fg:** cells undergoing fragmentation; **c:** cytoplasm; **nc:** nucleus of **central** cell; **np:** nucleus of **peripheral** cell; **s:** secretion; ∗**:** broken secretion granules; **in:** irregular nucleus; **rs:** remnants of secretion; **ein:** enlarged and irregular nucleus; **pn:** picnotic nucleus; **en:** enlarge nucleus; **lu:** lumen; **sc:** cytoplasmic shrinkage; **fn:** fragmenting nucleus; **dashed arrow:** nucleus of the **central** cell with chromatin margination; **double dashed arrow:** nucleus of **peripheral** cell with chromatin margination; **arrow head:** fragmented nucleus; **cm:** cytoplasmic mass; **n:** nucleus; **va:** vacuole.

Bars: 8, **10** and **35**= 10 μm; **1-7**, **9** and **11-34**= 20 μm.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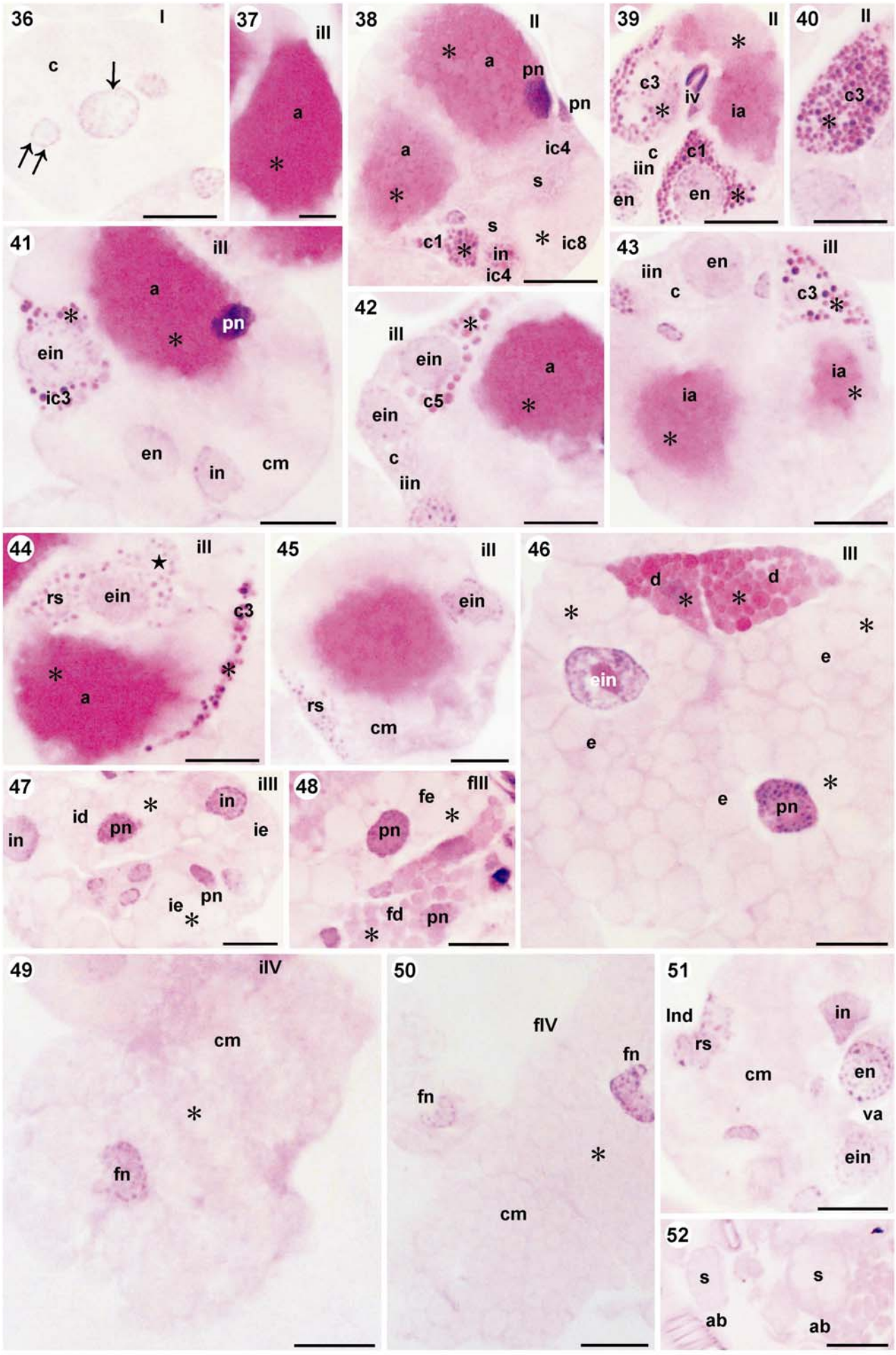
FIGURES 36-52:

Histological sections of salivary glands of *Rhipicephalus sanguineus* males at day seven post-detachment stained with Hematoxylin-Eosin. **36**. Type I acinus undergoing degeneration. **37-45.** Type II acini undergoing degeneration, note in **37** and **41-45**, irregular shaped type II acini (**iII**). **46-48.** Type III acini undergoing degeneration, observe in **47**, irregular shaped type III acinus (**iIII**) and **48**, type III undergoing fragmentation (**fIII**). **49** and **50.** Type IV undergoing degeneration, in **49**, irregular shaped type IV acinus (**iIV**) and **50**, Type IV acinus undergoing fragmentation (**fIV**). **51. Indeterminate** acinus (**Ind**). **52.** Apoptotic bodies (**ab**).

I-III: acini with regular shape; **a**, **c1**, **c3**, **c5**, **d** and **e:** cells with cuboidal shape; **iin: indeterminate** cuboidal cell; **ia**, **ic3**, **ic4**, **ic8**, **id** and **ie:** cells with irregular shape; ⋆ **:** irregular shaped **indeterminate** cell; **fd** and **fe:** cells undergoing fragmentation; **c:** cytoplasm; **arrow:** enlarged nucleus of the **central** cell with chromatin margination; **double arrow:** enlarged nucleus of **peripheral** cell with chromatin margination; **s:** secretion; **∗:** broken secretion granules; **in:** irregular nucleus; **pn:** picnotic nucleus; **iv:** intraacinar valve; **en:** enlarged nucleus; **ein:** enlarged and irregular nucleus; **cm:** cytoplasmic mass; **rs:** remnants of secretion; **fn:** fragmenting nucleus; **va:** vacuole.

Bars: 37= 10 μm; **36** and **38-52**= 20 μm.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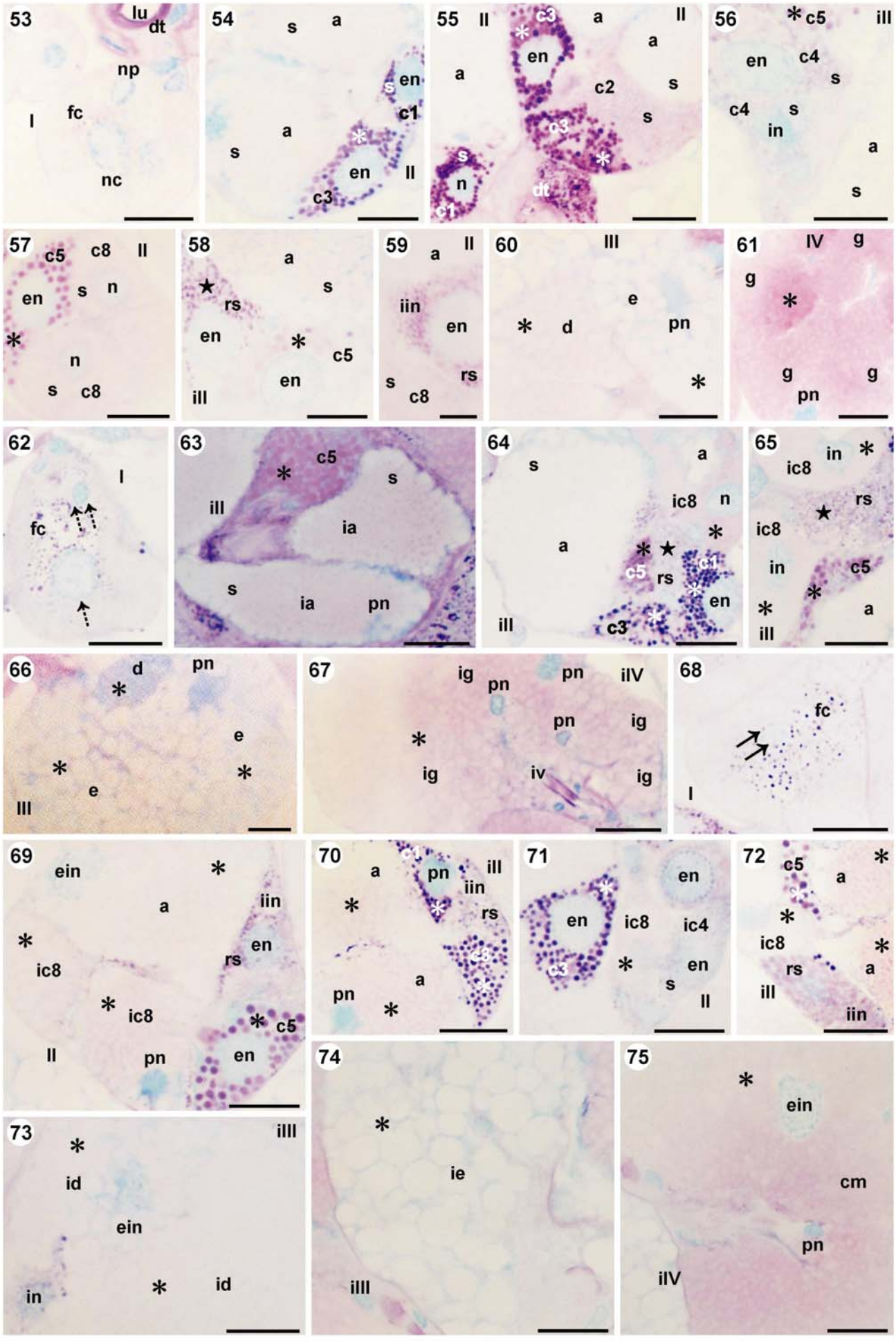
FIGURES 53-74:

Histological sections of salivary glands of *Rhipicephalus sanguineus* males stained with PAS and counterstained with Methyl Green. **53-61.** Males at day seven post-attachment. **53.** Intact type I acinus. **54-59.** Type II acini undergoing degeneration, note in **56** and **58** irregular shaped type II acini (**iII**). Observe in **55**, intact cell **c1** (**c1**) and in **57**, intact cell **c8** (**c8**). **60.** Type III acinus undergoing degeneration. **61.** Type IV acinus undergoing degeneration. **62-67.** Males at day three post-detachment. **62.** Type I acinus undergoing degeneration. **63-65.** Type II acini undergoing degeneration and with irregular shape (**iII**). **66.** Type III acinus undergoing degeneration. **67.** Type IV acinus undergoing degeneration and with irregular shape (**iIV**). **68-75.** Males at day seven post-detachment. **68.** Type I acinus undergoing degeneration. **69-72.** Type II acini undergoing degeneration, note in **70** and **72**, irregular shaped type II acini (**iII**). **73** and **74.** Type III acini undergoing degeneration and with irregular shape (**iIII**). **75.** Type IV acinus undergoing degeneration and with irregular shape (**iIV**).

I-IV: acinis with regular shape; **a**-**c5**, **c8**, **d**, **e** and **g:** cells with cuboidal shape; **iin: indeterminate** cuboidal cell; ⋆ **:** irregular shaped **indeterminate** cell; **ig:** irregular shaped **g** cell; **fc:** fibrillar cytoplasm; **nc:** nucleus of **central** cell; **np:** nucleus of **peripheral** cell; **dt:** ducto; **lu:** lumen; **s:** secretion; **en:** enlarged nucleus; **n:** nucleus; **in:** irregular nucleus; ∗**:** broken secretion granules; **rs:** remnants of secretion; **dashed arrow:** nucleus of the **central** cell with chromatin margination; **double dashed arrow:** nucleus of **peripheral** cell with chromatin margination; **pn:** picnotic nucleus; **iv:** intraacinar valve; **double arrow:** enlarged nucleus of **peripheral** cell with chromatin margination; **s:** secretion; **ein:** enlarged and irregular nucleus; **cm:** cytoplasmic mass.

Bars: 59= 10 μm; **66**= 15 μm; **53-58**, **60-65** and **67-75**= 20 μm.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DISCUSSION

This morphological and histochemical study detected degenerative characteristics in the cells of salivary glands of *Rhipicephalus sanguineus* males at day seven post-attachment, and at day three and seven post-detachment from the host, shedding light on the changes undergone by this organ during the degenerative process.

The onset of this process in the individuals examined caused a reduction in the secretory capacity of salivary glands, indicated by a decrease in secretion in cells. This was also observed in *Amblyomma hebraeum* and *R. sanguineus* females, as reported by Harris and Kaufman (1984) and Furquim (2005), respectively.

These results revealed that the degenerative process in the glands of *R. sanguineus* males is asynchronous among different types of acini, with more prominent changes observed in types III and IV acini, as well as acini of the same type. Asynchrony among cell types in the same acini was also observed. Cells at different stages of degeneration coexisted and some of them could not be identified, as reported by Till (1961), L'Amoreaux et al. (2003), Nunes et al. (2006), and Furquim (2005) for females of *R. appendiculatus*, *D. variabilis*, *R. (Boophilus) microplus* and *R. sanguineus*, respectively.

The present study clearly showed that the degenerative process in glands of *R. sanguineus* males followed the same pattern observed in females of the same species (Furquim, 2005). The onset of degeneration began in the posterior region of the gland, first affecting types II, III, and IV acini, which were more distally located. Gland degeneration advanced as time away from the host progressed. More prominent changes were therefore observed in males at day seven post-detachment from the host than in those at day seven post-attachment, as in the latter, type I acini were intact, while types II, III, and IV were undergoing degeneration. Thus, if *R. sanguineus* males at days three and seven post-detachment were allowed to reattach to a host, they would probably not resume feeding, since their salivary glands were in advanced stages of degeneration. Kaufman (1990), on the other hand, observed a reduction of 62% in the secretory capacity of glands of *A. hebraeum* males removed from the host for four days compared to those examined immediately after removal. However, despite being reduced, the secretory capacity was reversed when males resumed feeding.

The advance in the process of salivary gland degeneration in *R. sanguineus* males as time away from the host progressed confirmed the data obtained by Furquim (2005) for females of this species. In the latter, more prominent characteristics of gland degeneration were observed at day three post-engorgement than in engorged individuals; and at day seven post-engorgement, the salivary gland was completely degenerated (Furquim, 2005). In males, gland degeneration less intense than in females, as female glands at day seven post-engorgement were already degenerated (Furquim, 2005) while in males at day seven post-detachment, some secretory cells were still observed, although the entire tissue was undergoing degeneration.

Also, the salivary glands of *R. sanguineus* males exhibited less prominent changes than those of females (Furquim, 2005). In the latter, one of the factors triggering the onset of gland degeneration is the "critical weight" (Weiss and Kaufman, 2001). This does not occur in males, since the small amount of blood ingested does not allow the tick to reach the weight ("critical weight") to induce gland degeneration. Therefore, salivary gland degeneration in these individuals is not controlled by the volume of blood consumed. In females, degeneration may also be triggered by hormones (Lomas et al., 1998) synthesized and released in function of the "critical weight" (Weiss and Kaufman, 2001) and the release of the "male factor" (Weiss and Kaufman, 2004), which is transferred from the male to the female during matting (Weiss and Kaufman, 2004). The synthesis and release of hormones in females increase until the post-engorgement phase (Lomas, 1993 apud Lomas et al., 1998). In males, on the other hand, hormonal levels are constant, regardless of the feeding condition (Kaufman, 1990), and therefore without a stimulus to maintain the degenerative process.

In *R. sanguineus* males at day seven post-attachment examined in this study, type I acinus did not exhibited changes, unlike the observed in those at day three and seven post-detachment from the host. Morphological changes, such as changes in size, were observed. At day seven post-detachment from the host, acini were larger than those of males at day seven post-detachment, confirming the data obtained for females of the same species at day three post-engorgement (Furquim, 2005).

Type II acinus of *R. sanguineus* males at day seven post-attachment and at day three post-detachment exhibited both intact and changed cells, suggesting a functional

asynchrony among them. These cells may be active in different moments during feeding, as well as become inactive at different stages, as a result of several degrees of cell degeneration.

In males at day seven post-attachment, among identified cells, **c1** and **c8** were intact or undergoing degeneration. All cells **a** and **c2–c5** were undergoing degeneration, unlike the observed in females of the same species. In the latter, type II acini did not exhibit intact cells, and only cells **a**, **c1** and **c3** were observed (Furquim, 2005). At day three and seven post-detachment from the host, males exhibited cells **a**, **c1–c5** and **c8,** and **a**, **c1**, **c3–c5** and **c8**, respectively, unlike the observed in females at day three post-engorgement, which presented only cells **a** and **c3** (Furquim, 2005). The cell types still present in type II acini at the end of the degeneration process in the males examined in our study might be associated with the distinct behavior between males and females, confirming their distinct needs regarding the activity of different secretory cell types of their salivary glands.

In some type III acini of males at day seven post-attachment, the presence of degenerating and fragmenting cells **d**, **e** and **f** partially agree with the results obtained in engorged *R. sanguineus* females (Furquim, 2005). Contrary to the observed in the present study, fragmentation of type III acinus and broken secretion granules in cells **d** were not reported in these females. In males with three and seven days post-detachment from the host, type III acini were still undergoing degeneration, unlike females at day three post-engorgement. Type III acini of the latter were in such advanced stages of degeneration that cells could no longer be identified (Furquim, 2005).

Type IV acinus of males examined in the three conditions exhibited changes that became more prominent as time away from the host progressed, contrary to the reported by Gill and Walker (1987). These authors described in *Hyalomma anatolicum anatolicum* only morphological changes in the secretion granules of cells in final feeding stages (120 to 144 hours of feeding).

In general, the results obtained in the present study showed that the degenerative changes in the salivary glands of male ticks were not the same in different cells. Some exhibited reduction in secretion (interruption of synthesis) and in some cases, broken granules that appeared as an amorphous mass. Thereafter, we observed changes in shape and size of nuclei of cells, as well as condensation level of chromatin, followed

by cytoplasmic shrinkage, loss of cell shape, loss of cell boundaries, and cytoplasmic vacuolation and consequently breakdown of acini, formation and release of apoptotic bodies. These characteristics clearly indicate that the cells of salivary glands of *R. sanguineus* males most likely die by apoptosis, similarly to glands of females of this species (Furquim, 2005).

ACKNOWLEDGMENTS

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REFERENCES

Bechara, G.H., Szabó, M.P.J., Ferreira, B.R., Garcia, M.V., 1995. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditons. Brazil. J. Vet. Parasitol. 4, 61-66.

Binnington, K.C., 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, *Boophilus microplus*. Int. J. Parasitol. 8, 97-115.

Furquim, K.C.S., 2005. Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados. 2º Relatório Científico CAPES, Instituto de Biociências, UNESP, Rio Claro (S.P.), Brasil, 40 p.

Furquim, K.C.S., 2006. Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados. 3º Relatório Científico CAPES, Instituto de Biociências, UNESP, Rio Claro (S.P.), Brasil, 27 p.

Gill, H.S., Walker, A.R., 1987. The salivary glands of *Hyalomma anatolicum anatolicum*: structural changes during attachment and feeding. Int. J. Parasitol. 17 (8), 1381-1392.

Harris, R.A., Kaufman, W.R., 1984. Neural involvement in the control of salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*. J. Exp. Biol. 109, 281-290.

Kaufman, W.E., 1990. Effect of 20-hydroxiecdysone on the salivary glands of the male tick, *Amblyomma hebraeum*. Exp. Appl. Acarol. 9, 87-95.

L'Amoreaux, W.J., Junaid, L., Trevidi, S., 2003. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. Tissue Cell 35, 95-99.

Lomas, L.O., 1993. The influence of a male-derived protein factor on salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum* Koch. PhD thesis, University of Alberta, Edmond, Canada.

Lomas, O.L., Gelman, D., Kaufman, W. R., 1998. Ecdysteroid regulation of salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*: a reconciliation of *in vivo* and *in vitro* observations. Gen. Comp. Endocrinol. 109, 200-211.

McManus, J.F.A., 1946. Histological demonstration of mucin after periodic acid. Nature 158, 202.

Nunes, E.T., Camargo-Mathias, M.I., Bechara, G.H., 2006. Structural and cytochemical changes in the salivary glands of the *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae) tick female during feeding. Vet. Parasitol. 140, 114-123.

Sanders, M.L., Scott, A.L., Glass, G.E., Schwartz, B.S., 1996. Salivary gland changes and host antibody responses associated with feeding of male lone star ticks (Acari: Ixodidae). J. Med. Entomol. 33 (4), 628-634.

Till, W.M., 1961. A Contribution to the Anatomy and Histology of the Brown Ear Tick *Rhipicephalus appendiculatus* Neumann. Memoirs of the Entomological Society of Southern Africa, Pretoria, 6, 1-124.

Walker, A.R., Fletcher, J.D., Gill, H., 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. Int. J. Parasitol. 15 (1), 81-100.

Wang, H., Henbest, P.J., Nuttall, P.A., 1999. Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by re-programming of salivary gland protein expression. Parasitology 119, 143-149.

Weiss, L.B., Kaufman, W.R., 2001. The relationship between 'critical weight' and 20-hydroxiecdysone in the female ixodid tick, *Amblyomma hebraeum*. J. Insect Physiol. 47, 1261-1267.

Weiss, L.B., Kaufman, W.R., 2004. Two feeding-induced proteins from the male gonad trigger engorgement of the female tick *Amblyomma hebraeum*. Proc. Natl. Acad. Sci. S.A. 101 (16), 5874-5879.

Capítulo 6

CAPÍTULO 6

TITLE: The process of cell death in salivary glands of males of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae).

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RESUMO

As glândulas salivares de machos de carrapatos *Rhipicephalus sanguineus* nos estados de jejum, sete dias de infestação, três e sete dias pós-remoção do hospedeiro foram submetidas a testes citoquímicos para análises enzimática e da viabilidade celular. No estudo comparativo do tecido glandular nestas quatro situações observou-se que a marcação nas células dos ácinos IV, III, II e I foi diferente, afetados pela degeneração nesta sequência. Este estudo, também revelou: alterações nucleares, na intensidade da marcação da fosfatase ácida e de ATPase e na permeabilidade da membrana plasmática, sendo que a positividade da reação para fosfatase foi inversamente proporcional à da ATPase e a positividade da reação para a ATPase foi proporcional à integridade da membrana.

O tecido glandular dos machos em jejum apresentou alta atividade metabólica com células com núcleo e membrana plasmática íntegros. Conclui-se que a presença de fosfatase ácida nestes indivíduos faz parte da fisiologia de alguns ácinos, visto que este tecido não está em degeneração. Nos machos com sete dias de infestação foi observada membrana íntegra nas células dos ácinos I, II e na maioria dos III e IV, bem como maior marcação para fosfatase ácida, alterações nucleares e queda na atividade da ATPase, alterações que fazem parte do processo degenerativo. Naqueles com três e sete dias pós-remoção do hospedeiro o processo degenerativo estava mais avançado, com perda da integridade da membrana nas células de alguns ácinos I e II e na maioria dos III e IV nos machos com três dias pós-remoção do hospedeiro, nas de poucos ácinos I e II, na maioria dos III e em todos os ácinos IV naqueles com sete dias pós-remoção do hospedeiro. Houve também alteração nuclear, marcante redução da atividade ATPásica, aumento da atividade da fosfatase ácida naqueles com três dias e diminuição desta nos com sete dias pós-remoção do hospedeiro.

Nestas glândulas a morte celular ocorreu numa seqüência onde as alterações nucleares precederam as citoplasmáticas, como segue: a) alteração no núcleo, b) perda da atividade da ATPase, c) perda da integridade da membrana plasmática e d) aumento da atividade da fosfatase ácida. Esta última estaria relacionada com a degradação tardia de porções citoplasmáticas, caracterizando assim o processo de morte celular em glândulas de machos de *R. sanguineus* como apoptose atípica ou não clássica.

PALAVRAS-CHAVE: *Rhipicephalus sanguineus*, glândulas salivares, carrapatos, machos, atividade enzimática, apoptose.

ABSTRACT

The salivary glands of males of the tick *Rhipicephalus sanguineus* at four feeding stages: unfed, at day seven post-attachment, and at day three and seven post-detachment from the host were subjected to cytochemical methods of enzymatic analysis and cell viability. Comparing gland tissues at these stages, we observed different staining patterns in the cells of the types IV, III, II acini, which were affected by degeneration in this sequence. This study also revealed changes in: nuclei, staining intensity for acid phosphatase and ATPase activities, and permeability of the plasmic membrane. Acid phosphatase activity was inversely proportional to that of ATPase, while ATPase activity was proportional to membrane integrity.

The salivary gland tissue of unfed males exhibited high metabolic activity and cells with intact nucleus and plasmic membrane, suggesting that the presence of acid phosphatase detected in these individuals may participate in the normal physiology of some acini, as they were not undergoing degeneration. In males at day seven post-attachment, we observed intact membranes in the cells of types I, II and most types III and IV acini, as well as stronger staining for acid phosphatase, nuclear changes, and decrease in ATPase activity. These changes were associated with the degenerative process. At day three and seven post-detachment from the host, degeneration progressed to more advanced stages. Loss of membrane integrity was observed in the cells of a few types I and II acini and most types III and IV acini of males at day three post-detachment; and few types I and II acini, most type III acini, and all type IV acini of males at day seven post-detachment from the host. Nuclear changes, prominent decrease in ATPase activity, and increase in acid phosphatase activity were also observed at day three post-detachment, while acid phosphatase activity decreased at day seven post-detachment from the host.

During the death of cells in these glands, alterations in the nucleus preceded cytoplasmic ones in the following sequence: a) nuclear changes, b) loss of ATPase activity, c) loss of integrity of the plasmic membrane, and d) increase in acid phosphatase activity. The latter might be associated with the late degradation of cytoplasmic remnants, characterizing the process of cell death in glands of *R. sanguineus* males as atypical or non-classic apoptosis.

KEY WORDS: *Rhipicephalus sanguineus*, salivary glands, ticks, males, enzymatic activity, apoptosis.

INTRODUCTION

The onset of characteristics associated with salivary gland degeneration in males of the tick *Rhipicephalus sanguineus* results in a decrease in the secretory capacity of this organs, and the progression of this process leads to the degradation of the glandular tissue (Furquim et al., paper in preparation).

The process of gland degeneration in males exhibits similarities and differences with that of females of the same species, as in the former, gland degeneration is less prominent than in females (Furquim et al., paper in preparation).

According to some authors, the degenerative process is regulated by the ecdysteroid hormone in both male and female ticks [1, 2]. In males, however, the synthesis of this hormone does not increase during and after the completion of feeding [1], causing a delay in the process of gland degeneration and less prominent cell changes compared to those observed in females (Furquim et al., paper in preparation). This might be due to the fact that males, in addition to not reaching the "critical weight", do not receive the "male factor", a substance transferred to the female during mating [3]. Both conditions promote the synthesis of ecdysteroids and therefore are instrumental in gland degeneration [3, 4].

It was reported that salivary gland degeneration in female ticks is genetically programmed [5], which may also occur in the males examined in our study. The literature has shown that cells die by two main overlapping processes: apoptosis or autophagy [6]. The latter has been commonly observed during insect metamorphosis [7, 8, 9-12].

In ticks, studies have demonstrated that cells of salivary glands die by apoptosis [13, 14], and in *R. sanguineus* females, apoptotic death involves acid phosphatase to completely degrade the cytoplasm, characterizing an atypical apoptosis (Furquim et al., paper in preparation).

Several specific methodologies have been described to detect and characterize the process of cell death. ATPase activity is a good indicator of cells in late stages of apoptosis; nuclear breakdown indicates cells in early stages of apoptosis while loss of membrane integrity signals late stages [15, 16]. Acid phosphatase activity is a good

indicator of autophagic death, since it plays an essential role in this type of death [7, 8, 9, 6, 10, 11, 12].

Based on the presented informations, the purpose of this study was to describe the cell changes resulted from salivary gland degeneration in *R. sanguineus* males at day seven post-attachment, and at days three and seven post-detachment from the host, comparing them with unfed males, and identifing the type of cell death in these organs.

MATERIAL AND METHODS

For this study, were utilized males of the tick *Rhipicephalus sanguineus* in the following feeding stages: unfed, at day seven post-attachment, and at day three and seven post-detachment from the host.

Unfed individuals were provided by Dr. Gervásio Henrique Bechara of the Department of Veterinary Pathology of UNESP, Jaboticabal campus (São Paulo), from a colony maintained under controlled conditions (29^{o} C, 80% humidity, and 12 hour photoperiod) in BOD incubator.

A group of unfed individuals was assigned to cytochemical methods, while another group was placed with females in a feeding chamber previously glued to the shaved back of the host (rabbit) according to technique described elsewhere [17]. At day seven post-attachment, males were collected and a group was assigned to cytochemical procedures. Another group was maintained alive for three and seven days in closed containers with breathing holes to be later analyzed.

After the completion of the time periods examined in this study, salivary glands were removed in saline solution and processed according to the following methods described below for observation under light and fluorescence microscope.

For light microscopy, the material was fixed in 10% buffered neutral formalin and acetone (9:1) for one hour and thirty minutes at 4^{o} C, then processed according to the methods described for detection of acid phosphatase and ATPase activities [18]. The material was then dehydrated in increasing concentrations of ethanol (70%, 80%, 90% and 95%), embedded in Leica resin, and sectioned at a thickness of 7 μm. Sections were placed on glass slides, counterstained with Hematoxylin for 2 minutes, and mounted in

Canada balsam for later examination under light microscope. For the demonstration of acid phosphatase activity, total preparations of some glands were also examined.

In both enzymatic experiments, control samples were incubated without substrate.

For fluorescence microscopy, after dissection, salivary glands were placed on glass slides and received two drops of Acridine Orange (100 μg/mL) and Ethidium Bromide (100 μg/mL) both in PBS [16], to demonstrate cell viability and detection of apoptotic and/or necrotic cells. Slides were then covered with cover glasses and immediately examined under fluorescence microscope with a 488 nm excitation filter. Healthy cells are homogeneously green (cytoplasm and nucleus) or the nucleus is homogeneously green and the cytoplasm orange/red. Cells in early stages of apoptosis exhibit green or red-orange cytoplasm and green nucleus with clusters of bright green condensed chromatin. Cells in late stages of apoptosis present red cytoplasm and nucleus with bright orange condensed chromatin. Necrotic cells exhibit red cytoplasm and homogeneously orange nucleus.

RESULTS

Unfed males

In the salivary glands of unfed *R. sanguineus* males, cells are intact, strongly stained for RNA (Figs. $2A_1$, A_2) and ATPase (Figs. $1A_1$-B_1), and in general, weakly stained for acid phosphatase (Figs. $3A_1$-B_3). The plasmic membrane and nuclei are intact (Figs. $2A_1$-A_4).

Type I acinus

The acini basal membrane is strongly stained for ATPase (Fig. $1A_1$) and the plasmic membranes are intact (Fig. $2A_1$). The cytoplasm is strongly positive for RNA (Fig. $2A_1$), weakly positive for ATPase (Fig. $1A_1$), and moderately positive for acid phosphatase (Fig. $3A_6$).

Type II acinus

The acini basal membrane and the plasmic membranes are strongly positive for ATPase (Figs. $1A_2$-A_4). The cytoplasm is strongly positive for RNA (**undifferentiated** cells) (Fig. $2A_2$), and in most cells, strongly positive for ATPase (Figs. $1A_2$, A_3) and negative for acid phosphatase (Fig. $3B_1$).

Type III acinus

The acini basal membrane and plasmic membranes are strongly positive for ATPase (Figs. $1A_5$, A_6). The cytoplasm is negative for RNA (Fig. $2A_3$) and, in most cells, strong staining for ATPase (Figs. $1A_5$, A_6) and no staining for acid phosphatase (Fig. $3B_2$) are observed.

Type IV acinus

The acini basal membrane is strongly stained for ATPase (Fig. $1B_1$) and the plasmic membranes are intacts (Fig. $2A_4$). The cytoplasm is negative for RNA (Fig. $2A_4$), moderately positive for ATPase (Fig. $1B_1$), and weakly positive for acid phosphatase (Fig. $3B_3$).

Males at day seven post-attachment

Degenerative characteristics are observed in *R. sanguineus* males at day seven post-attachment (Figs. $1B_5$-D_1, $2B_1$-C_1, $3B_6$-C_1, $3C_3$-C_5). Type I acini are intact (Figs. $1B_3$, B_4, $2A_5$, $3B_5$, C_2) and types II (Figs. $1B_5$-B_8, $3B_6$, C_3), III (Figs. $1C_1$-C_4, $2B_2$, B_3, $3B_7$, C_4), and IV (Figs. $1C_5$, D_1, $2B_4$, C_1, $3C_1$, C_5) exhibit changes. In general, staining for ATPase is weaker (Figs. $1B_4$-D_1), while for acid phosphatase is stronger (Figs. $3B_5$-C_5).

Type I acinus

The acini basal membranes of most acini is moderately positive for ATPase (Fig. $1B_4$). The plasmic membranes of all cells are intacts (Fig. $3A_5$). The cytoplasm is weakly positive for ATPase (Figs. $1B_3$, B_4) and moderately positive for acid phosphatase (Fig. $3C_2$). Nuclei are intact (Fig. $2A_5$)

Type II acinus

The acini basal membrane of most acini is moderately stained for ATPase (Figs. 1B$_7$, B$_8$), while the plasmic membranes of most cells are negative for ATPase (Figs. 1B$_6$, B$_8$) and intacts (Fig. 2B$_1$). The cytoplasm of most cells is weakly positive for ATPase (Figs. 1B$_5$, B$_6$, B$_8$) and moderately positive for acid phosphatase (Fig. 3C$_3$).

Type III acinus

The acini basal membrane of most acini is strongly positive for ATPase (Figs. 1C$_1$, C$_2$). In most cells, the plasmic membranes are moderately positives for ATPase (Figs. 1C$_1$, C$_3$) and intacts (Fig. 2B$_2$). The cytoplasm of most cells is weakly positive for ATPase (Fig. 1C$_2$, C$_3$) and moderately positive for acid phosphatase (Fig. 3C$_4$). Nuclei exhibit changes (Fig. 2B$_2$).

Type IV acinus

The acini basal membrane of most acini is moderately positive for ATPase (Figs. 1C$_5$). The plasmic membranes are negatives for ATPase (Figs. 1C$_5$, D$_1$) and intacts in most cells (Fig. 2B$_4$). The cytoplasm is moderately positive for acid phosphatase (Fig. 3C$_5$) and, in most cells, weakly positive for ATPase (Fig. D$_1$). Nuclei exhibit changes (Figs. 2B$_4$, C$_1$).

Males at day three post-detachment

Degeneration has progressed to more advanced stages in types I (Figs. 1D$_4$, 2C$_2$, C$_3$, 3D$_2$, E$_1$), II (Figs. 1D$_5$-E$_1$, 2D$_1$, 3D$_3$, E$_2$), III (Figs. 1E$_3$, E$_4$, 2D$_3$, 3D$_4$, E$_3$), and IV (Figs. 1F$_1$, 2E$_1$, 3D$_5$, E$_4$) acini. Types III (Figs. 1E$_3$-E$_4$, 2D$_3$, 3D$_4$, E$_3$) and IV (Figs. 1F$_1$, 2E$_1$, 3D$_5$, E$_4$) acini are the most affected. In general, staining is weaker for ATPase (Figs. 1D$_4$-E$_1$, E$_3$, E$_4$, F$_1$) and stronger for acid phosphatase (Figs. 2D$_2$-E$_4$).

Type I acinus

The acini basal membrane of most acini is moderately positive for ATPase (Fig. 1D$_4$) and in most cells, the plasmic membranes are intacts. (Fig. 2C$_2$) The cytoplasm is strongly positive for acid phosphatase (Fig. 3E$_1$) and, in most cells, no staining for ATPase is observed (Fig. 1D$_4$). Nuclei exhibit changes (Figs. 2C$_2$, C$_3$).

Type II acinus

The acini basal membrane of most acini is moderately positive for ATPase (Figs. $1D_6$, E_1). In most cells, the plasmic membranes are negatives for ATPase (Figs. $1D_5$, D_6, E_1) and intacts (Fig. $2C_4$), and the cytoplasm is negative for ATPase (Figs. $1D_5$, D_6) and strongly positive for acid phosphatase (Fig. $3E_2$). Nuclei exhibit changes (Fig. $2D_1$).

Type III acinus

The acini basal membrane of most acini is strongly positive for ATPase (Fig. $1E_2$). In most cells, the plasmic membranes are negatives for ATPase (Figs. $1E_3$, E_4) and has lost their integrity (Fig. $2D_3$); the cytoplasm is negative for ATPase (Figs. $1E_2$-E_4) and strongly positive for acid phosphatase (Fig. $3E_3$). Nuclei exhibit changes (Figs. $2D_2$, D_3).

Type IV acinus

The acini basal membrane of most acini is moderately stained for ATPase in some areas and not stained in others (Fig. $1F_1$). The plasmic membranes are negatives for ATPase (Figs. $1E_5$, F_1) and in most cells, has lost their integrity (Figs. $2D_4$, E_1). The cytoplasm is strongly positive for acid phosphatase (Fig. $3E_4$) and in most cells, negative for ATPase (Fig. $1F_1$). Nuclei exhibit changes (Fig. $2E_1$).

Males at day seven post-detachment

In these males, degeneration has progressed and types III (Figs. $1H_1$, $2F_2$, F_3) and IV (Fig. $1H_3$, $2F_4$) are the most affected acini. In general, weaker staining for ATPase (Figs. $1F_5$, G_2, H_1, H_3) and acid phosphatase (Figs. $3F_2$-G_4) are observed.

Type I acinus

The acini basal membrane of most acini is moderately positive for ATPase (Fig. $1F_4$) and in most cells, the plasmic membranes are intacts (Fig. $2E_2$). The cytoplasm is moderately positive for acid phosphatase (Fig. $3G_1$) and, in most cells, weakly positive for ATPase (Figs. $1F_3$, F_4). Nuclei exhibit changes (Figs. $2E_2$, E_3).

Type II acinus

The acini basal membrane of most acini is moderately positive for ATPase (Fig. $1G_1$). In most cells, the plasmic membranes are negatives for ATPase (Figs. $1G_1$, G_2) and intacts (Fig. $2E_4$), and the cytoplasm is negative for ATPase (Figs. $1F_6$-G_2) and acid phosphatase (Fig. $3G_2$). Nuclei exhibit changes (Figs. $2E_4$, E_5)

Type III acinus

The acini basal membrane of most acini is strongly positive for ATPase (Figs. $1G_3$, G_4). In most cells, the plasmic membranes are negatives for ATPase (Figs. $1G_4$, H_1) and has lost their integrity (Figs. $2F_2$, F_3); and no ATPase (Figs. $1G_3$-H_1) and acid phosphatase (Fig. $3G_3$) activity is observed in the cytoplasm. Nuclei exhibit changes (Figs. $2F_1$, F_2).

Type IV acinus

The acini basal membrane of most acini is moderately stained for ATPase in some areas and not stained in others (Fig. $1H_3$). The plasmic membranes are negatives for ATPase (Figs. $1H_2$, H_3) and is not intacts (Fig. $2F_4$). The cytoplasm of most cells is negative for ATPase (Figs. $1H_2$, H_3) and acid phosphatase (Fig. $3G_4$). Nuclei exhibit changes (Fig. $2F_4$).

To better compare the results, the data are summarized in Tables **1** and **2**.

Table 1: Enzymatic activity and cell viability of salivary glands of unfed *Rhipicephalus sanguineus* males and males at day seven post-attachment.

		Type I Acini	Type II Acini	Type III Acini	Type IV Acini
Unfed	**Basal Membrane of Acinus**	- ATPase +++ (Fig. 1A$_1$);	- ATPase +++ (Figs. 1A$_2$-A$_4$);	- ATPase +++ (Figs. 1A$_5$, A$_6$);	- ATPase +++ (Fig. 1B$_1$);
	Plasmic Membrane	- intact (Fig. 2A$_1$);	- ATPase +++ (Figs. 1A$_2$-A$_4$); - intact (Fig. 2A$_2$);	- ATPase +++ (Figs. 1A$_5$, A$_6$); - intact (Fig. 2A$_3$);	- intact (Fig. 2A$_4$);
	Cytoplasm	- ATPase + (Fig. 1A$_1$), - fa ++ (Fig. 3A$_6$);	- ATPase +++ (Figs. 1A$_2$, A$_3$) and – (Figs. 1A$_2$, A$_4$); - fa + (Fig. 3B$_1$) and – (Fig. 3B$_1$);	- ATPase +++ (Fig. 1A$_5$, A$_6$) and – (Fig. 1A$_5$, A$_6$); - fa + (Fig. 3B$_2$) and – (Fig. 3B$_2$);	ATPase ++ (Fig. 1B$_1$); - fa + (Fig. 3B$_3$);
	Nucleus	- intact (Fig. 2A$_1$);	- intact (Fig. 2A$_2$);	- intact (Fig. 2A$_3$);	- intact (Fig. 2A$_4$);
Day 7 Post-Attachment	**Basal Membrane of Acinus**	- ATPase +++ (Fig. 1B$_3$) and ++ (Fig. 1B$_4$);	- ATPase +++ (Figs. 1B$_5$, B$_6$) and ++ (Figs. 1B$_7$, B$_8$);	- ATPase +++ (Figs. 1C$_1$, C$_2$) and ++ (Figs. 1C$_3$, C$_4$);	- ATPase ++ (Fig. 1C$_5$) and +/– (Fig. 1D$_1$);
	Plasmic Membrane	- intact (Fig. 2A$_5$);	- ATPase ++ (Figs. 1B$_5$, B$_7$) and – (Figs. 1B$_6$, B$_8$); - intact (Fig. 2B$_1$);	- ATPase ++ (Figs. 1C$_1$, C$_3$) and – (Figs. 1C$_2$, C$_4$); - > intact (Fig. 1B$_2$); - < not intact (Fig. 1B$_3$);	- ATPase – (Figs. 1C$_5$, D$_1$); - > intact (Fig. 2B$_4$); - < not intact (Fig. 2C$_1$)
	Cytoplasm	- ATPase + (Figs. 1B$_3$, B$_4$); - fa ++ (Fig. 3C$_2$);	- ATPase +++ (Fig. 1B$_5$), ++ (Fig. 1B$_7$), + (Figs. 1B$_5$, B$_6$, B$_8$) and – (Figs. 1B$_5$, B$_6$, B$_8$); - fa ++ (Fig. 3C$_3$) and – (Fig. 3C$_3$);	- ATPase + (Figs. 1C$_2$, C$_3$) and – (Figs. 1C$_1$-C$_4$); - fa ++ (Fig. 3C$_4$) and – (Fig. 3C$_4$);	- ATPase ++ (Fig. 1C$_5$) and + (Fig. 1D$_1$); - fa ++ (Fig. 3C$_5$);
	Nucleus	- intact (Fig. 2A$_5$);	- not observed;	- ! (Fig. 2B$_2$);	- ! (Figs. 2B$_4$, C$_1$) and ? (Fig. 2C$_1$);

(+++) strongly positive; (++) moderately positive; (+) weakly positive; (–) negative; (+/–) irregular staining; (**af**) acid phosphatase; (**>**) most cells; (**<**): some cells; (**!**) irregular nucleus; (**#**) nucleus with clusters of condensed chromatin; (**?**) picnotic nucleus.

Table 2: Enzymatic activity and cell viability of salivary glands of *Rhipicephalus sanguineus* males at day three and seven post-detachment from the host.

		Type I Acini	Type II Acini	Type III Acini	Type IV Acini
Day 3 Post-Detachment	**Basal Membrane of Acinus**	- ATPase +++ (Fig. 1D$_3$) and ++ (Fig. 1D$_4$);	- ATPase +++ (Fig. 1D$_5$) and ++ (Figs. 1D$_6$, E$_1$);	- ATPase +++ (Fig. 1E$_2$), ++ (Fig. 1E$_3$) and +/– (Fig. 1E$_4$);	- ATPase ++ (Fig. 1E$_5$) and +/– (Fig. 1F$_1$);
	Plasmic Membrane	- > intact (Fig. 2C$_2$); - < not intact (Fig. 2C$_3$);	- ATPase ++ (Figs. 1D$_5$, D$_6$) and – (Figs. 1D$_5$-E$_1$); - > intact (Fig. 2C$_4$); - < not intact (Fig. 2D$_1$);	- ATPase ++ (Fig. 1E$_2$) + (Fig. 1E$_3$) and – (Fig. 1E$_4$); - > not intact (Fig. 2D$_3$); - < intact (Fig. 2D$_2$);	- ATPase – (Figs. 1E$_5$, F$_1$); - > not intact (Figs. 2D$_4$, E$_1$); - < intact (Fig. 2D$_4$);
	Cytoplasm	- ATPase + (Fig. 1D$_3$) and – (Fig. 1D$_4$); - fa +++ (Fig. 3E$_1$);	- ATPase +++ (Fig. 1D$_5$), ++ (Fig. 1 D5, E1), + (Figs. 1D$_6$, E$_1$) and – (Figs. 1D$_5$, D$_6$); - fa +++ (Fig. 3E$_2$) and – (Fig. 3E$_2$);	- ATPase +++ (Fig. 1E$_2$), ++ (Fig. 1E$_4$) and – (Figs. 1E$_2$-E$_4$); - fa +++ (Fig. 3E$_3$) and – (Fig. 3E$_3$);	-ATPase ++ (Fig. 1E$_5$) and – (Fig. 1F$_1$); - fa +++ (Fig. 3E$_4$);
	Nucleus	- # (Figs. 2C$_2$, C$_3$);	- ? (Fig. 2D$_1$);	- ! (Fig. 2D$_2$) and ? (Fig. 2D$_3$);	- !, ? and * (Fig. 2E$_1$);
Day 7 Post-Detachment	**Basal Membrane of Acinus**	- ATPase +++ (Fig. 1F$_3$), ++ (Fig. 1F$_4$) and +/– (Fig. 1F$_5$);	- ATPase +++ (Fig. 1F$_6$), ++ (Fig. 1G$_1$) and +/– (Fig. 1G$_2$);	- ATPase +++ (Figs. 1G$_3$, G$_4$), ++ (Fig. 1G$_5$) and +/– (Fig. 1H$_1$);	- ATPase ++ (Fig. 1H$_2$) and +/– (Fig. 1H$_3$);
	Plasmic Membrane	- > intact (Fig. 2E$_2$); - < not intact (Fig. 2E$_3$);	- ATPase ++ (Figs. 1F$_6$, G$_1$) and – (Figs. 1G$_1$, G$_2$); - > intact (Fig. 2E$_4$); - < not intact (Fig. 2E$_5$);	- ATPase ++ (Figs. 1G$_3$, G$_5$) and – (Figs. 1G$_4$, H$_1$); - > not intact (Figs. 2F$_2$, F$_3$); - < intact (Fig. 2F$_1$);	- ATPase – (Figs. 1H$_2$, H$_3$); - not intact (Fig. 2F$_4$);
	Cytoplasm	- ATPase + (Figs. 1F$_3$, F$_4$) and – (Fig. 1F$_5$); - fa ++ (Fig. 3G$_1$);	- ATPase ++ (Figs. 1F$_6$, G$_2$) and – (Figs. 1F$_6$-G$_2$); - fa ++ (Fig. 3G$_2$) and – (Fig. 3G$_2$);	- ATPase +++ (Figs. 1G$_4$, G$_5$), ++ (Fig. 1G$_4$) and – (Figs. 1G$_3$-H$_1$); - fa ++ (Fig. 3G$_3$) and – (Fig. 3G$_3$);	- ATPase ++ (Fig. 1H$_2$) and – (Figs. 1H$_2$, H$_3$); - fa ++ (Fig. 3G$_4$) and – (Fig. 3G$_4$);
	Nucleus	- # (Figs. 2E$_2$, E$_3$);	- ? (Figs. 2E$_4$, E$_5$);	- ! (Figs. 2F$_1$, F$_2$);	- ? (Fig. 2F$_4$);

(+++) strongly positive; (++) moderately positive; (+) weakly positive; (–) negative; (+/–) irregular staining; (**>**) most cells; (**<**): some cells; (**af**) acid phosphatase; (**!**) irregular nucleus; (**#**) nucleus with clusters of condensed chromatin; (**?**) picnotic nucleus; (*****) fragmenting nucleus.

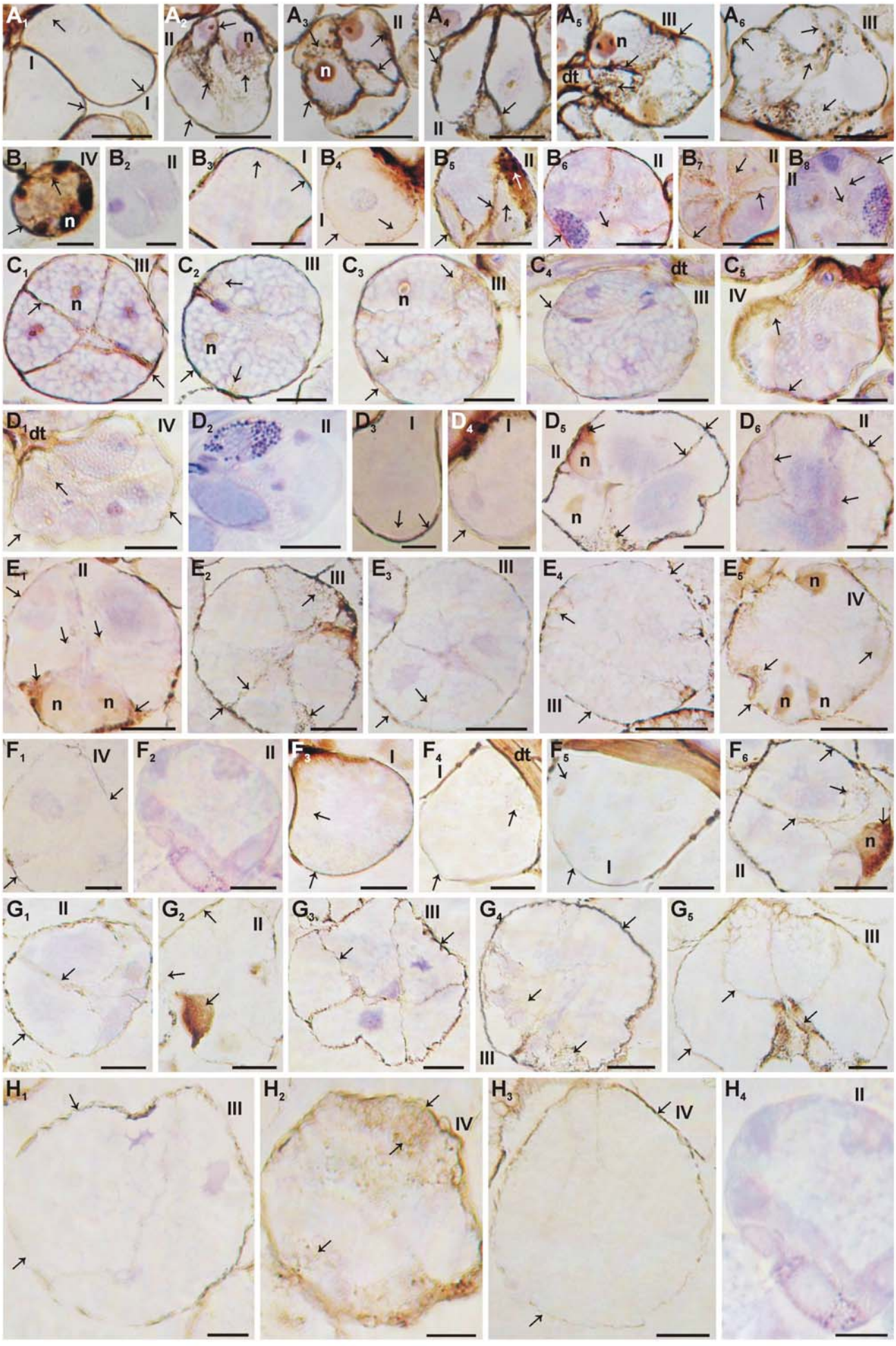
FIGURES

FIGURE 1:

ATPase activity in the salivary glands of *Rhipicephalus sanguineus* males. **A_1-B_2.** Unfed male. Observe intense staining for ATPase (**arrow**) in the basal membrane and cells of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini. **B_2.** Negative control. **B_3-D_2.** Male at day seven post-attachment. Note decrease in ATPase activity (**arrow**) in the four types of acini (**I**, **II**, **III**, and **IV**). In **B_6**, **B_8**, **C_2**, **C_4-D_1**, absence of ATPase activity in the plasmic membranes of cells of types II (**II**), III (**III**), and IV (**IV**) acini. Note in **D_1**, irregular staining for ATPase (**arrow**) in the basal membrane of type IV acinus (**IV**). **D_2.** Negative control. **D_3-F_2.** Male at day three post-detachment. Observe weaker staining for ATPase (**arrow**) in types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini. Note in: **E_4** and **F_1**, irregular staining for ATPase (**arrow**) along the basal membrane of cells of types III (**III**) and IV (**IV**) acini; **D_5-E_1** and **E_3-F_1**, absence of ATPase activity in the membrane of cells of types II (**II**), III (**III**) and IV (**IV**) acini. **F_2.** Negative control. **F_3-H_4.** Male at day seven post-detachment. Observe even weaker staining for ATPase (**arrow**). Note in: **F_5**, **G_2**, **H_1**, and **H_3**, irregular staining for ATPase (**arrow**) along the basal membrane of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini, and **G_2**, **G_4**, **H_1-H_3**, no staining for ATPase in the plasmic membrane of all cells of types II (**II**), III (**III**), and IV (**IV**) acini. **H_4.** Negative control.

I-IV: types acini; **arrow:** staining for ATPase; **n:** staining for ATPase in the nucleus; **dt:** duct.

Bars: B_1, **B_2**, **D_3** and **D_4**= 12, 5 μm; **A_1-A_6**, **B_3-D_2**, **D_5-E_2** and **F_1-H_4**= 25 μm; **E_3-E_5**= 50 μm.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A3 n II
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A5 n III dt
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B3 I
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B6 II
B7 II
B8 II
C1 III n
C2 III n
C3 n III
C4 dt III
C5 IV
D1 dt IV
D2 II
D3 I
D4 I
D5 II n n
D6 II
E1 II n n
E2 III
E3 III
E4 III
E5 n IV n n
F1 IV
F2 II
F3 I
F4 I dt
F5 I
F6 n II
G1 II
G2 II
G3 III
G4 III
G5 III
H1 III
H2 IV
H3 IV
H4 II

FIGURE 2:

Total preparation of salivary glands of *Rhipicephalus sanguineus* males stained with Ethidium Bromide and Acridine Orange. **A_1-A_4.** Unfed male. Note healthy cells of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini with intact plasmic membrane and nuclei (**tn**). **A_5-C_1.** Male at day seven post-attachment. Observe in: **A_5**, intact type I acinus (**I**); **B_1**, type II acinus (**II**) undergoing degeneration, although the plasmic membrane is still intact; **B_2** and **B_3**, type III acinus (**III**) undergoing degeneration with cells in **B_3** exhibiting loss of membrane integrity; **B_4** and **C_1**, type IV acinus (**IV**) undergoing degeneration with cells in **C_1** exhibiting loss of cell membrane integrity. **C_2-E_1.** Male at day three post-detachment. Observe types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini undergoing degeneration. Note in: **C_3**, **D_1**, and **D_3**, loss of integrity of the plasmic membrane of cells of types I (**I**), II (**II**), and III (**III**) acini; **D_4**, loss of membrane integrity of some cells of type IV acinus (**IV**), and **E_1**, loss of membrane integrity of all cells of type IV acinus (**IV**). **E_2-F_4.** Male at day seven post-detachment. Observe in **E_3**, **E_5** and **F_2-F_4**, loss of integrity of the plasmic membrane of cells of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini.

I-IV: types acini; **arrow:** cytoplasmic RNA; **tn:** intact nucleus; **in:** irregular nucleus; **n:** nucleus; **pn:** picnotic nucleus; **dashed arrow:** nucleus with clusters of condensed chromatin; ***:** fragmenting nucleus.

Bars: **A_1-A_4**= 12, 5 μm; **A_5-F_4**= 25 μm.

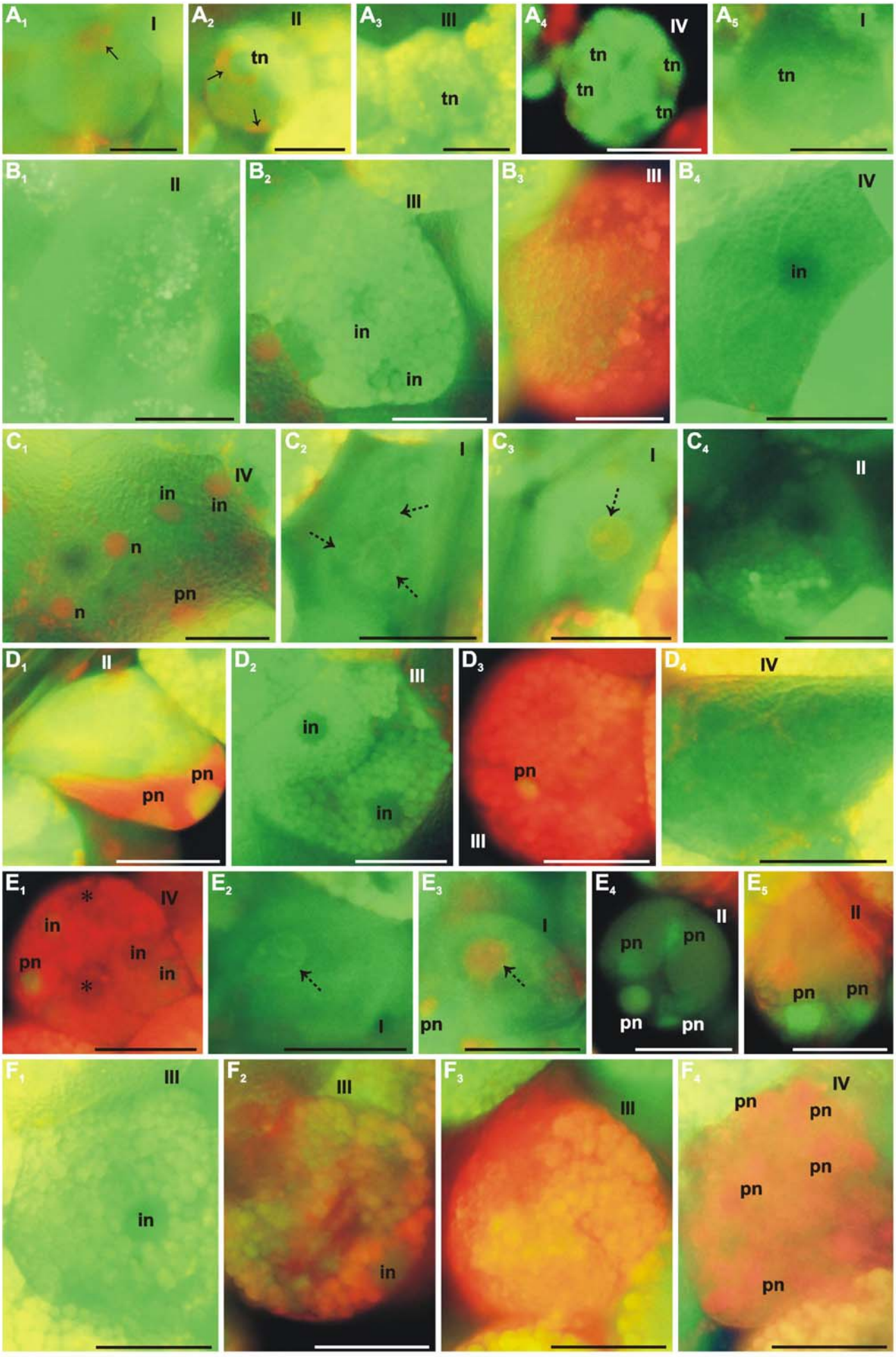
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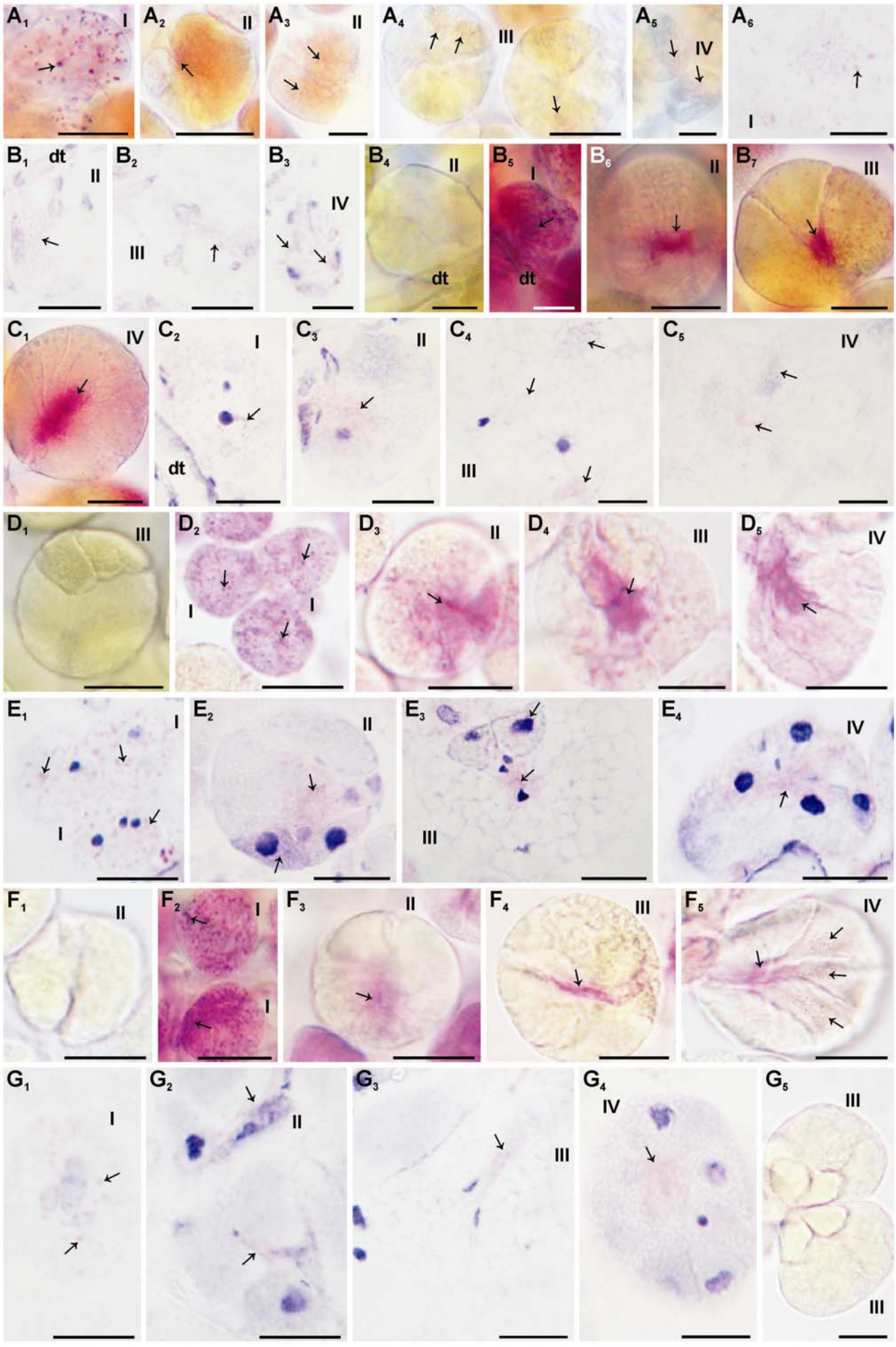
FIGURE 3:

Acid phosphatase activity in the salivary glands of *Rhipicephalus sanguineus* males. **A_1-B_4.** Unfed male. In **A_1-A_5**, whole mount preparation of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini showing low phosphatase activity (**arrow**). **A_6-B_3.** Histological sections of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini exhibiting weak staining for acid phosphatase (**arrow**) in the cytoplasm of cells. **B_4.** Negative control. **B_5-D_1.** Male at day seven post-attachment. **B_5-C_1.** Total preparation of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini showing more intense phosphatase activity (**arrow**). **C_2-C_5.** Histological sections of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini. **D_1.** Negative control. **D_2-F_1.** Male at day three post-detachment. **D_2-D_5.** Total preparation of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini, note even more intense phosphatase activity (**arrow**). **E_1-E_4.** Histological sections of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini. Observe in **E_1-E_4**, cytoplasm of cells of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini strongly stained for acid phosphatase (**arrow**). **F_1.** Negative control. **F_2-G_5.** Male at day seven post-detachment. **F_2-F_5.** Total preparation of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini. **G_1-G_4.** Histological sections of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini. Note decrease in intensity of staining for acid phosphatase. **G_5.** Negative control.

Bars: **A_3**, **A_5**, **B_4** and **B_5**= 10 μm; **B_3**= 12, 5 μm; **A_1**, **A_2**, **A_4**, **B_6-C_1** and **D_1**= 20 μm; **A_6-B_2**, **C_2-C_5** and **D_2-G_5**= 25 μm.


A1 I
A2 II
A3 II
A4 III
A5 IV
A6 I
B1 dt II
B2 III
B3 IV
B4 II dt
B5 I dt
B6 II
B7 III
C1 IV
C2 I dt
C3 II
C4 III
C5 IV
D1 III
D2 I I
D3 II
D4 III
D5 IV
E1 I I
E2 II
E3 III
E4 IV
F1 II
F2 I I
F3 II
F4 III
F5 IV
G1 I
G2 II
G3 III
G4 IV
G5 III III

DISCUSSION

The comparative analysis of the salivary glands of unfed *Rhipicephalus sanguineus* males, at day seven post-attachment, and at days three and seven post-detachment revealed structural and enzymatic changes as a result of the process of gland degeneration.

The glandular tissue of unfed individuals was intact and metabolically active, as confirmed by the integrity of nuclei, intense staining for ATPase in the plasmic membrane and cytoplasm of cells. The latter was also strongly positive for RNA and weakly positive for acid phosphatase, confirming the observed in *R. sanguineus* females at the same feeding stage (Furquim et al., paper in preparation).

In this study, the ATPase activity detected in different compartments of the cells indicated a consumption of energy for the maintenance of the integrity of plasmic membrane, contributing for the functioning of its ion pumps, as well as for metabolic processes, such as protein synthesis in types I and II acini.

Acid phosphatase was detected in the four types of acini of unfed *R. sanguineus* males. The most intense staining for acid phosphatase was observed in type I acinus, as reported for females of the same species (Furquim et al., paper in preparation). Acid phosphatase activity in type IV might be associated with the normal metabolism of cells. In type I acinus, this enzyme may be involved in the synthesis of structural proteins, as suggested by the large quantity of RNA, confirming the observed in hypopharyngeal glands of *Scaptotrigona postica* [19] and in *R. sanguineus* females by Furquim et al. (paper in preparation). In types II and III acini, which exhibit cells in pre-secretory phase, acid phosphatase might be involved in secretion maturation, as observed in hypopharyngeal glands of *Scaptotrigona postica* [19] and in in *R. sanguineus* females (Furquim et al., paper in preparation).

The salivary gland cells of *R. sanguineus* males at day seven post-attachment, and three and seven days post-detachment from the host underwent changes in the nucleus, cytoplasm (acid phosphatase and ATPase), and plasmic membrane (maintenance of integrity). These changes became more prominent as time away from the host progressed, suggesting that the degenerative process in salivary gland *R. sanguineus* males is asynchronous among different types of acini. Types III and IV

acini were the most affected, as also reported for females of the same species (Furquim et al., paper in preparation).

In males at day seven post-attachment, and at days three and seven post-detachment from the host, we observed a gradual decrease in ATPase activity. At day seven post-attachment and day three post-detachment, acid phosphatase activity increased. These changes were accompanied by a gradual loss of the integrity of the plasmic membrane, which started at day seven post-attachment. Similar results obtained elsewhere [20] showed a simultaneous increase in acid and basic phosphatase activities and a decrease in ATPase activity. These results also support those obtained in engorged and at day three post-engorgement females of *R. sanguineus*, in which an increase in acid phosphatase and decrease in ATPase were observed, in addition to loss of integrity of the plasmic membrane (Furquim et al., paper in preparation).

In males at day seven post-detachment from the host, the decrease in acid phosphatase probably occurred because it was utilized and not synthesized during the degenerative process. Some reports [1], shows in males, the hormone (ecdysteroid) controlling gland degeneration does not reach levels as high as those of females (Lomas, 1993, PhD thesis), and therefore, the stimulus is not as intense to maintain gland degeneration and consequently, the synthesis of acid phosphatase. This hypothesis was confirmed by the less intense degenerative process observed in the males examined in the present study compared to the observed for females of the same species. In the latter, acid phosphatase activity was much higher, resulting in more prominent degradation (vacuolation) of the cytoplasm (Furquim et al., paper in preparation), as well as decrease or absence of ATPase activity and loss of membrane integrity, affecting more cells and acini during the degenerative process.

In the present study, we observed irregular staining patterns for ATPase in the basal membrane in type IV acinus of males at day seven post-attachment, types III and IV acini of males at day three post-detachment from the host, and types II, III and IV acini of males at day seven post-detachment. In these acini, stained areas alternated with non-stained ones, confirming the results obtained for engorged and at day three post-engorgement females of *R. sanguineus* (Furquim et al., paper in preparation).

Type II acinus of *R. sanguineus* males at day seven post-attachment, and at days three and seven post-detachment, and type IV acinus of males at day seven post-

attachment and at day three post-detachment from the host exhibited a characteristic not observed in the other two types of acini, which was also reported for type II acinus of females of the same species (Furquim et al., paper in preparation). In the cells of some types II and IV acini, the plasmic membrane was intact, but ATPase activity was absent. This could be due to the presence of a functional cell membrane only in the basal portion that still could not be distinguished from the basal membrane of the acinus. Thus, only an ATPase positive halo was observed around it.

The enzymatic changes observed in the present study were accompanied by morphological ones (Furquim et al., paper in preparation), such as a) decrease in the amount of secretion in the cells, b) nuclear breakdown, c) cytoplasmic shrinkage, d) loss of cell shape, e) loss of cell boundaries (cell individuality), cytoplasmic vacuolation, and f) cell fragmentation resulting in apoptotic bodies enclosed by membrane, containing cytoplasmic and nuclear remnants. The decrease in the amount of secretion and nuclear breakdown preceded all other morphological and enzymatic changes, characterizing a classic apoptosis, as also observed elsewhere [21, 22, 23, 10, 24].

However, early loss of ATPase activity and integrity of the plasmic membrane during the degenerative process and the presence of acid phosphatase do not characterize a classic apoptotic death. In fact, participation of acid phosphatase has been associated with autophagic death [7, 21, 9, 6, 10, 11, 12], in which the plasmic membrane is still functional in newly formed apoptotic bodies [25, 23].

On the other hand, it was reported that during cell death in the salivary glands of *Calliphora vomitoria*, ATPase activity ceased at some point and a massive cytoplasmic vacuolation was observed [24]. This indicates that ATPase activity and preservation of the integrity of the membrane is not always observed until the late stages of the process, as suggested by other author [23, 25]. Similarly, several studies have described the presence of hydrolytic enzymes (acid phosphatase) during apoptotic death [26, 21, 6, 27, 28, 10, 29, 11, 30]. Based on this information, our findings indicate that the salivary gland cells of all acini in *R. sanguineus* males exhibited characteristics of classic apoptosis and autophagic death. The latter included the involvement of hydrolytic enzymes and the presence cytoplasmic vacuoles, characterizing the cell death as an atypical apoptosis. This was also proposed for females of the same species, in which the late involvement of acid phosphatase was associated with the removal of cytoplasmic

remnants, as well as fragmentation of gland cells (Furquim et al., paper in preparation). In the present study, acid phosphatase activity was low and did not degrade large areas of cytoplasm.

In this case, acid phosphatase probably had an endogenous origin, as we did not observe hemocytes adhered to the tissue, as described for *Calliphora erythrocephala* [26, 8]. However, it was suggested that acid phosphatase detected in gland cells of *R. (Boophilus) microplus* females might come from the hemolymph [14].

Thus, these results, supported by those obtained previously by Furquim et al. (paper in preparation), indicate that the degenerative process of salivary glands of *R. sanguineus* males occurs by atypical apoptosis, characterized by a) early nuclear breakdown, mainly by chromatin condensation, b) loss of cell shape, c) decrease and/or absence of ATPase activity and loss of integrity of the plasmic membrane, d) cytoplasm shrinkage and loss of cell boundaries, e) low acid phosphatase activity, resulting in limited degradation (vacuolation) of the cytoplasm, and f) fragmentation of acini with release of apoptotic bodies.

ACKNOWLEDGMENTS

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References

[1] Kaufman WE. 1990. Effect of 20-hydroxiecdysone on the salivary glands of the male tick, *Amblyomma hebraeum*. Exp Appl Acarol 1990; 9: 87-95.

[2] Lomas OL, Gelman D, Kaufman W R. Ecdysteroid regulation of salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*: a reconciliation of *in vivo* and *in vitro* observations. Gen Comp Endocrinol 1998; 109: 200-211.

[3] Weiss LB, Kaufman WR. The relationship between 'critical weight' and 20-hydroxiecdysone in the female ixodid tick, *Amblyomma hebraeum*. J Insect Physiol 2001; 47: 1261-1267.

[4] Weiss LB, Kaufman WR. Two feeding-induced proteins from the male gonad trigger engorgement of the female tick *Amblyomma hebraeum*. Proc Natl Acad Sci SA 2004; 101 (16): 5874-5879.

[5] Bowman AS, Sauer JR. Tick salivary glands: function, physiology and future. Parasitology 2004; 129: S67-S81.

[6] Zakeri Z, Bursch W, Tenniswood M, Lockshin RA. Cell death: programmed, apoptosis, necrosis, or other? Cell death Differ 1995; 2: 87-96.

[7] Pipan N, Rakovec V. Cell death in the midgut epithelium of the worker honey bee (*Apis mellifera carnica*) during metamorphosis. Zoomorphologie 1980; 97: 217-224.

[8] Armbruster L, Levy M, Mathieu N, Bautz AM. Acid phosphatase activity in the hemolymph, hemocytes, fat body and salivary glands during larval and prepupal development in *Calliphora erythrocephala* (Diptera: Calliphoridae). Comp Biochem Physiol 1986; 84B (3): 349-354.

[9] Cummings LA, Bowen ID. The localization of acid phosphatase as a prelude to cell death in the blow-fly *Calliphora erithrocephala*. Micron Microsc Acta 1992; 23 (3): 357-358.

[10] Lockshin AR, Zakeri Z. The Biology of Cell Death and Its Relationship to Aging. In: Holbrook N, Martin GR, Lockshin RA, editors. Cellular Aging and Cell Death. New York: Wiley-Liss, 1996: 167-180.

[11] Jochová J, Zakeri Z, Lockshin RA. Rearrangement of the tubulin and actin cytoskeleton during programmed cell death in *Drosophila* salivary glands. Cell Death Differ 1997; 4: 140-149.

[12] Gregorc A, Bowen ID, Pogacnik A. Acid phosphatase activity in the midgut of honeybee (*Apis melliphera* L.) larvae. Apidologie 1998; 29: 579-584.

[13] L'Amoreuax WJ, Junaid L, Trevidi S. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. Tissue Cell 2003; 35: 95-99.

[14] Nunes ET, Camargo-Mathias MI, Bechara GH. *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae): acid phosphatase and ATPase activities localization in salivary glands of females during the feeding period. Exp Parasitol 2006; 114: 109-117.

[15] Bowen ID, Worrill NA, Winters CA, Mullarkey K. The use of backscattered electron imaging, X-ray microscopy in demonstrating physiological cell death. Scanning Microsc 1988; 2: 1453-1462.

[16] MacGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ. The End of the (Cell) Line: Methods for the Study of Apoptosis *in vitro*. In: Schwartz LM, Osborne BA, editors. Cell Death. San Diego: Academic Press, 1995: 153-185.

[17] Bechara GH, Szabó MPJ, Ferreira BR, Garcia MV. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Brazil J Vet Parasitol1995; 4: 61-66.

[18] Hussein MA, Bowen ID, Lewis GH. The histochemical localization of ATPase, cholinesterase and acid phosphatase activity in *Culex pipiens* (Diptera, Culicidae) larvae using a methacrylate embedding technique. Cell Biol Int Rep 1990; 14 (9): 775-781.

[19] Costa RAC, Cruz-Landim C. Distribution of acid phosphatases in the hypopharyngeal glands from workers, queens and males of a Brazilian stingless bee *Scaptotrigona postica* Latreille: an ultrastructural cytochemical study. Histochem Journal 2001; 33: 653-662.

[20] Weber R. Tissue Involution and Lysosomal Enzimes During Anuran Metamorphosis. In: Dingle JT, Fell HB, editors. Lysosomes in Biology and Pathology, vol. 2. North Amsterdam and London: Holland Publishing Co, 1969: 437-461.

[21] Clarke PGH. Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 1990; 181: 195-213.

[22] Bowen ID. Apoptosis or programmed cell death? Cell Biol Int 1993; 17 (4): 365-380.

[23] Kerr JFR, Gobé GC, Winterford CM, Harmon B. Anatomical Methods in Cell Death. In: Schwartz LM, Osborne BA, editors. Cell Death. San Diego: Academic Press, 1995: 1-27.

[24] Häcker G. The morphology of apoptosis. Cell Tissue Res 2000; 301: 5-17.

[25] Bowen ID, Bowen SM. Programmed Cell Death in Tumours and Tissues, Chapman and Hall: London, 1990.

[26] Levy M, Bautz AM. Degeneration of larval salivary glands during metamorphosis of the blowfly, *Calliphora erythrocephala* Meigen (Diptera: Calliphoridae). Int J Insect Morphol Embryol 1985; 14 (5): 281-290.

[27] Bowen ID, Mullarkey K, Morgan SM. Programmed cell death during metamorphosis in the blow-fly *Calliphora vomitoria*. Micros Res Tech 1996; 34: 202-217.

[28] Dai J, Gilbert LI. Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. Insect Biochem Mol Biol 1997; 27 (1): 69-78.

[29] Gregorc A, Bowen ID. Programmed cell death in the honey-bee (*Apis mellifera* L.) larvae midgut. Cell Biol Int 1997; 21 (3): 151-158.

[30] Yamamoto S, Sawada K, Shimomura H, Kawamura K, James TN. On the nature of cell death during remodeling of hypertrophied human myocardium. J Mol Cell Cardiol 2000; 32: 161-175.

Capítulo 7

CAPÍTULO 7

TITLE: Cytoplasmic and nuclear changes detected cytochemically during the degeneration of salivary glands of the tick *Rhipicephalus sanguineus* (LATREILLE, 1806) (Acari, Ixodidae).

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RESUMO

O presente estudo analisou citoquimicamente glândulas salivares de fêmeas (em jejum, ingurgitadas e com três dias pós-ingurgitamento) e de machos (em jejum, com sete dias de infestação, três e sete dias pós-remoção do hospedeiro) de carrapatos *Rhipicephalus sanguineus*. Os resultados revelaram a ocorrência de alterações nucleares nas células secretoras de fêmeas em estágios ingurgitado e com três dias pós-ingurgitamento e nos machos em todos os estágios (exceto em jejum), sendo mais intensas nas fêmeas. Além disso demonstrou a presença de alterações citoplasmáticas nas células de todos os ácinos de fêmeas e de machos.

Nos ácinos II e III de fêmeas ingurgitadas houve alterações na forma do núcleo (arredondados, irregulares, com "blebbs", fragmentando-se ou fragmentados), no tamanho (normal, dilatados ou reduzidos), na disposição da cromatina e no grau de condensação da mesma (por todo o núcleo, marginalizada ou em blebbs). Os nucléolos também sofreram alterações na forma (arredondados, fragmentando-se ou fragmentados), no tamanho (normais ou dilatados), na localização (centrais, marginais ou em blebbs), e alguns ainda estavam compactados ou desorganizados. Nas fêmeas com três dias pós-ingurgitamento todos os ácinos apresentaram alterações semelhantes às verificadas nas fêmeas ingurgitadas. A marcação para presença de RNA foi maior nas células das fêmeas ingurgitadas do que nas com três dias após-ingurgitamento.

Nos machos com sete dias de infestação os ácinos II, III e IV apresentaram células com tamanho do núcleo e grau de condensação da cromatina alterados semelhantes às das fêmeas. Por outro lado com forma arredondada, irregular ou fragmentando-se e a cromatina disposta por todo núcleo ou marginalizada. As alterações no nucléolo foram semelhantes às das fêmeas, isto é no tamanho e na organização, já a forma arredondada permaneceu e a localização foi central. Nos machos com três dias pós-remoção do hospedeiro todas as células de todos os ácinos apresentaram alterações nucleares semelhantes às dos machos com sete dias de infestação, além da fragmentação do nucléolo. Naqueles com sete dias pós-remoção do hospedeiro as alterações ocorreram em todos os ácinos semelhante às verificadas naqueles com sete dias de infestação. Quanto a presença de RNA citoplasmático, este

foi fortemente marcado nos machos com sete dias de infestação e fracamente naqueles com sete dias pós-remoção do hospedeiro.

Tanto nas fêmeas quanto nos machos as diferentes intensidades de marcação do RNA no citoplasma, bem como as alterações nucleares caracterizaram a ocorrência de morte celular do tipo apoptótica.

PALAVRAS-CHAVE: *Rhipicephalus sanguineus*, glândulas salivares, fêmeas, machos, cromatina, nucléolo, apoptose.

ABSTRACT

The present study reports cytochemistry data about salivary gland of females (unfed, engorged, and at days three post-engorgement) and males (unfed, at day seven post-attachment, and at day three and seven post-detachment from the host) of the tick *Rhipicephalus sanguineus*. The results revealed nuclear changes in engorged females and at day three post-engorgement, and in males in all stages (except unfed). These changes were more prominent in females. Cytoplasmic changes were also observed in cells of all acini of males and females.

In types II and III acini of engorged females, nuclear changes were observed in the shape (round, irregular, with blebs, fragmenting or fragmented), size (normal, enlarged or reduced), and arrangement and condensation level of chromatin (throughout the nucleus, marginal or as blebs). Changes were also detected in nucleoli, regarding their shape (round, fragmenting or fragmented), size (normal or enlarged), and location (central, marginal or as blebs). Some nucleoli were also compacted or disorganized. In females at day three pos-engorgement, all acini exhibited similar changes to those observed in engorged females. RNA staining was stronger in cells of engorged females than those at day three post-engorgement.

In males at day seven post-attachment, cells of types II, III, IV acini presented changes in the size of the nucleus and condensation level of chromatin similar to those of females. The shape of the nucleus was round, irregular or undergoing fragmentation, and the chromatin was located marginal or throughout the nucleus. The changes in the nucleolus were similar to those of females, regarding size and organization, although round-shaped and in the center location. In males at day three post-detachment, cells of all acini exhibited nuclear changes similar to those of males at day seven post-attachment, in addition to the fragmentation of the nucleolus. At day seven post-detachment, changes were detected in all acini similar to the observed in males at day seven post-attachment. Regarding cytoplasmic RNA, staining was prominent in males at day seven post-attachment and weak in those at day seven post-detachment from the host.

In females as well as males, different RNA staining patterns in the cytoplasm and nuclear changes characterized apoptotic cell death.

KEY WORDS: *Rhipicephalus sanguineus*, salivary glands, females, males, chromatin, nucleolus, apoptosis.

INTRODUCTION

Female and male ticks present salivary glands that secrete substances responsible for the attachment of the parasite to the host to allow feeding (Binnington, 1978; Walker et al., 1985; Gill and Walker, 1987).

Previous studies on the tick *Rhipicephalus sanguineus* have demonstrated that the salivary glands undergo periods of intense and low secretory activity. During low activity, the morphology and cytochemistry of these gland cells revealed degenerative features characterizing an atypical type of apoptosis observed in males as well as females of this species, although more prominent in the latter (Furquim, 2006).

Data available in the literature indicate that during apoptotic death, nuclear breakdown is one of the first changes observed, characterized by fragmentation, compression, and margination of chromatin, appearance of blebs and finally fragmentation of the nucleus itself (Bowen and Bowen, 1990; Bowen, 1993; Kerr et al., 1995; Häcker, 2000).

Apoptotic death may also require RNA and protein synthesis (Bowen and Bowen, 1995; Zakeri et al., 1995; Lockshin and Zakeri, 1996; Tata, 1966 apud Häcker, 2000) during the early stages of the process (*de novo* synthesis) (Bowen and Bowen, 1990)

Due to the nuclear changes during apoptotic death (Bowen, 1990; Bowen and Bowen, 1990; Clarke, 1990; Kerr et al., 1995; Zakeri et al., 1995; Lockshin and Zakeri, 1996; Häcker, 2000), as well as *de novo* synthesis (Bowen and Bowen, 1995; Zakeri et al., 1995; Lockshin and Zakeri, 1996; Tata, 1966 apud Häcker, 2000), the utilization of specific techniques is instrumental to examine the nucleus and nucleolus, the organelles affected by the degenerative process, as well as the RNA present in the cytoplasm of these cells.

The application of cytochemical techniques such as the Feulgen reaction (Feulgen and Rossenbeck, 1924) and a variant of the Critical Electrolyte Concentration (CEC) (Mello et al., 1993) to examine the nucleus and nucleolus, respectively, have been widely reported in the literature. The Feulgen reaction is utilized to visualize characteristics of the chromatin (Mello, 1983; Mello et al., 1995; Vidal et al., 1998; Mello and Vidal, 2000; Moraes et al., 2005), as well as in studies on cell death (Mello et

al., 2003; Nunes et al., 2006). The variant of the Critical Electrolyte Concentration (CEC) has been used in studies involving the identification, morphometry, and changes in the structure of the nucleolus (Mello et al., 1993); the identification of different stages of mitosis (Mello, 1995); cells in high secretory activity or undergoing degeneration in the venom glands of *Apis mellifera* (Abreu et al., 2004).

Thus, the purpose of this study was to examine, using cytochemical techniques, the cytoplasmic RNA and nuclear changes in cells of salivary glands of females (unfed, engorged, and at day three post-engorgement) and males (unfed, at day seven post-attachment, at days three and seven post-detachment from the host) of the tick *Rhipicephalus sanguineus*.

MATERIAL AND METHODS

In this study, were utilized females and males of the tick *Rhipicephalus sanguineus*. Were examined unfed, engorged, and at days three post-engorgement females; and unfed, at day seven post-attachment, and at day three and seven post-detachment from the host males. Unfed individuals were provided by Dr. Gervásio Henrique Bechara of the Department of Veterinary Pathology of UNESP, Jaboticabal campus (São Paulo), from a colony maintained under controlled conditions (29^{o} C, 80% humidity, and 12 hour photoperiod) in BOD incubator.

A group of unfed adult ticks (females as well as males) was assigned to cytochemical methods, while another group was placed in a feeding chamber previously glue with an atoxic and non-lesive preparation (Britannia Adhesive-Unit 4, UK) to the shaved back of the host (rabbit) according to technique described elsewhere (Bechara et al., 1995). A group of engorged females and males removed from the host after seven days post-attachment was assigned for cytochemical procedures, while another group was maintained alive in closed containers with breathing holes for three days (females and males), and seven days (only males) to be later analyzed.

After the completion of the time periods examined in this study, salivary glands were processed according to the seven conditions analyzed, removed in 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 minutes. The material was then dehydrated

in increasing concentrations of ethanol (70%, 80%, 90% and 95%), embedded and included in Leica resin, and sectioned at a thickness of 3 μm. Sections were placed on glass slides and processed for the variant of the Critical Electrolyte Concentration (CEC) (Mello et al., 1993) and the Feulgen reaction (Feulgen and Rossenbeck, 1924).

For the variant of the CEC, slides were stained with 0.025% Toluidine Blue in McIlvane's buffer (pH 4.0) for 20 minutes. The material was then immersed in an aqueous solution of 0.05M MgCl for 2, 5, 7, and 10 minutes to detect the ideal time at which metachromasy is abolished, due to the removal of Toluidine Blue molecules bound to the chromatin by $MgCl_2$. At this point, only RNA metachromasy is maintained, consequently staining the nucleolus and demonstrating the presence of cytoplasmic RNA (violet color) (Mello et al., 1993). Slides were then washed with distilled water and mounted in Permount for later observation under light microscope.

For the Feulgen reaction, slides were immersed in 1N HCl solution at 60° C for 11 minutes, then washed in distilled water and stained with Schiff's reagent for two hours in the dark. Slides were counterstained with eosin for 5 minutes and mounted in Canada balsam for later observation under light microscope.

RESULTS

The results obtained with the CEC method revealed that the point in which metachromasy is abolished (chromatin stains green and nucleolus violet) is 5 minutes (Fig. 1).

1. Content of cytoplasmic RNA

In unfed females (Figs. $1A_1$-A_3) and males (Figs. $1D_5$-E_3) of *R. sanguineus*, RNA is observed mainly in the cytoplasm of **undifferentiated** cells of type II acini (Figs. $1A_2$, E_1) and cells of type III acini (Figs. $1A_3$, E_2). In general, RNA staining is more prominent in the gland tissue of females (Figs. $1A_1$-A_3) than those of males (Figs. $1D_5$-E_3).

In engorged females, RNA staining is observed in the cytoplasm of different cells of all acini (Figs. $1A_4$-C_1). Staining is stronger than that observed in the previous condition (Figs. $1A_1$-A_3).

At day three post-engorgement, staining is less prominent (Figs. $1C_2$-D_4) than that of cells in the previous condition (Figs. $1A_4$-C_1), as it is only observed in the cells or cytoplasmic mass of some acini (Figs. $1C_2$, C_4, D_1, D_4).

In males at day seven post-attachment (Figs. $1E_4$-F_2), and at days three (Figs. $1F_3$-G_1) and seven (Figs. $1G_2$-G_4) post-detachment from the host, RNA is detected in the cytoplasm of cells of types II and type III acini. In males at day seven post-attachment (Figs. $1E_4$-F_2), staining is stronger than that of males at day seven post-detachment (Figs. $1G_2$-G_4).

2. Nuclear Characteristics

The changes observed in the nuclei of salivary glands of females and males are characterized by changes in shape (Figs. $2B_1$, B_4, C_1, C_5, D_3, E_1-E_3, F_3, $3B_3$, C_1, C_2, D_2, D_3, D_6, E_2, G_1, G_2, G_5, I_1, I_2), size (Figs. $2B_1$-B_3, C_1, C_5, D_1, D_2, E_1-E_3, F_3, $3B_2$-B_4, C_2, C_4, D_3, E_2, E_6, F_6-G_2, H_1, H_2, I_2), condensation level (Figs. $2B_3$, B_4, D_1, D_2, $3B_4$, C_3, D_4, E_1, E_3, E_6, E_7, F_2, F_4, G_2, G_3, H_1, H_3, I_3, I_4) and arrangement of the chromatin (Figs. $2B_2$, C_2, C_3, D_2-D_4, E_3, F_1-F_3, $3B_5$, B_6, C_4, D_5, D_6, E_4, F_5, F_6, H_2, H_4, I_3, I_4), as well as fragmentation of the nucleus (Figs. $2C_1$, C_4, E_3, F_2, F_3, $3C_3$-C_5, E_5, F_1, F_2, G_4, H_5, H_6). In the nucleolus, we observed changes in shape (Figs. $1B_4$, G_1) and size (Figs. $1B_3$, C_4, F_1, F_2, F_4, F_5, G_3, G_4), disorganization of its structure (Figs. $1B_2$-B_4, C_3, C_4, E_5, F_2, F_5, G_4) as well as fragmentation (Figs. $1B_5$, C_1, C_5, D_1-D_4).

In unfed females and males, cells of types I (Figs. $1A_1$, D_5, $2A_1$, $3A_1$), II (Figs. $1A_2$, E_1, $2A_2$, $3A_2$), and III (Figs. $1A_3$, E_2, $2A_3$, $3A_3$) acini exhibit intact nuclei and nucleoli, with normal size, round shape, and uncondensed chromatin. In type IV acinus of males, nuclei present reduced size and condensed chromatin (Fig. $3A_4$), and nucleoli were not observed (Fig. $1E_3$).

In engorged females, only cells of type I acinus exhibit round nuclei (Fig. $2A_4$) with intact nucleoli (Fig. $1A_4$), normal size, and uncondensed chromatin (Fig. $2A_4$).

At day three post-engorgement, types I (Fig. $2D_4$), II (Fig. $2E_1$) and **Indeterminate** (Figs. $1C_3$-D_4, $2E_2$-F_3) acini exhibit nuclear changes. Type III acini are not observed.

In males at day seven post-attachment, type I acini do not present nuclear changes (Figs. $1E_4$, $3B_1$), unlike types II (Figs. $1E_5$, $3B_2$-B_6), III (Figs. $1F_1$, F_2, $3C_1$-C_5), and IV (Figs. $3D_1$-D_5) acini.

In males at days three (Figs. $1F_4$, F_5, G_1, $3D_6$-F_5) and seven (Figs. $1G_3$, G_4, $3F_6$-I_4) post-detachment from the host, all types of acini exhibit nuclear changes.

FIGURES

FIGURE 1:

Histological sections of salivary glands of *Rhipicephalus sanguineus* submitted to a variant of the Critical Electrolyte Concentration (CEC) **A_1-A_3.** Unfed female. Observe intact nucleoli (**nu**) in types I (**I**), II (**II**), and III (**III**) acini. **A_4-C_1.** Engorged female. Note that among females, the most prominent staining for RNA was observed in engorged females (**∗**) in the cytoplasm of cells of types I (**I**), II (**II**), and III (**III**) acini. **C_2-D_4.** Female at day three post-engorgement. **D_5-E_3.** Unfed male. Note in: **D_5-E_2**, nucleoli (**nu**) of cells of types I (**I**), II (**II**), and III (**III**) acini with no changes, and **E3**, nuclei (**n**) of cells of type IV acinus (**IV**) without clearly visible nucleolus. **E_4-F_2.** Male at day seven post-attachment. Note that among males, the most prominent staining for RNA was observed at seven days post-attachment (**∗**) in the cytoplasm of cells of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini. **F_3-G_1.** Male at day three post-detachment. **G_2-G_4.** Male at day seven post-detachment.

I-IV: types acini; **Ind: Indeterminate** acinus; **∗:** cytoplasmic RNA; **nu:** intact nucleolus; **✦:** disorganized nucleolus; **✪:** enlarged and disorganized nucleolus; **curved arrow:** nucleolus undergoing fragmentation; **fnu:** fragmented nucleolus; **double dashed arrow:** nucleolar fragments located in the margin of the nucleus; **double arrow:** nucleolar fragments in blebs in the nuclear envelope; **enu:** enlarged nucleolus.

Bars: **A_1-G_4**= 12, 5 μm.

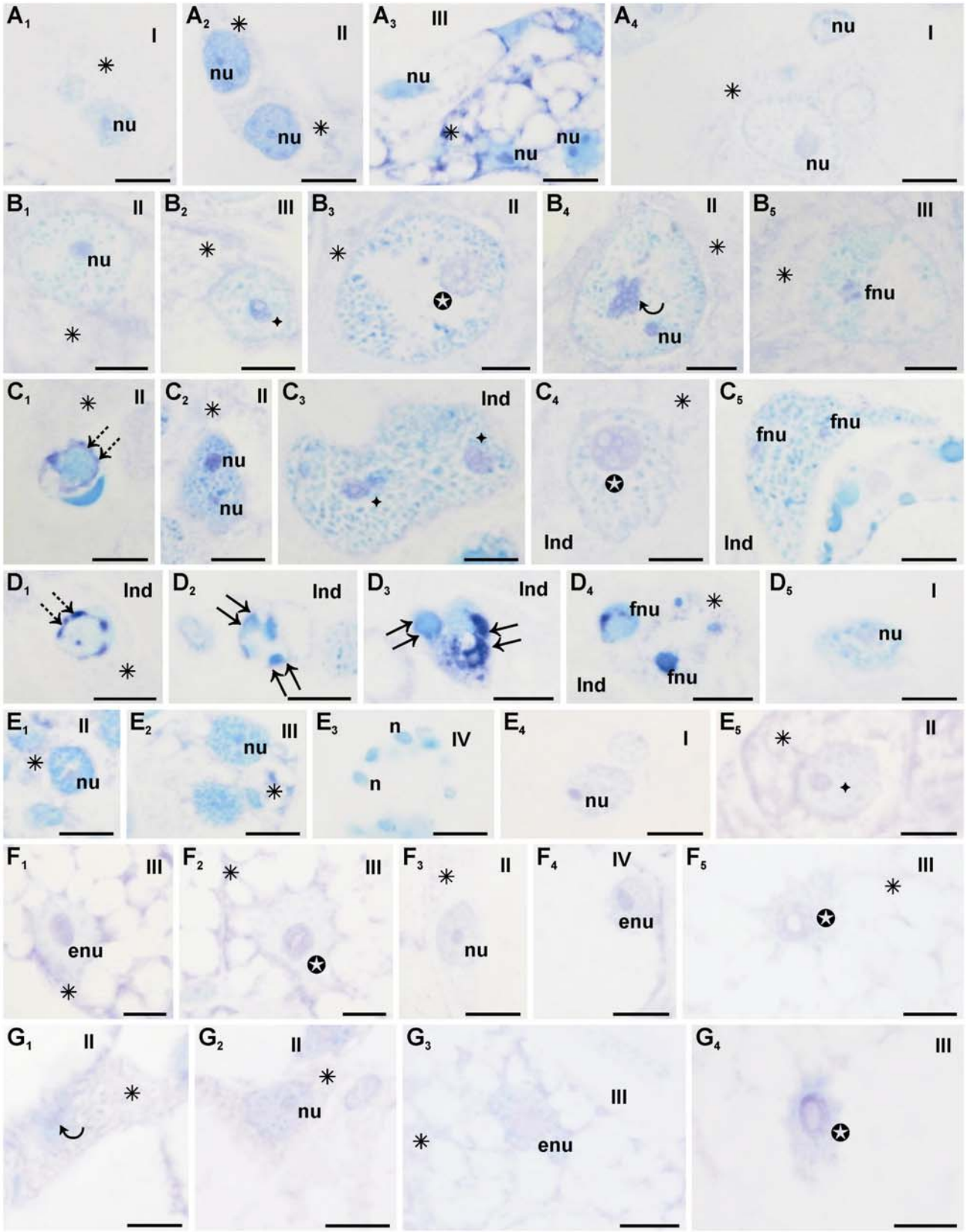
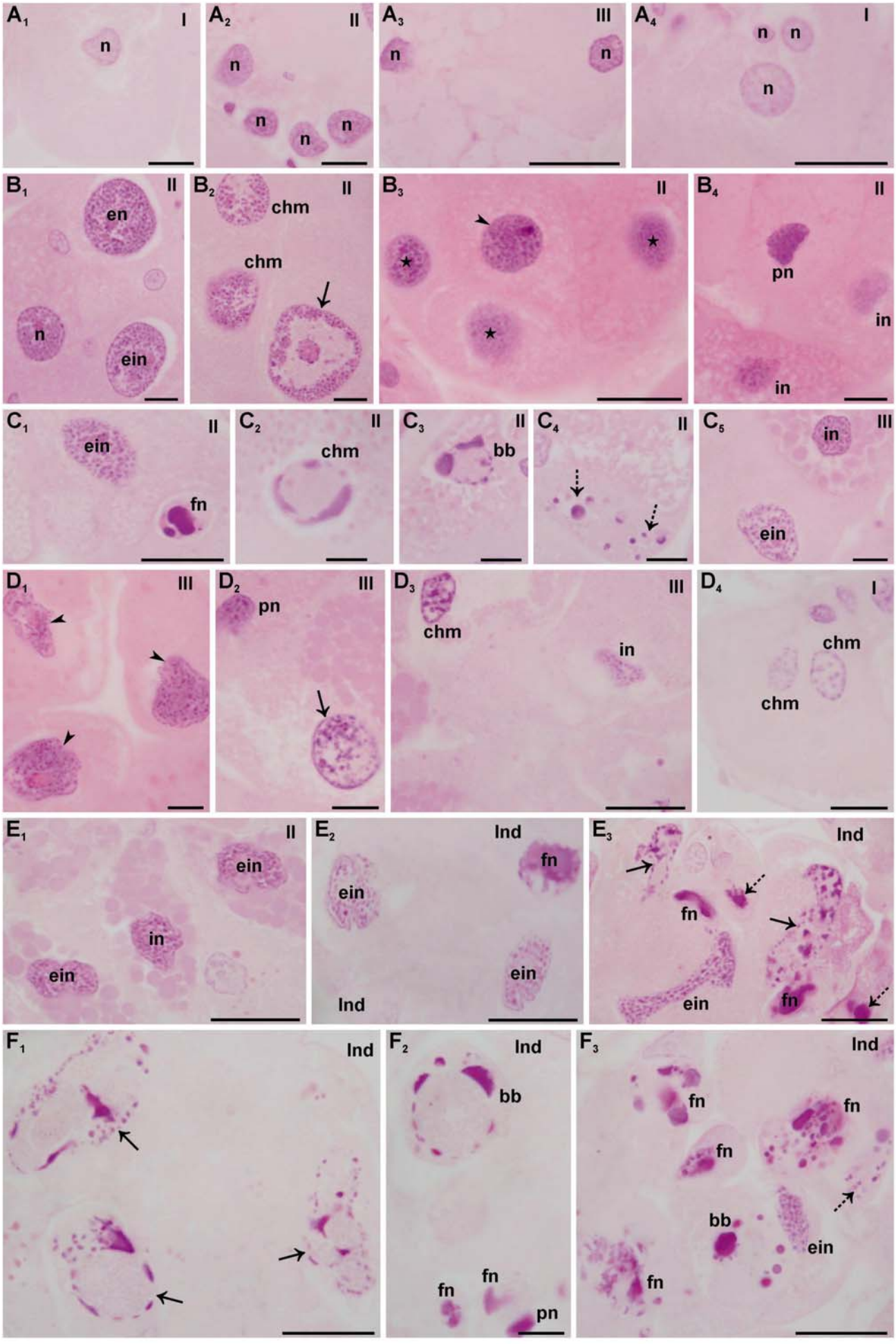

A1 I nu
A2 II nu nu
A3 III nu nu nu
A4 I nu nu
B1 II nu
B2 III
B3 II
B4 II nu
B5 III fnu
C1 II
C2 II nu nu
C3 Ind
C4 Ind
C5 fnu fnu Ind
D1 Ind
D2 Ind
D3 Ind
D4 fnu Ind fnu
D5 I nu
E1 II nu
E2 III nu
E3 n IV n
E4 I nu
E5 II
F1 III enu
F2 III
F3 II nu
F4 IV enu
F5 III
G1 II
G2 II nu
G3 III enu
G4 III

FIGURE 2:

Histological sections of salivary glands of *Rhipicephalus sanguineus* females submitted to the Feulgen reaction. **A_1-A_3.** Unfed: observe intact nuclei (**n**) of cells of types I (**I**), II (**II**) and III (**III**) acini. **A_4-D_3.** Engorged: note in: **A_4**, cell of type I acinus (**I**) with intact nucleus (**n**), and **B_1-D_3**, nuclei of cells of types II (**II**) and III (**III**) acini with changes. **D_4-F_3.** Day three post-engorgement: observe changes in nuclei of cells of types I (**I**), II (**II**), and **Indeterminate** (**Ind**) acini.

I-III: types acini; **Ind: Indeterminate** acinus; **n:** intact nucleus; **en:** enlarged nucleus; **ein:** enlarged and irregular nucleus; **arrow:** enlarged nucleus with chromatin margination; **⋆ :** regular sized nucleus and condensed chromatin; **arrow head:** enlarged nucleus with condensed chromatin; **pn:** picnotic nucleus; **in:** irregular nucleus; **fn:** fragmenting nucleus; **chm:** normal sized nucleus and with chromatin margination; **bb:** blebs; **dashed arrow:** fragmented nucleus.

Bars: **A_1**, **A_2**, **B_1**, **B_2**, **B_4**, **C_2-D_2**, **D_4** and **F_2**= 12, 5 μm; **A_3**, **A_4**, **B_3**, **C_1**, **D_3**, **E_1-F_1** and **F_3**= 25 μm.


A1 I
n
A2 II
n
n
n
A3 III
n
n
A4 I
n n
n
B1 II
en
n
ein
B2 II
chm
chm
B3 II
B4 II
pn
in
in
C1 II
ein
fn
C2 II
chm
C3 II
bb
C4 II
C5 III
in
ein
D1 III
D2 III
pn
D3 III
chm
in
D4 I
chm
chm
E1 II
ein
in
ein
E2 Ind
fn
ein
ein
Ind
E3 Ind
fn
ein
fn
F1 Ind
F2 Ind
bb
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F3 Ind
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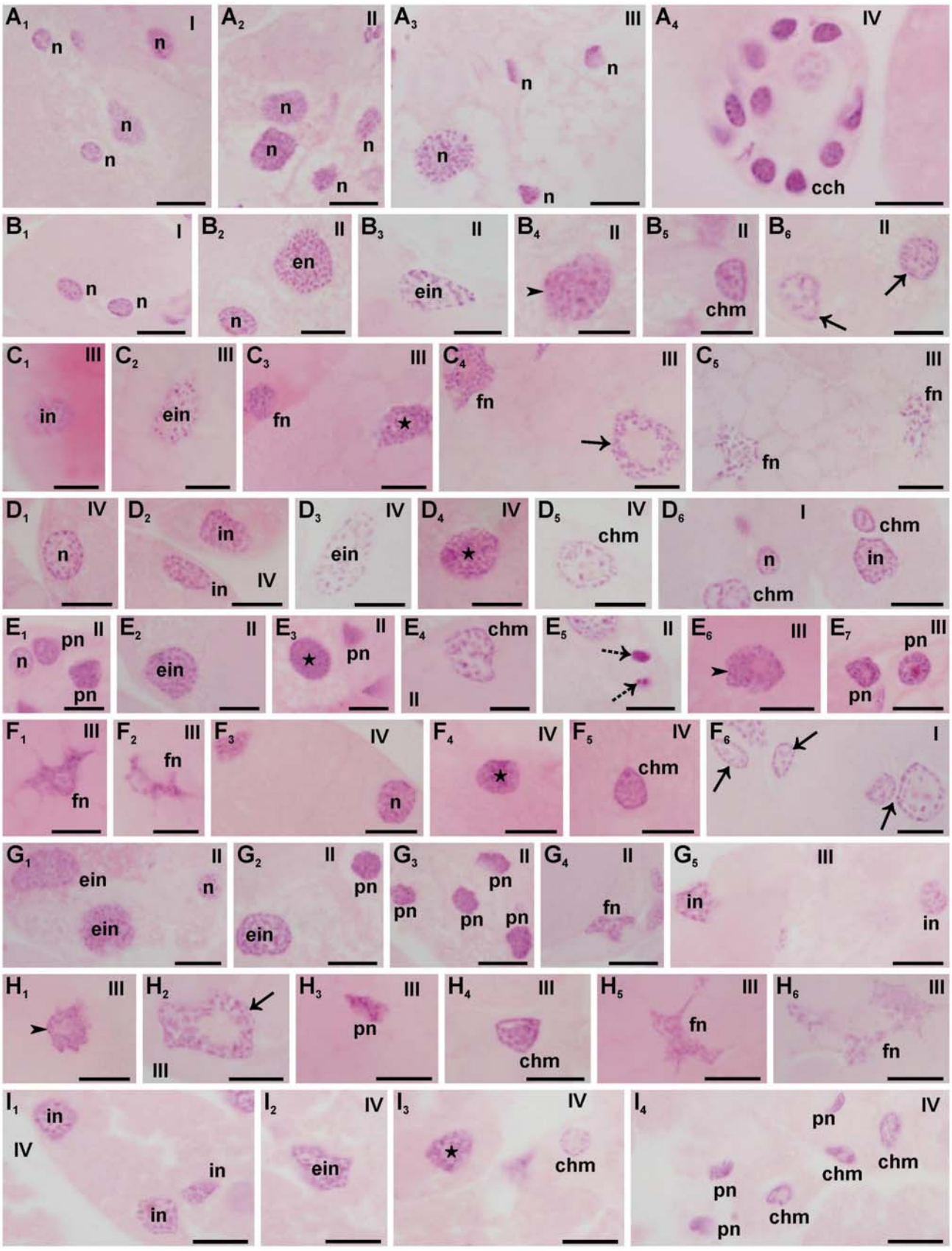
FIGURE 3:

Histological sections of salivary glands of *Rhipicephalus sanguineus* males submitted to the Feulgen reaction. **A_1-A_4.** Unfed: observe in **A_1-A_3**, absence of changes in nuclei (**n**) of cells of types I (**I**), II (**II**), and III (**III**) acini, and **A_4**, cells of type IV acinus (**IV**) with condensed chromatin (**star**). **B_1-D_5.** Day seven post-attachment: note in **B_1**, type I acinus (**I**) with intact nuclei (**n**) and **B_2-D_5**, nuclei of cells of types II (**II**), III (**III**) and IV (**IV**) acini exhibiting changes. **D_6-F_5.** Day three post-detachment: note nuclei of cells of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini exhibiting changes. **F_6-I_4.** Day seven post-detachment: observe nuclei of types I (**I**), II (**II**), III (**III**), and IV (**IV**) acini exhibiting changes.

I-IV: types acini; **n:** intact nucleus; **cch:** nucleus with condensed chromatin; **en:** enlarged nucleus; **ein:** enlarged and irregular nucleus; **arrow head:** enlarged nucleus with condensed chromatin; **chm:** normal sized nucleus and with chromatin margination; **arrow:** enlarged nucleus with chromatin margination; **in:** irregular nucleus; ⋆ **:** regular-sized nucleus and condensed chromatin; **fn:** fragmenting nucleus; **pn:** picnotic nucleus; **dashed arrow:** fragmented nucleus.

Bars: A_1-I_4= 12, 5 μm.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A2 II n n n n n
A3 III n n n n
A4 IV cch
B1 I n n
B2 II en n
B3 II ein
B4 II
B5 II chm
B6 II
C1 III in
C2 III ein
C3 III fn
C4 III fn
C5 III fn fn
D1 IV n
D2 in in IV
D3 IV ein
D4 IV
D5 IV chm
D6 I chm n in chm
E1 II pn n pn
E2 II ein
E3 II pn
E4 chm II
E5 II
E6 III
E7 III pn pn
F1 III fn
F2 III fn
F3 IV n
F4 IV
F5 IV chm
F6 I
G1 II ein n ein
G2 II pn ein
G3 II pn pn pn pn
G4 II fn
G5 III in in
H1 III
H2 III
H3 III pn
H4 III chm
H5 III fn
H6 III fn
I1 in IV in in
I2 IV ein
I3 IV chm
I4 IV pn chm chm pn chm pn

DISCUSSION

The present study revealed changes in the nucleus and RNA content of the salivary gland tissue of unfed, engorged females and at day three post-engorgement; and males at day seven post-attachment, and at days three and seven post-detachment of the tick *Rhipicephalus sanguineus*.

In gland cells of unfed females and males of *R. sanguineus*, there were no changes in the nucleus, the chromatin was uncondensed (granular texture) and intact nuclei and nucleoli were observed in types I, II, and III acini. These nuclear characteristics indicate synthesis of mRNA and rRNA, which was confirmed by RNA staining observed in the cytoplasm of some cells of types I, II and III acini, as also reported by other studies on *R. sanguineus* (Furquim, 2006). These RNA molecules might be utilized in the synthesis of acid phosphatase in unfed individuals (Furquim, 2006), and/or be stored for later use in the secretory phase, a period during which a high synthesis of proteins occurs (Binnington, 1978, Walker et al., 1985).

In type IV acinus of unfed males of *R. sanguineus*, the presence of reduced nuclei, condensed chromatin, and absence of nucleoli and cytoplasmic RNA supports the results obtained by Furquim (2006) with males of *R. sanguineus*, indicating absence of transcription and cell inactivity (Till, 1961; Binnington, 1978; Walker et al., 1985; Fawcett et al., 1986; Gill and Walker, 1987; Sonenshine, 1991; Serra-Freire and Olivieri, 1993).

The most prominent staining of cytoplasmic RNA was observed in engorged females and males at day seven post-attachment, as observed by Bowen and Bowen (1995), Zakeri et al. (1995), Lockshin and Zakeri (1996), and Tata (1966) apud Hacker (2000). These authors reported that apoptosis might require synthesis of RNA and proteins during the early stages of the apoptotic process (Bowen and Bowen, 1990). RNA molecules might also be used in the synthesis of acid phosphatase, as the most intense staining for this enzyme was detected in gland cells of females at day three post-engorgement and males at day three post-detachment from the host (Furquim, 2006). This supports the hypothesis suggested in previous studies that acid phosphatase

detected in the degenerating salivary glands of females and males of *R. sanguineus* might have an endogenous origin.

In the gland cells of females and males examined in this study, the nuclear changes became more prominent as time progressed after the tick was detached from the host, especially in types II, III, and IV acini. These changes were more evident in females than males.

In engorged females, only type I acinus did not exhibit changes. At day three post-engorgement, cells of types I, II and **Indeterminate** acini presented nuclear changes, while type III acini were no longer observed, confirming the obtained by Furquim (2005) that reported histological changes in nucleoli.

In males at day seven post-attachment, only cells of type I acinus did not present nuclear changes, unlike those at days three and seven days post-detachment from the host, in which all cells exhibited changes.

Our findings on the structural organization of the nucleus of cells of degenerating salivary glands of males and females of *R. sanguineus* confirm the obtained by Nunes et al. (2006) that reported in females of *R. (Boophilus) microplus*, enlarged nuclei with irregular shape, different condensation levels and margination of the chromatin, and fragmentation.

In this study, the presence of condensed and marginal chromatin, blebs, and nuclear fragmentation, as well as fragmentation of the nucleolus confirmed the results obtained by Kerr et al. (1995). These authors described nuclear changes in apoptotic cells, emphasizing that fragments of the nucleolus are found in nuclei or in their fragments, as observed in our study. In the cells of degenerating gland tissues examined, the presence of enlarged nucleus with uncondensed chromatin and dilated and or disorganized nucleoli clearly demonstrates the breakdown of chromatin, including the DNA regions responsible for the organization of the nucleolus (DNAr). This might be due to DNA cleavage, a characteristic commonly observed during apoptotic death (Bowen and Bowen, 1990; Bowen, 1993; Lockshin and Zakeri, 1996; Zakeri and Ahuja, 1997; Häcker, 2000).

Lockshin and Zakeri (1996), and Häcker (2000) reported that chromatin fragmentation (DNA cleavage) is an early sign of the apoptotic process associated with

the morphological changes in the nucleus (Häcker, 2000). The several nuclear changes observed in the present study may be the result of biochemical and morphological changes in the nucleus during apoptotic death (Bowen and Bowen, 1990; Bowen, 1993; Lockshin and Zakeri, 1996; Zakeri and Ahuja, 1997; Häcker, 2000). These changes might be due to asynchronism of the degeneration process among cells of a same acinus as well as among different acini (Furquim, 2005), indicating that nuclei with uncondensed chromatin and enlarged and/or disorganizing nucleoli may represent nuclear changes previous to condensation and margination of chromatin, formation of blebs, and nuclear fragmentation.

ACKNOWLEDGMENTS

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REFERENCES

Abreu, R.M.M., Silva de Maraes, R.L.M., Taboga, S.R., Camargo-Mathias, M.I., 2004. Cytochemical studies of the nuclei of the venom glands' cells of *Apis mellifera* (Hymenoptera, Apidae). Acta Histochemica Cytochemica 37 (5), 295-299.

Bechara, G.H., Szabó, M.P.J., Ferreira, B.R., Garcia, M.V., 1995. *Rhipicephalus sangineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Brazilian Journal of Veterinary Parasitology 4, 61-66.

Binnington, K.C., 1978. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, *Boophilus microplus*. International Journal for Parasitology 8, 97-115.

Bowen, I.D., 1993. Apoptosis or programmed cell death ? Cell Biology International 17 (4), 365-380.

Bowen, I.D., Bowen, S.M., 1990. Programmed Cell Death in Tumours and Tissues. Chapman and Hall, London.

Clarke, P.G.H., 1990. Developmental cell death: morphological diversity and multiple mechanisms. Anatomy and Embryology 181, 195-213.

Fawcett, D.W., Binnington, K., Voigt, W.P., 1986. The cell biology of the ixodid tick salivary gland. In: Sauer, J.R., Hair, A. (Eds.), Morphology, physiology, and behavioral biology of ticks. Ellis Horwood, Chichester, pp. 22-45.

Feulgen, R., Rossenbeck, H., 1924. Mikroskopisch- Chemischer Nachweis einer Nucleinsaure vom Typus der Thymonucleinsaure und die Darauf Beruhend Elective Farbung von Zillkernen in Mikroskopischen Praparaten Hoppe-Seylers. Zeitschrift fur Physiologische Chemie 135, 203-248.

Furquim, K.C.S., 2005. Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados, 2º Relatório Científico CAPES, Instituto de Biociências-UNESP, Rio Claro (S.P.), Brasil, 40 p.

Furquim, K.C.S., 2006. Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados. 3º Relatório Científico CAPES, Instituto de Biociências, UNESP, Rio Claro (S.P.), Brasil, 27 p.

Gill, H.S., Walker, A.R., 1987. The salivary glands of *Hyalomma anatolicum anatolicum*: structural changes during attachment and feeding. International Journal for Parasitology 17 (8), 1381-1392.

Häcker, G., 2000. The morphology of apoptosis. Cell and Tissue Research 301. 5-17.

Kerr, J.F.R., Gobé, G.C., Winterford, C.M., Harmon, B., 1995. Anatomical Methods in Cell Death. In: Schwartz, L.M., Osborne, B.A. (Eds.), Cell Death. Academic Press, San Diego, pp. 1-27.

Lockshin, A.R., Zakeri, Z., 1996. The Biology of Cell Death and Its Relationship to Aging. In: Holbrook, N., Martin, G.R., Lockshin, R.A. (Eds.), Cellular Aging and Cell Death. Wiley-Liss, New York, pp. 167-180.

Mello, M.L.S., 1983. Cytochemical properties of euchromatin and heterochromatin. Histochemical Journal 15, 739-751.

Mello, M.L.S., Vidal, B.C.V., 2000. Chromatin organization in normal and cancer cells. Journal of Women's Cancer 2 (2), 73-78.

Mello, M.L.S., Vidal, B.C., Dantas, M.M., Monteiro, A.L.P. 1993. Discrimination of the nucleolus by a critical eletrolyte concentration method. Acta Histochemica et Cytochemica 26 (1), 1-3.

Mello, M.L.S., Contente, S., Vidal, B.C., Planding, W., Schenck, U. 1995. Modulation of *ras* transformation affecting chromatin supraorganization as assessed by image analysis. Experimental Cell Research 220, 374-382.

Mello, M.L.S., Barbisan, L.F., Lareef, M.H., Russo, J., Vidal, B.C. 2004. Cell death evaluation in benzo[α]pyrene-transformed human breast epithelial cells after microcell-mediated transfer of chromosomes 11 and 17. Mutation Research 546 (1-2), 39-43.

Moraes, A.S., Vidal, B.C., Guaraldo, A.M.A., Mello, M.L.S. 2005. Chromatin supraorganization and extensibility in mouse hepatocytes following starvation and refeeding. Cytometry Part A 63A, 94-107.

Nunes, E.T., Camargo-Mathias, M.I., Bechara, G.H., 2006. Structural and cytochemical changes in the salivary glands of the *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae) tick female during feeding. Veterinary Parasitology 140, 114-123.

Serra-Freire, N.M., Olivieri, J.A., 1993. Structure of the salivary glands of the unfed male tick *Amblyomma cajennense* (Fabricius) (Acari: Ixodidae). Memórias do Instituto Oswaldo Cruz 88 (2), 207-213.

Sonenshine, D.E., 1991. Biology of Ticks. Oxford University Press, New York.

Tata, J.R., 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. Developmental Biology 13, 77-94.

Till, W.M., 1961. A Contribution to the Anatomy and Histology of the Brown Ear Tick *Rhipicephalus appendiculatus*. Memoirs of the Entomological Society of Southern Africa, 6, 1-124.

Vidal, B.C., Russo, J., Mello, M.L.S. 1998. DNA content and chromatin texture of benzo[α]pyrene-transformed human breast epithelial cells as assessed by image analysis. Experimental Cell Research 244, 77-82.

Walker, A.R., Fletcher, J.D., Gill, H., 1985. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. International Journal for Parasitology 15 (1), 81-100.

Zakeri, Z.F., Ahuja, H.S., 1997. Cell death/apoptosis: normal, chemically induced, and teratogenic effect. Mutation Research 396, 149-161.

Zakeri, Z., Bursch, W., Tenniswood, M., Lockshin, R.A., 1995. Cell death: programmed, apoptosis, necrosis, or other? Cell death and Differentiation 2, 87-96.

Discussão Geral

V. DISCUSSÃO GERAL

O presente estudo comparou as glândulas salivares de fêmeas e de machos de carrapatos *Rhipicephalus sanguineus* em diferentes condições de alimentação: fêmeas em jejum, semi-alimentadas (com dois e quatro dias de ingurgitamento), alimentadas (ingurgitadas) e pós-alimentadas (com três e sete dias pós-ingurgitamento); machos em jejum, com dois, quatro e sete dias de infestação e com três e sete dias pós-remoção do hospedeiro, registrando as alterações sofridas desde o início do ciclo secretor até a completa degeneração do órgão.

Nos indivíduos de ambos os sexos, em jejum, observou-se alguma atividade celular, pois apenas poucas células continham secreção, enquanto outras já estavam com o citoplasma reduzido, supostamente células em baixa atividade de síntese ou mesmo inativas. Já nas fêmeas com dois dias de alimentação e nos machos com dois dias de infestação as glândulas estavam com suas células ativas, corroborando dados obtidos por Sanders et al. (1996) em *Amblyomma americanum*, que demonstraram que o início da alimentação estimularia o desenvolvimento das glândulas, aumentando nas células principalmente a síntese de vários tipos de proteínas.

Nas fêmeas com quatro dias de alimentação as glândulas se mostraram ainda mais desenvolvidas, ao contrário do observado nos machos com quatro dias de infestação, sugerindo que, diferentemente das fêmeas, nestes últimos estes órgãos estariam no final de um dos ciclos secretores pelos quais passam devido às sucessivas refixações ao hospedeiro.

O presente estudo mostrou que em *R. sanguineus* os ácinos I, tanto de fêmeas quanto de machos, independentemente do estágio de alimentação do indivíduo, não

sofreram alterações nem no tamanho e nem na histologia, corroborando Binnington (1978) e Walker et al. (1985), que relataram que a forma e a histologia deste tipo de ácino persistiriam durante a alimentação. Os resultados para fêmeas com três dias pós-ingurgitamento e para machos com três dias pós-remoção do hospedeiro indicaram que estes seriam os estágios de alimentação onde primeiro surgiriam as características degenerativas em *R. sanguineus*.

Os ácinos II, nos dois sexos de *R. sanguineus*, apresentaram um número maior de tipos de células com função secretora do que aquele relatado na literatura para outras espécies (BINNINGTON, 1978; WALKER et al., 1985; GILL; WALKER, 1987; MARZOUK; DARWISH, 1994), ou seja, existem ainda as células "**indiferenciadas**"(fêmeas e machos em jejum), **indefinidas 1** e **2** (fêmeas e machos em jejum), **c5** e **c6** (fêmeas com dois e quatro dias de alimentação e machos com dois e quatro dias de infestação) e **c7** e **c8** (machos com dois dias de infestação).

Nos indivíduos aqui estudados no estado de jejum, além das células "**indiferenciadas**", que provavelmente seriam ativadas somente depois do início da alimentação, ocorreriam também as **indefinidas 1** e **2** e as **a**, **c1** e **c3**, discordando dos dados de Binnington (1978) e Gill e Walker (1987), que observaram nestes mesmos tipos de ácinos, também em indivíduos em jejum de *Boophilus microplus* e *Hyalomma anatolicum* anatolicum, respectivamente, um número maior de tipos celulares.

O presente trabalho mostrou também que em fêmeas com dois dias de alimentação e machos com dois de infestação, portanto ambos no estágio inicial do período alimentar, todas as células dos ácinos II intensificaram o processo de síntese e secreção, corroborando Walker et al. (1985), que afirmaram que as glândulas salivares não se tornariam completamente ativas até o iniciar da alimentação.

Nas fêmeas com quatro dias de alimentação os ácinos II estavam ainda mais desenvolvidos quando comparados aos daquelas com dois dias de alimentação, isso devido ao grande acúmulo de secreção nas suas células. Estes resultados indicaram que a partir deste período a glândula salivar participaria mais intensamente no processo de alimentação do parasita. Neste estágio de alimentação as células do tipo **c6** não foram mais observadas, provavelmente devido à regressão e concomitante perda de função das mesmas. Nos machos com quatro dias de infestação, além dos ácinos II estarem menos

ativos, as células **c6**, **c7** e **c8** também não foram mais observadas, sugerindo interrupção ou finalização de suas funções.

Ainda nos ácinos II de fêmeas alimentadas e com três dias pós-alimentação observou-se o início da degeneração celular, sendo possível identificar: nas primeiras apenas os tipos celulares **a**, **c1** e **c3**, nas com três dias pós-alimentação somente os **a** e **c3** e nas com sete dias pós-ingurgitamento não se observaram mais ácinos do tipo II. Nos machos com sete dias de infestação somente algumas células dos tipos **c1** e **c8** estavam íntegras; naqueles com três e sete dias pós-remoção todos os tipos estavam em degeneração e especificamente naqueles com sete dias pós-remoção os **c2** já tinham degenerado, uma vez que não foram mais observados, dados estes primeiramente relatados para machos de carrapatos.

Os ácinos III das fêmeas, diferentemente do observado nos machos, aumentaram progressivamente de tamanho com o decorrer da alimentação e suas células, principalmente na fase de grande consumo de sangue do carrapato, sofreram rápidas transformações, passando de piramidais a pavimentosas, provocando assim um aumento no lúmen do ácino e corroborando Binnington (1978), Walker et al. (1985), Fawcett et al. (1986) e Sonenshine (1991). No caso específico dos machos a ausência de alterações nos ácinos III dar-se-ia devido ao pequeno volume de sangue por eles consumido.

Ainda nos ácinos III dos carrapatos aqui estudados as células **f**, ao contrário das **d** e **e**, estavam com citoplasma reduzido. Na condição de alimentação as células **d** e **e** permaneceram repletas de secreção e as **f** apresentaram diferenças dependendo do estágio alimentar considerado, como por exemplo: nas fêmeas com dois dias de alimentação elas estavam ativas e nas com quatro dias não continham mais secreção, provavelmente devido à perda de atividade secretora e atuação agora na osmorregulação, corroborando dados de Binnington (1978) e Gill e Walker (1987). Já nos machos na situação de alimentação, estas células apresentaram a mesma morfologia encontrada nos machos em jejum, sugerindo a não participação das mesmas nem no processo de secreção e nem no de osmorregulação, tendo sido consideradas por Binnington (1978) como inativas, ao contrário do que ocorreria nas fêmeas.

Os ácinos III começaram a apresentar sinais de degeneração nas fêmeas alimentadas, embora neles ainda pudessem ser identificados todos os tipos celulares descritos na literatura. Naquelas com três dias pós-alimentação poucos ácinos III foram

identificados. Aqueles com sete dias pós-alimentação não apresentaram mais os ácinos III. Nestes ácinos de machos o início da degeneração ocorreu nos indivíduos com sete dias de infestação, onde estavam presentes os três tipos celulares e naqueles com três e sete dias pós-remoção as células **f** não foram mais observadas.

No caso dos ácinos IV, específicos dos machos, em *R. sanguineus* as células começaram a sintetizar e secretar intensamente apenas com o iniciar da alimentação, enquanto naqueles com quatro dias de infestação, onde as glândulas estavam completando um dos seus ciclos secretores, observou-se uma queda nestes processos. Os indícios de degeneração surgiram, portanto, primeiramente nos machos com sete dias de infestação. Os dados aqui obtidos corroboram parcialmente aqueles de Binnington (1978), Walker et al. (1985), Fawcett et al. (1986), Gill e Walker (1987) e Sonenshine (1991).

O acompanhamento da atividade secretora das glândulas salivares de fêmeas e machos de *R. sanguineus* mostrou que estes órgãos começaram a degenerar após a secreção de substâncias que fixariam o carrapato ao hospedeiro e que manipulariam a resposta imunológica deste último. Este estudo revelou ainda que a degeneração glandular se intensificar-se-ia à medida que o parasita passasse mais tempo fora do hospedeiro, ocorrendo nas fêmeas as alterações mais significativas.

De forma geral o processo de degeneração glandular em *R. sanguineus* foi assincrônico entre os diferentes ácinos, bem como entre os diferentes tipos de células de um mesmo ácino. Os I foram os últimos afetados, permanecendo, portanto, íntegros nas fêmeas ingurgitadas e nos machos com sete dias de infestação, corroborando os dados de Till (1961), L'Amoreaux et al. (2003) e Nunes et al. (2006b), em *R. appendiculatus*, *D. variabilis* e *R (Boophilus) microplus*, respectivamente. Os resultados confirmaram que o processo de degeneração em *R. sanguineus* teve início na região posterior da glândula salivar, onde as características de degeneração foram primeiro observadas. Na anterior, no entanto, ainda foram encontrados ácinos íntegros, padrão este também descrito para a degeneração glandular na maioria dos insetos (Silva de Moraes, 1998; Abreu et al., 2004).

Os dados aqui obtidos também indicaram que as glândulas salivares perderiam sua capacidade secretora, mostrada pela diminuição de grânulos e pelo surgimento de características degenerativas nas fêmeas alimentadas e nos machos com sete dias de

infestação. Harris e Kaufman (1984) também relataram para *A. hebraeum* a redução de mais de 90% da capacidade secretora máxima de glândulas de fêmeas desta espécie.

O estudo da degeneração glandular em *R. sanguineus*, sob o aspecto citoquímico, mostrou que as células morreriam por apoptose, corroborando L'Amoreaux et al. (2003), Bowman e Sauer (2004), Nunes et al. (2005) e Nunes et al. (2005, 2006a, b), apresentando também características de morte autofágica, confirmada pela presença da fosfatase ácida, a qual neste caso atuaria secundariamente, auxiliando na remoção de restos citoplasmáticos das células glandulares, bem como fragmentando e contribuindo, desta forma, para a desorganização dos ácinos e formação de corpos apoptóticos, o que caracterizaria a apoptose atípica.

A forte marcação RNA positiva no citoplasma das células glandulares das fêmeas alimentadas e dos machos com sete dias de infestação foi a primeira indicação da ocorrência de morte por apoptose nestes indivíduos. Segundo alguns autores a síntese de RNA e as proteínas surgiriam já no início do processo apoptótico, sendo estas últimas consideradas as promotoras da apoptose (BOWEN; BOWEN, 1990; BOWEN; BOWEN, 1995; ZAKERI et al., 1995; LOCKSHIN; ZAKERI, 1996; TATA, 1966 apud HÄCKER, 2000).

Outros indicativos aqui observados, que confirmariam a apoptose nas glândulas salivares, seriam as alterações nucleares precoces, bem como a retração citoplasmática sofrida por suas células secretoras (CLARKE, 1990; BOWEN, 1993; KERR et al., 1995; LOCKSHIN; ZAKERI, 1996; HÄCKER, 2000).

Por outro lado, o presente estudo revelou, além da queda precoce da atividade ATPásica, perda da integridade da membrana e aumento da atividade da fosfatase ácida, dados que não caracterizariam no entanto a apoptose clássica. Mullarkey (1987), apud Bowen e Bowen (1990); Bowen et al. (1988), apud Bowen e Bowen (1990); Bowen e Bowen (1990); Kerr et al. (1995) afirmaram que na morte apoptótica a perda da atividade ATPásica e da integridade da membrana seriam eventos observados somente nas fases muito avançadas da degeneração ou após a formação dos corpos apoptóticos e segundo Pipan e Ravoc (1980), Clarke (1990), Cummings e Bowen (1992), Bowen (1993), Zakeri et al. (1995), Lockshin e Zakeri (1996), Jochová et al. (1997) e Gregorc et al. (1998) a fosfatase ácida estaria principalmente envolvida com a morte autofágica. Os dados aqui obtidos sobre a atividade da ATPase e da fosfatase ácida concordaram

com Bowen et al. (1993), que estudando o processo de morte nas glândulas salivares de *Calliphora vomitoria* detectaram um momento em que a atividade da ATPase cessaria, indicando que na morte celular programada nem sempre ocorreria atividade ATPásica e preservação da integridade da membrana até os estágios tardios do processo.

Neste estudo, nas fêmeas alimentadas e nos machos com sete dias de infestação detectou-se aumento da atividade da fosfatase ácida, a qual se intensificou nas fêmeas com três dias pós-alimentação, provocando maior vacuolização nas células. Ao contrário, nos machos com três dias pós-remoção do hospedeiro houve um aumento na atividade da fosfatase ácida, que diminuiu naqueles com sete dias pós-remoção. A queda da fosfatase nas glândulas dos machos ocorreu devido a sua utilização e não mais produção no processo de degeneração, pois nestes indivíduos, ao contrário do que ocorreria nas fêmeas, a degeneração não seria hormonalmente estimulada (Kaufman, 1990; Lomas, 1993 apud Lomas et al., 1998).

De forma geral o presente estudo com as glândulas salivares de carrapatos da espécie *R. sanguineus*, além de confirmar algumas informações já existentes na literatura, trouxe à luz alterações que as mesmas sofrem no decorrer do processo de alimentação, como as resumidas nas páginas 211 à 218.

ÁCINO I					
	Jejum			**Jejum**	
		idem			idem
	2 Dias de Alimentação	idem		**2 Dias de Infestação**	idem
	4 Dias de Alimentação	idem		**4 Dias de Infestação**	idem
FÊMEAS	**Alimentada**	idem	**MACHOS**	**7 Dias de Infestação**	idem
	3 Dias após	idem, início da degeneração com alterações nucleares, pouca ou nenhuma atividade ATPásica e grande atividade fosfatásica		**3 Dias após**	idem, início da degeneração com alterações nucleares, pouca ou nenhuma atividade ATPásica e grande atividade fosfatásica
	7 Dias após	degenerado		**7 Dias após**	idem, com pouca atividade fosfatásica

ÁCINO I					
	Jejum	célula **central** e células **periféricas**		**Jejum**	célula **central** e células **periféricas**
FÊMEAS	**2 Dias de Alimentação**	idem	**MACHOS**	**2 Dias de Infestação**	idem
	4 Dias de Alimentação	idem		**4 Dias de Infestação**	idem
	Alimentada	idem		**7 Dias de Infestação**	não descrito na literatura
	3 Dias após	não descrito na literatura		**3 Dias após**	não descrito na literatura
	7 Dias após	não descrito na literatura		**7 Dias após**	não descrito na literatura

idem: refere-se sempre ao estágio alimentar anterior;

■ descrição segundo Binnington (1978);

■ dados apresentados neste estudo;

■ dados apresentados neste estudo referentes ao processo de morte celular;

ÁCINO II					
	Jejum	células indiferenciadas, **indefinidas 1** e **2, a**, **c1** e **c3**		**Jejum**	células **indiferenciadas**, **indefinidas 1** e **2**, **a**, **c1** e **c3**
	2 Dias de Alimentação	células **a**, **b**, **c1-c6**		**2 Dias de Infestação**	células **a**, **b**, **c1-c8**
	4 Dias de Alimentação	**c5** regrediu e o restante persistiu		**4 Dias de Infestação**	**c6**, **c7** e **c8** regrediram e o restante persistiu
FÊMEAS	**Alimentada**	presença de **a**, **c1**, **c3** e **indeterminadas** em degeneração, com alterações nucleares, pouca atividade ATPásica e grande atividade fosfatásica	**MACHOS**	**7 Dias de Infestação**	**c1** e **c8** ou integras ou em degeneração **a**, **c2-c5** e **indeterminadas** em degeneração, com alterações nucleares, pouca atividade ATPásica e grande atividade fosfatásica
	3 Dias após	presença de **a**, **c3** e **indeterminadas** em degeneração, com pouca ou nenhuma atividade ATPásica, grande atividade fosfatásica e corpos apoptóticos		**3 Dias após**	**c1-c5**, **c8** e **indeterminadas** também em degeneração, com alterações nucleares, pouca ou nenhuma atividade ATPásica, grande atividade fastásica e corpos apoptóticos
	7 Dias após	degenerado		**7 Dias após**	**c2** ausente **a**, **c3-c5** e **indeterminadas** persistiram pouca atividade fosfatásica

			ÁCINO II		
	Jejum	células **a**, **b** e **c1-c4**		**Jejum**	células **a**, **b**, **c1**, **c3** e **c4**
	2 Dias de Alimentação	idem		**2 Dias de Infestação**	células **a**, **b** e **c1-c4**
	4 Dias de Alimentação	não descrito na literatura		**4 Dias de Infestação**	não descrito na literatura
	Alimentada	células **a**, **b**, **c1** e **c3**		**7 Dias de Infestação**	não descrito na literatura
FÊMEAS	**3 Dias após**	células **b** e **c3**	**MACHOS**	**3 Dias após**	não descrito na literatura
	7 Dias após	não descrito na literatura		**7 Dias após**	não descrito na literatura

ÁCINO III					
	Jejum	idem		**Jejum**	idem
	2 Dias de Alimentação	idem		**2 Dias de Infestação**	idem
	4 Dias de Alimentação	**d** e **e** pavimentosas e granulares **f** pavimentosas e agranulares		**4 Dias de Infestação**	idem
FÊMEAS	**Alimentada**	**d**, **e** e **f** em degeneração, com alterações nucleares, pouca ou nenhuma atividade ATPásica e grande atividade fosfatásica	**MACHOS**	**7 Dias de Infestação**	**d**, **e** e **f** em degeneração, com alterações nucleares, pouca ou nenhuma atividade ATPásica e grande atividade fosfatásica
	3 Dias após	degenerado		**3 Dias após**	**f** ausentes **d** e **e** persistiram grande atividade fosfatásica e corpos apoptóticos
	7 Dias após	degenerado		**7 Dias após**	**d** e **e** em degeneração, com pouca atividade fosfatásica

		ÁCINO III			
	Jejum	**d** e **e** cúbicas e granulares **f** cúbicas e agranulares		**Jejum**	**d** e **e** cúbicas e granulares **f** cúbicas e agranulares
	2 Dias de Alimentação	**d**, **e** e **f** cúbicas e granulares		**2 Dias de Infestação**	idem
	4 Dias de Alimentação	não descrito na literatura		**4 Dias de Infestação**	idem
FÊMEAS	**Alimentada**	**d** e **e** pavimentosas e granulares **f** pavimentosas e agranulares	**MACHOS**	**7 Dias de Infestação**	não descrito na literatura
	3 Dias após	não descrito na literatura		**3 Dias após**	não descrito na literatura
	7 Dias após	não descrito na literatura		**7 Dias após**	não descrito na literatura

	ÁCINO IV	
MACHOS	**Jejum**	idem
	2 Dias de Infestação	idem
	4 Dias de Infestação	idem
	7 Dias de Infestação	células **g** em degeneração, com alterações nucleares, pouca ou nenhuma atividade ATPásica e grande atividade fosfatásica
	3 Dias após	células **g** em degeneração, com grande atividade fosfatásica e corpos apoptóticos
	7 Dias após	células **g** em degeneração, sem atividade ATPásica e com pouca atividade fosfatásica

	ÁCINO IV	
MACHOS	**Jejum**	células **g** agranulares
	2 Dias de Infestação	células **g** granulares
	4 Dias de Infestação	idem
	7 Dias de Infestação	não descrito na literatura
	3 Dias após	não descrito na literatura
	7 Dias após	não descrito na literatura

Conclusões

VI. CONCLUSÕES

1) Os ácinos I das glândulas salivares de carrapatos *Rhipicephalus sanguineus* não sofreram alterações morfológicas nem histológicas no decorrer da alimentação, e adquiriram características degenerativas somente após o término da alimentação (desprendimento do hospedeiro);

2) Este trabalho descreveu pela primeira vez nos ácinos II quatro novos tipos celulares, que foram aqui denominados de "**indiferenciadas**", **indefinidas 1** e **2**, **c5**, **c6**, **c7** e **c8**, sendo estes dois últimos observados somente nos machos;

3) As células secretoras dos ácinos II e III das fêmeas e II, III e IV dos machos permaneceram inativas até que os carrapatos iniciassem a alimentação;

4) Nos ácinos do tipo II de fêmeas e de machos e nos III de fêmeas os diferentes tipos celulares secretaram assincronicamente ao longo do ciclo glandular e conseqüentemente atuaram em momentos diferentes do processo de alimentação;

5) Nos machos as células das glândulas salivares passaram por vários ciclos secretores diferentemente das fêmeas, sofrendo no final de cada ciclo uma reprogramação na sua expressão e voltando a ter a mesma morfologia observada naqueles em jejum;

6) Nas glândulas salivares dos machos somente as células **a**, **c1** e **c3** nos ácinos II e as **d** e **e** nos III permaneceram continuamente ativas;

7) No decorrer do ciclo secretor das glândulas de fêmeas e machos as células secretoras (fêmeas **c5** e machos **c6**, **c7** e **c8**) deixaram de ser observadas por regredirem e provavelmente perderem sua função;

8) O surgimento de características degenerativas no tecido glandular das fêmeas provocou redução da sua capacidade secretora, o que fez com que estes indivíduos finalizassem seu processo alimentar;

9) A degeneração das glândulas salivares de fêmeas e machos de *R. sanguineus* foi detectada no final da alimentação e se intensificou à medida que ficaram mais tempo fora do hospedeiro;

10) As alterações degenerativas das glândulas salivares foram observadas com mais intensidade nas fêmeas do que nos machos;

11) A degeneração glandular, tanto de fêmeas quanto de machos de *R. sanguineus*, foi assincrônica entre os diferentes ácinos e entre as diferentes células de um mesmo ácino, afetando primeiramente aqueles da região posterior da glândula;

12) Durante a degeneração das glândulas salivares detectou-se ausência da atividade ATPásica e perda da integridade da membrana plasmática em momento diferente (precocemente) daquele relatado na literatura;

13) A fosfatase ácida que atuou apenas na remoção dos restos citoplasmáticos, nas glândulas de *R. sanguineus*, provavelmente teve origem endógena (produção pela própria célula), contrariando registros da literatura que informam origem exógena (na hemolinfa);

14) A degeneração nas glândulas salivares em *R. sanguineus* apresentou características de mais de um tipo de morte celular (apoptose e morte autofágica), caracterizando assim uma apoptose atípica;

Referências

VII. REFERÊNCIAS

ABREU, R.M.M.; SILVA DE MORAES, R.L.M.; TABOGA, S.R.; CAMARGO-MATHIAS, M.I. Cytochemical studies of the nuclei of the venom glands' cells of *Apis mellifera* (Hymenoptera, Apidae). **Acta Histochemica et Cytochemica**, Kyoto, v. 37, n. 5, p. 195-299, 2004.

ARMBRUSTER, L.; LEVY, M.; MATHIEU, M. N.; BAUTZ, A. M. Acid phosphatase activity in the hemolymph, hemocytes, fat body and salivary glands during larval and prepupal development in *Calliphora erythrocephala* (Diptera: Calliphoridae). **Comparative Biochemistry and Physiology**, Tarrytown, v. 84B, n.3, p. 349-354, 1986.

AZEREDO-OLIVEIRA, M. T. V.; MELLO, M. L. Mg^{2+}-dependent ATPase activity investigated on heterochromatin, euchromatin and nucleoli of *Triatoma infestans.* **Revista Brasileira de Genética**, Ribeirão Preto, v. IX, n. 2, p. 223-229, 1986.

BALASHOV, Yu. S. **Bloodsucking ticks (Ixodoidea)- vectors of diseases of man and animals**. Miscellaneous Publications of the Entomological Society of America, Lanham, v.8, p. 161-376, 1972.

BALASHOV, Yu. S. **An Atlas of Ixodid Tick Ultrastructure**. In: RAIKHEL, A. S.; HOOGSTRAAL, I. I. (ed.). Entomological Society of America (Special Publication), Lanham, 1983. 289p.

BARKER, D. M.; OWNBY, C. L.; KROLAK, J. M.; CLAYPOOL, P.L.; SAUER, J. R. The effects of attachment, feeding and mating on the morphology of type I alveolus of salivary glands of the lone star tick, *Amblyomma americanum.* **International Journal for Parasitology**, Oxford, v. 70, p. 99-113, 1984.

BECHARA, G.H.; SZABÓ, M.P.J.; FERREIRA, B.R.; GARCIA, M.V. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. **Brazilian Journal of Veterinary Parasitology**, São Paulo, v. 4, n. 2, p. 61-66, 1995.

BINNINGTON, K. C. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick *Boophilus microplus.* **International Journal for Parasitology**, Oxford, v. 8, p. 97-115, 1978.

BIOR, A.D.; ESSEMBERG, R.C.; SAUER, J.R. Comparison of differentially expressed genes in the salivary glands of male ticks, *Amblyomma americanum* and *Dermacentor andersoni*. **Insect Biochemistry and Molecular Biology**, Oxford, v. 32, p. 645-655, 2002.

BISHOP, R.; LAMBSON, B.; WELLS, C.; PANDIT, P.; OSASO, J.; NKONGE, C.; MORZARIA, S.; MUSOKE, A.; NENE, V. A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory *e* cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. **International Journal for Parasitology**, Oxford, v. 32, p. 833-842, 2002.

BOWEN, I. D. Apoptosis or programmed cell death? **Cell Biology International**, London, v. 17, n. 4, p. 365-380, 1993.

BOWEN, I. D.; BOWEN, S. M. **Programmed cell death in tumours and tissues**. London: Chapman and Hall, 1990. 289p.

BOWEN, I. D.; MORGAN, S. M.; MULLARKEY, K. Cell death in the salivary glands of metamorphosing *Calliphora vomitoria*. **Cell Biology International Reports**, London, v. 17, p. 13-34, 1993.

BOWEN, I. D.; MULLARKEY, K.; MORGAN, S. M. Programmed cell death in the salivary gland of the blow fly *Calliphora vomitoria*. **Microscopy Research and Technique**, New York, 34: 202-217. 1996.

BOWEN, I. D.; WORRILL, N.A.; WINTERS, C. A.; MULLARKEY, K. The use of backscattered electron imaging, X-ray microscopy in demonstrating physiological cell death. **Scanning Microscopy, Chicago**, v. 2, p. 1453-1462, 1988.

BOWMAN, A. S.; SAUER, J. R. Tick salivary glands: function, physiology and future. **Parasitology**, Cambridge, v. 129, p. S67-S81, 2004.

BRITTO, F. B.; CAETANO, F. H. Morphological features and occurrence of degenerative characteristics in the hypopharyngeal glands of the paper wasp *Polistes versicolor* (Olivier) (Hymenoptera: Vespedae). **Micron**, New York, v. 37, p. 742-747, 2006.

CLARKE, P. G. H. Developmental cell death: morphological diversity and multiple mechanisms. **Anatomy and Embriology**, Berlin, v. 181, p. 195-213, 1990.

COONS, L. B.; L'AMOREAUX, W. J. Developmental changes in the salivary glands of male and female *Dermacentor variabilis* (Say) during feeding. In: BOROVSKY, D.; SPIELMAN, A. (eds.). **Host Regulated Developmental Mechanisms in Vector Arthropods**. v. 2, University of Florida-IFAS, Vero Beach, 1986. p. 86-92.

COSTA, R. A. C.; CRUZ-LANDIM, C. Distribution of acid phosphatases in the hypopharyngeal glands from workers, queens and males of a Brazilian stingless bee *Scaptotrigona postica* Latreille: an ultrastructural cytochemical study. **Histochemical Journal**, London, v. 33, p. 653-662, 2002.

CUMMINGS, L. A.; BOWEN, I. D. The localization of acid phosphatase as a prelude to cell death in the blow-fly *Calliphora erithrocephala*. **Micron and Microscopica Acta**, New York, v. 23, n. 3, p. 357-358, 1992.

DAI, J. D., GILBERT, L. I. Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. **Insect Biochemistry and Molecular Biology**, Oxford, v. 27, n. 1, p. 69-78, 1997.

FAWCETT, D. W.; BINNINGTON, K. C.; VOIGHT, W. R. The cell biology of the ixodid tick salivary gland. In: SAUER, J. R.; HAIR, J. A. (ed.). **Morphology, Physiology and Behavioral Biology of Ticks**. Chichester: Ellis Horwood, 1986. p. 22-45.

FELDMAN-MUHSAM, B.; BORUT, S.; SALITERNIK-GIVANT, S.. Salivary secretion of the male tick during copulation. **Journal of Insect Physiology**, Oxford, v. 16, p. 1945-1949, 1970.

FLECHTMANN, C. H. W. **Ácaros de importância médico-veterinária**. Editora Livraria Nobel S.A., São Paulo, 1973. 192 p.

FEULGEN, R.; ROSSENBECK, H. Mikroskopisch – Chemischer Nachweis einer Nucleinsaure vom Typus der Thymonucleinsaure und Die Darauf Beruhend Elective Farbung von Zillkernen in Mikroskopischen Praparaten Hoppe-Seylers. **Zeitschrift fur Physiologische Chemie**, Strassburg, v.135, p.203-248, 1924.

FURQUIM, K. C. S. 2004. **Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados**. 2004. 37 pp. 1º Relatório Científico CAPES, Instituto de Biociências, UNESP, Rio Claro, 2004.

FURQUIM, K. C. S. 2005. **Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados**. 2005. 40 pp. 2º Relatório Científico CAPES, Instituto de Biociências, UNESP, Rio Claro, 2005.

FURQUIM, K. C. S. 2006. **Processos degenerativos em glândulas salivares de carrapatos *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) em jejum, em alimentação e alimentados**. 2006. 27 pp. 3º Relatório Científico CAPES, Instituto de Biociências, UNESP, Rio Claro, 2006.

FURQUIM, K. C. S.; CAMARGO-MATHIAS, M. I.; SILVA DE MORAES, R. L. M. Morphological modifications induced by an artificial diet on the hypopharyngeal glands of *Apis mellifera* (Hymenoptera, Apidae) during their degenerative process. **Sociobiology**, Chico, v. 43, n. 2, p. 279-291, 2004.

GAEDE, H.; KNULLE, W. On the mechanism of water vapour sorption from unsaturated atmospheres by ticks. **The Journal of Experimental Biology**, Cambridge, v. 200, p. 1491-1498, 1997.

GILL, H. S.; WALKER, A. R. The salivary glands of *Hyalomma anatolicum anatolicum*: nature of salivary gland components and their role in tick attachment and feeding. **International Journal for Parasitology**, Oxford, v. 18, n. 1, p. 83-93, 1987.

GREGORC, A.; BOWEN, I. D. Programmed cell death in the honeybee (*Apis mellifera*) larvae midgut. **Cell Biology International**, London, v. 21, n. 3, p. 151-158, 1997.

GREGORC, A.; BOWEN, I.D.; POGACNIK, A. Acid phosphatase activity in the midgut of honeybee (*Apis melliphera* L.) larvae. **Apidologie**, Versailles, v. 29, p. 579-584, 1998.

HÄCKER, G. The morphology of apoptosis. **Cell and Tissue Research**, New York, v. 301. p. 5-17, 2000.

HARRIS, R. A.; KAUFMAN, W. R. Neural involvement in the control of salivary gland degeneration in the ixodid tick *Amblyomma hebraeum.* **Journal of Experimental Biology**, Cambridge, v. 109, p. 281-290, 1984.

HARRIS, R. A.; KAUFMAN, W. R. Ecdysteroids: possible candidates for the hormone which triggers salivary gland degeneration in the tick, *Amblyomma hebraeum.* **Experientia**, Basel, v. 41, p. 740-741, 1985.

HURLE, J.; HINCHLIFFE, J. R. Cell death in the posterior necrotic zone (PNZ) of the chick wing-bud: a setereoscan and ultrastructural survey of autolysis and cell fragmentation. **Journal of Embryology and Experimental Morphology**, London, v. 43, p. 123-136, 1978.

HUSSEIN, M. A.; BOWEN, I. D.; LEWIS, G. H. The histochemical localization of ATPase, cholinesterase and acid phosphatase activity in *Culex pipiens* (Diptera, Culicidae) larvae using a methacrylate embedding technique. **Cell Biology International Reports**, London, v. 14, n. 9, p. 775-781, 1990.

JIANG, C.; BAEHRECKE, E. B.; THUMMEL, C. S. Steroid regulated programmed cell death during *Drosophila* metamorphosis. **Development**, Cambridge, v. 124, p. 4673-4683, 1997.

JOCHOVÁ, J.; ZAKERI, Z.; LOCKSHI, R. A. Protein synthesis, DNA degradation, and morphological changes during programmed cell death in labial glands of *Manduca sexta*. **Developmental Genetics**, New York, v. 21, p. 249-257, 1997.

JONES, H. E.; BOWEN, I. D. Acid phosphatase activity in the salivary glands of developing *Drosophila melanogaster*. **Cell Biology International**, London, v. 17, p. 305-315, 1993.

JUNQUEIRA, L. C. U.; JUNQUEIRA, L. M. M. S. **Técnicas básicas de citologia e histologia**. São Paulo: Editora Santos, 1983. p. 48-81.

JUNQUEIRA, L. C.; CARNEIRO, J. **Histologia básica- Texto/Atlas**, 10 ed. Rio de Janeiro: Guanabara Koogan, 2004, 488 p.

KAUFMAN, W.E. Effect of 20-hydroxiecdysone on the salivary glands of the male tick, *Amblyomma hebraeum*. **Experimental and Applied Acarology**, Amsterdam, v. 9, p. 87-95, 1990.

KAUFMAN, W. R.; LOMAS, L. O. Male factors in ticks: Their role in feeding and egg development. **Invertebrate Reproduction and Development**, Rehovot, v. 30, p. 191-198, 1996.

KAUFMAN, W. R.; SAUER, J. R. Ion and water balance in feeding ticks: mechanism of tick excretion. In: OBENCHAIN, F. D., GALUN, R. (eds.), **Physiology of Ticks**. Pergamon Press, Oxford, 1982. p. 213-243.

KERR, J. F. R.; GOBÉ, G. C.; WINTERFORD, C. M.; HARMONT, B. V. Anatomical Methods in Cell Death. In: SCHWARTZ, L. M.; OSBORNE, B. A. (ed.). **Cell Death**. San Diego: Academic Press, 1995. p. 1-27.

KRSTIC, R.; PEXIEDER, T. Ultrastructure of cell death in bulbar cushions of chick embryo heart. **Zeitschrift für Anatomie und Entwicklungsgeschichte**. Berlin, v. 140, p. 337-350, 1973.

L'AMOREAUX, W. J.; JUNAID, L.; TREVIDI, S. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. **Tissue and Cell**, Edinburgh, v. 35, p. 95-99, 2003.

LEVY, M.; BAUTZ, A. M. Degeneration of larval salivary glands during metamorphosis of the blowfly *Calliphora erythrocephala* Meigen (Diptera: Calliphoridae). **International Journal of Insect Morphology and Embriology**, Oxford, v. 14, p. 281-290, 1985.

LOCKSHIN, A. R.; ZAKERI, Z. The Biology of Cell Death and Its Relationship to Aging. In: HOLBROOK, N.; MARTIN, G. R., LOCKSHIN, R. A. (eds.). **Cellular Aging and Cell Death**. Wiley-Liss, New York, 1996, p. 167-180.

LOMAS, L.O. **The influence of a male-derived protein factor on salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum* Koch**. 1993. 197 p. Tese (PhD), University of Alberta, Edmond, 1993.

LOMAS, L. O.; GELMAN, D.; KAUFMAN, W. R. Ecdysteroid regulation of salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*: a reconciliation of *in vivo* and *in vitro* observations. **General and Comparative Endocrinology***,* Orlando, v. 109, p. 200-211, 1998.

LOMAS, L. O.; KAUFMAN, W. R. An indirect mechanism by which a protein from the male gonad hastens salivary gland degeneration in the female ixodid tick *Amblyomma hebraeum*. **Archives of Insect Biochemistry and Physiology**, Bonn, v. 21, p.169-178, 1992.

MACGAHON, A. J.; MARTIN, S. J.; BISSONNETTE, R. P.; MAHBOUBI, A.; SHI, Y.; MOGIL, R. J. The End of the (Cell) Line: Methods for the Study of Apoptosis *in vitro*. In: SCHWARTZ, L. M.; OSBORNE, B. A. (eds.). **Cell Death**. San Diego: Academic Press, 1995. p. 153-185.

MAO, H.; KAUFMAN, W. R. Profile of the ecdysteroid hormone and its receptor in the salivary gland of the adult female tick, *Amblyomma hebraum*. **Insect Biochemistry and Molecular Biology**, Oxford, v. 29, p. 33-42, 1999.

MAO, H.; MACBLAIN, W. A.; KAUFMAN, W. R. Some properties of the ecdysteroid receptor in the salivary gland of the ixodid tick, *Amblyomma hebraeum*. **General and Comparative Endocrinology**, San Diego, v. 99, p. 340-348, 1995.

MARZOUK, A. S.; DARWISH, Z. E. A. Changes in the salivary glands of female *Hyalomma (Hyalomma) dromedarii* during and after feeding. **Journal of the Egyptian Society of Parasitology**, Cairo, v. 24, n. 1, p. 39-57, 1994.

MCMANUS, J. F. A. Histological demonstration of mucin after periodic acid. **Nature**, London, v. 158, p. 202, 1946.

MCMULLEN, H. L.; SAUER, J. R.; BURTON, R. L. Possible role in uptake of water vapour by ixodid ticks salivary glands. **Journal of Insect Physiology**, Oxford, v. 22, p. 1281-1285, 1976.

MELLO, M. L. S. Cytochemical properties of euchromatin and heterochromatin. **Histochemical Journal**, London, v. 15, p. 739-751, 1983.

MELLO, M. L. S.; BARBISAN, L. F.; LAREEF, M. H.; RUSSO, J.; VIDAL, B. C. Cell death evaluation in benzo[α]pyrene-transformed human breast epithelial cells after microcell-mediated transfer of chromosomes 11 and 17. **Mutation Research**, Amsterdam, v. 546, n. 1-2, p. 39-43, 2004.

MELLO, M. L. S.; CONTENTE, S.; VIDAL, B. C.; PLANDING, W.; SCHENCK, U. Modulation of *ras* transformation affecting chromatin supraorganization as assessed by image analysis. **Experimental Cell Research**, New York, v. 220, p. 374-382, 1995.

MELLO, M. L. S.; VIDAL, B. C. V. Chromatin organization in normal and cancer cells. **Journal of Women's Cancer**, Rydal, v. 2, n. 2, p. 73-78, 2000.

MELLO, M. L. S.; VIDAL, B. C.; DANTAS, M. M.; MONTEIRO, A. L. P. Discrimination of the nucleolus by a critical eletrolyte concentration method. **Acta Histochemica et Cytochemica**, Kyoto, v. 26, n. 1, p. 1-3, 1993.

MORAES, A. S.; VIDAL, B. C.; GUARALDO, A. M. A.; MELLO, M. L. S. Chromatin supraorganization and extensibility in mouse hepatocytes following starvation and refeeding. **Cytometry,** Hoboken, part A, v. 63A, p. 94-107, 2005.

MULLARKEY, K. **Gene expression in the salivary gland of the blowfly (*Calliphora vomitoria*) larva**. 1987. 156 p. Tese (PhD), University of Exerter, Exerter, 1987.

NEEDHAM, G. R.; GREENWALD, L.; FREDA, T. J.; COONS, L. B. The ultrastructure of the tick (*Amblyomma americanum*) salivary gland acini responsible for secretion of the hygroscopic fluid and localization of Na^+/K^+ = ATPase. **American Zoologist**, Lawrence, v. 23, p.1013, 1983.

NUNES, E. T.; CAMARGO-MATHIAS, M. I.; BECHARA, G. H. *Rhipicephalus (Boophilus) microplus* (Canestrine, 1887) (Acari: Ixodidae): acid phosphatase and ATPase activities localization in salivary glands of females during the feeding period. **Experimental Parasitology**, San Diego, v. 114, p. 109-117, 2006a.

NUNES, E. T.; CAMARGO-MATHIAS, M. I.; BECHARA, G. H. Structural and cytochemical changes in the salivary glands of the *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae) tick female during feeding. **Veterinary Parasitology**, Amsterdam, v. 140, p. 114-123, 2006b.

OLIVEIRA, P. R.; BECHARA, G. H.; DENARDI, S. E.; SAITO, K. C.; NUNES, E. T.; SZABÓ, M. P. J.; CAMARGO-MATHIAS, M. I. Comparison of the external morphology of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) ticks from Brazil and Argentina. **Veterinary Parasitology**, Amsterdam, v. 129, p.139-147, 2005.

OLIVER, J. H. JR. Relationship among feeding, gametogenesis, mating and syngamy in ticks. In: BOROVSKY, D.; SPIELMAN, A. (eds.). **Host Regulated Developmental Mechanism in Vector Arthropods**. Vero Beach: University of Florida-IFAS, 1986. pp. 93-99.

OLIVIERI, J. A.; SERRA-FREIRE, N. M.. Structure of the salivary glands of the unfed female tick *Amblyomma cajennense* (Fabricius) (Acarina: Ixodidae). **Memórias do Instituto Oswaldo Cruz**, Rio de Janeiro,v. 87, suppl. I, p. 167-174, 1992.

PAUTOU, M.; KIENY, M. Sur les mecanismes histologiques et cytologiques de la necrose morphogene interdigitale chez I'embrion de poulet. **Comptes Rendus Hebdomadaires des Seances de l' Academie des Sciences, Sciences Naturelles**, **Série D**, Paris, v. 272, p. 2025-2028, 1971.

PELT-VERKUIL, E. V. Hormone mediated induction of acid phosphatase activity in the fat body of *Calliphora erythrocephala* prior to metamorphosis. **Journal of Insect Physiology**, Oxford, v. 25, p. 965-973, 1979.

PIPAN, N.; RAKOVEC, V. Cell death in the midgut epithelium of the worker honey bee (*Apis mellifera carnica*) during metamorphosis. **Zoomorphologie**, Berlin, v. 97, p. 217-224, 1980.

REY, L. **Parasitologia**. Rio de Janeiro: Guanabara Koogan, 1973. p. 633-641.

RIBEIRO, A. L.; FACCINI, J. L. H.; DAEMON, E. Estudo das variações morfológicas de *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) no Brasil. **Revista da Universidade Federal Rural do Rio de Janeiro, Série Ciências da Vida**, Itaguai, v.18, n. 1-2, p. 25-33, 1996.

RIBEIRO, J. M. C.; MAKOUL, G.T.; LEVINE, J; ROBINSON, D. K.; SPILMAN, A. Antihaemostatic, antiinflammatory and immunosupressive properties of the saliva of a tick, *Ixodes dammini*. **Journal of Experimental Medicine**, New York, v. 161, p. 332-344, 1985.

ROSENAU, W.; GOLDBERG, M. L.; BURKE, G. C. Early biochemical alterations induced by lymphotoxin in target cells. **The Journal of Immunology**, Baltimore, v. 111, p. 1128-1135, 1973.

RUDOLPH, D. KUNLLE, W. Site of the mechanism of water vapour uptake from the atmosphere in ixodid ticks. **Nature**, London, v. 249, p. 84-85, 1974.

RUDOLPH, D.; KNÜLLE, W. Uptake of water vapour from air: process, site, and mechanism in ticks. In: SCHMIDT-MIELSEN, K.; BOLIO, L.; MADDRELL, S. H. P. (eds.). **Comparative Physiology: *Water, Ions and Fluid Mechanics***. Cambridge: Cambridge University Press, 1978. p. 84-85.

SANDERS, M. L.; SCOTT, A. L.; GLASS, G. E.; SCHWARTZ, B. S. Salivary gland changes and host antibody responses associated with feeding of male lone star ticks (Acari: Ixodidae). **Journal of Medical Entomology**, Lanham, v. 33, n. 4, p. 628-634, 1996.

SAUER, J. R; ESSENBERG, R. C.; BOWMAN, A. S. Salivary glands in ixodid ticks: control and mechanism of secretion. **Journal of Insect Physiology**, Oxford, v. 46, p. 1069-1078, 2000.

SCHUMAKER, T. T.; SERRA-FREIRE, N. M. Histologia das glândulas salivares de adultos de *Argas (Persicargas) miniatus* (Koch, 1844) (Ixodoidea: Argasidae) em jejum, em alimentação e alimentados. **Revista Brasileira de Entomologia**, São Paulo, v. 35, p. 49-72, 1991.

SERRA-FREIRE, N. M.; OLIVIERI, J. A. Structure of the salivary glands of the unfed male tick *Amblyomma cajennense* (Fabricius) (Acari: Ixodidae). **Memórias do Instituto Oswaldo Cruz**, Rio de Janeiro, v. 88, n. 2, p.207-213, 1993.

SILVA DE MORAES, R. L. M. **Morte celular nas glândulas hipofaríngeas de *Apis mellifera* (Hymenoptera, Apidae)**. 1998. 97p. Tese (Livre Docência), Instituto de Biociências, UNESP, Rio Claro, Brasil, 1998.

SILVA DE MORAES, R. L. M.; BOWEN, I. D. Modes of cell death in the hypopharyngeal gland of the honey bee (*Apis mellifera*). **Cell Biology International**, London, v. 24, n. 10, p. 737-743, 2000.

SONENSHINE, D. E. **Biology of ticks**. New York: Oxford University Press, 1991. p. 141-158.

TATA, J. R. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. **Developmental Biology**, San Diego, v. 13, p. 77-94, 1966.

TILL, W. M. A contribution to the anatomy and histology of the brown ear tick *Rhipicephalus appendiculatus* Neumann. **Memoirs of the Entomological Society of Southern Africa**, Pretoria, v. 6, p. 1-124, 1961.

VIDAL, B. C.; RUSSO, J.; MELLO, M. L. S. DNA content and chromatin texture of benzo[α]pyrene-transformed human breast epithelial cells as assessed by image analysis. **Experimental Cell Research**, New York, v. 244, p. 77-82, 1998.

WALKER, A. Arthropods of domestic animals. A guide to preliminary identification. London: **Chapman and Hall**, 1994. p. 25-29.

WALKER, A.; FLETCHER, J. D.; GILL, H. S. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. **International Journal for Parasitology**, Oxford, v. 15, n. 1, p. 81-100, 1985.

WALKER, S. M.; LUCAS, Z. J. Cytochemical activity of lymphocytes: II. Studies on mechanism of lymphotoxin-mediated cytotoxicity. **The Journal of Immunology**, Baltimore, v. 109, p. 1233-1244, 1972.

WANG, H.; HENBEST, P. J.; NUTTALL, P. A. Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (Ixodidae) is accompanied by re-programming of salivary gland protein expression. **Parasitology**, Cambridge, v. 119, p. 143-149, 1999.

WEBER, R. Tissue involution and lysosomal enzymes during anuran metamorphosis. In: DINGLE, J. T.; FELL, H. B. (eds.). **Lysosomes in Biology and Pathology**, Amsterdam: North Holland Publishing Co., vol.2., 1969. p. 437-461.

WEISS, B. L.; KAUFMAN, W. R. The relationship between 'critical weight' and 20-hydroxyecdysone in the female ixodid tick *Amblyomma hebraeum.* **Journal of Insect Physiology**, Oxford, v. 47, p. 1261-1267, 2001.

WEISS, B. L.; KAUFMAN, W. R. Two feeding-induced proteins from the male gonad trigger engorgement of the female tick *Amblyomma hebraeum*. **Proceedings of the National Academy of Sciences**, Allahabad, v. 101, n. 16, p. 5874-5879, 2004.

WIKEL, S. K. The induction of host resistance to tick infestation with a salivary gland antigen. **American Journal of Tropical Medicine Hygiene**, Northbrook, v. 30, p. 284-288, 1981.

YAMAMOTO, S.; SAWADA, K.; SHIMOMURA, H.; KAWAMURA, K.; JAMES, T. N.. On the nature of cell death during remodeling of hypertrophied human myocardium. **Journal of Molecular and Cellular Cardiology**, London, v. 32, p. 161-175, 2000.

ZAKERI, Z. F.; AHUJA, H. S. Cell death/apoptosis: normal, chemically induced, and teratogenic effect. **Mutation Research**, Amsterdam, v. 396, p. 149-161, 1997.

ZAKERI, Z.; BURSCH, W.; TENNISWOOD, M.; LOCKSHIN, R. A. Cell death: programmed, apoptosis, necrosis, or other? **Cell Death and Differentiation**, [S. I.], v. 2, p. 87-96, 1995.

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