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

CIÊNCIAS BIOLÓGICAS

ELEN FERNANDA NODARI

AVALIAÇÃO DOS EFEITOS DO PIRETRÓIDE PERMETRINA NAS GLÂNDULAS SALIVARES DE FÊMEAS DE CARRAPATOS *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI: IXODIDAE)

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Orientadora: PROFa. Dra. MARIA IZABEL CAMARGO MATHIAS

Co-orientadora: Dra. GISLAINE CRISTINA ROMA

Trabalho de Conclusão de Curso apresentado ao Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho" - Câmpus de Rio Claro, para obtenção do grau de Bacharela e Licenciada em Ciências Biológicas.

Rio Claro
2013

595.42 Nodari, Elen Fernanda
N761a Avaliação dos efeitos do piretróide permetrina nas glândulas salivares de fêmeas de carrapatos Rhipicephalus sanguineus (Latreille, 1806) (Acari: Ixodidae) / Elen Fernanda Nodari. - Rio Claro, 2013
87 f. : il., figs., gráfs.

Trabalho de conclusão de curso (bacharelado - Ciências Biológicas) - Universidade Estadual Paulista, Instituto de Biociências de Rio Claro
Orientador: Maria Izabel Camargo Mathias
Coorientador: Gislaine Cristina Roma

1. Ácaro. 2. Carrapato R. sanguineus. 3. Acaricida. 4. Glândula salivar. I. Título.

Ficha Catalográfica elaborada pela STATI - Biblioteca da UNESP
Campus de Rio Claro/SP

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AGRADECIMENTOS

Hoje, na conclusão de mais uma etapa da minha vida, gostaria de dividir e agradecer a muitos por mais esta conquista. Primeiramente gostaria de agradecer a Deus e aos meus amados pais, Rute Maria Polido Nodari e Rodney André Ming Nodari, por sempre me apoiaram em minhas decisões e acreditarem que eu conseguiria entrar em uma universidade pública. Pela formação da pessoa que sou hoje, pelo carinho e amor de todos os dias.

Gostaria de agradecer muito ao meu noivo e amigo de sempre, Leonardo Phelipe Dragoni, por me apoiar e me esperar pacientemente, me ajudando nos estudos, tendo paciência em ouvir sobre carrapatos a cada nova apresentação em congresso, pela companhia nos fins de semana de experimento, por ser meu companheiro de todos os momentos, me ajudando, me animando e me erguendo quando eu desanimava ou encontrava dias difíceis e estressantes, sempre limpando as minhas lágrimas e trazendo sorrisos aos meus lábios, sem contar nos inúmeros chocolates para ajudar nessa tarefa.

Agradeço o apoio de toda a minha família, minhas avós Eva de Souza Polido e Janete Aparecida Nodari, meu avô Nereu Nodari, aos meus sogros Rosa Maria Campeão Dragoni e Leonildo Dragoni Sobrinho, a minha cunhada Laís Cristina Dragoni, por sempre me apoiarem, me ajudarem nos momentos difíceis, sempre me darem amor e carinho.

Agradeço aos meus irmãos Alan e Francieli por existirem em minha vida, e apesar das nossas diferenças, eu os amo muito. Ao meu pequeno sobrinho, Rafael, que sempre se interessou por meus estudos, por sua curiosidade na biologia, e em saber o que a sua tia estava fazendo, ouvindo pacientemente, mesmo quando essa louca falava como se estivesse contando algo a um adulto.

Agradeço a minha amiga Camila, que conheço desde muito pequena, pelas conversas e amizade incondicionais, que mesmo quando estávamos longe a nossa amizade nunca mudou, sempre compreensiva de que a universidade estava me ocupando, mas que nunca a esqueci.

Agradeço aos meus compadres Dailene e André, pelas fugas do meio científico, e pelo maravilhoso presente, minha afilhada Emanuely, que sempre ocupou parte dos meus dias, quando eu podia sair e cuidar dessa pequena.

Agradeço aos meus outros amigos Kátia, Emerson, Roberto, Helen, Andréia, pelas incansáveis conversas, pelas madrugadas, sempre torcendo por mim, em tudo o que eu fosse enfrentar. Amigos verdadeiros.

Agradeço a minha orientadora e amiga a professora doutora Maria Izabel Camargo Mathias, pela orientação, por me ensinar a conviver no meio acadêmico, sempre me apoiando,

e ouvindo as minhas dúvidas, agradeço por assistir e me acompanhar nas apresentações orais, torcer e vibrar a cada conquista nossa. Agradeço pelas conversas descontraídas na sala de café e agradeço também pelos momentos mais sérios e as puxadas de orelha, o que me tornou uma pessoa melhor e principalmente uma pesquisadora melhor. Agradeço os incentivos e por sempre acreditar em mim. Agradeço pelas oportunidades que sempre me ofereceu e aos amigos que me apresentou.

Agradeço o apoio e ajudas intermináveis de todos do grupo BCSTM (Brazilian Central of Studies on Ticks Morphology), principalmente a minha co-orientadora Gislaine Roma, que sempre me ensinou e corrigiu pacientemente cada relatório, a Karim Furquim pelas intermináveis seções de tirar dúvidas sobre glândulas e pelas conversas, a Patrícia Rosa, amiga sempre disposta a me ajudar, que me salvou em vários momentos de sufoco, a Izabela Calligaris pelas conversas e parcerias, e a todos os demais que tiveram uma participação na minha formação, e que sempre ajudaram a fazer com que o nosso grupo funcionasse.

Agradeço a Pró-Reitoria de Pesquisa (PROPe) da UNESP, na pessoa da professora doutora Maria José Soares Mendes Giannini, Pró-Reitora de Pesquisa e da professora doutora Maysa Furlan, Coordenadora Executiva do PIBIC/CIC/UNESP, pelo apoio as pesquisas na Iniciação Científica, pelo belíssimo trabalho nos Congressos de Iniciação Científica, e pelas premiações, que me proporcionaram viver experiências novas, incluindo as de participar em congressos científicos internacionais, conhecendo novos pesquisadores, diferentes países e suas culturas.

Agradeço a todos os professores que fizeram parte da minha vida, e que sempre me ensinaram algo de bom, seja para a biologia ou para a vida, pois os professores são uma das peças mais importantes da construção do saber. Agradeço aos técnicos dos laboratórios, que sempre estiveram dispostos a me ajudar, ao Pablo Nunes, a Monika, ao Antônio, e em especial ao Gerson que tornava os dias no laboratório e na sala de café bem mais divertidos.

Quero agradecer também e muito as minhas amigas e companheiras de graduação, de laboratório, de aulas práticas, de inúmeros trabalhos, de dias e noites de estudo, uma sempre apoiando a outra, Aline Catae, Laís Sommaggio e Paula Maldonado. Amigas que tornaram a minha passagem nesta graduação muito melhor, pois sempre nos ajudamos e demos força umas as outras. Agradeço as escapadas dos nossos compromissos, indo ao cinema, comendo um brigadeiro, ou festejando os nossos aniversários, agradeço pelas festas de finalização de semestre, sempre com muitas guloseimas e conversas. Obrigada meninas, pois vocês também fazem parte desta conquista. Agradeço também aos colegas de sala, mesmo que às vezes

tenha havido algum desentendimento principalmente por datas de prova, éramos uma sala unida, e muitos sempre se ajudaram.

Obrigada mais uma vez a todos, que direta ou indiretamente fizeram parte desta conquista, desculpa se me esqueci de alguém e sintam-se mais uma vez agradecidos.

RESUMO

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Devido à grande importância médico-veterinária dos carrapatos e a ampla utilização de produtos químicos sintéticos para o seu controle, tais como a permetrina, o presente estudo trouxe uma análise morfofisiológica, citoquímica e da reorganização do citoesqueleto durante os processos de degeneração e de morte celular que ocorreram nas glândulas salivares de fêmeas semi-ingurgitadas de carrapatos *Rhipicephalus sanguineus* quando expostas a 206, 1031 e 2062 ppm de permetrina. Os resultados obtidos mostraram que a permetrina é um potente agente químico que interfere na morfofisiologia do tecido glandular desta espécie, causando severas alterações na forma dos ácinos, bem como intensa vacuolização citoplasmática nas células acinares e acentuada desorganização do tecido glandular, culminando num avançado processo de degeneração, com consequente formação de corpos apoptóticos. Além disso, este piretróide comprometeu a capacidade de secreção das glândulas salivares, visto a drástica redução de proteínas, lipídeos e polissacarídeos nas diferentes células acinares. O material genético do tecido glandular também mostrou severas alterações induzidas pela permetrina, tais como: na forma e no tamanho do núcleo das células acinares, marginalização da cromatina, fragmentação nuclear, bem como surgimento de núcleos picnóticos, principalmente quando aplicadas as maiores concentrações do produto. Com relação à reorganização do citoesqueleto, os resultados revelaram que a permetrina foi capaz de modificar o citoesqueleto das glândulas salivares de *R. sanguineus* por meio de um processo gradual de desintegração à medida que as concentrações do acaricida aumentaram. Nestas células glandulares, os processos de morte celular ocorreram num ritmo mais acelerado do que em condições naturais, nas quais esse processo dar-se-ia somente no final do ingurgitamento da fêmea. Como consequência da exposição a este agente tóxico, os ácinos das glândulas salivares perderam sua forma original (estrutura típica de rede ao redor das células), visto que o citoesqueleto das células acinares sofreu extrema desorganização transformando-se em uma massa amorfa. Dessa forma, pode-se concluir que a permetrina induziu a degeneração precoce do tecido glandular, por meio de significativas alterações morfofisiológicas nas células acinares, bem como intensificou a produção de enzimas relacionadas aos processos de morte celular, comprometendo consequentemente as integridades celular e nuclear deste tecido, bem como sua fisiologia.

Palavras-chave: *Rhipicephalus sanguineus.* Carrapatos. Acaricida. Permetrina. Glândula salivar.

SUMÁRIO

INTRODUÇÃO

1 INTRODUÇÃO

Os carrapatos, artrópodes ectoparasitas de vertebrados, constituem um dos mais importantes grupos dos pontos de vista médico e veterinário, por provocarem lesões nos hospedeiros, seja no processo de repasto sanguíneo ou pela transmissão de agentes patogênicos como arboviroses, ricketsioses, espiroquetoses e protozoários (KAUFMAN, 1989). Esse parasitismo compromete todo o desenvolvimento do hospedeiro através da espoliação direta causada pelo hematofagismo, que permite a entrada de microrganismos responsáveis por infecções secundárias, como miíases cutâneas ou paralisia, provocadas pela ação tóxica da saliva deste parasita (WALL; SHEARER, 1997).

O sucesso dos carrapatos como vetores de microrganismos se deve às características biológicas que esses ectoparasitas apresentam como: hematofagismo em todas as fases do desenvolvimento; fixação profunda das partes bucais nos hospedeiros; ingurgitamento lento, havendo tempo para inoculação de patógenos; adaptação a diferentes espécies de hospedeiros; resistência à adversidade climática e longevidade nos diversos ambientes, propiciando tempo para multiplicação de patógenos (HARWOOD; JAMES, 1979).

O carrapato da espécie *Rhipicephalus sanguineus* está distribuído por todos os continentes, devido às migrações humanas pelo mundo levando consigo o cão doméstico, seu principal hospedeiro (WALKER et al., 2000). Essa espécie é representante da família Ixodidae possuindo, portanto, três formas parasitárias dentro de seu ciclo de vida: larva, ninfa e adulto, sendo esta última a única com dimorfismo sexual. Cada estágio parasita o hospedeiro por alguns dias, alimentando-se principalmente de sangue, mas também de linfa e de restos tissulares da derme e/ou epiderme lesada. No final do período parasitário as larvas e ninfas ingurgitadas se desprendem do hospedeiro para realizar a ecdise. Já as fêmeas adultas, fertilizadas pelos machos ainda no hospedeiro, se desprendem para a postura dos ovos no solo (cerca de 1000 a 3000 ovos) que depois de incubados por algumas semanas, darão origem às larvas. Os machos que ficam no hospedeiro ainda por dias ou semanas, não ingurgitam ou não aumentam significativamente de tamanho, mas podem fertilizar várias fêmeas neste período (LABRUNA, 2004).

O carrapato *R. sanguineus* tem assumido cada vez mais um papel relevante entre as espécies de carrapatos de importância mundial. Nos cães, além dos danos diretos, ele é responsável pela transmissão de *Ehrlichia canis*, *Babesia canis*, *Haemobartonella canis* e *Hepatozoon canis*. Nos humanos esta espécie ainda é vetora de *Rickettsia conorii* na Europa e de *R. rickettsii* no Arizona – EUA (BORGES et al., 2007). No Brasil é também considerado

como transmissor potencial deste último agente. Esta confirmação assume especial importância, uma vez que há relatos no Brasil de parasitismo humano por este carrapato (DANTAS-TORRES et al., 2006).

Outro ponto de muita discussão é sobre as alterações climáticas e seu impacto sobre os carrapatos e sobre a eco-epidemiologia de doenças transmitidas pelos mesmos (GRAY et al., 2009)

A biologia e a ecologia dos carrapatos estão sob a influência direta de fatores climáticos, tais como temperatura e umidade. Embora o aquecimento global possa afetar a sobrevivência de algumas espécies de carrapatos que estão adaptados a viver em ambientes úmidos (por exemplo, Mata Atlântica), este evento climático provavelmente terá pequeno (se houver) impacto negativo sobre os carrapatos da espécie *R. sanguineus*, visto serem indivíduos menos dependentes de um habitat rico em umidade para a sobrevivência (YODER et al., 2006) e mais resistentes a condições de dessecação (KOCH; TUCK, 1986).

Dessa forma, o aquecimento global poderá induzir o estabelecimento de populações de carrapatos em áreas anteriormente livres. Tem-se especulado, por exemplo, que um aumento de cerca de 2-3ºC na temperatura média de abril-setembro poderá resultar no estabelecimento de populações de *R. sanguineus* nas regiões de clima temperado do norte da Europa (GRAY et al., 2009). No entanto, o impacto real do aquecimento global sobre carrapatos *R. sanguineus* ainda é discutido. Curiosamente, estudos recentes têm demonstrado que carrapatos expostos a altas temperaturas tem se fixado mais rapidamente em coelhos e humanos (PAROLA et al., 2008; SOCOLOVSCHI et al., 2009), sugerindo que o risco de parasitismo humano poderá aumentar em áreas que estiverem experimentando verões mais quentes, o que resultará em última análise, no aumento do risco de transmissão de patógenos, como *Ricketsia. conorii* (PAROLA et al., 2008).

As glândulas salivares dos Ixodidae são órgãos vitais diretamente relacionados com o sucesso biológico deste grupo, visto desempenharem várias funções, como produção de substâncias necessárias à fixação e à alimentação dos ectoparasitas (BINNINGTON, 1978; GILL; WALKER, 1987; WALKER et al., 1985)

Segundo Sonenshine (1991) a saliva atua durante os períodos de parasitismo e de não parasitismo. As glândulas salivares, nos machos e nas fêmeas, 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) e sendo constituídas por uma porção secretora e uma excretora, além de serem desprovidas de

reservatório para armazenamento da secreção. A porção secretora é formada por diferentes ácinos: **I**, **II**, **III** e **IV**, este último presente somente nos machos (BINNINGTON, 1978; GILL; WALKER, 1987; OLIVIERI; SERRA-FREIRE, 1992; SERRA-FREIRE; OLIVIERI, 1993; SONENSHINE, 1991; WALKER et al., 1985). A porção excretora é composta por um sistema de ductos ramificados, sendo um principal ou excretor comum que leva a secreção para a cavidade oral do carrapato. Deste partem ductos intermediários ou secundários (calibre menor), que se subdividem ao longo do comprimento da glândula em pequenos canalículos ou ductos acinares que coletam diretamente do ácino a secreção nele produzida (BALASHOV, 1972; FAWCETT et al., 1986; NUNES et al., 2005; TILL, 1961; WALKER et al., 1985).

Os ácinos do tipo **I** localizam-se ao longo do ducto excretor principal das glândulas dos machos e das fêmeas. São agranulares (as células não produzem secreção salivar) e possuem função osmorregulatória, sendo que através destes ácinos o carrapato é capaz de secretar sal proveniente da hemolinfa na região oral para captação de água do ar insaturado. Os demais ácinos (**II**, **III** e **IV**) são formados por células granulares, e estão distribuídos ao longo de um sistema ramificado de ductos, sendo responsáveis pela secreção de produtos relacionados com a fixação do carrapato no hospedeiro; digestão de tecidos (WALKER et al., 1985); inibição da coagulação sanguínea (PAESEN et al., 1999; RIBEIRO; MATHER, 1998; WALKER et al., 1985) e secreção de quinases que catalisam a bradicinina, o que explicaria, em parte, a ausência de dor no hospedeiro (RIBEIRO, 1987). A saliva excretada pelo carrapato é produzida, na sua maior parte, pelo ácino **III** durante o processo de alimentação (COONS; L'AMOREAUX, 1986). Os ácinos **IV**, restritos aos machos, provavelmente estejam relacionados com os processos reprodutivos (SAUER et al., 1995).

Vitzhum (1943) trabalhando com carrapatos Ixodidae verificou que nas fêmeas adultas completamente ingurgitadas e já copuladas ocorreria atrofia seguida de degeneração das glândulas salivares. Posteriormente, Till (1961) confirmou que durante a alimentação dos carrapatos, as glândulas salivares passariam por modificações morfológicas e fisiológicas, primeiramente por meio do aumento no tamanho das mesmas, seguido de redução nos Argasidae e de degeneração nos Ixodidae, assim que fosse completada a fase de ingurgitamento.

Na literatura, trabalhos específicos sobre o processo de degeneração das glândulas salivares de carrapatos são escassos como os de L'Amoreaux et al. (2003). Os últimos e mais recentes têm sido desenvolvidos por pesquisadores do BCSTM (Brazilian Central of Studies on Ticks Morphology) e dentre eles podem ser citados, Furquim et al. (2008a; b; c), Nunes et al. (2006a; b) e Pereira et al. (2009; 2011) que realizaram estudos sobre a degeneração das

glândulas salivares de fêmeas de *R. (Boophilus) microplus* e *R. sanguineus*, revelando indícios de morte apoptótica atípica nas células glandulares.

Segundo Lomas et al. (1998), a degeneração das glândulas salivares seria um processo controlado por um hormônio ecdisteróide com regulação, em parte, pela ecdisona, resultando na degeneração deste tecido.

A degeneração das glândulas salivares das fêmeas, além de ser um processo hormonalmente controlado (LOMAS et al., 1998), seria também programado (BOWMAN; SAUER, 2004). Segundo Nunes et al. (2006b), este processo proporcionaria aos carrapatos uma economia energética, visto que estas estruturas não lhes seriam mais necessárias depois de finalizado o processo de alimentação. Segundo Sauer et al. (2000) não existem informações sobre o controle da degeneração das glândulas salivares de carrapatos durante as mudas larva-ninfa e ninfa-adulto.

De forma geral, no processo de degeneração estariam envolvidos dois tipos de morte celular geneticamente programados: 1) morte celular autofágica e 2) apoptose (BOWEN, 1993; CLARKE, 1990; JIANG et al., 1997; ZAKERI; AHUJA, 1997). Ambos apresentando características morfológicas e citoquímicas típicas, podendo ocorrer ou não ao mesmo tempo (ZAKERI et al., 1995).

A morte autofágica seria caracterizada pelo aumento do nível da atividade de hidrolases ácidas (fosfatase ácida), surgimento de extensos e numerosos vacúolos autofágicos; o que causaria a destruição da célula (ARMBRUSTER et al., 1986; CLARKE, 1990; CUMMINGS; BOWEN, 1992; GREGORC et al., 1998; JOCHOVÁ et al., 1997;LOCKSHIN; ZAKERI, 1996; PIPAN; RAKOVEC, 1980; ZAKERI et al., 1995), a ocorrência tardia de colapso nuclear (BOWEN, 1993; LOCKSHIN; ZAKERI, 1996; ZAKERI et al., 1995), bem como a remoção dos restos celulares por heterofagia (KRSTIC; PEXIEDER, 1973 apud CLARKE, 1990; PAUTOU; KIENY, 1971 apud CLARKE, 1990).

Já a apoptose seria caracterizada pelo colapso nuclear precoce (KERR et al., 1995; LOCKSHIN; ZAKERI, 1996; ZAKERI et al., 1995), onde o DNA, através da ação de endonucleases, seria clivado nas regiões internucleossômicas (BOWEN, 1993; HÄCKER, 2000; LOCKSHIN; ZAKERI, 1996; ZAKERI; AHUJA, 1997; ZAKERI et al., 1995). Esta clivagem no material genético seria responsável pela condensação e marginalização da cromatina, além de agir na formação de bolhas no envoltório nuclear (HÄCKER, 2000; KERR et al., 1995; ZAKERI et al., 1995). Além disso, ocorreria retração citoplasmática, devido à perda de água (BOWEN, 1993; CLARKE, 1990; KERR et al., 1995; ZAKERI; AHUJA, 1997) e formação de corpos apoptóticos, resultantes da fragmentação celular, os

quais seriam fagocitados (BOWEN; BOWEN, 1990; HÄCKER, 2000; KERR et al., 1995; LOCKSHIN; ZAKERI; AHUJA, 1997; ZAKERI, 1996).

Apesar de existirem vários estudos sobre os processos de morte celular, é difícil determinar qual o tipo exato estaria ocorrendo em um determinado tecido, visto que as alterações celulares não são específicas de nenhum dos tipos conhecidos. Além disso, pode haver sobreposição destas alterações num mesmo tecido, caracterizando, por exemplo, morte apoptótica com envolvimento de hidrolases (CLARKE, 1990; ZAKERI et al., 1995; YAMAMOTO et al., 2000).

Diversos métodos têm sido pesquisados para um efetivo controle de carrapatos, como vacinas, sprays acaricidas (drogas utilizadas topicamente) (BECHARA, 2003; PETER; BROSSARD, 1998), uso de predadores naturais como a garça vaqueira *Egretta bis* (ALVES-BRANCO et al., 1983) e parasitas como bactérias *Escherichia coli*, *Cedecea lapagei* e *Enterobacter agglomerans* (BRUM, 1988; LIPA, 1971), controle biológico com o emprego de fungos como o *Metarhizium anisopliae* (BITTENCOURT et al., 1994; DA COSTA et al., 2002; GARCIA et al., 2005), uso de feromônios (DEBRUYNE; GUERIN, 1994), no caso dos bovinos, a rotação de pastagens (LIPA, 1971), o controle climático (LIPA, 1971) e o cruzamento genético de diferentes raças de gado visando um aumento natural da resistência aos ectoparasitas (PETER; BROSSARD, 1998). Porém, atualmente o mais eficaz ainda é o uso de acaricidas sintéticos, método que apresenta inconvenientes, primeiro pelo seu alto custo devido a aquisição de produtos químicos, instalações e mão-de-obra especializada para a aplicação, e segundo por causar danos ao meio ambiente e à saúde pública, por meio dos resíduos químicos (FREITAS et al., 2005).

Uma das substâncias que compõe os acaricidas amplamente utilizados no controle de carrapatos, especialmente do carrapato do cão *R. sanguineus*, é a permetrina, um piretróide sintetizado pela primeira vez em 1973 (GARCIA-GARCIA et al., 2005), que provoca impulsos nervosos em série resultado da alteração da permeabilidade das membranas ao sódio, com consequente reação dos órgãos sensoriais e das terminações nervosas. Dessa forma, os ectoparasitas passam por um estado nítido de excitação com tremores e espasmos, seguidos de paralisia e morte (MENCKE et al., 2003).

Até agora registros sobre a influência direta dos acaricidas em outros sistemas dos carrapatos, que não o nervoso, são escassos. Alguns estudos como os realizados por Mohamed et al. (2000) revelaram que a permetrina estimularia o aumento da atividade da glândula salivar de *Hyalomma dromedarii*, entretanto, Pereira et al. (2009, 2011) estudando a ação do fipronil (Frontline®) em glândulas salivares de *R. sanguineus*, revelaram alterações,

como degeneração precoce. Da mesma forma, Roma et al. (2010a) estudaram os efeitos da permetrina nos ovários de fêmeas de *R. sanguineus* e relataram alterações morfológicas significativas neste tecido. As concentrações de permetrina utilizadas no presente estudo foram as mesmas determinadas por Roma et al. (2009)

Assim, de acordo com o acima exposto e considerando a importância médico-veterinária dos carrapatos *R. sanguineus* e o prejuízo econômico causado por estes ectoparasitas, além da grande dificuldade de controle dos mesmos, torna-se necessário o desenvolvimento de estudos detalhados sobre a influência de compostos químicos na morfofisiologia dos principais sistemas dos carrapatos, como o glandular, por exemplo, na tentativa de encontrar alternativas que sejam viáveis no controle dos mesmos. As informações resultantes auxiliarão na descrição de metodologias de controle mais eficazes e, ao mesmo tempo, menos agressivas tanto para os hospedeiros (organismos não-alvo), como para o meio ambiente em geral.

OBJETIVOS

2 OBJETIVOS

O presente estudo teve por objetivos estudar as glândulas salivares de fêmeas adultas semi-ingurgitadas de carrapatos *R. sanguineus* submetidas às concentrações de 206, 1031 e 2062 ppm de permetrina (doses previamente determinadas por Roma et al., 2009), a fim de analisar, através de técnicas morfológicas e histoquímicas, os possíveis efeitos deste composto químico nas glândulas salivares dessa espécie de carrapato. Além disso, este estudo analisou como ocorreria os processos de degeneração e de morte celular neste tecido, fazendo uso de técnicas citoquímicas e de microscopia confocal de varredura a laser.

MATERIAIS E MÉTODOS

3 MATERIAIS E MÉTODOS

3.1 Materiais

3.1.1 Carrapatos Rhipicephalus sanguineus

Para o presente estudo foram utilizadas, para cada concentração de permetrina previamente estabelecida, baseados em Roma et al.(2009), bem como para o controle, 15 fêmeas semi-ingurgitadas de carrapatos *R. sanguineus*, coletadas a partir de colônias mantidas nos laboratórios da "Brazilian Central of Studies on Ticks Morphology" (BCSTM) da Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP, campus de Rio Claro, SP, Brasil, em condições controladas (28 ± 1 ºC, 80% de umidade e fotoperíodo de 12 horas) em estufa BOD (Biological Oxygen Demand) Eletrolab EL 202.

Os carrapatos foram alimentados em coelhos New Zealand White isentos de contato prévio com os ectoparasitas, mantidos em gaiolas de contenção e alimentados com ração apropriada e água *ad libitum* (Protocolo nº 5442, aprovado pelo Comitê de Ética no Uso de Animal, UNESP, de Rio Claro/ CEUA-IB-UNESP – **anexo A**).

Todos os procedimentos de construção e fixação da câmara de alimentação nos hospedeiros (coelhos), bem como alocação dos carrapatos nestas câmaras foram realizados segundo Bechara et al. (1995).

3.1.2 Permetrina (nº CAS: 52645-53-1)

O composto químico permetrina (3-phenoxybenzyl (1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), fórmula bruta: $C_{21}H_{20}Cl_2O_3$ usada no presente estudo foi produzida pela empresa Fersol Indústria e Comércio S/A, Mairinque, SP, Brasil e adquirida por via comercial.

3.2 Métodos

Foram utilizados as dependências e os equipamentos disponíveis nos laboratórios de Histologia, de Microscopia Eletrônica e da "Brazilian Central of Studies on Ticks Morphology (BCSTM)" do Departamento de Biologia da UNESP, campus de Rio Claro, SP, Brasil.

3.2.1 Ensaio com a permetrina

As concentrações de permetrina utilizadas neste estudo foram baseadas na CL_{50} (Concentração Letal do ingrediente ativo do acaricida requerida para matar 50% dos carrapatos testados) de 2062 ppm (ROMA et al. 2009). As doses corresponderam a 10% (206 ppm) e 50% (1031 ppm) da CL_{50} e a própria CL_{50} (2062 ppm). O grupo controle foi exposto apenas ao solvente (água destilada).

Sessenta fêmeas semi-ingurgitadas de *R. sanguineus*, após lavadas em peneira com água corrente, foram secas em papel absorvente macio. Em seguida, 45 delas (três grupos com 15 fêmeas), permaneceram imersas durante 5 minutos em placas de Petri identificadas, contendo as diferentes concentrações de permetrina (206 ppm, 1031 ppm e 2062 ppm). O grupo controle foi composto também por 15 fêmeas, que foram imersas apenas em água destilada também por 5 minutos. Logo após, os carrapatos foram secos em papel absorvente e colocados em estufa BOD durante 7 dias. Períodoi estabelecido, para que os efeitos do acaricida (que na maioria das vezes, não ocorrem imediatamente) agisse na fisiologia do ectoparasita. Além disso, essas doses foram utilizadas a fim de que se pudesse acompanhar a ação da permetrina nas glândulas salivares de *R. sanguineus*, evitando a morte imediata dos indivíduos. Aqueles que morreram foram descartados dos bioensaios.

3.2.2 Análise morfológica

3.2.2.1 Microscopia Eletrônica de Varredura (MEV)

Para os estudos de MEV, 15 fêmeas semi-ingurgitadas de *R. sanguineus* de cada grupo de tratamento, foram dissecadas e as glândulas salivares retiradas e fixadas em Karnovsky (paraformaldeído 2% e glutaraldeído 2.5% em tampão Sorensen 0.1 M), durante 24 horas. A seguir, o material foi lavado em água destilada e desidratado em concentrações crescentes de acetona (70%, 90% e 95%) e finalmente acetona pura, cada banho com duração de 5 minutos. Logo após, o material foi processado em "Critical Point Drying", para completar a desidratação. Terminada esta etapa, o material foi colado, com auxílio de fita adesiva dupla face, em suportes de alumínio e metalizado com ouro através de "Sputtering".

3.2.2.2 Inclusão das glândulas salivares em historesina e coloração pela hematoxilina de Harris e eosina aquosa (JUNQUEIRA; JUNQUEIRA, 1983)

Os carrapatos foram dissecados em placas de Petri contendo solução fisiológica tamponada com fosfato-PBS (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2). Com o auxílio de estereomicroscópio, as glândulas salivares foram retiradas com pinças e micro-tesouras e fixadas por 24 horas em solução de paraformaldeído a 4% e NaCl a 0.9% em tampão fosfato 10% (0.1 M - pH 7.5). O material, a seguir, foi desidratado em soluções crescentes de etanol a 70, 80, 90 e 95% durante 15 minutos cada. Logo após, foi transferido para solução de resina (JB-4 Polaron Instruments/Bio Rad) na ausência de catalisador, durante 24 horas em geladeira. Posteriormente, as amostras foram transferidas para moldes plásticos previamente preenchidos com resina contendo catalisador. Os moldes foram selados com suportes de alumínio para microtomia. Depois de polimerizados os blocos foram seccionados com auxílio de micrótomo Leica RM 2255. Os cortes com 4 μm de espessura foram hidratados e recolhidos em lâminas de vidro, que depois de secas, foram submetidas à coloração pela hematoxilina de Harris e eosina aquosa durante 10 e 5 minutos, respectivamente. Em seguida foram novamente secas e montadas em bálsamo do Canadá para observação e documentação em fotomicroscópio Motic BA 300.

3.2.3 Histoquímica

As técnicas histoquímicas foram aplicadas nas secções histológicas, abtidas do material, que após ser dissecado, foi submetido aos diferentes fixadores específicos para preservação de proteínas, polissacarídeos e lipídios.

3.2.3.1 Técnica do azul de bromofenol para detecção de proteínas totais (PEARSE, 1985)

O material depois de dissecado foi fixado em paraformaldeído a 4% e NaCl a 0.9% em tampão fosfato 10% (0.1 M - pH 7.5) durante 24 horas. As secções foram coradas com solução de azul de bromofenol à temperatura ambiente durante 1 hora, sendo em seguida lavadas em solução aquosa de ácido acético 0.5%, durante 5 minutos. Logo após as lâminas foram passadas no álcool butílico terciário por 5 minutos. Em seguida, foram secas e montadas em bálsamo do Canadá.

3.2.3.2 Reação pelo PAS (Ácido Periódico-Schiff) (MCMANUS, 1946) e Contra-Coloração com verde de metila

Para realização deste procedimento as glândulas salivares 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. As secções depois de recolhidas em lâminas de vidro e reidratadas por 1 minuto em água destilada foram transferidas para solução de ácido periódico por 10 minutos. Novamente foram lavadas em água destilada por 1 minuto e, na sequência, colocadas no reagente de Schiff por 1 hora. A seguir, o material foi lavado por 30 minutos em água corrente e contra-corado por 20 segundos com verde de metila. Depois de lavadas as secções foram secas e montadas em Bálsamo do Canadá.

3.2.3.3 Técnica de Baker para detecção de lipídios (modificada por GIOVANETTI, comunicação pessoal)

As glândulas salivares foram fixadas em formol cálcio por 15 horas. Para este teste histoquímico todas as lâminas contendo as secções foram tratadas durante 18 horas com bicromato de cálcio. Após, foram lavadas em água destilada e permaneceram durante 5 horas em solução de hemateína. Em seguida, as lâminas foram novamente lavadas e diferenciadas rapidamente na mistura de Weigert. Logo após foi realizada lavagem do material com água destilada. Após a secagem, as lâminas foram montadas em glicerina.

3.2.4 Citoquímica

3.2.4.1 Reação de Feulgen para análise do nível de compactação cromatínica (FEULGEN; ROSSENBECK, 1924).

As glândulas salivares foram fixadas em mistura de álcool etílico e ácido acético na proporção de 3:1 por 12 minutos, desidratadas em concentrações crescentes de álcool (70,80,90 e 95%) com duração de 15 minutos cada banho, transferidas para a resina de embebição e incluídas. O material foi seccionado em micrótomo (3μm de espessura) e os cortes foram recolhidos em lâminas de vidro. As lâminas contendo as secções permaneceram por 45 minutos em solução de HCl 4N. Na sequência o material foi lavado em água destilada e colocado no reativo de Schiff por 1 hora e meia no escuro. Então o material foi lavado em

água corrente por 20 minutos. Em seguida as secções foram contra-coradas com eosina aquosa durante 5 minutos, foram lavadas em água corrente, secas e montadas em balsamo do Canadá, para observação e documentação em fotomicroscópio MOTIC BA 300.

3.2.4.2 *Detecção da atividade da enzima fosfatase ácida (HUSSEIN et al., 1990)*

A técnica para detecção da atividade de fosfatase ácida foi utilizada para confirmação da ocorrência ou não de morte autofágica no tecido glandular.

As glândulas salivares 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 4ºC. Posteriormente, foram lavadas em tampão acetato de sódio (0.05 M, pH 4.8) e incubadas por 45 minutos a 37^{o}C no seguinte meio: naftol AS-TR fosfato, DMSO (dimetil sulfoxido), tampão acetato de sódio (0.05 M, 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 foi acrescentado 0.2 mL de cloreto de manganês 10% mais 6 mg do sal vermelho violeta e, para finalizar, a solução final foi vigorosamente agitada.

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%), com duração de 15 minutos cada banho, transferido para resina de embebição, incluído e seccionado. As secções com 7 µm de espessura foram recolhidas em lâminas de vidro, reidratadas por 1 minuto em água destilada, contra-coradas por 1 minuto com hematoxilina de Harris, secas e montadas em bálsamo do Canadá para observação ao fotomicroscópio de luz.

3.2.5 Microscopia Confocal de Varredura a Laser

A aplicação da técnica de microscopia confocal foi utilizada a fim de se analisar a ocorrência de alterações no rearranjo do citoesqueleto durante os processos de morte na celulas das glândulas salivares de fêmeas de carrapatos expostas à permetrina.

Os indivíduos expostos, bem como aqueles do grupo controle, foram dissecados para a retirada das glândulas salivares, que foram fixadas em paraformaldeído 4%. A seguir, o material foi lavado 2 vezes em PBS (5 min cada banho), permeabilizado com triton-x 0.1% por 20 minutos e lavado mais 2 vezes em PBS (5 min cada banho). Após, o material foi

incubado em solução de "Alexa Fluor 488 Faloidina" (5 µL da solução estoque de "Alexa Fluor 488 faloidina" + 200 µL de PBS + 2 µL de BSA) durante 30 minutos em recipiente tampado e à temperatura ambiente. Posteriormente, o material foi lavado 2 vezes em PBS (5 min cada banho).

As glândulas salivares das fêmeas de carrapatos foram então lavadas em PBS + BSA para bloqueio de marcação inespecífica e incubadas overnight com anticorpos monoclonais (MOUSE) anti-tubulina (alfa (5µL/mL) e beta (3µL/mL)). O material foi novamente lavado em PBS (3 vezes de 5 min cada banho) e incubado durante 1 hora com anticorpos secundários (GOAT ANTI-MOUSE) conjugados com Cy5 (10µg/mL), e novamente lavado por 3 vezes em PBS. Após este processo o material foi montado utilizando meio de montagem ProLong® Gold reagent contendo DAPI (para marcação de núcleo). As imagens foram obtidas através de Microscópio de Varredura Confocal a Laser Leica TCS-SP5 II.

AVALIAÇÃO DOS RESULTADOS

4 AVALIAÇÃO DOS RESULTADOS

Os resultados obtidos neste estudo foram avaliados através de técnicas de microscopia de luz convencional e de varredura a laser, sendo realizada uma comparação entre o tecido glandular dos indivíduos expostos à permetrina com aqueles do grupo controle, no intuito de se obter respostas adicionais mais precisas que auxiliassem no entendimento de como dar-se-iam as alterações (neste tecido) provocadas pela ação deste produto sintético nas glândulas salivares desta espécie de carrapato.

Todos os dados obtidos através deste trabalho foram apresentados em eventos científicos, bem como submetidos à publicação em revistas internacionais e especializadas na área, possibilitando assim a ampla divulgação no meio acadêmico-científico.

RESULTADOS

5 RESULTADOS

Os resultados obtidos no presente trabalho estão sendo apresentados sob a forma de capítulos que correspondem a 3 artigos publicados (1, 2, e 3) e 1 artigo submetido (4) para publicação todos em periódicos internacionacionais e especializados na área de morfologia.

Capítulo 1: "Cytotoxic effects of permethrin in salivary glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females"

Capítulo 2: "Action of permethrin on *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females: Morpho-physiological evaluation of salivary glands"

Capítulo 3: "Degenerative Process and Cell Death in Salivary Glands of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) Semi-Engorged Female Exposed to the Acaricide Permethrin"

Capítulo 4: "Action of permethrin in the tubulin and actin cytoskeleton of acinary cells of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) salivary glands in semi-engorged females."

CAPÍTULO 1

Experimental Parasitology 128 (2011) 151–158

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

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

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ARTICLE INFO

Article history:
Received 13 October 2010
Received in revised form 25 January 2011
Accepted 1 February 2011
Available online 23 February 2011

Keywords:
Rhipicephalus sanguineus
Ticks
Acaricide
Salivary glands
Cytotoxic effects

ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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0014-4894/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2011.02.003

2. Material and Methods

2.1. Rhipicephalus sanguineus ticks

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

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

2.2. Dilution assays of permethrin (CAS n°: 52645–53-1)

Permethrin (3-phenoxybenzyl (1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) used in this study was purchased from Fersol Indústria e Comércio S/A (Mairinque, SP, Brazil). The permethrin concentrations were based on LC_{50} of 2062 ppm determined previously in a pilot test by Roma et al. (2009). The doses correspond to 10% of the LC_{50} (206 ppm), 50% of the LC_{50} (1031 ppm) and the normal LC_{50} (2062 ppm). A control group was exposed only to distilled water.

R. sanguineus females, after being washed in a sieve with tap water, were dried on soft absorbent paper. Afterwards, 45 females were divided into three groups of 15 females each and immersed for 5 min in Petri dishes containing the different concentrations of permethrin above. The control group was also composed of 15 females that were immersed in distilled water for the same period. Ticks were then dried on absorbent paper and placed in the BOD incubator for 7 days. The observation period was established since the effect of the acaricide is often not immediate, acting slowly on the physiology of the ticks.

2.3. Scanning Electron Microscopy (SEM)

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

2.4. Histology

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

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

3. Results

3.1. Scanning Electron Microscopy (SEM)

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

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

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

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

3.2. Histology

3.2.1. Group I (control)

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

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

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

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

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

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

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

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

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

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

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

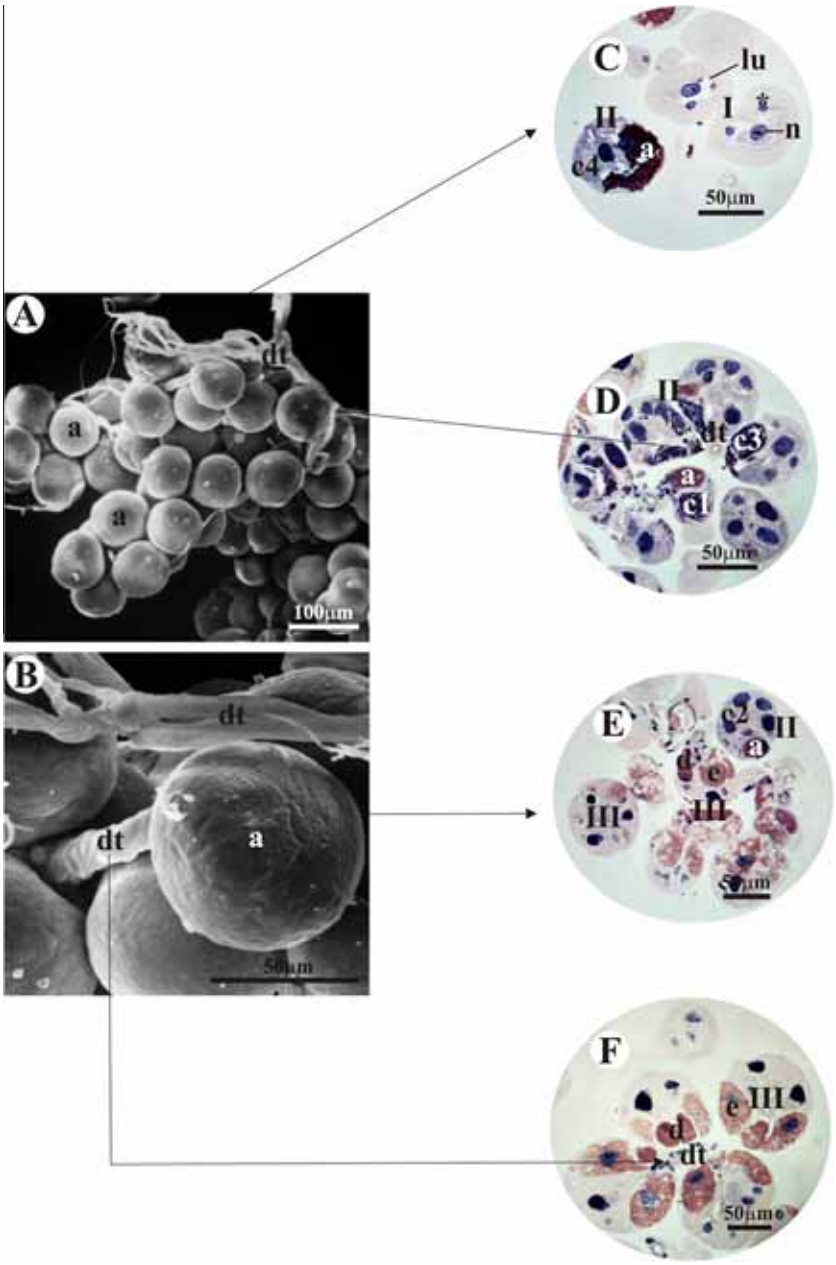

Fig. 1. (**A** and **B**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged female of the control group. (**A**) General view and (**B**) detail of the glandular acini (**a**) showing duct system (**dt**). (**C**–**F**) Histological sections of the *R.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

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

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

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

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

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

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

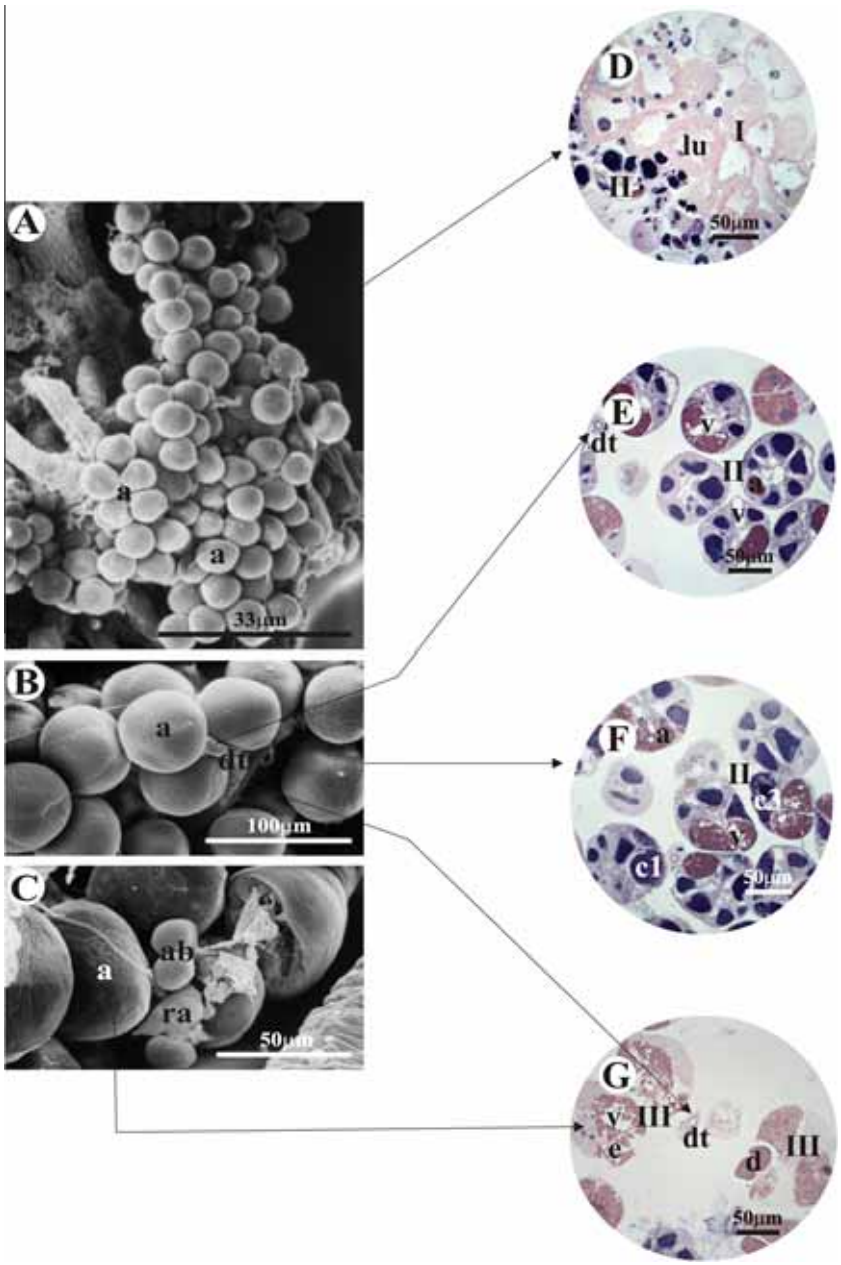

Fig. 2. (**A**–**C**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged female exposed to 206 ppm of permethrin. (**A**) General view and (**B** and **C**) detail of the acini (**a**) and duct (**dt**). Note in (**C**) the apoptotic bodies (**ab**) near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

For comparison of results, see Fig. 5.

4. Discussion

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

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

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

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

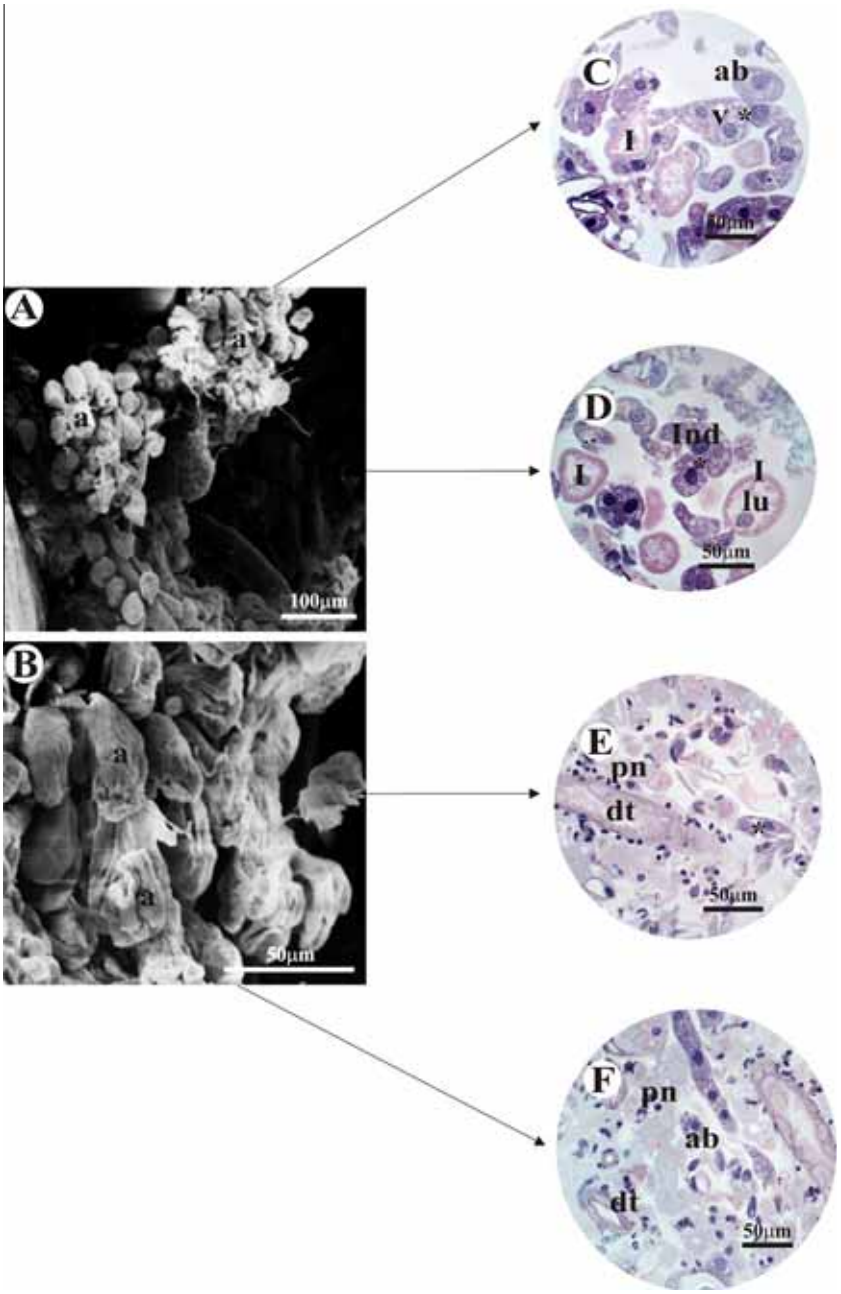

Fig. 3. (**A** and **B**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged female exposed to 1031 ppm of permethrin. (**A**) General view and (**B**) detail of the irregular acini (**a**). Note that acini seem to have "withered", thus modifying their initial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

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

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

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

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

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

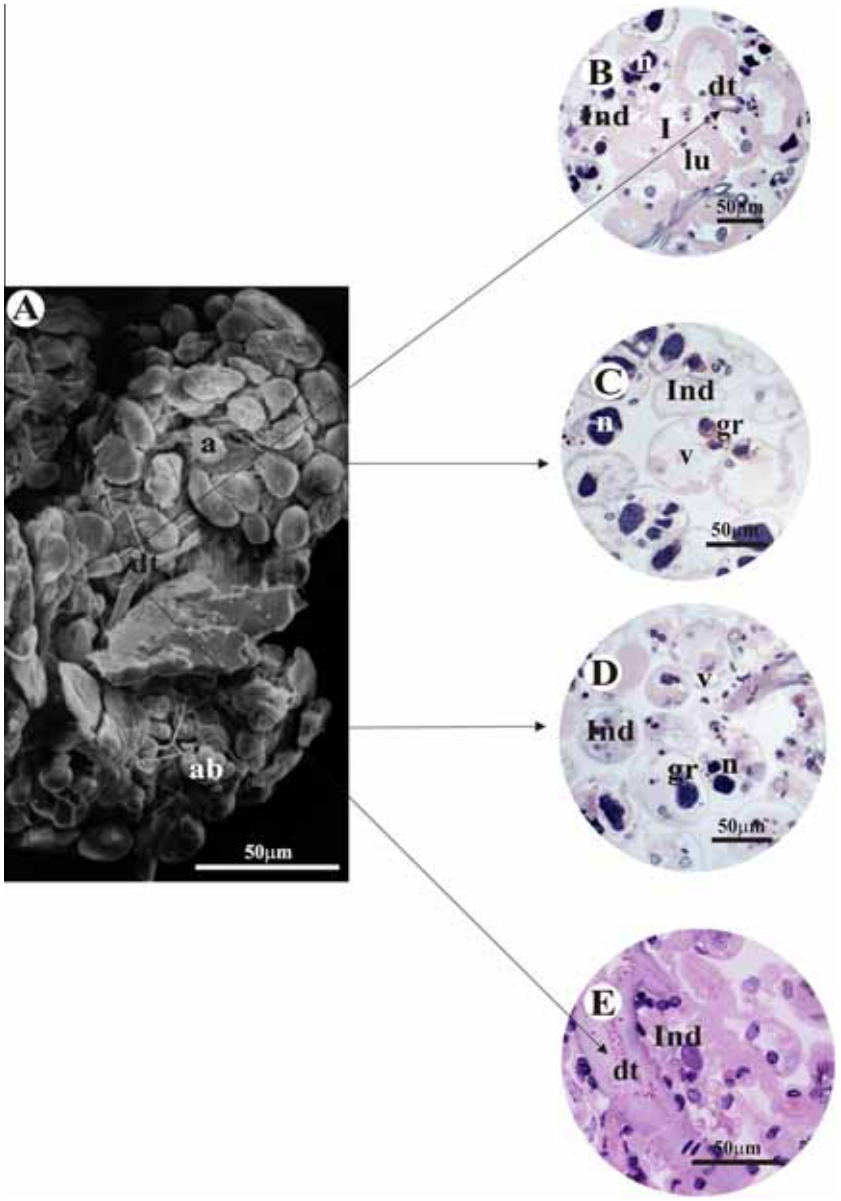

Fig. 4. (**A**) Scanning Electron Microscopy (SEM) of salivary glands of *Rhipicephalus sanguineus* semi-engorged females exposed to 2062 ppm of permethrin. (**A**) General view of the irregular acini (**a**), apoptotic body (**ab**) and ducts (**dt**). (**B**–**E**) Histological sections of the *R.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

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

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

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

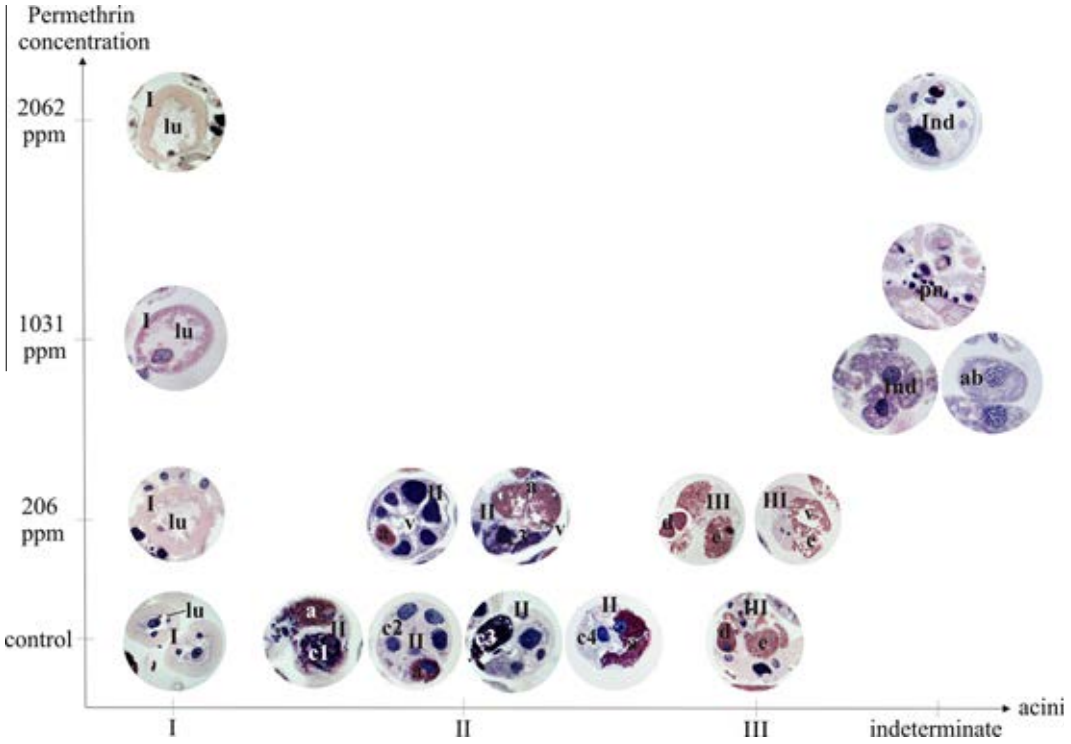

Fig. 5. Permethrin-induced morphological changes in salivary of *Rhipicephalus sanguineus* semi-engorged females. **I** = type I acinus, **II** = type II acinus, **III** = type III acinus, **Ind** = **indeterminate** acinus, **lu** = lumen, **a** = a cell, **c1** = c1 cell, **c2** = c2 cell, **c3** =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

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

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

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

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

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

Authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo -FAPESP (Grants n° 2009/13854-4, 07/57809-5 and 07/59020-0) and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Grant n° 308733/2006-1 and G.H. Bechara and M.I. Camargo Mathias academic carrier research fellowships) for the financial support and to Mr. Antonio Teruyoshi Yabuki and Mr. Gérson de Mello Sousa for technical support.

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CAPÍTULO 2

Ticks and Tick-borne Diseases 3 (2012) 219–226

Contents lists available at SciVerse ScienceDirect

Ticks and Tick-borne Diseases

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Original article

Action of permethrin on *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females: Morpho-physiological evaluation of salivary glands

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ARTICLE INFO

Article history:
Received 21 July 2011
Received in revised form 22 March 2012
Accepted 23 March 2012

Keywords:
Rhipicephalus sanguineus
Permethrin
Salivary glands
Histochemistry
Cytotoxicity

ABSTRACT

Currently the most effective method of tick control is the use of acaricides, among which stands out permethrin (active ingredient of acaricide Advantage® Max3, Bayer), a neurotoxic pyrethroid. However, assessments of their effects on other tick systems such as glandular are still scarce. Thus, this study provides information, through histochemical techniques, about the toxic effect of this pyrethroid on the morphophysiology of salivary glands of semi-engorged *Rhipicephalus sanguineus* females exposed to different concentrations of permethrin (206, 1031, and 2062 ppm). The results showed that permethrin caused significant changes in the salivary gland metabolism accelerating the process of glandular degeneration, an event which would occur naturally and with great intensity only in the final engorgement stage. Furthermore, this study pointed out that permethrin reduces the salivary gland secretion ability through a drastic reduction of proteins, lipids, and polysaccharides in acinar cells. These changes impair the females to finalize the feeding process, what indirectly affects the reproductive process.

Introduction

The economic importance of ticks is widely acknowledged. When feeding, many species of ticks transmit diseases to man and other animals caused by protozoa, virus, bacteria (e.g., rickettsias and spirochetes) as well as induce the appearance of dermatosis and other severe infections (Rey, 1973).

Among the species belonging to the family Ixodidae is the brown dog tick *Rhipicephalus sanguineus*, a species of great medical and veterinary importance, which produces debilitating effects to the host due to blood loss in heavy infestations and transmission of several pathogens (Borges et al., 2007).

In the literature, there are studies on the bio-ecology of these ectoparasites as well as descriptions of more effective and less harmful control methods for both environment and hosts (Kelly and Colley, 1988; Kaufman, 1989; Leal et al., 2003; Labruna, 2004). However, specific studies on the ticks' cell biology are still scarce. Thus, the group Brazilian Central of Studies on Ticks Morphology (BCSTM) have performed several studies mainly aimed at the morphohistology of the main systems of these ectoparasites (Denardi et al., 2004; Saito et al., 2005; Oliveira et al., 2005, 2007; Nunes et al., 2006, 2008; Furquim et al., 2008a,b,c, 2010). This group has also analyzed the functional changes in the glandular and reproductive system of ticks exposed to stressful conditions, such as the effects of chemical, synthetic, and natural compounds (Oliveira et al., 2008, 2009; Roma et al., 2009, 2010a,b, 2011; Arnosti et al., 2011; Pereira et al., 2009, 2011; Nodari et al., 2011).

It was pointed out that the most effective method of tick control is still the use of acaricides, although they have drawbacks such as environment and public health damages through contamination with chemical residues (Freitas et al., 2005). Among the widely used synthetic acaricides to control ticks, especially the dog tick *R. sanguineus*, there is permethrin (active ingredient in Advantage® Max3, Bayer), a chemical compound with neurotoxic action that leads to ectoparasite death (Mencke et al., 2003).

Data on the direct influence of acaricides in other systems of the tick with the exception of the nervous system are scarce. Few works as those performed by Mohamed et al. (2000) described that permethrin would act by stimulating salivary gland activity in *Hyalomma dromedarii*. However, Pereira et al. (2009) studying the action of fipronil (Frontline®) in the *R. sanguineus* salivary glands, revealed an impairment in the tissue metabolic activity caused by this chemical compound. Thus, based on this information, this study aimed to investigate through morphological and histochemical techniques the action of permethrin on the salivary glands of *R. sanguineus* semi-engorged females, in an attempt to provide new

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http://dx.doi.org/10.1016/j.ttbdis.2012.03.008

information for the development and/or improvement of control methods which are less aggressive and less toxic to non-target organisms and to the environment in general.

Material and methods

Rhipicephalus sanguineus ticks

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

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

Dilution assays of permethrin (CAS no.: 52645-53-1)

Permethrin [3-phenoxybenzyl (1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] used in this study was purchased from Fersol Indústria e Comércio S/A (Mairinque, SP, Brazil). The permethrin concentrations were based on an LC_{50} of 2062 ppm determined previously in a pilot test by Roma et al. (2009). The doses correspond to 10% of the LC_{50} (206 ppm), 50% of the LC_{50} (1031 ppm), and the normal LC_{50} (2062 ppm). The control group was exposed only to the placebo (distilled water).

R. sanguineus females, after being washed in a sieve with tap water, were dried on soft absorbent paper. After that, 45 females were divided into 3 groups of 15 females each and immersed for 5 min in Petri dishes containing the above-mentioned different concentrations of permethrin. The control group – also consisting of 15 females – was immersed in distilled water for the same period. Ticks were then dried on absorbent paper and placed in the BOD incubator (28 ± 1 °C, 80% relative humidity, and 12 h photoperiod) for 7 days. The observation period was established because frequently the effect of the acaricide is not immediate, but slowly acting on the physiology.

Histochemistry

The salivary glands of *R. sanguineus* were removed and fixed in paraformaldehyde 4% and 0.9% NaCl in 10% phosphate buffer (0.1 M, pH 7.5), Bouin's solution, and calcium formol for detection of protein, polysaccharide, and lipid, respectively. The material was then dehydrated in an alcoholic series (70%, 80%, 90%, and 95%) at 15 min of intervals. Infiltration was made with Leica resin and the material was embedded in plastic molds at 4 °C to delay prepolymerization. The molds with material were filled and covered with Leica resin and the polymerization was completed at room temperature (about 37 °C).

Sections of 4 μm thickness were mounted on glass slides and stained with bromophenol blue (Pearse, 1985) for protein detection, PAS (McManus, 1946) for polysaccharides detection, and counterstained with Methyl Green and Baker for lipid detection (modified by Giovanetti, pers. communication). Slides were mounted with Canadian balsam and examined and photographed in a Motic BA 300 photomicroscope. This device and other equipments were from the Histology Laboratory of the Biology Dept. at the Bioscience Institute, UNESP, Rio Claro, SP, Brazil.

Results

Protein detection

Group I (control)

The salivary glands of *R. sanguineus* semi-engorged females from the control group present acinus I (agranular) and the cell f from acinus III weakly positive for protein (Fig. 1A and H), whilst in the types II and III acini, the secretion granules of the cells a, c1, and c3 (acinus II) (Fig. 1D and F) and d and e (acinus III) (Fig. 1H) are strongly positive for protein.

Group II (treated with 206 ppm of permethrin)

R. sanguineus females exposed to this concentration of permethrin showed, in general, salivary glands with the same histochemical characteristics as those observed in the control group (Fig. 2A, D, and I). However, in the glandular tissue of this group, the early damage caused by permethrin can be already observed as cytoplasm vacuolation and the presence of indeterminate acini (Fig. 2L). In addition, some acini III showed a higher concentration of protein elements compared to the control group (Fig. 2I).

Group III (treated with 1031 ppm of permethrin)

The glandular tissue of these females was highly disorganized, with major changes caused by permethrin compared to other treatment groups. Due to the formation of an amorphous mass, identification of the types of acini was no longer possible. These were termed indeterminate (Fig. 3A and D) showing an advanced stage of degeneration, with few acini I, which can be distinguished among other unidentified ones (Fig. 3A and D). There was a drastic reduction in protein (moderately positive secretion granules) compared to other groups. Degenerative processes in salivary glands resulted in the formation of apoptotic bodies and residues of nuclei (Fig. 3A and D).

Group IV (treated with 2062 ppm of permethrin)

The glandular tissue showed significant changes, but less intense than in group III. The acini I were weakly positive (Fig. 3G). The remaining acini observed (indeterminate) had cells with border loss and a remarkable reduction of protein content, with few strongly positive granules (Fig. 3J and K). Some of the indeterminate acinar nuclei were fragmented (Fig. 3J and K).

Polysaccharide detection

Group I (control)

The salivary glands of the control group females had the acini I, II, and III with a regular shape and intact cells (Fig. 1B, E and I). The acini I were weakly positive for polysaccharides (Fig. 1B). In acini type II, cells a were PAS-negative (Fig. 1E) as well as the d and e cells from acini III (Fig. 1I). On the other hand, the acini type II cells c1 and c3 reacted with strong positivity (Fig. 1E), and the f cell of acini type III sheltered strongly positive secretion (Fig. 1I).

Group II (treated with 206 ppm of permethrin)

In females exposed to this concentration of permethrin, the acini I had few polysaccharides, except for the lumen of their cells with strongly positive cytoplasm markings (Fig. 2B). The acini II showed the same histochemical characteristics as observed in the control group, with c4 PAS-negative cells (Fig. 2E and F), but in some of these acini the c1 were broken releasing the secretion (strongly positive granules) to the lumen (Fig. 2E). In the acini III, cells d and e were PAS-negative as in the control group. The f cells were weakly positive for polysaccharide (Fig. 2J). Indeterminate acini with

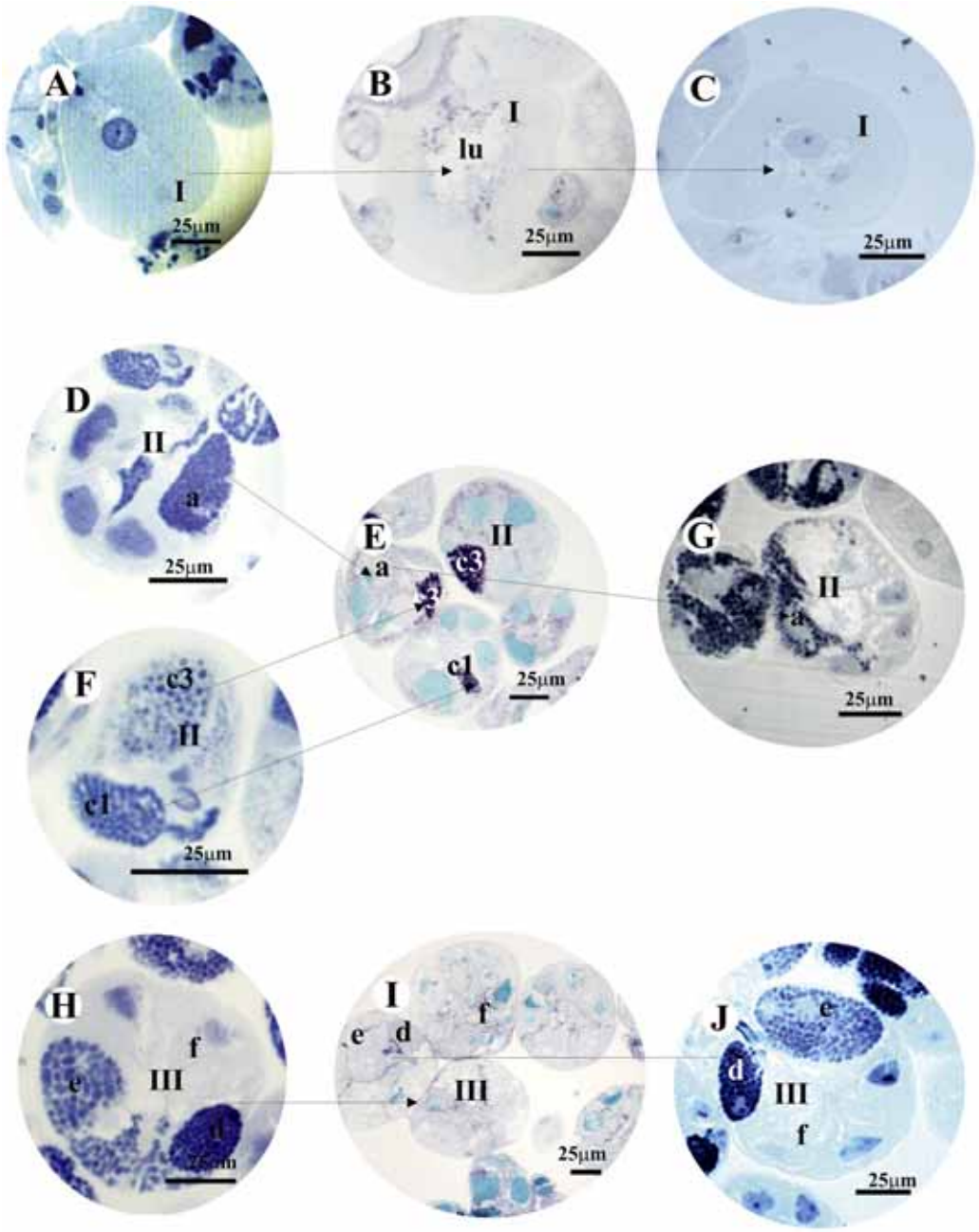

Fig. 1. Histological sections of the *R. sanguineus* salivary glands of the control group stained with bromophenol blue (A, D, F, and H), PAS/Methyl Green (B, E, and I) and Baker (C, G, and J). (A–C) I (type I acinus); (D–G) II (type II acinus) and (H–J) III (type III acinus). lu = lumen, a = a cell, c1 = c1 cell, c3 = c3 cell, d = d cell, e = e cell, and f = f cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

secretion strongly positive for polysaccharides could also be observed (Fig. 2M).

Group III (treated with 1031 ppm of permethrin)

The females in this group presented salivary glands with distinct changes such as severe histological disorganization. The acini formed an amorphous mass, and identification of the different types of acini was not possible (indeterminate) (Fig. 3B and E). Many apoptotic cells were observed, confirming an advanced degeneration stage of the tissue (Fig. 3B and E). Polysaccharides could not be detected in the salivary glands of this group (Fig. 3B and E).

Group IV (treated with 2062 ppm of permethrin)

The salivary glands of females exposed to this concentration exhibited indeterminate PAS-negative acini (Fig. 3L). Only a few acini type I could be recognized; they showed distinct changes from their original form, such as a dilated lumen, and they reacted weakly PAS-positive (Fig. 3H). There were picnotic nuclei, apoptotic bodies, and the secretion of indeterminate acini remained PAS-negative (Fig. 3L).

Lipid detection

Group I (control)

The female's salivary glands of the control group present acini I weakly positive for lipids (Fig. 1C). The cells a of the acini type II were strongly positive for lipids (Fig. 1G). The cells d of the acini type III were strongly positive, the e cells were moderately positive, whilst the f cells were lipid-negative (Fig. 1J).

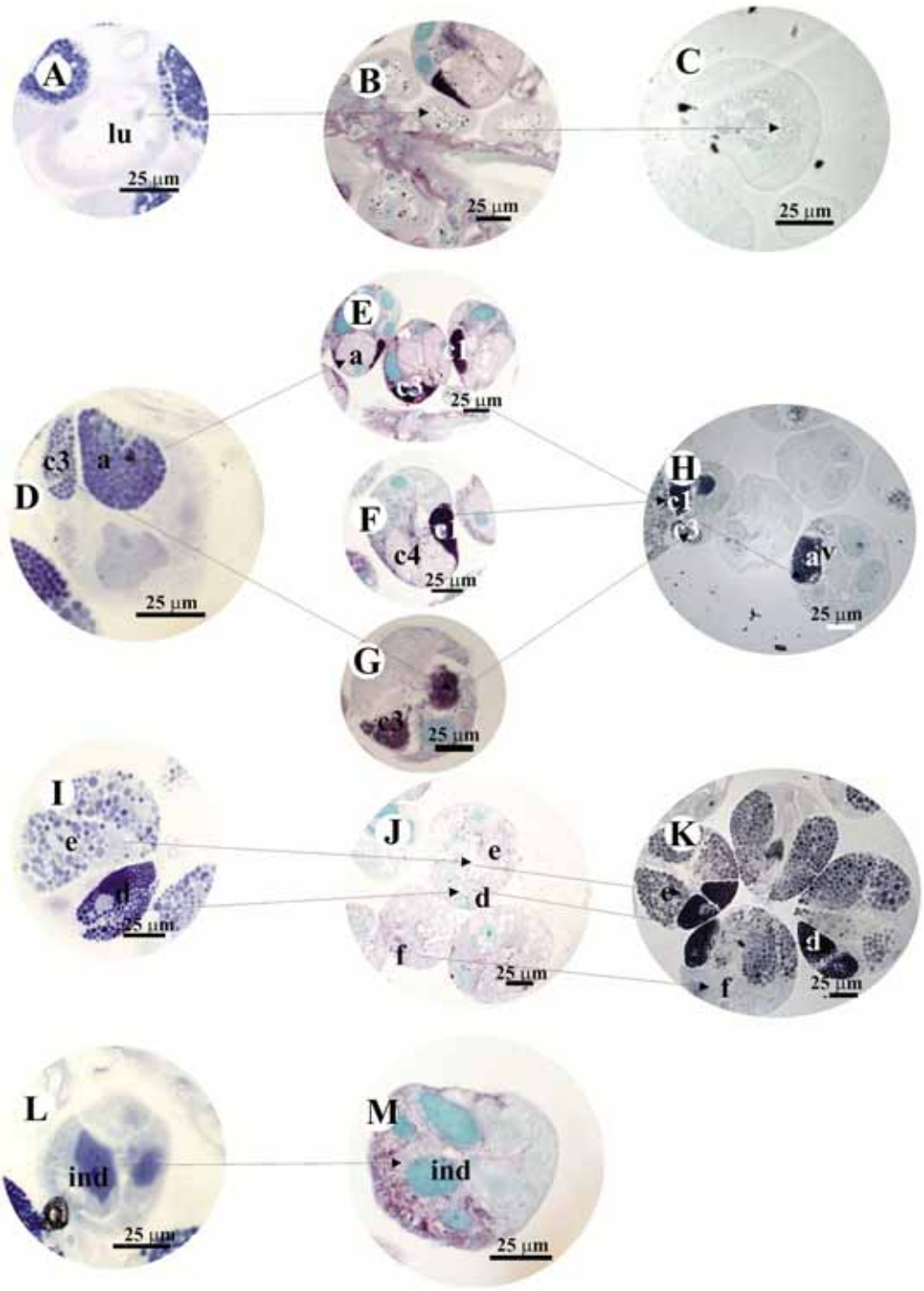

Fig. 2. Histological sections of the *R. sanguineus* salivary glands exposed to 206 ppm of permethrin stained with bromophenol blue (A, D, I, and L), PAS/Methyl Green (B, E–G, J, and M), and Baker (C, H, and K). (A–C) I (type I acinus); (D–H) II (type II acinus); (I–K) III (type III acinus); and (L–M) indeterminate acini (ind). lu = lumen, a = a cell, c1 = c1 cell, c3 = c3 cell, c4 = c4 cell, d = d cell, e = e cell, f = f cell, and v = vacuoles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Group II (treated with 206 ppm of permethrin)

The salivary glands of these females featured the same histochemical characteristics as observed in the control group (Fig. 2C, H, and K). In the acini type II, a, c1, and c3 cells were strongly positive for lipids (Fig. 2H). In this group, the lipids were in greater concentration than in the control group (Fig. 2H and K). However, there were some signs of early damage caused by permethrin, e.g., cytoplasm vacuolation (Fig. 2H).

Group III (treated with 1031 ppm of permethrin)

In females exposed to this concentration there was only an amorphous mass, with remnants of glandular tissue, with no presence of lipids detected (Fig. 3C and F).

Group IV (treated with 2062 ppm of permethrin)

Few acini type I could be detected which were weakly positive for lipids as well as indeterminate negative acini (Fig. 3I and M). In

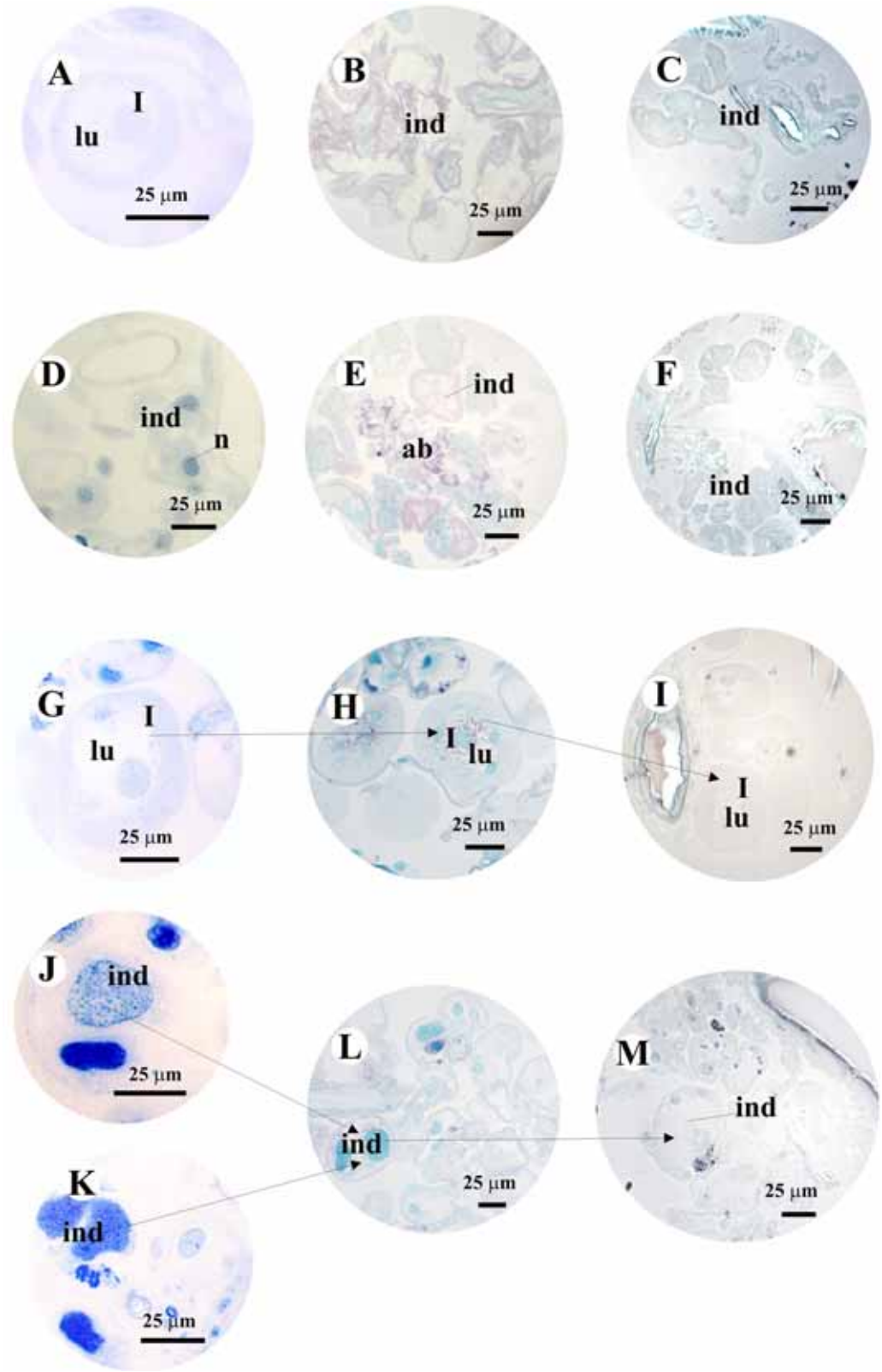

Fig. 3. Histological sections of the *R. sanguineus* salivary glands exposed to 1031 ppm (A–F) and 2062 ppm (G–M) of permethrin. (A and D) Details of the salivary gland stained bromophenol blue, (B and E) PAS/Methyl Green and (C and F) Baker. (G, J, and K) Details of the salivary gland stained bromophenol blue, (H and L) PAS/Methyl Green and (I and M) Baker. I = type I acinus, ind = indeterminate acini, ab = apoptotic body, and lu = lumen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

some indeterminate acini, a strong lipid-containing secretion could be observed (Fig. 3M).

Table 1 summarizes the results above.

Discussion

Ticks are one of the most important systematic groups from the veterinary and medical points of view, because they transmit pathogens to animals and humans (Kaufman, 1989). Researchers have tried various methods for an effective control of these ectoparasites, although the most effective method is still the use of neurotoxic acaricides. However, these compounds cause damage to the environment and public health (Freitas et al., 2005). Few studies have examined the effect of synthetic acaricides on ticks' physiology (Taylor et al., 1991; Mencke et al., 2003; Dong, 2007). These works describe that the toxic action of these compounds affect mainly the nervous system of the ectoparasite. Recent data suggest that other the glandular and reproductive systems of ticks would also be severely susceptible these products (Oliveira et al., 2008, 2009; Pereira et al., 2009; Roma et al., 2009, 2010a,b, 2011).

Because of the widespread use of permethrin and the scarcity of data in the literature about its action on female ticks' glandular system, this study provides information about the histochemical changes on salivary glands in semi-engorged *R. sanguineus* females exposed to different permethrin concentrations. Our results here confirm and complement those of Nodari et al. (2011) which showed that the acaricide, even at low concentrations, is able to cause changes in the tick salivary glands, vital organs for these parasites.

In the control group, we verified that the acini type I synthesize mainly lipid and protein and a few polysaccharide compounds. In the literature, information about the real function of these acini is still scarce and contradictory. Some authors have reported a possible role in osmoregulation (excretory function) (Balashov, 1972; Coons and Roshdy, 1973). According these authors acini type I are osmoregulatory and, through saliva, they remove the toxic compound from the hemolymph. The results obtained in the present study showing the morphological changes observed in type I acini suggest that these structures might be involved in the elimination of toxic permethrin, present in the circulating hemolymph. The increase in lumen diameter suggests a role in osmoregulation of type I acini, via salivation, through the removal of the toxic compound from the hemolymph. However, Rudolph and Knülle (1974) and McMullen et al. (1976) demonstrated that the mouthparts of ticks are the site in which the absorption of atmospheric water vapor takes place. In addition, these authors suggested that the salivary glands produce the water vapor-absorbing substance. Rudolph and Knülle (1974), McMullen et al. (1976), Needham and Coons (1984), Needham and Teel (1986), and Kahl et al. (1990) provided strong experimental evidence that the acini type I are responsible for producing that hygroscopic saliva in non-feeding ticks.

Based on data from the acini II and III from *R. sanguineus* salivary glands of the control group, it was inferred that they synthesize and secrete proteins, lipids, and polysaccharides, which play a key role in setting the tick to the host and in the process of tick feeding. These data corroborate those of Furquim et al. (2010) for semi-engorged females of the same tick species.

The results of this study show that acini II and III of *R. sanguineus* salivary glands from the control group synthesize and secrete protein, lipid, and polysaccharide. According to Furquim et al. (2010), these elements play a key role in fixing the tick to the host through the production of a cement cone that attaches the tick to the host skin as well as in the process of tick feeding.

Overall, the acini II synthesize protein, lipid, and polysaccharide elements through the a cells (protein and lipid synthesis) and the c1 and c3 cells (protein and polysaccharide synthesis). According to Binnington (1978), Walker et al. (1985), Fawcett et al. (1986), and Sonenshine (1991), the protein and lipid synthesis by the a cell is related to the production of elements that are used in the cement cone formation; a crucial structure for tick attachment to the host. The c1 and c3 cells contain protein and polysaccharide elements. According to Sonenshine (1991), these compounds have anticoagulant activity and are released during tick feeding.

In the female salivary glands of the control group, the d and e cells from acini III contained lipid and protein elements, which may be related to the formation of the cement cone. These data corroborate those described by Binnington (1978), Fawcett et al. (1986), Walker et al. (1985), Sonenshine (1991), and Bishop et al. (2002) for other species of ticks. In fact, these acini synthesize aminopeptidases, like the a cells of the acini type II, which according to Walker et al. (1985) participate in the cement infiltration into the host skin.

In our study, strongly PAS-positive f cells were found in the acini type III. According to Binnington (1978) in a study of salivary glands of female *Rhipicephalus (Boophilus) microplus* ticks, the role of these cells in female *Rhipicephalus (Boophilus) microplus*, in the early stages of feeding, is associated with the blood consumption by the tick, in which the cells are completely filled with granules. However, after approximately 72 h of attachment, these granules were released, and the cytoplasm was strongly stained by PAS. Then, the f cells lost their secretory function and transported fluids from the hemolymph to the saliva (Binnington, 1978; Coons and L'Amoreaux, 1986; Fawcett et al., 1986; Sonenshine, 1991).

The salivary glands of female *R. sanguineus* exposed to 206 ppm permethrin showed the same histochemical characteristics as the control group, suggesting that permethrin was not able to change the chemical nature of the secretion synthesized by acinar cells. However, some first signs of structural cellular damages appeared (cytoplasmic vacuolation and the appearance of indeterminate acini) mainly in the acini type II, in which this process would take place with greater intensity when compared to other acini. In some acini type III there was a higher concentration of protein over those of control group. These results suggest that these acini synthesize a larger amount of protein elements to supply the precocious degeneration of the acinus type II.

The data above confirm those from Nodari et al. (2011) showed that even at low concentrations, the permethrin would already be able to interfere with the physiology of glandular tissue, stimulating the early degeneration.

In the salivary glands of female *R. sanguineus* exposed to 1031 and 2062 ppm of permethrin, we observed severe morphophysiological changes, which was more evident in the intermediate concentration (1031 ppm), suggesting that this was significantly more toxic to the glandular tissue (Nodari et al., 2011). Moreover, in this intermediate concentration, there was a highly disorganized glandular tissue with the presence of many apoptotic bodies, acinar remains, indeterminate acini, and marked reduction of protein elements, besides complete absence of both polysaccharide and lipid. In the salivary glands of females exposed to 2062 ppm of permethrin, the same stages of advanced and intense degeneration, and granules containing proteins, lipids, and polysaccharides were observed, although in reduced amounts when compared to the control group and that exposed to 206 ppm of permethrin.

These data corroborate those of Nodari et al. (2011) in that glandular degeneration, which occurs in natural conditions only in the final engorgement process (Furquim et al., 2008b), would occur early in the semi-engorged stage. This process might occur by the same way as described by Furquim et al. (2008b), i.e., the degeneration of this tissue would occur through an atypical cell death process by apoptosis (fragmentation) and by autophagy (vacuolation), both happening simultaneously. Our results agree with those reported by Pereira et al. (2009) who studied the action of fipronil

Table 1
Protein, polysaccharide and lipid detection in the salivary glands of *R. sanguineus* semi-engorged females submitted to permethrin treatment.

Permethrin concentrations (ppm)	Acinus	Cell	Proteins	Polysaccharides	Lipids
Control	I		+ (Fig. 1A)	+ (Fig. 1B)	+ (Fig. 1C)
		a	+++ (Fig. 1D)	– (Fig. 1E)	+++ (Fig. 1G)
		b	⌀	⌀	⌀
	II	c1	+++ (Fig. 1F)	+++ (Fig. 1E)	⌀
		c3	+++ (Fig. 1F)	+++ (Fig. 1E)	⌀
		c4	⌀	⌀	⌀
		d	+++ (Fig. 1H)	– (Fig. 1I)	+ (Fig. 1J)
	III	e	+++ (Fig. 1H)	– (Fig. 1I)	++ (Fig. 1J)
		f	+ (Fig. 1H)	+++ (Fig. 1I)	– (Fig. 1J)
	Indetermined		⌀	⌀	⌀
206	I		+ (Fig. 2A)	+ (Fig. 2B)	+ (Fig. 2C)
		a	+++ (Fig. 2D)	– (Fig. 2E)	+++ (Fig. 2H)
		b	⌀	⌀	⌀
	II	c1	⌀	+++ (Fig. 2E and F)	+++ (Fig. 2H)
		c3	+++ (Fig. 2D)	+++ (Fig. 2E and G)	+++ (Fig. 2H)
		c4	⌀	– (Fig. 2F)	⌀
		d	+++ (Fig. 2I)	– (Fig. 2J)	+++ (Fig. 2K)
	III	e	+++ (Fig. 2I)	– (Fig. 2J)	++ (Fig. 2K)
		f	⌀	+ (Fig. 2J)	– (Fig. 2K)
	Indetermined		++ (Fig. 2L)	+++ (Fig. 2M)	⌀
1031	I		+ (Fig. 3A)	⌀	⌀
		a	⌀	⌀	⌀
		b	⌀	⌀	⌀
	II	c1	⌀	⌀	⌀
		c3	⌀	⌀	⌀
		c4	⌀	⌀	⌀
		d	⌀	⌀	⌀
	III	e	⌀	⌀	⌀
		f	⌀	⌀	⌀
	Indetermined		++ (Fig. 3D)	– (Fig. 3E)	⌀
2062	I		+ (Fig. 3G)	+++ (Fig. 3H)	+ (Fig. 3I)
		a	⌀	⌀	⌀
		b	⌀	⌀	⌀
	II	c1	⌀	⌀	⌀
		c3	⌀	⌀	⌀
		c4	⌀	⌀	⌀
		d	⌀	⌀	⌀
	III	e	⌀	⌀	⌀
		f	⌀	⌀	⌀
	Indetermined		+++ (Fig. 3J and K)	– (Fig. 3L)	– (Fig. 3M)

+, weakly positive; ++, moderately positive; +++, strongly positive; –, negative; ⌀, cell type not observed.

on the secretory cycle and the salivary glands morphology of the same tick species.

According to Furquim (pers. communication), the salivary glands of fully engorged females of *R. sanguineus* showed a significant reduction in secretory capacity, marked by the loss of cytoplasm granules and the appearance of degeneration features. In these females, these changes would make the feeding process impossible, which was also reported from *Amblyomma hebraeum* by Harris and Kaufman (1985). These authors have also reported that gland degeneration is characterized by a reduction of over 90% of the tissue maximum secretion. Based on this information, it was possible to confirm that the same process was taking place (especially at higher concentration), but at the semi-engorged stage, due to a severe reduction of secretion granules in the exposed females glands when compared to the control group. The changes in the salivary glands caused by the pyrethroid in the females studied would certainly prevent females from completing feeding, which is indirectly reflected in yolk production, including reduced egg-laying which directly depends on the salivary glands' activity. These data also complement the analysis performed by Roma et al. (2010a,b, 2011) studying the permethrin action in the vitellogenesis process of *R. sanguineus*.

As described above, in the salivary glands of females exposed to 2062 ppm of permethrin, proteins secretory granules were more evident in the indeterminate acini in relation to the other experimental groups. The presence of protein elements in this stage of glandular degeneration, according to Bowen and Bowen (1990), Zakeri et al. (1995), Lockshin and Zakeri (1996), Tata (1966), and Hacker (2000) occur due to the synthesis of RNA and proteins that occurs in the early stages of apoptosis in engorged females, which could be also occurring here due to premature glandular tissue degeneration.

Based on these considerations, this study has confirmed that permethrin, besides its neurotoxic action (Mencke et al., 2003), acts directly on the physiology of the female salivary glands of *R. sanguineus* triggering early degeneration of this tissue, which under natural conditions would occur only after full engorgement. This pyrethroid acts by interfering and/or inhibiting the feeding process, even when administered at low concentrations.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP (Grants nos. 2009/13854-4, 07/57809-5 and 07/59020-0) and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (Grant no.

308733/2006-1), and G.H. Bechara and M.I. Camargo Mathias academic carrier research fellowships for the financial support and to Mr. Gérson de Mello Sousa for technical support.

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CAPÍTULO 3

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

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

ABSTRACT Ticks are ectoparasites of great medical and veterinary importance around the world and synthetic chemicals such as permethrin have been used for their control. This study provides a cytochemistry analysis of both degenerative and cell death processes in salivary glands of the brown dog tick *Rhipicephalus sanguineus* semi-engorged females exposed to 206, 1,031, and 2,062 ppm of permethrin. The results presented herein demonstrate that permethrin is a potent chemical acaricide that would act on the glandular tissue's morphophysiology in this tick species by eliciting severe changes in the acinus shape, intense vacuolation of the acinar cells' cytoplasm, marked glandular tissue disorganization, culminating in an advanced degenerative stage with consequent formation of many apoptotic bodies (cell death). In addition, permethrin induced major changes in the acinar cells' nucleus, such as a change both in its shape and size, chromatin marginalization, nuclear fragmentation, and appearance of picnotic nuclei, especially when the highest concentrations of the product were used. Thus, permethrin induced early degeneration of this tissue characterized by significant changes in the structure of acinar cells and production of enzymes related to the cell death process, in addition to interfering directly in the genetic material of these cells. *Microsc. Res. Tech. 75:1012–1018, 2012.*

INTRODUCTION

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

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

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

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

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Received 29 September 2011; accepted in revised form 19 January 2012

Contract grant sponsor: FAPESP—Fundação de Amparo à Pesquisa do Estado de São Paulo; Contract grant numbers: 2009/13854-4, 07/59020-0; Contract grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq; Contract grant number: 308733/2006-1; Contract grant sponsor: G.H. Bechara and M.I. Camargo Mathias Academic Carrier Research Fellowships.

DOI 10.1002/jemt.22025

Published online 10 March 2012 in Wiley Online Library (wileyonlinelibrary.com).

MATERIAL AND METHODS

Rhipicephalus sanguineus Ticks

A total of 60 semi-engorged females of *R. sanguineus*, weighing 27 mg in average, supplied by the colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) of the UNESP, at the Instituto de Biociências of Rio Claro, SP, Brazil, were used throughout the experiment. The ticks were kept under controlled conditions (28°C ± 1°C, 80% humidity, and 12 h photoperiod) in an Eletrolab EL 202 BOD (Biological Oxygen Demand) incubator and fed on New Zealand White rabbits (Project subjected and approved by the Ethics Committee on Use of Animal-CEUA, UNESP, Rio Claro, Brazil, Protocol no 5,442).

The feeding laboratorial conditions of *R. sanguineus* ticks in the hosts were followed according to Bechara et al. (1995) technique. In summary, the ticks were placed inside a feeding chamber consisting of a plastic tube (2.5 cm of diameter and 3 cm of height) glued with a non-hazardous preparation on to the shaved backs of the hosts. Elizabethan collars were used on the rabbits to prevent grooming. In order to avoid the escape of ticks during experiments, hosts were kept in cages placed in trays surrounded by a gutter filled with water and oil. Daily observations were performed on some biological parameters of the female ticks.

Dilution Assays of Permethrin (CAS no: 52645-53-1)

Permethrin (3-phenoxybenzyl (1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) used in this study was purchased from Fersol Indústria e Comércio S/A (Mairinque, SP, Brazil). The permethrin concentrations (diluted in distilled water) were based on LC_{50} of 2,062 ppm determined by Roma et al. (2009). The doses correspond to 10% of the LC_{50} (206 ppm), 50% of the LC_{50} (1,031 ppm) and the normal LC_{50} (2,062 ppm). A control group was exposed only to the placebo (distilled water).

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

Detection of Acid Phosphatase

The *R. sanguineus* ticks (control and treated groups) were dissected and the salivary glands were removed. Afterwards, the salivary glands were fixed in 10% buffered formalin and acetone (9:1) for 90 min at 4°C and processed according to the methods described by Hussein et al. (1990) for detection of acid phosphatase. Then, the material was dehydrated in increasing concentrations of ethanol (70, 80, 90, and 95%), for 15 min each, embedded in Leica resin, and sectioned (microtome Leica RM 2255) at 7 μm thickness. Sections were placed on glass slides, counterstained with Hematoxylin for 2 min, and mounted in Canada balsam and examined and photographed in a Motic BA 300 photomicroscope.

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

Feulgen Reaction

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

RESULTS

Acid Phosphatase

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

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

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

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

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

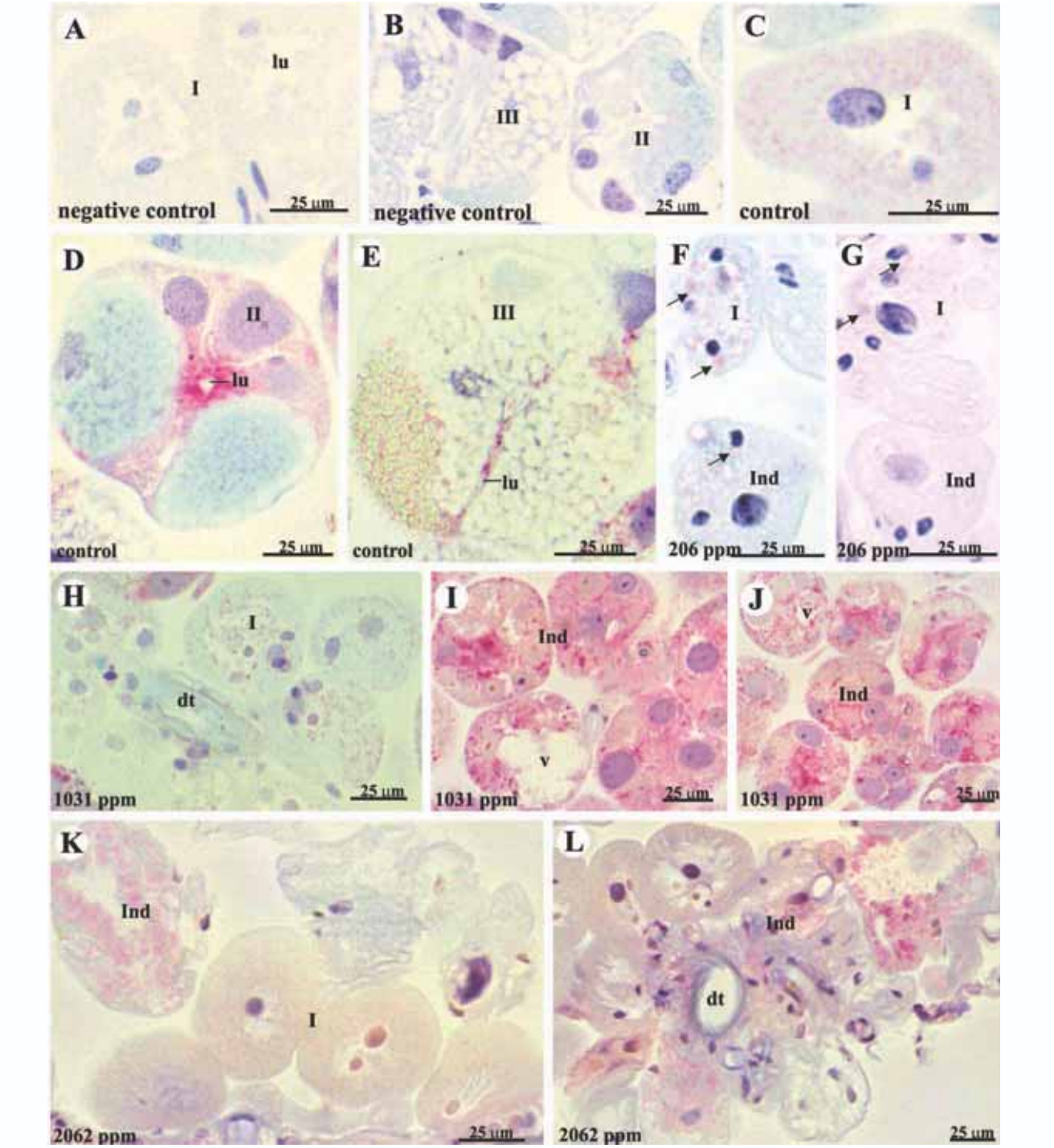

Fig. 1. Acid phosphatase activity in the salivary glands of semi-engorged females of *R. sanguineus* exposed to permethrin. **A** and **B**: Negative control. **C**–**E**: Salivary glands of the control group showing I, II, and III acini weakly positive for acid phosphatase. **F** and **G**: Histological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

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

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

Feulgen

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

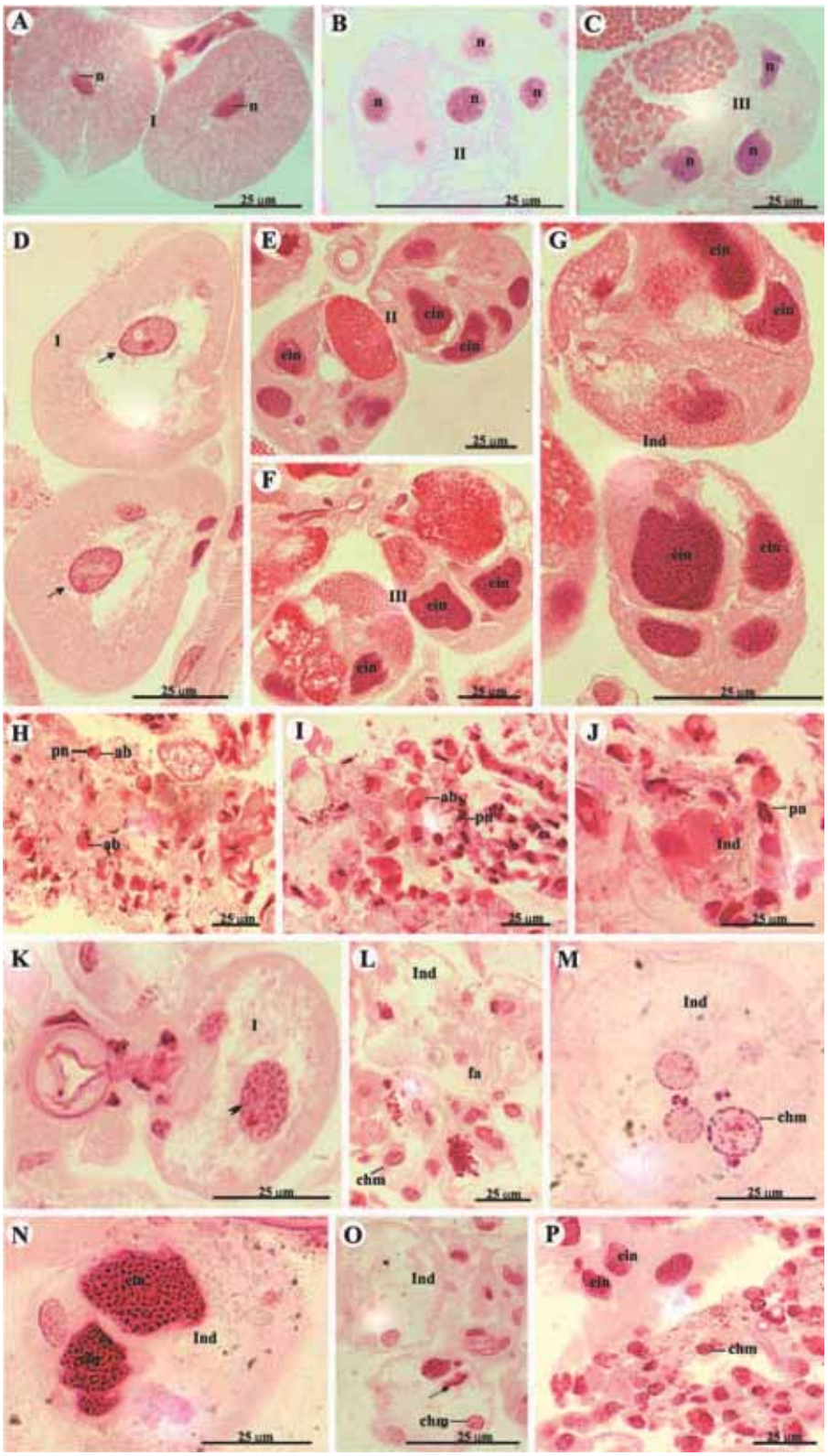

Fig. 2. Histological sections of the salivary glands of semi-engorged females of *R. sanguineus* (control groups and exposed to permethrin) subjected to the Feulgen reaction. **A–C**: Control group; observe intact nuclei (n) of cells from I (I), II (II), and III (III) acini. **D–G**: Salivary glands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

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

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

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

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

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

ACKNOWLEDGMENTS

Authors are grateful to Mr. Gérson de Mello Sousa for technical support.

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CAPÍTULO 4

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

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Running Title: Permethrin action in cytoskeleton of *R. sanguineus*

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Abstract

Considering the great medical-veterinary importance of the ticks and the wide use of synthetic chemical products to control them, such as permethrin, this study brings information about the reorganization of the cytoskeleton during the degeneration processes and cell death which occur on the salivary glands of *Rhipicephalus sanguineus* semi-engorged females subjected to 206, 1031 and 2062 ppm of permethrin. The results show that permethrin acts on the cytoskeleton of *R. sanguineus* salivary glands, modifying it through a gradual process of disintegration as the concentrations of the acaricide increased. In glandular tissue exposed to permethrin the cell death processes advance faster than in normal conditions, where this process occurs in the final of the engorgement. As a consequence of the exposure to this chemical, the acini of salivary glands lost their original shape (typical net structure around the cells) in relation to the control group; once the cytoskeleton (filaments of actin and tubulin) of the acinar cells is disorganized, becoming an amorphous mass. In addition, permethrin induce great changes in the nucleus of acinar cells, such as: in shape and size, causing nuclear fragmentation, mainly when higher concentrations of the product are applied. Thus, permethrin induces the precocious degeneration of the glandular tissue, causing significant changes, mainly in the structure of the cytoskeleton, such as the loss of some components, and consequently, affecting the cellular and nuclear integrity.

Keywords: *Rhipicephalus sanguineus*; permethrin; salivary glands; cell death, cytoskeleton, actin, tubulin

1. Introduction

Ticks, arthropods and ectoparasites of vertebrates, are a group of great medical-veterinary importance as they cause lesions on the hosts and transmit pathogenic agents [1]. Their salivary glands are vital organs and are directly related to the biological success of this group, once they have fundamental functions, such as the production of substances which are necessary for the fixation to the host and feeding [2, 3, 4].

The salivary glands of adult ticks (males and females) are paired structures [5, 6] anterolaterally extended in the ventral portion of the body cavity, reaching the oral cavity [3, 7, 8]. They are constituted by a secretory and an excretory portion, being unprovided of a reservatory to store secretion [2, 9]. The secretory portion is comprised of different acini: I, II, III and

IV, the latter present only in males [2, 9]. The excretory portion is comprised of a system of branched ducts, the one with the largest caliber named common excretory, which has the function of taking the secretion produced in the acini to the tick's oral cavity [3, 7, 10, 11, 12].

Physiologically, the ticks' salivary glands present a well-defined secretory cycle, marked by a phase of production and a phase of release of secretion, processes which are followed by the degeneration of this organ. In the ticks'females, the degeneration of the glandular tissue occurs naturally after the complete engorgement, where the cells die due to the occurrence of apoptosis (fragmentation) and autophagy (vacuolation) processes that occur simultaneously [13].

Due to the increasing medical-veterinary importance given to ticks today, several methods have been researched in order to find an effective control of these ectoparasites. The most efficient method today is still the chemical control (via synthetic acaricides), despite the high cost with the acquisition of the products, installations and adequate workforce for their application, in addition to the environmental contamination through chemical residues, with consequent risks to human health [14].

Permethrin (active ingredient of Advantage® Max3, Bayer) is one of the main compounds of the most used acaricides. This chemical is a pyrethroid with neurotoxic action, which triggers nervous impulses in series resulting from the change of permeability of the sodium membranes, with consequent reaction of the sensorial organs and nervous terminations which cause the ectoparasites a clear state of excitation with tremors and spasms, followed by paralysis and death [15, 16].

There are few specific studies in literature addressing the degeneration process of salivary glands (17, 18, 19), mainly about the influence of synthetic acaricides in the morphophysiology of this tissue [20, 21, 22, 23]. In this sense, this study aimed to analyze, through Laser Scanning Confocal Microscopy, changes in the structure of the cytoskeleton of salivary glands cells of *R. sanguineus* semi-engorged females when these ectoparasires are subjected to the action of permethrin at different concentrations (206, 1031 e 2062 ppm), once previous studies performed by Nodari et al. [20] showed that this pyrethroid accelerates the process of degeneration of this glandular tissue.

2. Material and Methods

2.1. *Rhipicephalus sanguineus* ticks

A total of 60 semi-engorged females of *R. sanguineus*, weighing 27 mg in average, supplied by the tick colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) of the UNESP, at the Instituto de Biociências of Rio Claro, SP, Brazil, were used throughout the experiment. The ticks were kept under controlled conditions (28 ± 1ºC, 80% relative humidity and 12 h photoperiod) in a Biological Oxygen Demand (BOD) incubator and blood fed on New Zealand White rabbits. The feeding laboratorial conditions of *R. sanguineus* ticks in the hosts were followed according Bechara et al. [24] technique. In summary, the ticks were placed inside a feeding chamber consisting of a plastic tube (2.5 cm of diameter and 3 cm of height) glued with a non-hazardous preparation on to the shaved backs of the hosts. Elizabethan collars were used on the rabbits to prevent grooming. In order to avoid the escape of ticks during experiments, hosts were kept in cages placed in trays surrounded by a gutter filled with water and oil. Daily observations were performed on some biological parameters of the female ticks.

This study was approved by the Ethics Committee in the Animal Use, CEUA, UNESP, Rio Claro, SP, Brazil, Protocol nº 5442.

2.2. Dilution assays of permethrin (CAS nº: 52645-53-1)

Permethrin (3-phenoxybenzyl (1RS, 3RS, 1RS, 3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) used in this study was purchased from Fersol Indústria e Comércio S/A (Mairinque, SP, Brazil). The permethrin concentrations were based on LC_{50} of 2062 ppm determined by Roma et al. [25]. The doses correspond to 10% of the LC_{50} (206 ppm), 50% of the LC_{50} (1031 ppm) and the normal LC_{50} (2062 ppm), considering that a permethrin was diluted in distilled water. A control group was exposed only to distilled water.

Rhipicephalus sanguineus semi-engorged females, after being washed in a sieve with tap water, were dried on soft absorbent paper. After that, 45 females were divided into three treated groups of 15 females each and immersed in Petri dishes containing the above different concentrations of permethrin, for 5 min. The control group was also composed of 15 females that had been immersed in distilled water for the same period. Ticks were then dried on absorbent paper and placed in a BOD incubator (28 ± 1°C, 80% relative humidity and 12 h photoperiod) for 7

days. The observation period was established because frequently the effect of acaricide is not immediate, but acts slowly on the physiology [25].

2.3. Laser Scanning Confocal Microscopy

Laser Scanning Confocal Microscopy was used to analyze if there were changes in the rearrangement of the cytoskeleton, as well as changes in the nucleus during the death processes of salivary glands in females ticks exposed to permethrin.

The individuals exposed to permethrin, as well as the ones from control group, were dissected for the removal of the salivary glands, which were fixed in 4% paraformaldehyde. Then, the material was washed in PBS twice (5 min each bath), permeabilized with Triton-X 0.1% for 20 min and washed in PBS (5 min each bath) twice again. The material was then incubated in "Alexa Fluor 488 Phalloidin" solution (5 µL of the stock solution of "Alexa Fluor 488 Phalloidin" + 200 µL de PBS + 2 µL of BSA) during 30 min in covered container and in ambient temperature. Posteriorly, the material was washed in PBS (5 min each bath) twice. The salivary glands of the female ticks were then washed in PBS + BSA to block unspecific staining and incubated overnight with monoclonal antibodies (MOUSE) anti-tubulin (alpha (5□L/mL) and beta (3□L/mL)). The material was again washed in PBS (3 times, 5 min each bath) and incubated during 1 h with secondary antibodies (GOAT ANTI-MOUSE) conjugated with CY5 (10µg/mL), and washed again for 3 times in PBS. After this process the material was mounted using ProLong® Gold reagent containing DAPI (for nucleus staining). The images were obtained using Laser Scanning Confocal Microscope Leica TCS-SP5 II.

3. Results

3.1. Confocal Microscopy

3.1.1. Group I (control)

The acini of salivary glands of females from the control group are spherical and present intact shape (Figs. 1A-H). There is a strong presence of actin (green), as well as tubulin (red), mainly in the central region of the acini. These two components of the cytoskeleton are also distributed in a well-defined net around the acinar cells, maintaining the round shape of the acini (Figs. 1A; C-E; G-H).

The nuclei (blue) of the acinar cells are spherical and intact (Figs. 1B, F).

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

In the acini of the salivary glands of the females exposed to 206 ppm of permethrin the staining for actin (green) is much less evident in relation to the control group, except for the central region of the acini and excretory ducts (excretory portion), which are strong stained (Figs. 2A, D; G-H). As for the tubulin (red), a slight reduction in its amount is observed in relation to the control group; however, in the ducts the staining for this component is intense (Figs. 2C-D; F, H). For this concentration of permethrin, both actin and tubulin present a more defined organization in relation to control group, mainly around the nuclei of acinar cells, forming a basket of microtubules (Figs. 2A, C-D; E-F).

The acini of the salivary glands do not present changes in their shape (Figs. 2A-H); however, the nuclei of the acinar cells show hypertrophy (increase in size) when compared with the nuclei of acinar cells of individuals from the control group, as well as irregular shape and non-uniform coloration (Figs. 2B, D-E, H).

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

In *R. sanguineus* females exposed to 1031 ppm of permethrin the staining for actin (green) is reduced in comparison with the previous groups; however, the central region of the acini, as well as the excretory ducts present strong staining (Figs. 3B, F). For this concentration, the actin net is disorganized with changes in relation to its original form, becoming an amorphous mass. In addition, the glandular acini also show changes, becoming irregular (Figs. 3B, D, F, H). As for tubulin (red), a drastic reduction in the staining of this component both in the acini and acinar ducts, as well as a severe disorganization, is observed. The tubulin filaments are no longer observed, only an amorphous mass (Figs. 3C, G-H).

The acini nuclei show great changes in relation to the previous groups, such as hypertrophy, change in the shape (irregular) and fragmentation (Figs. 3A, E, H).

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

As observed for the previous concentration, the staining for actin (green) in the salivary glands of *R. sanguineus* females exposed to 2062 ppm of permethrin is reduced in relation to the other groups, except for the ducts, strongly stained (Figs. 4B, D, F, H). In addition the actin

filaments are disorganized, becoming an amorphous mass which occupies the peripheral portion of the acini, not their central region (Figs. 4B, D, F, H). The presence of tubulin is no longer observed in the secretory cells of the glandular acini (irregular), except in the ducts, where this component is evident (Figs. 4C-D, G-H). The acini nuclei present more severe changes in relation to those observed in the other treatment groups, such as irregular shape and intense fragmentation, and intact nuclei are no longer observed (Figs. 4A, D, E, H).

4. Discussion

In literature, there are several studies on ticks based mainly on the bioeconomic and ecological aspects, in addition to research on the external anatomy, with a view on taxonomy [7, 26]. However, studies focusing the cell biology of this group of animals are scarce in literature, mainly on the action of synthetic acaricides on the main systems of the ticks, such as the glandular. In this sense, this study performs for the first time a detailed analysis of the action of the synthetic pyrethroid permethrin in the salivary glands of *R. sanguineus* females using laser scanning confocal microscopy. The results showed that permethrin is an active ingredient able to induce changes in the cytoskeleton of the salivary glands during cell death processes of this tissue, mainly in higher concentrations.

The cytoskeleton, in general, corresponds to a set of cellular elements responsible for the structural interaction of cells and for a wide variety of dynamics processes, such as the maintenance of cell shape. It comprises three types of filaments, each of them composed of different proteins: actin filaments, intermediate filaments and microtubules [27, 28, 29]. The actin filaments form a weft of thin and flexible filaments disperse throughout the cellular cytoplasm; however, there is a higher concentration of these filaments in the periphery of the cells, forming the actin cortex, responsible for the shape of the cells [27, 28, 29]. The microtubules are formed by the protein tubulin, and as the actin filaments, they are involved in the determination of cell shape, in the cytoplasmic organization, in the intracellular transportation of vesicles and organelles, and in a variety of cellular movements, such as the separations of chromosomes during cellular division [27, 28, 29]. The intermediate filaments are present in multicellular organisms and are composed of fibrous proteins which associate forming rigid structures, very resistant to traction forces [27, 28, 29].

Considering the exposed, it is suggested that the cytoskeleton would play an important role to start and develop processes of apoptosis (cell death) in different systems of Arthropoda; however this function still raises a lot of discussion among researchers [31]. In this sense, this study brings an important contribution on this issue, through the analysis of microtubules and actin filaments of salivary glands of semi-engorged *R. sanguineus* females exposed to permethrin, once preliminary studies performed by Nodari et al. [20, 21] showed that this pyrethroid would be able to induce the precocious degeneration (cell death) of the glandular tissue in this species of tick.

In the control group the salivary glands of *R. sanguineus* females presented intact acini and cell nuclei, demonstrating the absence of degeneration characteristics on this stage of development (semi-engorged females). These data corroborate Furquim et al. [13, 31], in studies on this same species of tick. Likewise, the components of the cytoskeleton (actin filaments and microtubules) are distributed as a well-defined net around the acinar cells functioning as a mechanical support for the maintenance of the shape of these secretory acini [29].

As for the salivary glands of individuals subjected to permethrin, the results obtained confirm those described by Nodari et al. [20, 21] showing that permethrin accelerates the degeneration process of the glandular tissue, even when applied in low concentrations. In addition, this study showed that the cytoskeleton of *R. sanguineus* salivary glands underwent a gradual process of disorganization as the concentrations of permethrin increased, accelerating the cell death processes. Consequently, the acini of salivary glands lost their original shape for losing their mechanical support (typical net structure around the cells) in relation to control group, once the actin and tubulin filaments became an amorphous mass.

In the salivary glands of females subjected to 206 ppm of permethrin a decrease in actin and tubulin staining was observed in relation to control group; however, the cytoskeleton of acinar cells showed a more defined organization, forming a type of basket around the nucleus, which could be interpreted as a way the cell found to isolate its genetic material, protecting it from the damage caused by the acaricide, in addition to giving mechanical support for the cells which would be directly involved in the process of

glandular secretion. These data are also described by Riparbelli et al. [32] and Jochová et al. [30] for *Drosophila*. These results still suggest that permethrin, even in low concentrations, would be able to cause damages to the structure of the cytoskeleton of acinar cells (actin and tubulin filaments), which would certainly impair the general structure of the acini, and consequently the physiological processes inherent to this tissue. In the same way, the nuclei of acinar cells presented hypertrophy when compared with the ones of control group, as well as changes in the shape (irregular), confirming the cytochemical analysis for cell death performed by Nodari et al. [21] for this same species of tick subjected to permethrin. These authors suggested that these nuclear characteristics are strong indicators of the beginning of degenerative process (apoptosis) of these glands.

In higher concentrations of permethrin (1031 and 2062 ppm) more severe changes were observed, both in the cytoskeleton and the nuclei of acinar cells. Thus, in salivary glands subjected to 1031 ppm of permethrin a drastic reduction on actin and tubulin staining was observed, which consequently caused the loss of the acinus' original shape, turning it into an amorphous mass. Based on these data, it can be suggested that, due to the precocious disorganization of the cytoskeleton structure, all the physiological processes of the salivary glands would also be impaired, once the mechanical support provided by the cytoskeleton elements (among other functions) would be an essential condition for the maintenance of the salivary secretion dynamics which occur in this tissue.

In the concentration of 2062 ppm of permethrin the loss of cytoskeleton elements of the salivary glands was more evident, once the actin filaments, in addition to having become an amorphous mass, were reduced in relation to other treatment groups. Tubulin was not observed in the glandular acini. This loss of cellular integrity, mainly microtubules, was previously described by Martin and Cotter [33] in studies on leukemia strain cells (HL-60) of mammals during apoptosis.

Other authors, as Pereira et al. [23], also observed a disarrange of the cytoskeleton elements of salivary glands of *R. sanguineus* exposed to fipronil. Ndozangue-Touriguine et al. [34] suggested that the depolarization of the proteins of the cytoskeleton would be a sign of its involvement with the preparation of the cell for the apoptosis phase. Considering the data presented by these authors and the results here obtained, it can be concluded that the cytoskeleton is directly involved in the processes of cell death by apoptosis which are occurring in the salivary glands of the female ticks here analyzed.

In higher concentrations of permethrin, (1031 and 2062 ppm) the nuclei of acinar cells showed significant changes in their shape, and some of them were fragmented. Besides this, in the concentration of 1031 ppm of permethrin some nuclei presented hypertrophy, while in 2062 ppm no intact nuclei were found. These data suggest an advance in the degenerative processes in the glandular tissue of the individuals exposed to higher concentrations of permethrin in relation to the other treatment groups. These severe and irreversible changes in the genetic material of acinar cells fatally lead the glandular tissue to a complete degeneration. The results here obtained corroborate the morphological analysis (light microscopy) by Nodari et al. [20; 21] performed for this same species when also exposed to permethrin.

According to Bowen and Bowen [35] and Hacker [36] such characteristics observed in the nuclei of the salivary glands cells would be the result of morphological and biochemical changes which would be occurring in the genetic material due to apoptosis processes. Moreover, Furquim et al. [31] suggested that the hypertrophy of the acinar cells nuclei would be a consequence of the chromatin breakdown, characteristic frequently observed during apoptosis [35; 36, 37, 38, 39].

The excretory ducts of salivary glands of *R. sanguineus* exposed to permethrin maintained the same structure observed in the control group; i.e., the structural organization of actin and tubulin filaments remained intact. These data are supported by the analysis by Till [7] on the salivary glands of *R. appendiculatus* ticks. According to this author, after the complete degeneration process of the salivary glands, only the ducts system remains, along with an amorphous mass resulting from the acini degeneration. The ducts would be the last to undergo apoptosis, which certainly occurred in this study.

Thus, considering the results here obtained and the comparison with other researchers' findings, it can be concluded that the permethrin, in addition to its proven neurotoxic action, [15], would also be responsible for causing severe and irreversible changes in the cytoskeleton of *R. sanguineus* ticks, which would lead to the acini disorganization and consequent damage to the salivary secretion dynamics. The loss of

cytoskeleton elements and consequently the nuclear and cellular integrity confirmed that permethrin accelerates the apoptosis process, without; however, altering the way this process occurs; i.e., by atypical cellular death, where the cell death by apoptosis and autophagy occur simultaneously [13].

Data obtained in this study will certainly serve as scientific basis to help researches in the creation of more efficient and less aggressive methodologies of control, less harmful to the hosts, non-target organisms and the environment in general.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

Authors are grateful to FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo (Grants nº 2009/13854-4 and 07/59020-0) and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Grant nº 308733/2006-1 and G.H. Bechara and M.I. Camargo Mathias academic carrier research fellowships) for the financial support and to Mr. Pablo Henrique Nunes for technical support.

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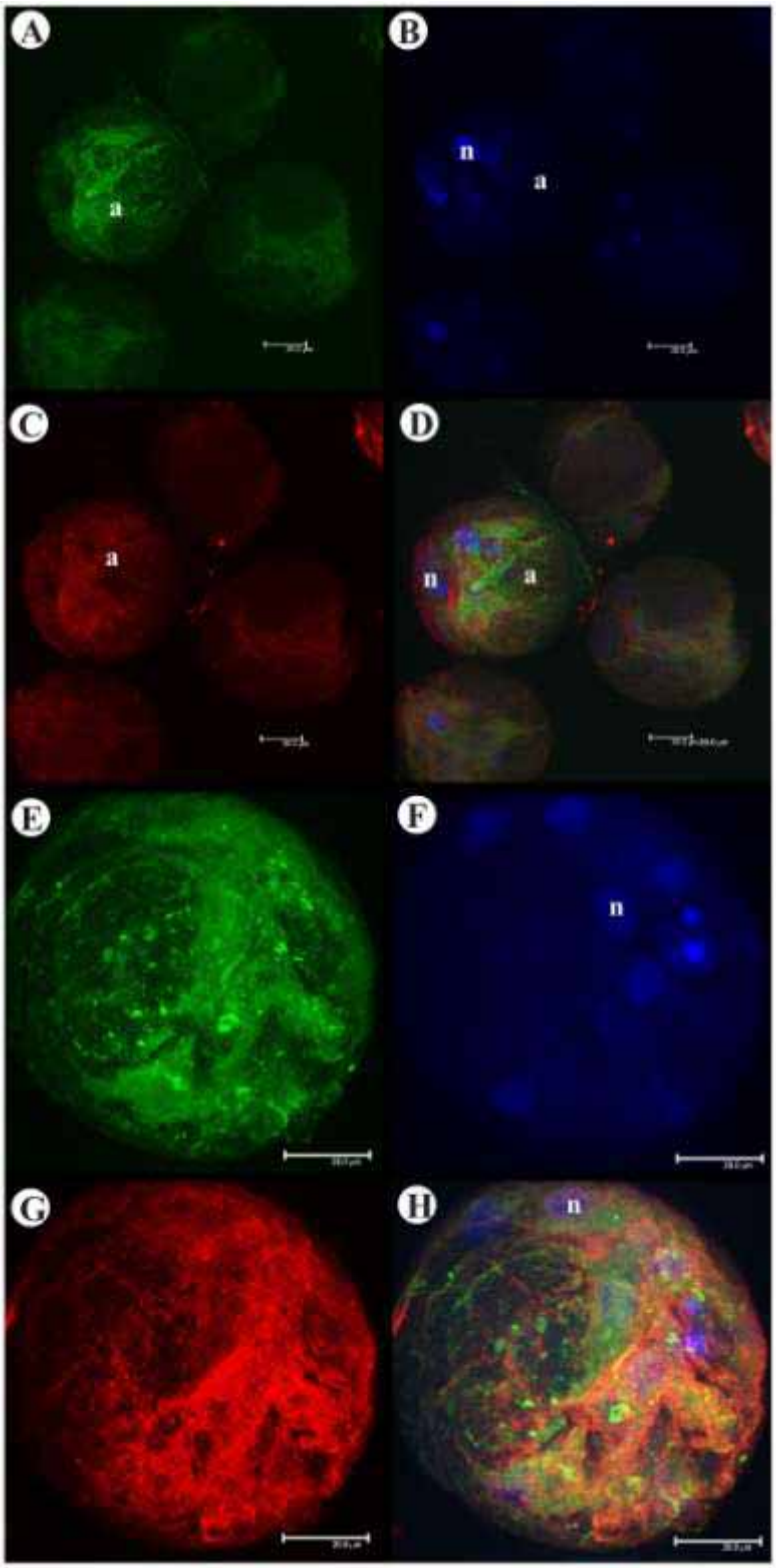

Figure 1: Laser scanning confocal microscopy of secretory portion (acini) of salivary glands from *R. sanguineus* females of control group . (**A**-**D**) General view and (E-H) detail of acini. (**A**; **E**) Observe the presence of actin (green) in acini, especially in the central portion. (**B**; **F**) Detail of nuclei (blue) of the acinar cells. (**C**; **G**) Observe the presence of tubulin (red) in glandular acini. (**D**; **H**) Maximum projections of optical sections of salivary glands showing the glandular acini (red and green) and the nuclei (blue) of acinar cells.
a= acini; n = nucleus

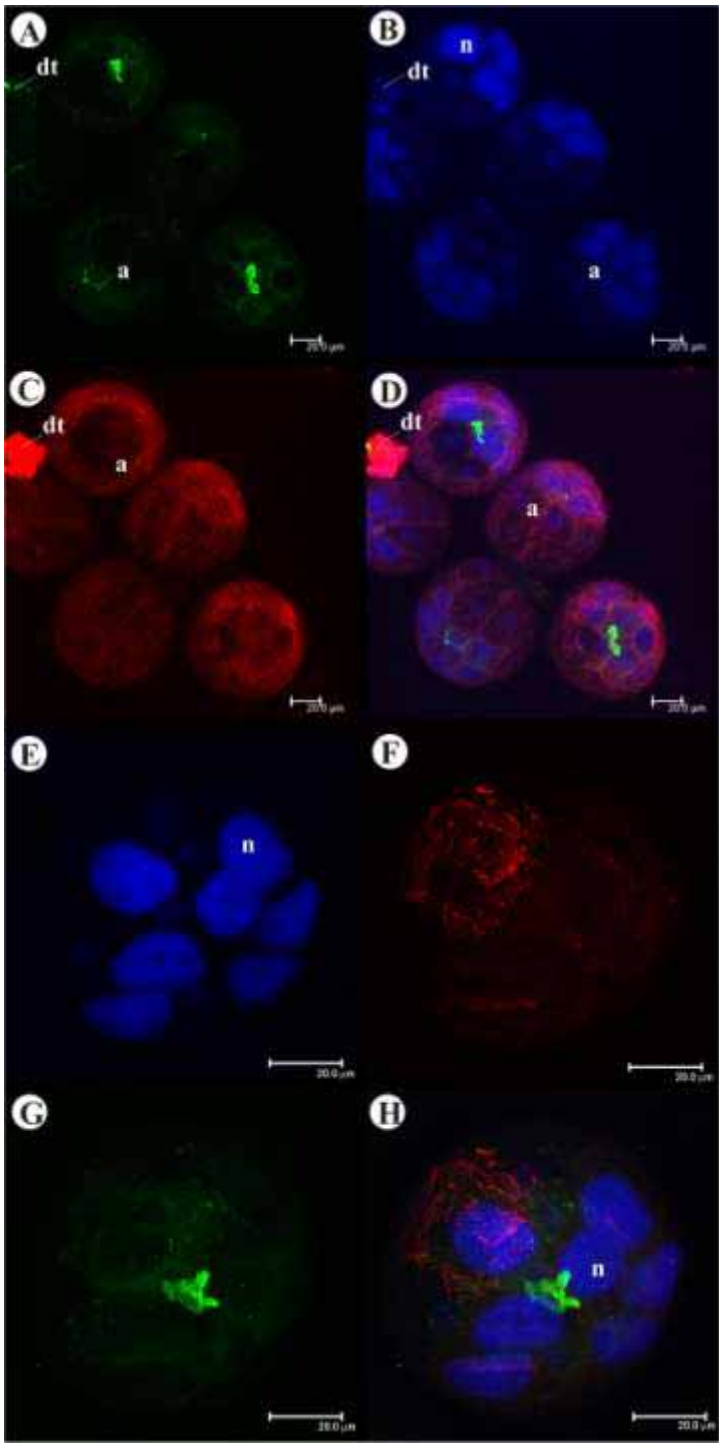

Figure 2: Laser scanning confocal microscopy of secretory (acini) and the excretory (ducts) portion of salivary glands from *R. sanguineus* females exposed to 206 ppm of permethrin. (**A**-**D**) General view and (**E**-**H**) detail of acini. (**A**; **G**) Observe that the levels of actin (green) are reduced when compared with the control group, but the central portion and the acinar ducts are strong stain. (**B**; **E**) Detail of nuclei (blue) of the acinar cells with changes (irregular shape, non-uniform staining and hypertrophy) in relation to the control group. (**C**; **F**) Presence of tubulin (red) in glandular acini; observe a slight reduction in its amount, except in ducts . (**D**; **H**) mMaximum projections of optical sections of salivary glands showing the glandular acini (red and green) and the nuclei (blue) of acinar cells.
a = acini; n = nuclei; dt = ducts

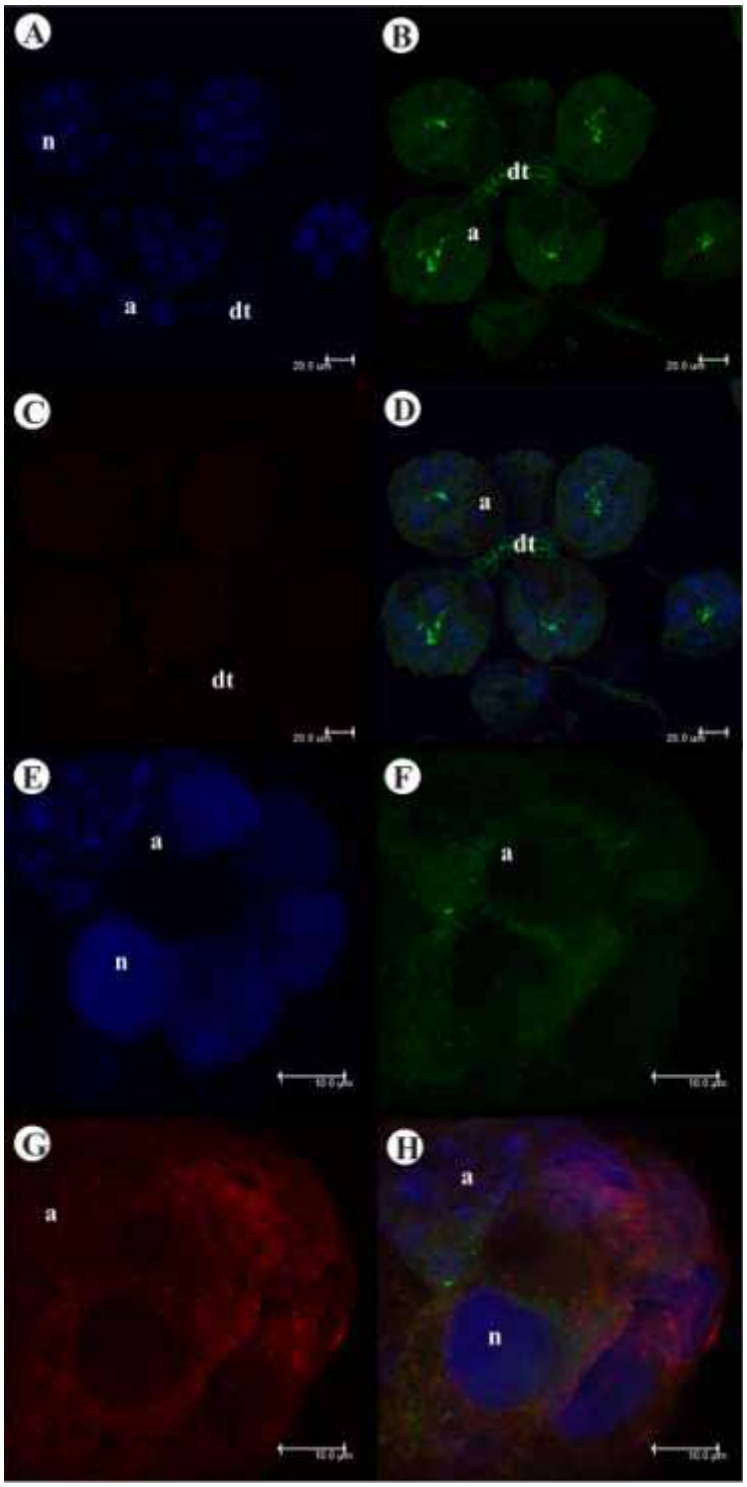

Figure 3: Laser scanning confocal microscopy of salivary glands of female *R. sanguineus* exposed to 1031 ppm of permethrin. (**A**-**D**) General view and (**E**-**H**) detail of acini. (**A**; **E**) Nuclei (blue) of acinar cells. (**B**; **F**).Observe in acini the reduction of actin (green) when compared to other groups, however, the central region of acini, as well as the ducts show strong stain. Observe the disorganization of actin, becoming an amorphous mass. (**C**; **G**) Observe the severe reduction in amount of tubulin (red) both in acini and acinar ducts. Filaments are not more observed, but only an amorphous mass. (**D**; **H**) Maximum projections of optical sections of salivary glands showing the glandular acini (red and green) and the nuclei of acinar cells.
a = acini n = nuclei dt = ducts

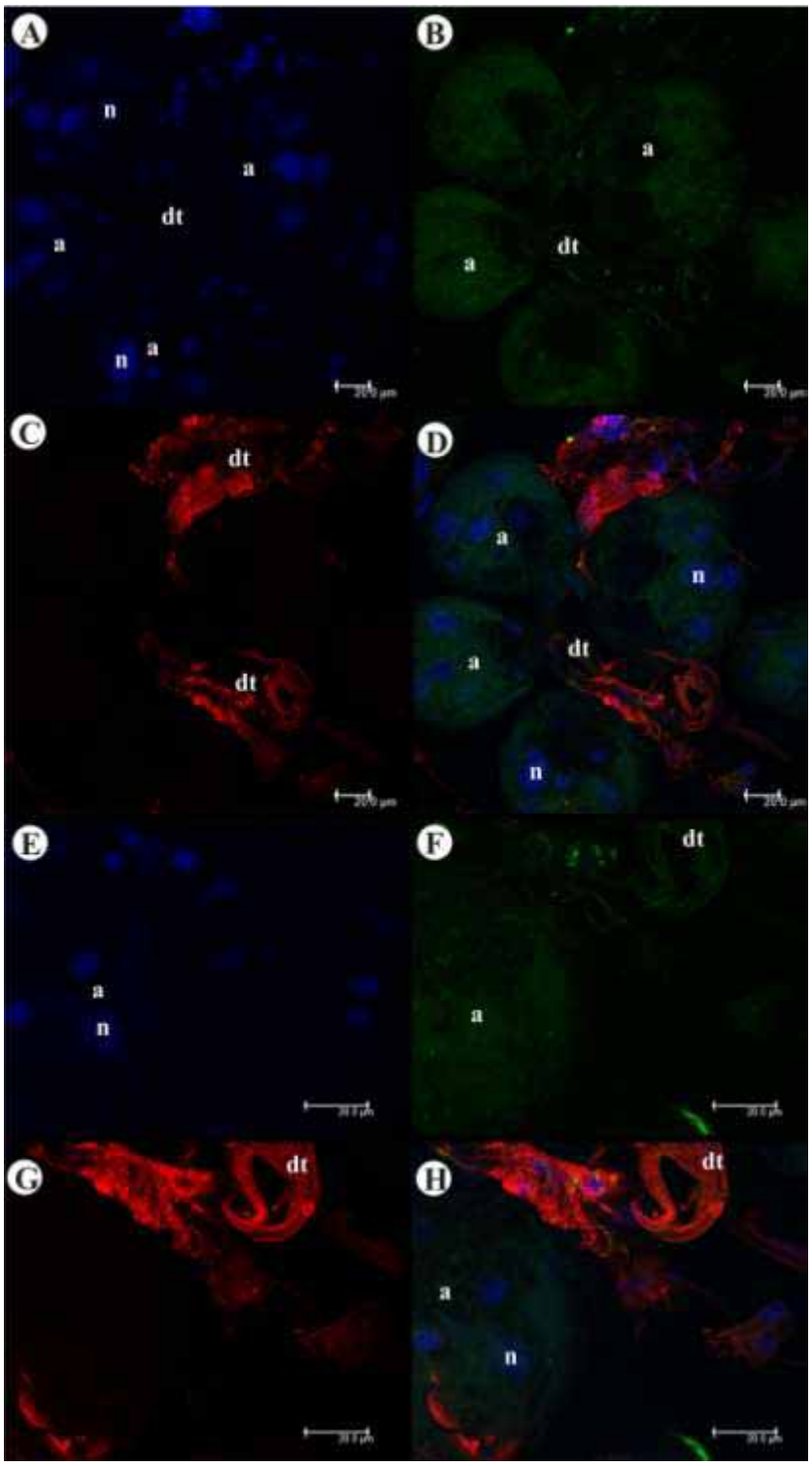

Figure 4: Laser scanning confocal microscopy of salivary glands of female *R. sanguineus* exposed to 2062 ppm of permethrin. (**A**-**D**) General view and (**E**-**H**) detail of acini. (**A**; **E**) Detail of nuclei (blue) of the acinar cells (irregular and fragmented). (**B**; **F**) Observe the reduction in amount of actin (green) in relation to the other groups. The ducts present strong staining for actin. (**C**; **G**) Observe the tubulin is no longer observed in the secretory cells of the glandular acini, except in the ducts, where this component is evident. (**D**; **H**) Maximum projections of optical sections of salivary glands showing the glandular acini (red and green) and the nuclei (blue) of acinar cells.
a = acini; n = nuclei; dt = ducts

DISCUSSÃO GERAL

6 DISCUSSÃO GERAL

O método mais eficaz para controlar infestações de carrapatos nos diversos hospedeiros é o uso de acaricidas sintéticos químicos com ação neurotóxica (DONG, 2007; MENCKE et al., 2003), porém, poucos trabalhos tem descrito as alterações provocadas pela ação tóxica desses compostos em outros sistemas dos carrapatos, como o glandular e o reprodutor (OLIVEIRA et al., 2008; 2009; PEREIRA et al., 2009; 2011; ROMA et al., 2009; 2010a; b; c). Assim, o presente estudo vem mostrar, através do emprego de técnicas morfo-histológicas, histoquímicas, citoquímicas para morte celular e de microscopia confocal, dados sobre as alterações provocadas pela permetrina (ingrediente ativo do acaricida Advantage® Max3, Bayer) nas glândulas salivares de fêmeas semi-ingurgitadas de carrapatos *Rhipicephalus sanguineus*.

Os resultados obtidos mostraram que ocorrem profundas alterações na morfofisiologia das glândulas salivares dessa espécie, quando estas são expostas a diferentes concentrações de permetrina. Além disso, ficou comprovado que já nas menores concentrações, este piretróide é capaz de provocar alterações nas células e no tecido glandular, comprometendo o metabolismo deste órgão que é vital para o sucesso biológico da espécie.

Neste trabalho as fêmeas do grupo controle apresentaram as glândulas salivares com as mesmas características já descritas por Furquim et al. (2008b) para a espécie, confirmando a organização das células secretoras em ácinos e estabelecendo a ocorrência de um ciclo secretor bem definido. Após o término das fases de produção e de liberação da secreção ocorreu a degeneração glandular natural, nos indivíduos em estágios mais avançados de alimentação.

No grupo controle foi também observado que os ácinos **I** teriam a função de sintetizar compostos lipoprotéicos e polissacarídicos. Na literatura, informações sobre a real função desses ácinos são escassas e contraditórias. Alguns autores relatam seu possível papel na osmorregulação (função excretora) (BALASHOV, 1972; COONS; ROSHDY, 1973), o que provavelmente também estaria aqui ocorrendo. Já os ácinos **II** e **III** estariam em intensa atividade de síntese e de secreção de compostos protéicos, polissacarídicos e lipídicos, os quais teriam participação na fixação do carrapato ao hospedeiro e nos processos de alimentação (consumo de sangue) do ectoparasita, dados estes que corroboraram os de Furquim et al. (2010), para fêmeas semi-ingurgitadas dessa mesma espécie.

De forma geral, os ácinos **II** seriam responsáveis pela síntese de compostos glicolipoprotéicos, pois as células **a** conteriam elementos protéicos e lipídicos e as **c1** e **c3** elementos protéicos e polissacarídicos. Segundo Binnington (1978), Fawcett et al., (1986), Sonenshine (1991) e Walker et al. (1985), a síntese dos elementos lipoprotéicos pelas células **a**, estaria relacionada com a produção de elementos que seriam utilizados na formação e na manutenção do cone de cemento, estrutura essencial para a fixação dos carrapatos nos hospedeiros. Já as células **c1** e **c3** conteriam grânulos glicoprotéicos, que segundo Sonenshine (1991) teriam potencial anticoagulante, sendo liberados durante a alimentação dos carrapatos.

Nas glândulas salivares das fêmeas do grupo controle as células **d** e **e** dos ácinos **III** conteriam grânulos lipoprotéicos, elementos estes que, segundo diversos autores (BINNINGTON, 1978; BISHOP et al., 2002; FAWCETT et al., 1986; SONENSHINE, 1991; WALKER et al., 1985) estariam, assim como as células **a** dos ácinos **II**, relacionados com a formação do cone de cemento, sintetizando aminopeptidases, que segundo Walker et al. (1985), participariam no processo de fixação do carrapato infiltrando o cemento na pele do hospedeiro. No presente estudo, no ácino **III** também foram observadas as células **f** com forte positividade para o PAS, assim como também relatado por Binnington (1978) em estudo com glândulas salivares de fêmeas de carrapatos *Boophilus microplus*. Segundo este autor, o papel destas células estaria associado com a alimentação do carrapato, através do transporte de fluidos da hemolinfa para a saliva (BINNINGTON, 1978; COONS; L'AMOREAUX, 1986; FAWCETT et al., 1986; SONENSHINE, 1991), o que provavelmente estaria também aqui ocorrendo.

Além disso, neste estudo observou-se tecido glandular das fêmeas do grupo controle, intensa marcação fosfatásica (somente no lúmen) dos ácinos **II** e **III**. Considerando que tais dados corroboraram aqueles de Furquim et al. (2008b), pode-se aqui inferir que a presença da enzima fosfatase ácida estaria diretamente relacionada com o processo de secreção glandular, ou seja, ela faria parte de muitos dos componentes presentes na saliva das fêmeas de *R. sanguineus* assim, como proposto por Furquim et al. (2008b). Essa hipótese teve suporte nos dados obtidos e apresentados por outros autores que estudaram diferentes espécies de carrapatos e demonstraram histoquimicamente que a fosfatase ácida seria um dos componentes da secreção salivar de indivíduos das espécies *R.* (*Boophilus) microplus* (BINNINGTON, 1978; NUNES et al. 2006a) e *R. appendiculatus* (WALKER et al., 1985).

Com relação aos núcleos das células acinares nos indivíduos do grupo controle, observou-se os mesmos íntegros, corroborando dados obtidos por Furquim et al. (2008a), que sugeriram que essa integridade seria devida a ausência de alterações degenerativas nas

glândulas salivares, informação esta que veio confirmar e ressaltar que a presença da fosfatase ácida nem sempre estaria associada aos processos de morte celular e sim poderia estar envolvida com os processos de produção e liberação de secreção por estas glândulas. De acordo com a literatura pertinente haveria na secreção salivar dos carrapatos enzimas hidrolíticas (grupo das fosfatases ácidas) as quais apresentariam função importante para formação da lesão de alimentação do carrapato no hospedeiro (BINNINGTON, 1978; BROSSARD e WIKEL, 2004; STEEN et al., 2006; WIKEL, 1996,1999).

Com relação ao citoesqueleto das células acinares no grupo controle, observou-se também a integridade dos ácinos, assim como dos componentes do citoesqueleto (filamentos de actina e microtúbulos), que mostraram-se distribuídos de modo a formar uma rede bem definida na periferia das células acinares, tendo como função o suporte mecânico para a manutenção da forma dos ácinos glandulares (NETO et al. 2007), demonstrando mais uma vez a ausência de características de degeneração nas glândulas salivares deste grupo.

O presente estudo ainda mostrou que as glândulas salivares dos indivíduos expostos à concentração de 206 ppm de permetrina apresentaram os ácinos **I** morfologicamente alterados (forma irregular e lúmen dilatado) quando comparados com os do grupo controle, corroborando dados de Pereira et al. (2009) que descreveram que ácinos I também seriam afetados quando fêmeas de *R. sanguineus* fossem expostas ao fipronil. Esses autores sugeriram ainda que os ácinos **I** seriam agentes osmorreguladores e que, através da salivação, conseguiriam retirar o composto tóxico da hemolinfa, fato este que seria comprovado pelo aumento do diâmetro do lúmen. Os resultados aqui obtidos sugeriram que a mesma dinâmica ocorreu com a permetrina, provavelmente com a finalidade de inibir a ação danosa da mesma no organismo do ectoparasita.

Os ácinos **II** dos indivíduos expostos à concentração de 206 ppm de permetrina foram os mais alterados, principalmente as células **c1** e **c3**, as quais apresentaram muitos vacúolos citoplasmáticos, contrariamente ao observado nas células **a**, demonstrando a ocorrência de um processo assincrônico de degeneração, no qual as células **a** seriam as últimas a sofrerem degeneração (NODARI et al., 2011).

Com base nos resultados aqui obtidos, sugeriu-se que a natureza dos vacúolos presentes no citoplasma das células acinares seria autofágica. Neste sentido, esses vacúolos estariam funcionando como agentes na degradação das organelas celulares danificadas pela permetrina, o que provavelmente culminaria na morte celular e consequente total degeneração do tecido glandular (NODARI et al., 2011).

Já as células dos ácinos **III** das glândulas expostas à 206 ppm de permetrina apresentaram vacuolização, especialmente as células **e**, que segundo Gill e Walker (1987) eWalker et al. (1985) juntamente com as **d** seriam as responsáveis por secretar os componentes do cone de cemento, fundamental no processo de fixação do carrapato ao hospedeiro. Assim, os resultados obtidos neste trabalho sugeriram que a permetrina agiria alterando a fisiologia das glândulas salivares, comprometendo a finalização da formação do cone de cemento. Porém, observou-se ainda que para esta concentração de permetrina as glândulas salivares apresentaram as mesmas características histoquímicas observadas no grupo controle, sugerindo que, nesta concentração, este acaricida não seria capaz de alterar a natureza química dos grânulos de secreção sintetizados pelas células dos ácinos **II** e **III**. Entretanto, em alguns ácinos **III** observou-se maior concentração de elementos protéicos em relação ao grupo controle, sugerindo uma tentativa de compensar a degeneração precoce dos ácinos **II**.

Nos núcleos das células acinares das fêmeas submetidas à 206 ppm de permetrina, também foram observadas alterações, na forma (irregular), tamanho (aumentados) e disposição da cromatina (início de marginalização). No entanto, verificou-se que para esta concentração de permetrina não houve mudança na marcação fosfatásica em relação àquelas do grupo controle. (NODARI et al 2012a)

Assim, embora tenham ocorrido alterações morfológicas no citoplasma e citoquímicas no núcleo das células das glândulas salivares expostas, não foram observadas alterações para marcação da fosfatase ácida, o que levou os autores a sugerirem que a exposição à permetrina na concentração de 206 ppm afetaria, num primeiro momento, somente a organização do citoplasma das células glandulares (vacúolos citoplasmáticos) e o estado fisiológico do núcleo das mesmas, mas não modificaria o comportamento hidrolítico celular (ação da fosfatase ácida) (NODARI et al 2012a).

Já com relação à marcação para os elementos do citoesqueleto (actina e microtúbulos) observou-se nos ácinos uma diminuição da marcação dos elementos em relação ao grupo controle, entretanto o citoesqueleto das células acinares mostrou organização mais definida, formando uma estrutura como cesta ao redor do núcleo, o que poderia ser interpretado como uma forma da célula isolar seu material genético, protegendo-o do dano causado pelo acaricida, além de dar suporte mecânico para as células envolvidas diretamente no processo de secreção glandular, dados estes também descritos por Jochová et al. (1997) e Riparbelli et al. (1993) para *Drosophila*. Estes resultados sugeriram ainda que a permetrina mesmo em baixas concentrações já seria capaz de induzir danos na estrutura do citoesqueleto

das células acinares, o que certamente comprometeria a estrutura geral dos ácinos, e consequentemente os processos fisiológicos inerentes a este tecido.

À medida que a concentração da permetrina aumentou (1031 ppm), as alterações nas glândulas salivares se intensificaram em relação ao grupo anterior, tratamento com 206ppm de permetrina, indicando que esta concentração seria aquela que causaria alterações mais significativas no tecido glandular. Este aumento na concentração de permetrina estimulou o avanço do processo de degeneração do tecido, resultando no surgimento de uma massa amorfa composta por restos de ácinos, o que impossibilitou a identificação (**indeterminados**), dos mesmos além de: a) presença de muitos corpos apoptóticos e de inúmeros núcleos com aspecto picnótico e/ou fragmentados; b) acentuada redução de elementos protéicos e c) completa ausência de polissacarídeos e lipídios (NODARI et al 2012b). Resultados semelhantes foram encontrados por Furquim et al. (2008b), Hacker (2000) e Kerr et al. (1995) que sugeriram que estas características seriam fortes indicativos da ocorrência de morte celular por apoptose, que ocorreria naturalmente (em situação de campo) durante a degeneração das glândulas salivares dos carrapatos logo após o total ingurgitamento. Os dados aqui obtidos corroboraram esses autores e mostraram ainda que a permetrina agiria acelerando a degeneração do tecido glandular, por meio da ocorrência de morte celular atípica, onde a morte celular por apoptose (fragmentação) e a morte celular por autofagia (vacuolização) ocorreriam simultaneamente. Os resultados aqui obtidos corroboraram também aqueles descritos por Pereira et al. (2009; 2011) que estudaram a ação do fipronil sobre o ciclo secretor e sobre a morfologia das glândulas salivares de carrapatos desta mesma espécie.

As glândulas salivares dos carrapatos expostos à 1031 ppm de permetrina mostraram a presença de poucos ácinos **I**, os quais estavam com grandes alterações morfológicas, confirmando os dados de Furquim et al. (2008b) que demonstraram que estes seriam, no processo normal de desativação da glândula salivar, os últimos a sofrerem degeneração, a qual ocorreria primeiramente nos ácinos **II** e **III**. Ainda, para esta concentração, o tecido glandular mostrou, em relação aos demais grupos de tratamento, acentuada redução de elementos protéicos e completa ausência de polissacarídeos e de lipídios (NODARI et al 2012b).

Da mesma forma, nas fêmeas expostas a 2062 ppm de permetrina, apenas alguns ácinos **I** foram identificados, sendo os demais aqui classificados como **indeterminados**, por terem perdido suas características morfo-histológicas devido ao avançado processo de degeneração. Em contraposição às alterações sofridas por este tecido nesta concentração, ainda puderam ser observados elementos protéicos, polissacarídicos e lipídicos, embora em quantidades reduzidas em relação aos demais grupos (NODARI et al 2012b). Segundo

terorias de Bowen e Bowen (1990), Hacker (2000), Lockshin e Zakeri (1996), Tata (1966) e Zakeri et al. (1995) nos estágios iniciais dos processos de apoptose de fêmeas totalmente ingurgitadas haveria a síntese de RNA e de proteínas nos ácinos **indeterminados**, sendo que a presença de elementos protéicos nos mesmos em fêmeas semi-ingurgitadas indicaria que esta síntese também estaria ocorrendo devido à degeneração precoce do tecido glandular.

Segundo Lomas et al. (1998), a degeneração natural das glândulas salivares de fêmeas de carrapatos seria controlada hormonalmente por um ecdisteróide. Estes autores sugeriram que a regulação dar-se-ia em parte pela participação da ecdisona, hormônio que provocaria a degeneração deste tecido (HARRIS; KAUFMAN, 1985; LINDSAY; KAUFMAN, 1988). A síntese e a liberação desse hormônio ocorreriam no começo do período alimentar atingindo um pico de produção logo após o desprendimento do ectoparasita (do hospedeiro) (LOMAS, 1993). Porém, com base nos dados aqui obtidos, observou-se que já no estágio de semi-ingurgitamento houve degeneração precoce do órgão, confirmando, portanto, que a permetrina acelerou o processo (NODARI et al 2011; 2012a, b).

Em relação às características enzimáticas, nas maiores concentrações de permetrina (1031 e 2062 ppm) foram observadas severas alterações tanto para a marcação da fosfatase ácida, que se intensificou, quanto no núcleo, que passou a apresentar alterações no tamanho (aumentado), na forma (irregular ou fragmentado) e no grau de condensação da cromatina (condensada e/ou marginalizada) (NODARI et al 2012a).

Os dados apresentados no presente estudo permitiram sugerir que a síntese da fosfatase ácida, relacionada ao processo de morte das células glandulares, seria intensificada pelas maiores concentrações de permetrina, demonstrando que a ação da mesma estaria primeiramente relacionada às alterações morfológicas das células glandulares e somente tardiamente atuaria no comportamento hidrolítico celular (NODARI et al 2012a). De acordo com Furquim et al. (2008b) a fosfatase ácida participaria na finalização do processo de morte por apoptose atípica, contribuindo para a fragmentação celular e removendo os restos citoplasmáticos. Sendo assim, o aumento na marcação fosfatásica frente à exposição à permetrina possivelmente causaria à aceleração do processo degenerativo glandular, o que em condições normais ocorreria somente no final do processo alimentar (FURQUIM et al., 2008b).

Além disso, as alterações nucleares aqui detectadas (picnose, marginalização cromatínica e fragmentação) também caracterizaram a ocorrencia de apoptose, pois de acordo com a literatura (BOWEN E BOWEN, 1990; BOWEN, 1993; FURQUIM et al., 2008a; HACKER, 2000; LOCKSHIN e ZAKERI, 1996; ZAKERI e AHUJA, 1997), tais eventos

seriam resultado das mudanças bioquímicas e morfológicas ocorridas no núcleo, decorrentes do processo apoptótico. Somando-se a isso, Furquim et al. (2008a) discutiram que a presença de núcleos aumentados nas células dos ácinos glandulares, (aqui também observado), sugeriria a quebra da cromatina, característica observada nos processos da apoptose (BOWEN E BOWEN, 1990; BOWEN, 1993; FURQUIM et al., 2008a; HACKER, 2000; LOCKSHIN e ZAKERI, 1996; ZAKERI e AHUJA, 1997)

Assim, com base nos dados obtidos no presente estudo, características do tecido glandular como: a) perda da forma do ácino, b) perda da integridade das membranas das células secretoras, c) vacuolização citoplasmática, d) presença de poucos grânulos secretores nas células e e) alterações nucleares (forma e tamanho), seriam eventos anteriores que culminariam com o rompimento dos ácinos, e com a consequente formação e liberação de corpos apoptóticos, que foram aqui observadas nas glândulas das fêmeas expostas à permetrina, corroborando Pereira et al. (2009) em estudos com glândulas salivares desta mesma espécie de carrapato quando expostas ao fipronil.

Ainda nas maiores concentrações de permetrina (1031 e 2062 ppm) foram observadas alterações severas no citoesqueleto. Assim, nas glândulas salivares submetidas a 1031 ppm de permetrina observou-se drástica redução na marcação da actina e da tubulina das células acinares, o que consequentemente provocou a perda da forma original do ácino transformando-o em uma massa amorfa. Com base nestes dados, sugeriu-se que devido à desorganização precoce da estrutura do citoesqueleto, processos fisiológicos das glândulas salivares destes carrapatos ficariam também comprometidos, visto que o suporte mecânico desempenhado pelos elementos do citoesqueleto (dentre outras funções) seria uma condição essencial para a manutenção da dinâmica da secreção salivar.

Na concentração de 2062 ppm de permetrina a perda dos elementos do citoesqueleto das células glândulares tornou-se mais evidente, visto que os filamentos de actina, além de terem se transformado em massa amorfa, foram reduzidos em relação aos demais grupos de tratamento. No caso da tubulina, esta não foi mais observada nas células dos ácinos glandulares. Esta perda da integridade celular, principalmente em relação aos microtúbulos, já haveria sido anteriormente descrita por Martin e Cotter (1990) em estudos com células de linhagem leucêmica (HL-60) de mamíferos, durante a apoptose.

Outros autores, tais como Pereira et al. (2011), também observaram a desorganização dos elementos do citoesqueleto nas células das glândulas salivares de *R. sanguineus* expostas ao fipronil. Ndozangue-Touriguine et al. (2008) sugeriram que a despolarização das proteínas do citoesqueleto, seria um sinal do seu envolvimento com a preparação da célula para a fase

da apoptose. Com base nos dados apresentados por estes autores e nos resultados aqui obtidos, pode-se concluir que o citoesqueleto estaria diretamente envolvido nos processos de morte celular por apoptose nas glândulas salivares das fêmeas dos carrapatos aqui analisadas.

Os ductos excretores das glândulas salivares dos carrapatos *R. sanguineus* expostos à permetrina em todas as concentrações aqui testadas mantiveram a mesma estrutura observada no grupo controle, com a organização estrutural dos filamentos de actina e de tubulina íntegras. Estes dados corroboraram Till (1961) que estudou as glândulas salivares de carrapatos *R. appendiculatus* e verificou que, após o processo completo de degeneração das glândulas salivares de carrapatos, restaria apenas o sistema de ductos, bem como uma massa amorfa resultando da degeneração dos ácinos. No caso dos ductos estes seriam as últimas estruturas a sofrerem apoptose nas glândulas salivares, o que certamente também foi observado neste estudo.

Dessa forma, os resultados aqui apresentados mais uma vez confirmaram que a permetrina, além de ter comprovada ação neurotóxica (MENCKE et al., 2003) também acelerou a degeneração do tecido glandular, evento este que naturalmente ocorreria somente após o total ingurgitamento da fêmea. Entretanto, estes resultados deixaram claro que nas fêmeas semi-ingurgitadas de *R. sanguineus* expostas às maiores concentrações de permetrina, as glândulas salivares mostraram, além de elevadas taxas de degeneração, também degeneração precoce, quando comparadas com as dos indivíduos do grupo controle, complementando as análises realizadas por Roma et al. (2010a; b; c) que tratou do estudo da ação da permetrina nos processos de vitelogênese de fêmeas de *R. sanguineus*. Sabe-se que quando as fêmeas de carrapatos ficam impossibilitadas de concluírem o seu processo alimentar, a vitelogênese também fica comprometida, bem como a postura de ovos, processos dependentes da atividade das glândulas salivares. Desta forma, a degeneração precoce das glândulas salivares devido ao uso de acaricidas causaria menores perdas de sangue nos hospedeiros, e também reduziria a taxa de transmissão de patógenos, veiculados via glândulas salivares.

As informações obtidas neste trabalho vieram, portanto, confirmar a necessidade de se realizar estudos abordando a morfo-fisiologia do sistema glandular exócrino de carrapatos expostos aos químicos sintéticos em geral, e ressaltar o uso desta ferramenta que é de grande importância na obtenção de informações que auxiliem no melhoramento e desenvolvimento de métodos de controle que sejam menos agressivos para os organismos não-alvo.

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ANEXOS

ANEXO A – APROVAÇÃO DO COMITÊ DE ÉTICA NO USO ANIMAL

unesp

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Rio Claro

COMISSÃO DE ÉTICA
NO USO DE ANIMAL
CEUA – IB – UNESP - CRC

Protocolo nº: 5442 (02-09-09)
Data Registro CEP: 03-09-2009

Rio Claro, 04 de novembro de 2009.

Ofício CEUA 008/2009

Prezada Senhora,

Aprovo "ad *referendum*" da Comissão de Ética no Uso de Animal, UNESP, Campus de Rio Claro (CEUA-IB-UNESP), o projeto de pesquisa intitulado *"Avaliação dos efeitos do piretróide permetrina (ingrediente ativo do Advantage Max 3-Bayer) nas glândulas salivares de fêmeas de carrapatos Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae)", sob sua responsabilidade, tendo como orientanda Elen Fernanda Nodari –colaboradores: Gislaine Cristina Roma e Gervásio Henrique Bechara.

Atenciosamente,

Prof. Dr. **Célio Fernando Baptista Haddad**
Coordenador

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Elen Fernanda Nodari

Orientada

Profa. Dra. Maria Izabel Camargo Mathias

Orientadora

Dra. Gislaine Cristina Roma

Co-Orientadora