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

ESTUDO DOS EFEITOS DE EXTRATOS DE GLÂNDULAS SALIVARES DE FÊMEAS DE *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidae) NA RESPOSTA IMUNOLÓGICA DE HOSPEDEIROS PÓS INOCULADOS

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

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À minha família, Luiz Fernando, meu esposo, Lucas e Cecília, meus queridos filhos, que puderam compreender o tempo que lhes neguei durante a realização deste trabalho... .

À "família Polivet", pelo apoio e empenho para que eu pudesse dedicar-me a este trabalho....

Dedico esta dissertação...

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À minha família e meus amigos que dela participam.

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A vida tem duas faces:
Positiva e negativa
O passado foi duro
mas deixou o seu legado
Saber viver é a grande sabedoria
Que eu possa dignificar
Minha condição de mulher,
Aceitar suas limitações
E me fazer pedra de segurança
dos valores que vão desmoronando.
Nasci em tempos rudes
Aceitei contradições
lutas e pedras
como lições de vida
e delas me sirvo
Aprendi a viver.
("Assim eu vejo a vida", Cora Coralina)

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SUMÁRIO

Resumo

Resumo

Este estudo abordou os efeitos de extratos de glândulas salivares de fêmeas de *Rhipicephalus sanguineus* com 2, 4 e 6 dias de alimentação (**EGS2-EGS6**) em coelhos New Zealand White virgens de infestação inoculados três vezes a intervalos de 21 dias. Os animais inoculados foram desafiados com carrapatos *R. sanguineus*, para verificar a influência dos extratos sobre a resposta imune-inflamatória dos hospedeiros. Foram estudadas as alterações histopatológicas na pele dos hospedeiros na região da lesão de fixação dos carrapatos, os parâmetros alimentar e reprodutivo das fêmeas obtidas da infestação desafio, bem como suas glândulas salivares aos 2, 4 e 6 dias de alimentação. Para obtenção dos extratos glandulares utilizou-se 55 fêmeas (110 glândulas salivares) com 2 dias de alimentação, 36 (72 glândulas) com 4 dias e 25 (50 glândulas) com 6 dias, de onde obteve-se os extratos: **EGS2**, a partir de glândulas salivares de fêmeas com 2 dias de alimentação; **EGS4** com 4 dias, e, **EGS6** com 6 dias. Os resultados revelaram que os diferentes extratos glandulares estimularam o desenvolvimento de resposta imune-inflamatória na pele dos hospedeiros comprometendo os processos alimentar e reprodutivo das fêmeas dos carrapatos. Os hospedeiros do grupo **GT (EGS4)** mostraram a formação de um intenso e precoce infiltrado de células inflamatórias, sinalizando que este foi o extrato mais eficaz no desenvolvimento da resposta imune-inflamatória local. Por outro lado, a resposta dos hospedeiros do grupo **GT (EGS6)** à infestação foi mais tardia e com menor ocorrência desse infiltrado. Já o processo inflamatório local nos hospedeiros do **GT (EGS2)** foi o menos intenso. O teste de desafio mostrou médias para os parâmetros alimentar e reprodutivo das fêmeas oriundas da infestação desafio que não foram significativamente diferentes, com exceção do peso médio de ingurgitamento das fêmeas do grupo **GT (EGS6)**. Contudo, considerou-se que estes efeitos ocorreram em virtude das respostas desenvolvidas pelos hospedeiros, visto que a análise histopatológica da pele bem como o estudo histológico das glândulas salivares revelaram diferenças em relação à imunização com os diferentes extratos. Neste sentido, verificou-se que os extratos **EGS2** e **EGS4** poderiam alterar os parâmetros alimentar e reprodutivo dos carrapatos infestantes, mostrando ação na imunização dos hospedeiros contra a infestação por carrapatos *R. sanguineus*. Embora com ação específica, o **EGS2** reduziu o número de descendentes (ação na reprodução) enquanto o **EGS4** reduziu o número de carrapatos que ingurgitaram por completo (ação na alimentação), principalmente devido à morte dos mesmos. Além disso, ambos os extratos reduziram o consumo de sangue pelos ectoparasitas. Já o extrato **EGS6** não foi eficaz, estimulando a alimentação dos ectoparasitas. Desta forma, embora estatisticamente as diferenças entre os parâmetros não tenham sido

significativas, nos animais dos grupos **GT**, estas diferenças poderiam ser reflexo da imunização dos hospedeiros com os extratos glandulares, as quais influenciaram na resposta imune-inflamatória, fornecendo informações importantes para estudos futuros sobre o desenvolvimento de métodos de controle da infestação por carrapatos (vacinas).

Abstract

Abstract

This study addressed the effects of salivary glands extracts of *Rhipicephalus sanguineus* females with 2, 4 and 6 days of feeding (**EGS2-EGS6**) in New Zealand White rabbits naive inoculated three times at intervals of 21 days. The inoculated animals were challenged with *R. sanguineus* ticks to verify the influence of extracts on the host's immune-inflammatory response. Changes in the hosts' skin at the feeding lesion, feeding and reproductive parameters as well salivary glands with 2, 4 and 6 days of feeding of females obtained from the challenge infestation were studied. To obtain the glandular extracts, we used 55 females (110 salivary glands) with 2 days of feeding, 36 (72 glands) with 4 days and 25 (50 glands) with 6 days, which were obtained the extracts: **EGS2**, from salivary glands of females with two days of feeding; **EGS4** with 4 days and **EGS6** with 6 days. The results revealed that the different glandular extracts stimulated the development of immune-inflammatory response in the hosts' skin compromising the feeding and reproductive processes of the female ticks. The hosts of the **TG** group **(EGS4)** showed the formation of an early and intense infiltration of inflammatory cells, indicating that this extract was more effective in developing the local inflammatory-immune response. On the other hand, the response of the **TG** group hosts **(EGS6)** to the infestation was delayed and with lower incidence of this infiltrate. The local inflammatory process in the **GT** hosts **(EGS2)** was the less intense. The challenge test showed averages for feeding and reproductive parameters of females from infestation challenge that were not significantly different, with the exception of the engorgement average weight of females from **TG** group **(EGS6)**. However, it was considered that these differences occurred because of the responses developed by the hosts, since the histopathology of the skin and the histological study of the salivary glands revealed these differences in relation to immunization with the different extracts. In this sense, it was found that the extracts **EGS2** and **EGS4** reduced feeding and reproductive parameters of infesting ticks, being effective in immunizing hosts against *R. sanguineus* tick infestation. Although acting specifically, the **EGS2** reduced the number of offspring (action on reproduction) while the **EGS4** reduced the number of ticks that engorged completely (action on feeding), mainly due to their death. Moreover, both extracts reduced the consumption of blood by ectoparasites. The **EGS6** extract was not effective, stimulating feeding of the ectoparasites. Thus, although statistically the differences between the parameters were not significant, in animals from **TG** groups the differences are reflections of hosts' immunization with glandular extracts that influenced the immune-inflammatory response, thus providing important information for future studies on the development of tick infestation control methods (vaccines).

Introdução Geral

1.Introdução

O carrapato *Rhipicephalus sanguineus* é popularmente conhecido como carrapato do cão por ter este animal como seu principal hospedeiro, embora também possa parasitar outros mamíferos, inclusive o homem (REY, 1973). É encontrado em regiões tropicais e temperadas (WALKER, 1994). Também é reconhecido como praga urbana por estar bem adaptado aos centros urbanos (LABRUNA; PEREIRA, 2001). Assim, é considerado um dos principais problemas parasitários em canis, ambientes domiciliar e peridomiciliar além de áreas rurais (PAZ et al. 2008).

A espécie *R. sanguineus* tem importância em saúde pública e veterinária, pois além da ação espoliativa sobre o hospedeiro, é conhecida como vetor de patógenos como a *Babesia canis* e a *Ehrlichia canis* (DANTAS-TORRES, 2008).

A alimentação dos carrapatos dá-se basicamente devido à ação combinada das estruturas bucais e da saliva, produzida pelas glândulas salivares (BALASHOV, 1972). Nos ixodídeos, as glândulas salivares são órgãos importantes para o sucesso biológico, atuando na produção de substâncias necessárias aos processos de fixação e alimentação (BINNINGTON, 1978; WALKER et al., 1985; GILL; WALKER, 1987) e também a evasão da resposta imune-inflamatória do hospedeiro (PECHOVÁ et al, 2002).

Segundo Sonenshine (1991), a saliva é uma mistura complexa de substâncias que apresentam diferentes funções, como a manipulação dos processos hemostático, inflamatório e imunológico do hospedeiro. Sua ação anti-hemostática dá-se por aumento do fluxo sangüíneo na região da lesão de fixação, por meio da secreção de agentes vasoativos e anticoagulantes (SAUER et al., 2000). Sua ação anti-inflamatória e imunossupressora dá-se por substâncias que inibem a ação de citocinas envolvidas na resposta imune inata e adquirida (SAUER et al., 2000).

A ação imunomoduladora da saliva dos carrapatos no hospedeiro acaba por atuar como facilitadora na transmissão de patógenos que encontram menores obstáculos à sua penetração e multiplicação (GILLESPIE et al., 2001; PECHOVÁ et al., 2002).

Segundo Turni et al. (2002) diversas informações podem ser obtidas sobre a resistência adquirida por hospedeiros sensibilizados a diferentes espécies de carrapatos ou mesmo a uma só espécie, pois as moléculas imunossupressoras sintetizadas pelas glândulas salivares são diferentemente expressas durante a alimentação dos carrapatos. Além disso, glândulas salivares de espécies diferentes devem apresentar diferentes antígenos, em quantidades e concentrações diferentes (JAWORSKI et al., 1990; INOKUMA et al., 1994).

A complexidade funcional das glândulas salivares, bem como a complexidade bioquímica da saliva é resultado da morfologia glandular. Assim, as glândulas salivares de fêmeas de carrapatos são compostas por ácinos dos tipos I, II e III (BINNINGTON, 1978; WALKER et al., 1985; GILL;

WALKER, 1987; SONENSHINE, 1991), onde aqueles do tipo I são agranulares e os II e III são granulares e atuam no processo de alimentação (BINNINGTON, 1978) e de osmorregulação do ectoparasita na fase de grande consumo de sangue (KAUFMAN; SAUER, 1982; SONENSHINE, 1991).

Os ácinos do tipo II são constituídos pelas células secretoras dos tipos **a**, **b**, **c1**, **c2**, **c3** e **c4** (BINNINGTON, 1978) e em fêmeas de *R. sanguineus*, além destas, foram recentemente descritas as **c5** e **c6** (FURQUIM et al., artigo submetido). Sabe-se que as **a** estão envolvidas com a secreção do cemento (BINNINGTON, 1978; WALKER et al., 1985; FAWCETT et al., 1986; GILL; WALKER, 1987), e as **b** e **c** com a modulação da resposta do hospedeiro (BINNINGTON, 1978; WALKER et al., 1985).

Os ácinos III, por sua vez, são constituídos por três tipos de células, as **d**, **e** e **f** (BINNINGTON, 1978; WALKER et al., 1985; GILL; WALKER, 1987; FURQUIM et al., artigo submetido), onde as **d** e **e** secretam componentes do cemento durante a fixação (BINNINGTON, 1978; WALKER et al., 1985; GILL; WALKER, 1987), e as **f**, substâncias relacionadas ao consumo de sangue pelo ectoparasita (BINNINGTON, 1978).

Atualmente, o controle deste ectoparasita baseia-se no uso de acaricidas, no entanto, a toxicidade desses produtos e seu potencial de contaminação residual levam à busca de métodos eficientes de controle com baixo impacto tanto para o hospedeiro quanto para o meio ambiente (SAITO et al., 2005, NUTTAL et al. 2006). Assim, uma das formas de controle que atualmente tem merecido especial atenção é a utilização de vacinas, produzidas com apoio de técnicas de biologia molecular e de imunologia, que auxiliam na identificação de novos antígenos, oriundos principalmente dos aparelhos digestório, reprodutor e das glândulas salivares dos carrapatos. Estes métodos têm desenvolvido melhor resposta imune do hospedeiro e, portanto, habilitando-os a controlar essa ectoparasitose (TELLAM et al., 1992; WILLADSEN, 1997; NUTTAL et al., 2006). Uma proteção vacinal do hospedeiro contra o carrapato poderia prover também proteção contra as doenças transmitidas por estes ectoparasitas já que comprometeria a capacidade imunossupressora da saliva do carrapato permitindo ao hospedeiro montar uma resposta imune eficiente dificultando a transmissão de patógenos pela saliva do artrópode (NAZARIO et al. 1998; GILLESPIE et al., 2000; NUTTAL et al. 2006).

A interação hospedeiro-ectoparasita envolvida no mecanismo de desenvolvimento de resistência aos carrapatos é bastante complexa (WILLADSEN, 1980; OBEREM, 1984). A ausência de resposta deletéria ao ácaro pode ser observada em algumas relações carrapato-hospedeiro (BRUMPT; CHABAUD, 1947; RANDOLPH, 1979). O cão doméstico, hospedeiro natural do *R. sanguineus*, não desenvolve resistência após repetidas infestações (CHABAUD, 1950; FERREIRA;

BECHARA, 1995; SZABÓ et al., 1995; BECHARA, 2006) ou após vacinação com extrato de carrapato adulto não alimentado seguida de infestação desafio com indivíduos da mesma espécie (BECHARA et al., 1995). No mesmo sentido, estudos realizados com o cachorro-do-mato *Cerdocyon thous* demonstraram que este ancestral do cão doméstico também não adquire imunidade à mesma espécie de carrapato (FERREIRA;BECHARA, 1995). Por outro lado, o carrapato *R. sanguineus* parece ser capaz de induzir marcada resistência em hospedeiros não habituais, como o coelho (CHABAUD, 1950) e cobaias submetidos à infestações sucessivas ou à vacinação com extrato de carrapato adulto não alimentado (BECHARA et al., 1995; SZABÓ et al., 1995; BECHARA, 2006).

Brossard e Wikel (1997) relataram em hospedeiros resistentes ao carrapato a presença de neutrófilos e basófilos nas amostras de pele. Da mesma forma, Monteiro e Bechara (2008) demonstraram a presença de basófilos na lesão de fixação do carrapato *Amblyomma cajennense* em cabras submetidas a várias infestações. Além disso, Veronez et al. (2010) mostraram a presença de células inflamatórias provenientes de cobaias resistentes no lumem do intestino de carrapatos *R. sanguineus* infestantes.

Diante dessas informações, este trabalho buscou obter dados que contribuíssem com o estudo do desenvolvimento de novas estratégias de controle do carrapato *R. sanguineus* através do desenvolvimento de resposta imune-inflamatória nos hospedeiros.

Objetivo

2. Objetivo

Geral: Avaliar a resposta imune-inflamatória de coelhos hospedeiros frente à inoculação de diferentes extratos (**EGS2-EGS6**) obtidos a partir de glândulas salivares de fêmeas de carrapato *Rhipicephalus sanguineus* com 2, 4 e 6 dias de alimentação.

Específico: Avaliar a resposta dos hospedeiros sensibilizados e desafiados com *R. sanguineus* considerando-se:

a) As alterações de caráter imune-inflamatório na lesão de fixação de fêmeas de *R. sanguineus* aos 2, 4 e 6 dias de alimentação nos hospedeiros do grupo teste bem como do grupo controle;

b) A alimentação e a reprodução das fêmeas utilizadas na infestação desafio;

c) As alterações no ciclo secretor de glândulas salivares de fêmeas com 2, 4 e 6 dias de alimentação fixadas em coelhos imunizados com os extratos (**EGS2**, **EGS4** e **EGS6**).

Material e Métodos

3. Material e Métodos

3.1. Material

3.1.1. Obtenção das Larvas de *Rhipicephalus sanguineus* (Início da Colônia)

Para iniciar a colônia de *R. sanguineus* foram utilizadas quatro fêmeas matrizes completamente ingurgitadas, oriundas de colônia mantida em laboratório em condições controladas (28^{o} C ± 1ºC, 80% de umidade e de fotoperíodo de 12 horas) em estufa BOD no Departamento de Patologia Veterinária da UNESP *campus* de Jaboticabal (SP) e cedidas pelo Prof. Dr. Gervásio Henrique Bechara. A colônia em questão foi estabelecida em sala do Biotério do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP). Estas fêmeas foram mantidas em tubos plásticos, cuja tampa continha pequenos orifícios para oxigenação, onde ocorrereu a postura de ovos (15 dias após o completo ingurgitamento).

Os ovos foram recolhidos e depositados no interior de um mecanismo inoculador desenvolvido pelo Prof. Dr. Gervásio Henrique Bechara (Fig. 1A).

3.1.1.1. Construção do Mecanismo Inoculador

Seringas de 3 mL tiveram 2 cm de suas extremidades opostas às do êmbolo removidas. A abertura foi então vedada com algodão e o êmbolo foi removido, a massa de ovos foi depositada no interior da seringa, a abertura correspondente ao encaixe do êmbolo também foi vedada com algodão, este levemente umedecido. Na seqüência, o êmbolo foi encaixado parcialmente (Figs. 1A, 1C).

O inoculador foi acondicionado em estufa BOD (Fig. 1B), até que ocorresse a eclosão das larvas, o que se deu em 7 dias.

3.1.2. Obtenção das Ninfas de *R. sanguineus*

As larvas presentes no inoculador foram alocadas (1 inoculador/hospedeiro) no interior de câmaras alimentadoras fixadas em coelhos virgens de infestação (Figs. 1E-G). As larvas permaneceram alimentando-se até o ingurgitamento (7 dias) quando foram coletadas e mantidas em frasco plástico com a tampa contendo orifícios para a oxigenação. Os frascos

foram mantidos em condições controladas (28^{o} C ± 1ºC, 80% de umidade e fotoperíodo de 12 horas) em estufa BOD, até a muda para o estágio de ninfas, o que ocorreu em 7 dias.

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

Um círculo de borracha fina de 9 cm de diâmetro foi cortado e revestido com tecido de algodão (que ficou em contato com a pele do hospedeiro). Em seguida outro círculo de 3.5 cm de diâmetro foi retirado do centro do círculo de 9 cm de diâmetro. Na borda deste foi fixado com cola plástica um tubo plástico de 2 cm de altura, que foi vedado internamente com a mesma cola e externamente com esparadrapo (Fig. 1D). Esse tubo plástico recebeu uma tampa com pequenos orifícios para que os carrapatos fossem supridos com ar (figs. 1E-G).

3.1.2.2. Fixação da Câmara Alimentadora (BECHARA et al., 1995)

O hospedeiro, virgem de infestação, teve uma área da região dorsal tosada que recebeu uma camada de cola plástica (brascoplast®) (Fig. 1D). Da mesma forma, a câmara alimentadora (na região revestida com tecido de algodão) recebeu uma camada desta cola, que foi fixada na pele do coelho. A fixação foi reforçada com esparadrapo, que cobriu parte da câmara e da região tosada.

Depois de fixada, a câmara alimentadora permaneceu 24 horas destampada para eliminar o odor da cola, para então serem depositados os carrapatos (larvas). Após a alocação das larvas, a primeira observação deu-se depois de 8 horas (tempo necessário para a acomodação dos parasitas), e a partir daí as seguintes deram-se diariamente. As larvas completamente ingurgitadas foram recolhidas, armazenadas nos inoculadores (Figs. 1H-I) e mantidas em condições controladas em estufa BOD (Fig. 1B), para a ecdise das ninfas, que ocorreu em 7 dias em média (Figs. 1K, L).

3.1.3. Obtenção das Fêmeas Adultas de *R. sanguineus*

As ninfas foram alocadas em câmaras alimentadoras para engurgitamento que foram fixadas em coelhos virgens de infestação (Fig. 1J), segundo metodologia descrita por Bechara

et al. (1995). A primeira observação deu-se após 8 horas, e a partir daí as seguintes deram-se diariamente.

Em 5 dias as ninfas completaram seu ingurgitamento (Figs. 1K-L), então foram coletadas e depositadas em tubos plásticos para sofrerem a muda para o estágio adulto (Fig. 1M), o que ocorreu em 5 dias.

3.2. Métodos

Foram utilizadas fêmeas de *R. sanguineus* em jejum e submetidas a alimentação em coelhos New Zealand White. Os indivíduos em jejum (machos e fêmeas) foram oriundos de colônia mantida em estufa BOD em condições controladas (28^{o} C +/- 1ºC, 80% de umidade e fotoperíodo de 12 horas) em sala do Biotério do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP).

As fêmeas em jejum foram utilizadas nas infestações **A**, **B** e **C**, que se realizaram em coelhos segundo procedimento descrito por Bechara et al. (1995).

Foram realizadas três infestações, a saber:

- Infestação A

Foi realizada em coelhos virgens de infestação utilizando-se de 25 casais de carrapatos *R. sanguineus*/câmara (Figs. 1N, O), para aquisição de fêmeas de *R. sanguineus* com 2, 4 e 6 dias de alimentação, para produção dos extratos glandulares: **EGS2**, obtido a partir de glândulas salivares de fêmeas com 2 dias de alimentação, **EGS4**, a partir de glândulas de fêmeas com 4 dias de alimentação, e, **EGS6**, a partir de fêmeas com 6 dias de alimentação (Figs. 2A-C).

Os períodos de alimentação escolhidos (2, 4 e 6 dias) foram determinados com base no ciclo secretor da glândula salivar, visto que indivíduos em jejum possuem as glândulas contendo ativas as células **a**, **c1**, **c3**, **d** e **e**, aqueles com dois dias de alimentação têm ativação das células **b**, **c2**, **c4**, **c5**, **c6** e **f**, com 4 dias as **c5** e **f** tornam-se inativas, permanecendo ativas apenas as **a**, **b**, **c1-c4** e **c6** e finalmente os indivíduos com seis dias de alimentação têm inativação das células **c6** (FURQUIM, artigo submetido).

Os extratos após serem preparados, foram adicionados de adjuvante completo de Freund e inoculados nos hospedeiros.

- Infestação B (grupo teste GT)

Foi realizada em 12 coelhos sensibilizados, sendo 4 inoculados com o extrato **EGS2**, 4 com o **EGS4** e 4 com o **EGS6**, os quais foram submetidos à infestação desafio com 15 casais de carrapatos *R. sanguineus* adultos/hospedeiro (Figs. 6C, 7A).

- Infestação C (grupo controle)

Foi realizada em 8 coelhos virgens de infestação, sendo que 4 foram inoculados com mistura de adjuvante de Freund completo e tampão fosfato (grupo controle 2= **GC2**) e 4 não foram inoculados (grupo controle 1= **GC1**). Então, estes animais foram submetidos à infestação desafio com 15 casais de *R. sanguineus* adultos/hospedeiro (Fig. 7B).

3.2.1 Preparação dos Extratos Glandulares

As fêmeas de *R. sanguineus* com 2, 4 e 6 dias de alimentação (infestação **A**) (Figs. 2A-C) foram retiradas do hospedeiro pela região do hipostômio com o auxílio de pinça cirúrgica por meio de movimentos circulares. A seguir, foram colocadas em placas de Petri para dissecção contendo solução salina (7,5 g de NaCl, 2,38 g de Na_2HPO_4, 2,72 g de KH_2PO_4 e 1000 mL de água destilada), onde procedeu-se a remoção das glândulas salivares.

Nas dependências do Laboratório de Biologia Molecular do Departamento de Biologia da UNESP Rio Claro (SP) as glândulas foram colocadas (separadamente por período de alimentação), em tubos eppendorfs contendo 200 µL de tampão fosfato pH 7.4, onde foram maceradas (Figs. 2D, E). Na seqüência os tubos foram centrifugados por 30 minutos a 10.000 xg (Fig. 2F), os sobrenadantes foram depositados em eppendorfs estéreis e encaminhados para a dosagem de proteínas, segundo metodologia descrita por Sedmark e Grossberg (1977) (método de Bradford) (Figs. 2G-I).

Após a determinação do conteúdo protéico de cada amostra (Tabela 1), no interior de capela de fluxo laminar vertical pré-estéril (Figs. 3A, B), cada extrato foi, separadamente, diluído com tampão fosfato (concentração final de 0,2 µg/µL) e filtrado com auxílio de

unidades filtrantes estéreis (JBR610303, unidade filtrante descartável Millex GV, membrana durapore PVDF, Millipore, MilliUni), de 0,22 µm e diâmetro de 13mm, acopladas a seringas hipodérmicas (Figs. 3C-E). Depois os extratos foram aliquotados em volumes de 50 µL e novamente armazenados em eppendorfs estéreis em freezer a -20 ^{o}C. Somente no momento das inoculações realizou-se a mistura de cada um dos extratos (50 µL de extrato/hospedeiro) com 50 µL de adjuvante de Freund completo (referência n^{o} F 5881, Sigma-Aldrich) (Fig. 3F).

Tabela 1: Valores da dosagem protéica dos extratos obtidos a partir de glândulas salivares de fêmeas de *Rhipicephalus sanguineus* com 2, 4 e 6 dias de alimentação.

	Tempo de Alimentação		
	2 dias	**4 dias**	**6 dias**
Concentração protéica	1,08 µg/µL	1,75 µg/µL	1,49 µg/µL

3.2.2 Inoculação dos Extratos nos Hospedeiros

Em sala do Biotério do Departamento de Biologia da UNESP de Rio Claro (SP) os coelhos dos grupos **GT (EGS2, 4 e 6)** (4 coelhos/ extrato glandular) e do **GC2** (4 coelhos) tiveram sua região dorso-lateral direita tosada (Fig. 4A) e subcutaneamente, via seringa hipodérmica, foram inoculados com os extratos **EGS2**, **EGS4** e **EGS6** (**GT**), bem como com AF+PBS (**GC2**) por três vezes a intervalos de 21 dias (Figs. 4B-D).

Somente após 15 dias da última inoculação, todos os hospedeiros dos grupos **GT (EGS2, 4 e 6)** e **GC1** e **2** foram submetidos à infestação desafio com 15 casais de *R. sanguineus*/hospedeiro (Figs. 4E-H, 5A-C).

3.2.3. Obtenção de Amostras de Pele da Região da Lesão de Fixação dos Carrapatos

Os animais submetidos às infestações **B** e **C** tiveram amostras de pele retiradas do local da lesão de fixação das fêmeas de *R. sanguineus* aos 2, 4 e 6 dias após o início da alimentação, coletadas utilizando-se "punch" (saca-bocados) com 0,3 cm de diâmetro em área previamente anestesiada com xilocaina 2% sem vasoconstritor. No momento da coleta, o ectoparasita fixado ao hospedeiro foi seccionado na região do capítulo, permanecendo

somente o hipostômio fixado à pele do coelho. Com o "punch" posicionado no local e por meio de movimentos circulares retirou-se o fragmento de pele. As amostras coletadas foram fixadas em solução de paraformaldeído a 4% para histopatologia.

3.2.4. Análise Histopatológica

Após a fixação, o material recolhido foi desidratado em concentrações crescentes de álcool (70%, 80%, 90% e 95%), banhos de 20 minutos cada, foi transferido para resina de embebição, incluído e seccionado. A embebição e a inclusão foram em resina Leica. Os cortes foram seccionados com espessura de 3 μm, recolhidos em lâminas de vidro e processados segundo a técnica de hematoxilina de Harris e eosina aquosa e Giemsa. As lâminas foram montadas em Bálsamo do Canadá e examinadas em fotomicroscópio Motic BA 300.

3.2.5. Teste Desafio com Avaliação dos Desempenhos Alimentares e Reprodutivos (Potencial Biótico) das Fêmeas de *R. sanguineus*

Foi realizado em sala do Biotério do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP) utilizando-se as fêmeas infestantes dos coelhos dos grupos **GT** (infestação **B**) e **GC1** e **2** (infestação **C**) (Figs. 5D-F, 6A-C, 7A-B). Para avaliação do efeito das imunizações foram considerados os seguintes parâmetros do ectoparasita: a) tempo necessário para as fêmeas completarem o ingurgitamento, b) porcentagem de recuperação de fêmeas em relação ao número liberado, c) avaliação do peso das fêmeas completamente ingurgitadas, d) tempo de oviposição, e) peso da massa de ovos, f) viabilidade dos ovos e g) índice de eficiência alimentar (peso da fêmea ingurgitada/tempo para fêmea completar o ingurgitamento) (JITTAPALAPONG et al., 2000a).

A média ± desvio padrão dos parâmetros alimentares e reprodutivos foram analisados estatisticamente por meio do teste ANOVA com pós-teste de TUKEY, utilizando-se nível de significância de 5% ($p < 0.05$).

3.2.6. Análise Histológica das Glândulas Salivares das Fêmeas de *R. sanguineus* Alimentadas nos Hospedeiros Imunizados com os Extratos

As fêmeas de carrapatos do grupo teste (**GT**) (infestação **B**) e controle (**GC1** e **GC2**) (infestação **C**) com 2, 4 e 6 dias de alimentação foram retiradas do hospedeiro pela região do hipostômio com o auxílio de pinça cirúrgica por meio de movimentos circulares. A seguir, os carrapatos foram colocados em placas de Petri para dissecção contendo solução salina (7.5 g de NaCl, 2.38 g de Na_2HPO_4, 2.72 g de KH_2PO_4 e 1000 mL de água destilada) para a remoção das glândulas salivares.

Na seqüência, as glândulas foram fixadas em paraformaldeido 4%, por 24 horas, a 4^{o}C. Após a fixação, o material foi desidratado em concentrações crescentes de álcool (70%, 80%, 90% e 95%), banhos de 20 minutos cada, foi transferido para resina (Leica) de embebição, incluído e seccionado. O material foi seccionado com 3 µm de espessura. As seções recolhidas em lâminas de vidro foram coradas com hematoxiclina e eosina, montadas com bálsamo do Canadá e fotografadas em microscópio Motic BA 300.

Figura 1: Imagens da colônia de carrapatos *Rhipicephalus sanguineus* estabelecida em sala no Biotério do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP).

A. Estufa BOD onde ovos, larvas, ninfas e adultos de carrapatos foram mantidos.
B. Inoculadores contendo ovos e acondicionados no interior da estufa BOD.
C. Detalhe do inoculador contendo larvas.
D. Material utilizado na preparação dos hospedeiros para receberem os carrapatos: máquina de tosa (**1**), cola plástica para fixação da câmara alimentadora (**2**), tubo plástico para confecção da câmara alimentadora (**3**) e câmara alimentadora para deposição dos ectoparasitas (**4**).
E-G. Câmara alimentadora fixada com cola plástica e esparadrapo no dorso do hospedeiro. **F-G.** Detalhe da câmara aberta (**F**) e vedada com tampa contendo orifícios para oxigenação (**G**).
H. Inoculador contendo larvas (**5**) e ninfas em jejum armazenadas em tubo plástico (**6**).
I. Detalhe de **H**.
J-L. Ninfas: (**J**) em início de alimentação no interior da camara alimentadora fixadas à pele do hospedeiro, (**K**) e (**L**) completamente ingurgitadas no interior de tubo plástico, onde as mesmas permanecem até se tornarem carrapatos adultos. **L.** Detalhe de **K**.
M. Machos e fêmeas em jejum armazenados no interior de um tubo plástico.
N-O. Machos e fêmeas em início de alimentação no interior da câmara alimentadora fixada à pele do hospedeiro. **O.** Detalhe de **N**.

FIGURA 1

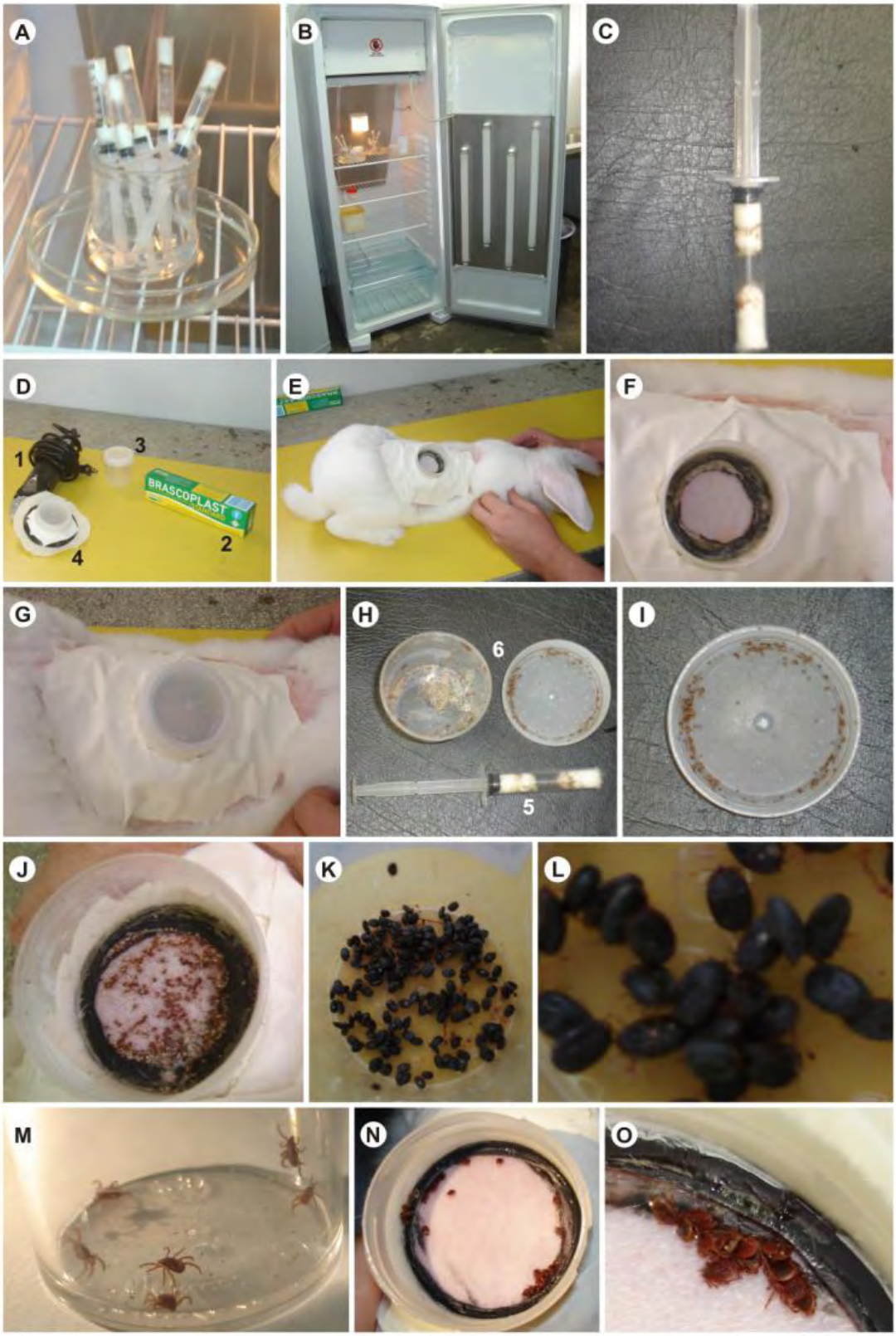

Figura 2: Imagens dos procedimentos para obtenção dos extratos glandulares (**EGS2-EGS6**) realizados no laboratório de Biologia Molecular do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP).

A-C. Carrapatos *Rhipicephalus sanguineus* no interior de câmara alimentadora fixada em coelho mantido em Biotério do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP). **A.** Infestação com carrapatos com 2 dias de alimentação. **B.** Infestação com carrapatos com 4 dias de alimentação. **C.** Com 6 dias de alimentação.
D. Maceração das glândulas salivares em tubos eppendorf contendo tampão fosfato de sódio.
E. Macerado glandular.
F. Centrifugação do macerado glandular para obtenção dos extratos glandulares.
G-H. Material e equipamento utilizado durante a dosagem protéica dos extratos glandulares. **G.** Cubetas onde o material teve a determinação da concentração protéica. **H.** Espectrofotometro para leitura e determinação do teor protéico.
I. Freezer para armazenamento das alíquotas de 50 µL de cada um dos extratos **EGS2-EGS6** (concentração final de 0,2 µg/µL).

FIGURA 2

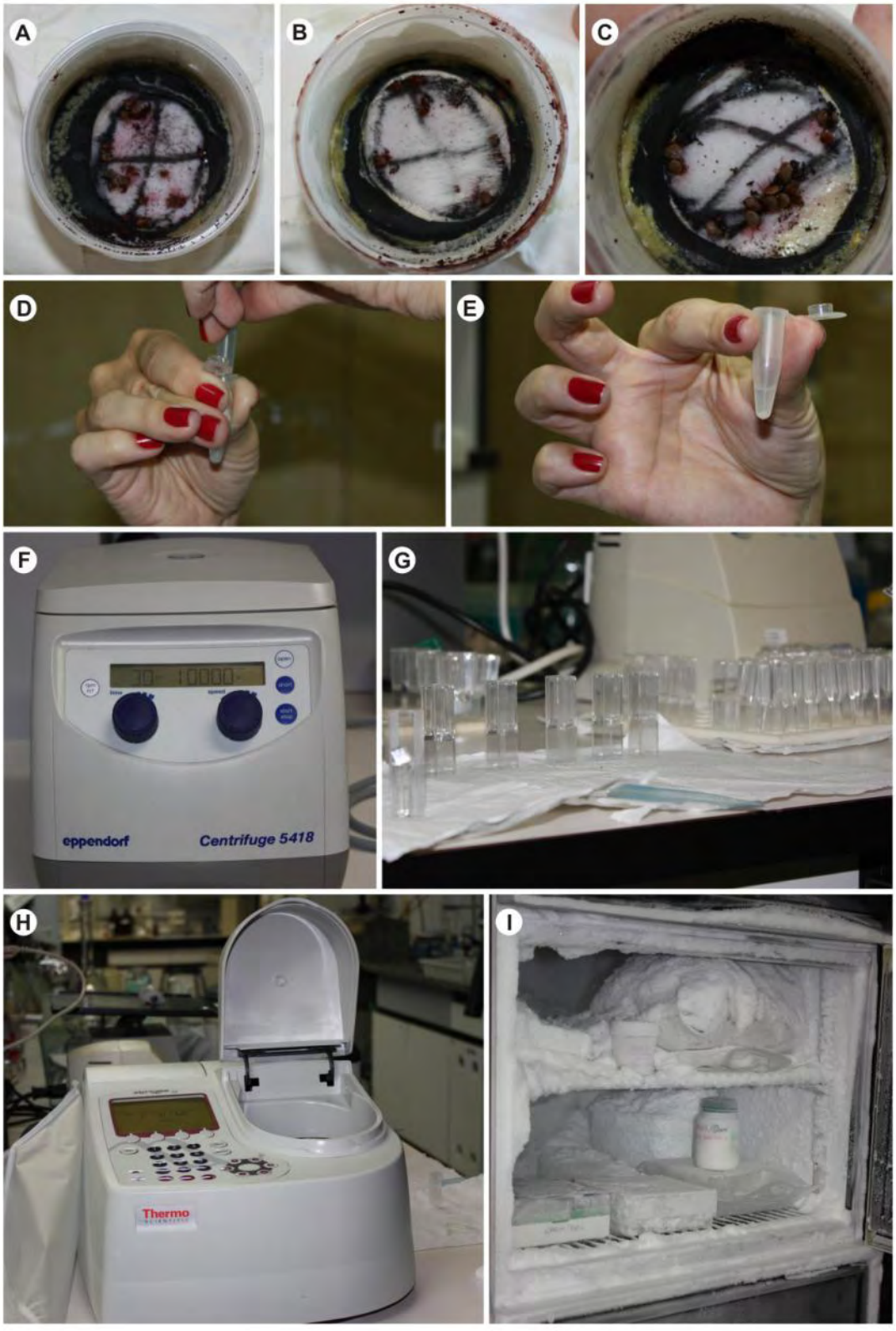

Figura 3: Imagens dos procedimentos realizados no interior de capela de fluxo laminar do laboratório de Biologia Molecular do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP) quando da preparação dos extratos glandulares **(EGS2-EGS6)**

A. Esterelização a frio (luz UV= **seta**) dos materiais utilizados para a filtragem dos extratos.

B. Fluxo laminar e seringas hipodérmicas (**1**), unidades filtrantes (**2**), tubos eppendorfs (**3**), pipeta (**4**) e ponteiras (**5**) para serem utilizados durante a filtragem dos extratos.

C. Coleta do extrato com o auxílio de seringa hipodérmica (**1**) a qual foi acoplada à unidade filtrante (**2**).

D. Seringa hipodérmica (**1**) e unidade filtrante (**2**).

E. Filtragem do extrato (50 µL) e deposição em eppendorf estéril (**3**) contendo 50µL de adjuvante de Freund.

F. Mistura (extrato e adjuvante) recolhida com auxílio de seringa hipodérmica estéril acoplada à agulha.

FIGURA 3

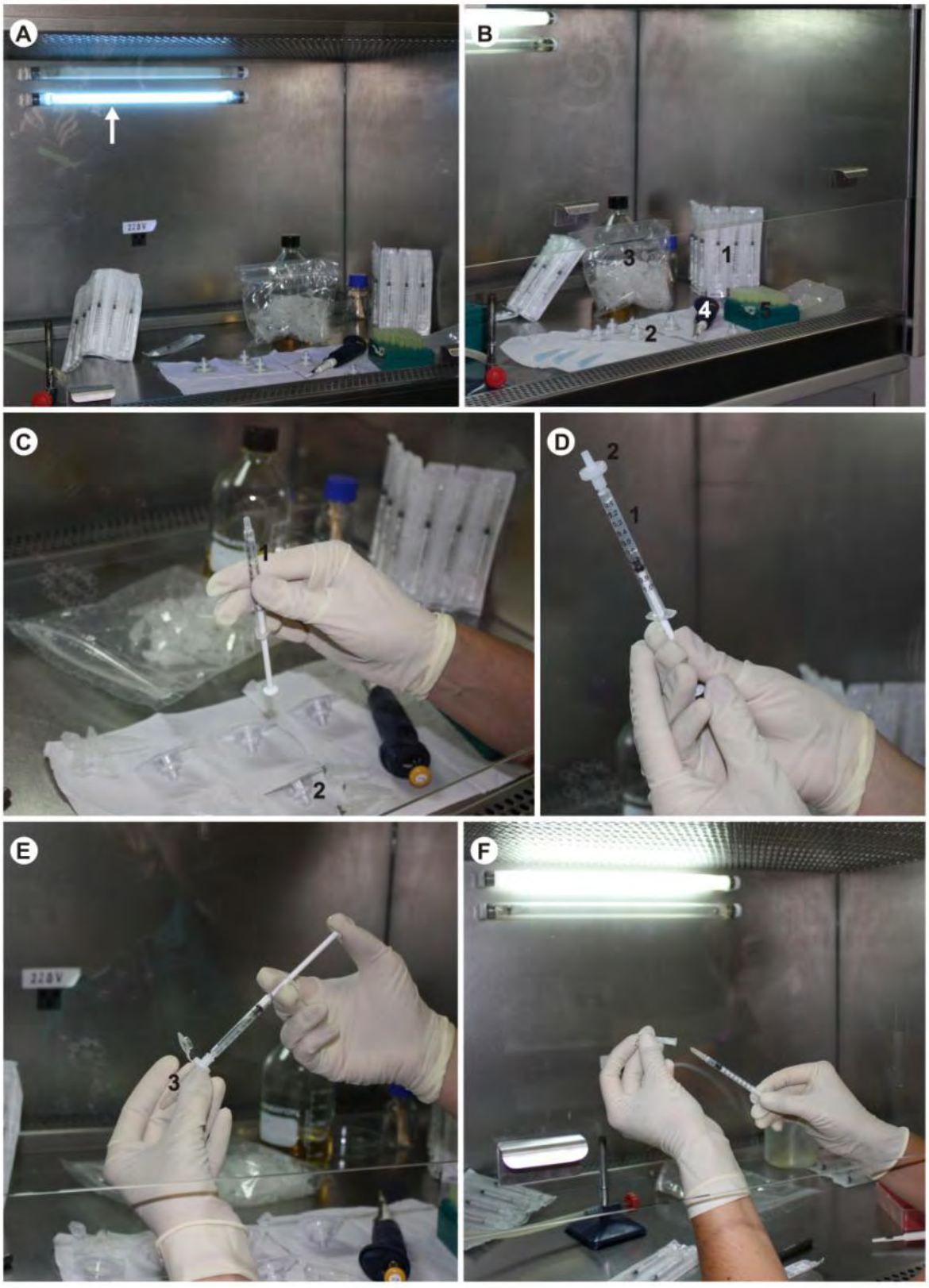

Figura 4: Imagens dos procedimentos de inoculação dos extratos glandulares nos coelhos utilizados para realização das infestações desafio.

A. Coelho hospedeiro utilizado na infestação desafio com a região dorso-lateral direita parcialmente tosada.

B. Inoculação do extrato glandular.

C e **D.** Desenvolvimento de granuloma (**círculo pontilhado**), devido ao processo inflamatório ali instalado após sucessivas inoculações dos extratos. **C.** Desenvolvimento de granuloma (**círculo pontilhado**) na pele de coelho após a realização da segunda inoculação. **D.** Desenvolvimento de granuloma (**círculo pontilhado**) após a realização da terceira inoculação

E-H. Realização das infestações desafio. **E.** Detalhe de duas câmaras alimentadoras, uma com e outra sem tampa, em um único coelho. **F-H.** Detalhe de camaras alimentadoras destampadas mostrando diferentes etapas do processo alimentar do *R. sanguineus*. **F.** Estágio inicial de alimentação. **G.** Estágio alimentar mais avançado que o da figura **F**. **H.** Estágio alimentar intermediário.

FIGURA 4

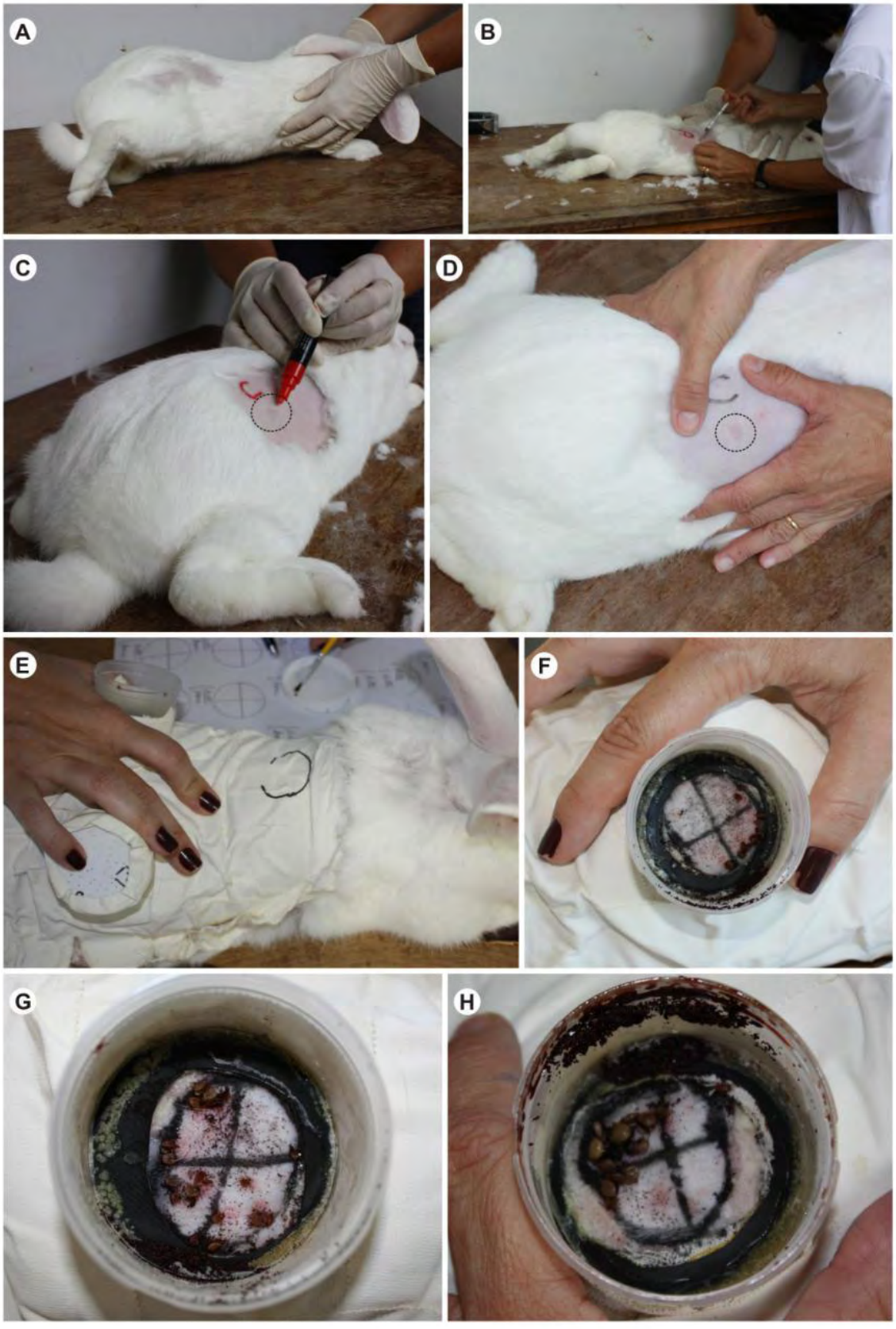

Figura 5: Imagens dos carrapatos *R. sanguineus* e dos coelhos utilizados nas infestações desafio.

A-C. Câmaras alimentadoras destampadas mostrando diferentes etapas da alimentação do *R. sanguineus*. **A.** Estágio alimentar intermediário. **B.** Estágio final da alimentação. **C.** Fêmeas de *R. sanguineus* completamente ingurgitadas.
D-F. Detalhe de câmaras alimentadoras fechadas em coelhos de diferentes grupos **GT (EGS2-EGS6)** utilizados para realização do teste de desafio. **D.** Coelho do grupo **GT (EGS2)**. **E.** Coelho do **GT (EGS4)**. **F.** Coelho representando aqueles do **GT (EGS6)**.

FIGURA 5

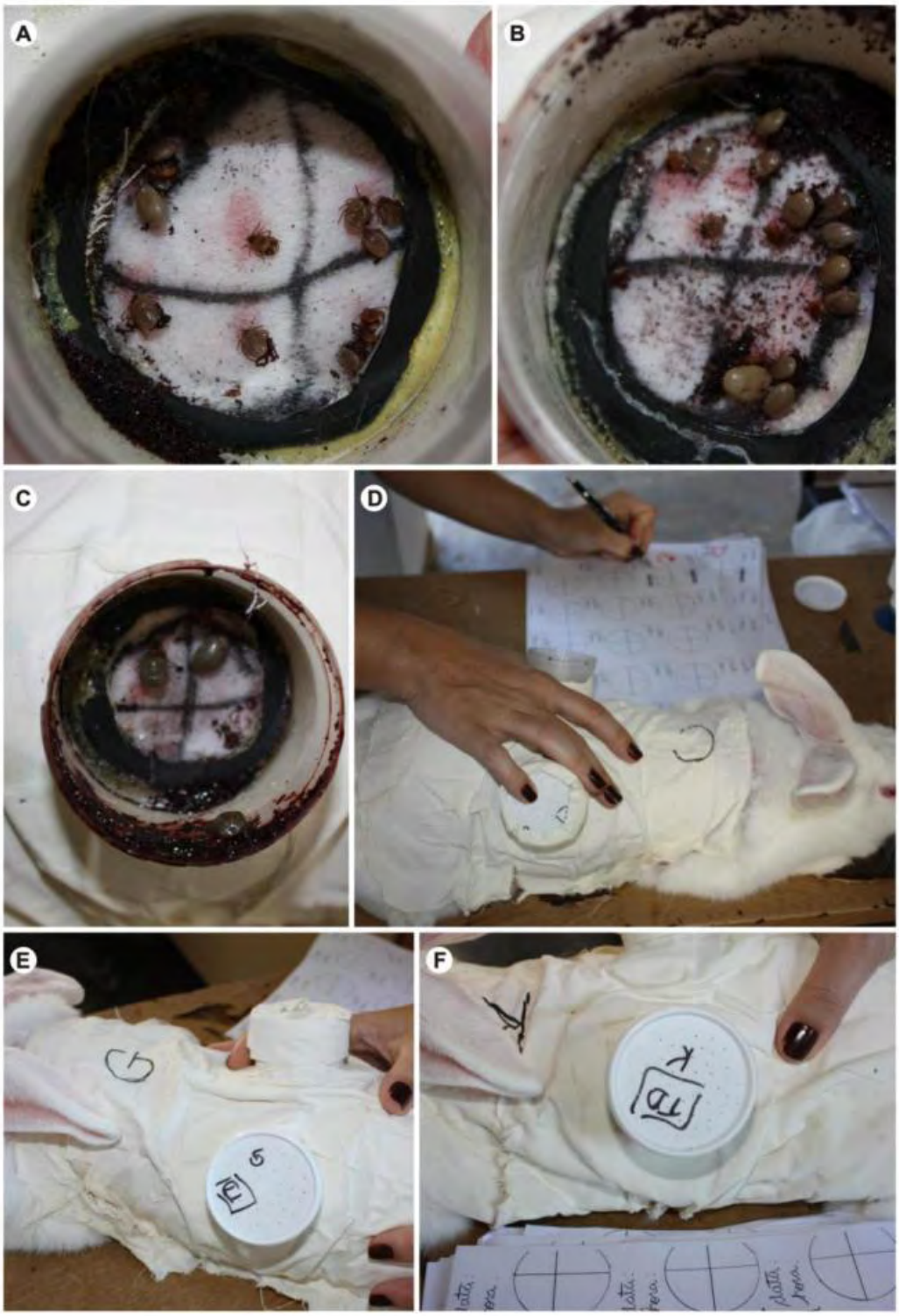

Figura 6: Imagens dos coelhos utilizados durante as infestações desafio realizadas no Biotério do Departamento de Biologia da UNESP de Rio Claro (SP).

A-B. Detalhe da câmara alimentadora fechada de coelhos dos grupos **GC1** e **GC2** utilizados para realização da infestação desafio (teste de desafio). **A.** Coelho do grupo **GC2**. **B.** Coelho do grupo **GC1.**

C. Todos os indivíduos (4 coelhos/grupo) do grupo **GT (EGS2-EGS6)** em suas respectivas gaiolas utilizadas para realização do teste de desafio.

FIGURA 6

Figura 7: Imagens dos coelhos dos grupos **GC1 e GC2** utilizados nas infestações desafio realizadas no Biotério do Departamento de Biologia da UNESP de Rio Claro (SP).

A. Coelhos dos grupos **CG2**.

B. Coelhos dos grupos **CG1**.

FIGURA 7

Resultados

4. Resultados

Os resultados obtidos no presente trabalho estão sendo apresentados na forma de capítulos, onde cada um contém um artigo submetido à publicação ou já publicado em periódico internacional especializado e com seletiva política editorial.

CAPÍTULO 1

Título: Protocol to obtain and inoculate salivary gland extracts of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari, Ixodidae) in rabbit hosts.

Autores: Karim Christina Scopinho Furquim, Maria Izabel Camargo Mathias, Letícia Maria Gráballos Ferraz Hebling, Danielli Thieza Giratto, Débora Laís Justo Jacomini, Márcia Regina Brochetto Braga and Gervásio Henrique Bechara

Periódico: Ticks and Tick-Borne Diseases
Situação: Submetido.

CAPÍTULO 2

Título: Inoculation of salivary gland extract obtained from female of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari, Ixodidae) with 2, 4 and 6 days of feeding in rabbit: I. Histopathology of the feeding lesion.

Autores: Letícia Maria Gráballos Ferraz Hebling, Karim Christina Scopinho Furquim, Gervásio Henrique Bechara and Maria Izabel Camargo Mathias

Periódico: Experimental and Applied Acarology
Situação: Submetido.

CAPÍTULO 3

Título: Inoculation of glandular extracts of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari, Ixodidae) with 2, 4 and 6 days of feeding in rabbits. II. Inflammatory cells in the feeding lesion.

Autores: Letícia Maria Gráballos Ferraz Hebling, Karim Christina Scopinho Furquim, Gervásio Henrique Bechara, Maria Izabel Camargo Mathias

Periódico: Tissue and Cell
Situação: Submetido.

CAPÍTULO 4

Título: *Rhipicephalus sanguineus* (Latreille, 1806) (Acari, Ixodidae) females fed on rabbits immunized with extracts of salivary glands in different periods of the secretory cycle. Analysis of the feeding and reproductive parameters.

Autores: Karim Christina Scopinho Furquim, Letícia Maria Gráballos Ferraz Hebling, Maria Izabel Camargo Mathias, Gislaine Cristina Roma, Leonardo Peres de Souza and Gervásio Henrique Bechara

Periódico: Experimental and Applied Acarology
Situação: Submetido.

CAPÍTULO 5

Título: Tick's response to feeding on host immunized with glandular extracts of *Rhipicephalus sanguineus* females fed for 2, 4 and 6 days. I. Inactivity or early degeneration of salivary glands?

Autores: Karim Christina Scopinho Furquim, Maria Izabel Camargo Mathias, Letícia Maria Gráballos Ferraz Hebling, Gislaine Cristina Roma and Gervásio Henrique Bechara

Periódico: Parasitology Research
Situação: Publicado (DOI: 10.1007/s00436-010-2238-7).

Capítulo I

PROTOCOL TO OBTAIN AND INOCULATE SALIVARY GLAND EXTRACTS OF *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) IN RABBIT HOSTS

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Resumo

Glândulas salivares de fêmeas de *Rhipicephalus sanguineus* com 2, 4 e 6 dias de alimentação foram usadas para o desenvolvimento deste protocolo. Extratos estéreis de glândulas salivares induziram resposta imune em hospedeiros com eles inoculados sem, contudo, induzir-lhes patologias. Foram utilizadas 55 fêmeas alimentadas por 2 dias (110 glândulas salivares), 36 fêmeas alimentadas por 4 dias (72 glândulas salivares) e 25 fêmeas alimentadas por 6 dias (50 glândulas) para a obtenção de **SGE2**= extrato de glândula salivar de fêmeas alimentadas por 2 dias, **SGE4**= extrato de glândula salivar de fêmeas alimentadas por 4 dias, e **SGE6**= extrato de glândula salivar de fêmeas alimentadas por 6 dias. Após a remoção, as glândulas separadas por situação foram imersas em 200 µL de solução tampão., maceradas e centrifugadas. O sobrenadante foi recuperado e transferido a tubos eppendorf estéreis, e a concentração protéica foi determinada. A concentração protéica encontrada foi de 1.08 µg/µL no **SGE2**, 1.75 µg/µL no **SGE4**, e 1.49 µg/µL no **SGE6**. Após a determinação da concentração protéica, numa câmara de fluxo laminar pré-estéril, cada extrato foi diluído separadamente com tampão fosfato (concentração final de 0.2 µg/µL) e filtrado por meio de unidades filtrantes estéreis com poros de 0.22µm e 13 mm de diâmetro acopladas a seringas hipodérmicas. Foram obtidas 12 alíquotas de 50 µL para a realização das inoculações dos extratos (**SGE2**, **SGE4**, and **SGE6**). Cada extrato foi inoculado três vezes em grupos de 4 coelhos New Zealand White a intervalos de 21 dias. Imediatamente antes da inoculação, cada alíquota de extrato (50 µL contendo 10 µg de proteína) foi adicionada de 50 µL de adjuvante completo de Freund. A inoculação subcutânea foi realizada utilizando-se seringas hipodérmicas na região dorso-lateral direita do hospedeiro. Decorridos 15 dias da última inoculação, os coelhos foram infestados com 15 casais de *R. sanguineus* em jejum. Esta metodologia descreveu um protocolo prático detalhado e procedimentos estandardizados para a realização de um experimento para a obtenção e inoculação de extratos glandulares que induzam uma resposta imune em hospedeiros sensíveis.

Palavras-chaves: *Rhipicephalus sanguineus*, extratos de glândulas salivares, inoculação, sensibilização, resistência.

Abstract

Salivary glands of two, four, and six-day-fed *Rhipicephalus sanguineus* females were used to develop the present protocol. Sterile salivary gland extracts induced an immune response in inoculated hosts without inducing pathological conditions. Fifty-five two-day-fed females (110 salivary glands), 36 four-day-fed females (72 salivary glands), and 25 six-day-fed females (50 glands) were used to obtain **SGE2**= salivary gland extracts of two-day-fed females, **SGE4**= salivary gland extracts of four-day-fed females, and **SGE6**= salivary gland extracts of six-day-fed females. After removal, glands of each group were immersed in 200 µL phosphate buffer, macerated, and centrifuged. The supernatant was recovered and transferred to sterile eppendorf tubes and protein concentrations were determined. The protein concentrations found were 1.08 µg/µL in **SGE2**, 1.75 µg/µL in **SGE4**, and 1.49 µg/µL in **SGE6**. After determining the protein concentration in a pre-sterile vertical laminar flow hood, each extract was separately diluted with phosphate buffer (final concentration of 0.2 µg/µL) and filtered in sterile filtering units with pore size of 0.22 µm and 13 mm in diameter attached to hypodermic syringes. Twelve aliquots of 50 µL were obtained to perform 12 inoculations/gland extract (**SGE2**, **SGE4**, and **SGE6**). Each extract was inoculated three times in four naive New Zealand White rabbits at 21-day intervals. Immediately prior to inoculations, each aliquot (50 µL, 10 µg of protein) of each extract was mixed to 50 µL of Freund's complete adjuvant. Subcutaneous inoculation was carried out with hypodermic syringes in the right dorsolateral region of each host. Fifteen days after the third inoculation, rabbits were infested with 15 couples of unfed *R. sanguineus*. This methodology described a practical protocol with detailed and standardized procedures on how to set up and perform an experiment to obtain and inoculate tick gland extracts to induce an immune response in sensitized hosts.

Keywords: *Rhipicephalus sanguineus*, salivary gland extracts, inoculation, sensitization, resistance.

Introduction

The salivary glands of ticks are organs that produce antigens that can stimulate defense mechanisms and evoke immune-inflammatory responses in their hosts (Sauer et al., 2000).

According Sonenshine (1991), the saliva is a complex mixture that plays many roles, mainly in the manipulation of the host hemostatic, inflammatory, and immunological processes.

Several studies have examined acquired resistance to ticks by hosts, as a response to successive infestations (Jittapalapong et al., 2000; Monteiro and Bechara, 2008; Monteiro et al., 2010; Caperucci et al., 2009, 2010; Veronez et al., 2010; Nunes et al., in press) or the inoculation of whole or parts (organs) of tick extracts (Wikel, 1981; Ferreira et al., 1996; Jittapalapong et al., 2000, 2008). Trager (1939) conducted the first study on acquired resistance to *Dermacentor variabilis* ticks in rabbits. Later, Shapiro et al. (1987) examined acquired resistance to *Rhipicephalus appendiculatus* in rabbits. Other studies on sensitized hosts with successive infestations (Jittapalapong et al., 2000; Monteiro and Bechara, 2008; Monteiro et al., 2010; Caperucci et al., 2009, 2010; Veronez et al., 2010; Nunes et al., in press) or the inoculation of molecules from salivary glands of three and five-day-fed ticks (Jittapalapong et al., 2000) demonstrated that the immunological response of hosts greatly affected the physiology of the ectoparasite, altering the feeding process and consequently the physiology of salivary glands and ovaries (Jittapalapong et al., 2000).

According to Turni et al. (2002), much can be learned from the acquired resistance by hosts to different tick species or even the same species, as the immunosuppressive molecules synthesized by salivary glands are differently expressed during tick feeding. In addition, salivary glands of different species contain different antigens and concentrations (Jaworski et al., 1990; Inokuma et al., 1994).

Studies on acquired resistance to ticks by hosts, especially those that induced by inoculation of antigens from ticks, are of special importance, as they are essential to more refined analysis that together with molecular and immunological techniques, can identify new molecules, especially from the digestive and reproductive systems of ticks and provide a framework for the development of vaccines.

Another important aspect is the failure to develop resistance by the domestic dog after successive infestations with *R. sanguineus* (Chabaud, 1950; Ferreira and Bechara, 1995;

Szabó et al., 1995; Bechara, 2006) or even after vaccination with extracts of unfed adult ticks followed by a challenge infestation (Bechara et al., 1994). According to Jittapalapong et al. (2000), since the domestic dog is the natural host of *R. sanguineus*, only immunization by inoculation of saliva or salivary gland extract could induce resistance.

Based on this information and the veterinary importance of *R. sanguineus* ticks, the present study describes a protocol to obtain and inoculate sterile salivary gland extracts capable of inducing resistance in inoculated hosts. The preparation of sterile extracts was used to avoid the induction of pathological conditions in host rabbits.

Materials and Methods

This experiment was approved by Comitê de Ética em Pesquisa e Mérito Científico – UNIARARAS, Protocol nº 021/2009.

Materials

Twenty-five unfed *R. sanguineus* couples per host were used (Fig. 1A). Ticks were obtained from a colony maintained in BOD incubator under controlled conditions (28ºC ± 1ºC, 80% relative humidity, and 12-h photoperiod). Couples were allowed to feed on naive New Zealand White rabbits, following the procedure described by Bechara et al. (1995). Two, four, and six-day-fed female ticks were collected (Fig. 1B) for the preparation of salivary gland extracts: **SGE2**= salivary gland extract of two-day-fed females, **SGE4**= salivary gland extract of four-day-fed females, and **SGE6**= salivary gland extract of six-day-fed females (Fig. 1C).

Fifty-five females (110 salivary glands) were used to obtain **SGE2**, 36 females (72 salivary glands) for **SGE4**, and 25 females (50 salivary glands) for **SGE6**, in order to obtain a protein level above 0.2 µg/µL (Table 1).

Table 1: Protein concentrations of salivary gland extracts of two, four, and six-day-fed *Rhipicephalus sanguineus* females.

	Feeding Time		
	2 days	**4 days**	**6 days**
Protein concentration	1.08µg/µL	1.75µg/µL	1.49µg/µL

Extracts **SGE2**, **SGE4**, and **SGE6** were inoculated in 12 naive New Zealand White rabbits (4 rabbits/extract) (Fig. 1D), followed by a challenge infestation (15 *R. sanguineus* couples/host) (Fig. 1E).

The feeding periods (two, four, and six days) were determined based on the secretion cycle of the salivary gland previously described by (Furquim et al., submitted paper). During this period, **b**, **c2**, **c4**, **c5**, **c6**, and **f** cells become active in two-day-fed ticks, **c5** and **f** become inactive in four-day-fed female ticks, and **c6** are inactive in six-day-fed female ticks.

Methods

Preparation of gland extracts

Two, four, and six-day-fed females were removed from the hosts by holding the region around the hypostome and making circular movements with the aid of tweezers. Ticks were placed in Petri dishes with colored paraffin, dissected under saline solution (7.5 g of NaCl, 2.38 g of Na_2HPO_4, 2.72 g of KH_2PO_4 and 1000 mL of distilled water), and salivary glands were removed. The removal of salivary glands differed depending on the feeding condition: in two-day-fed females, ticks were sectioned in half with the aid of tweezers (Fig. 2A, B), all organs were carefully removed (Fig. 2C) until salivary glands were exposed (Fig. 2D). In four and six-day-fed females, ticks were stocked with entomological pins with the dorsal region facing upward (Fig. 3). An incision was made in the central dorsal region with microscissors, from the posterior until the anterior portion of the body (Fig. 3A). To expose the organs, both dorsal halves were opened and secured with entomological pins (Fig. 3B). Intestine and ovaries were removed and the exposed salivary glands were collected (Fig. 3C).

The salivary glands, separated in groups according to the feeding times, were placed in eppendorf tubes containing 200 µL of phosphate buffer (pH 7.4), and macerated with a pestle. The material was centrifuged for 30 minutes at 10.000 xg. The supernatant was collected with a 100 µL pipette and placed in sterile eppendorf tubes. The protein concentration was determined according to Sedmark and Grossberg (1977) (Bradford method). The procedures were performed at the Laboratório de Biologia Molecular of the Departmento de Biologia of UNESP, Rio Claro, São Paulo State, Brazil.

After the protein content of each sample was determined (0.2 µg/µL) in a pre-sterile vertical laminar hood, the extracts were filtered with sterile filtering units (pore size of 0.22 µm, 13 mm in diameter, JBR610303, Millex GV disposable filtering unit, Durapore membrane PVDF, Millipore, MilliUni) attached to hypodermic syringes. Aliquots of 50µL of extracts were obtained and stored in sterile eppendorf tubes in a freezer at -20 ^{o}C. Immediately before inoculations, extracts (50 µL of extract/host) were mixed with 50 µL of Freund's complete adjuvant (reference # F 5881, Sigma-Aldrich).

Inoculation of gland extracts in hosts

Twelve naive New Zealand White rabbits (4 rabbits/ gland extract) were used. The right dorso-lateral region was shaved and each extract was inoculated three times subcutaneously with a hypodermic syringe (**SGE2**, **SGE4** or **SGE6**) at 21-day intervals (Fig. 1E). Fifteen days after the last inoculation, hosts were infested with 15 *R. sanguineus* couples/ host (Fig. 1F).

Figure 1

Sequence of procedures:

A. Females of *R. sanguineus* allowed to feed on hosts (rabbits);

B. Obtainment of two, four, and six-day fed females;

C. Removal of salivary glands of two, four, and six-day-fed females;

D. Preparation of gland extracts **SGE2-SGE6**;

E. Inoculation of extracts in hosts (4 rabbits/extract);

F. Infestation of inoculated rabbits with *R. sanguineus* ticks;

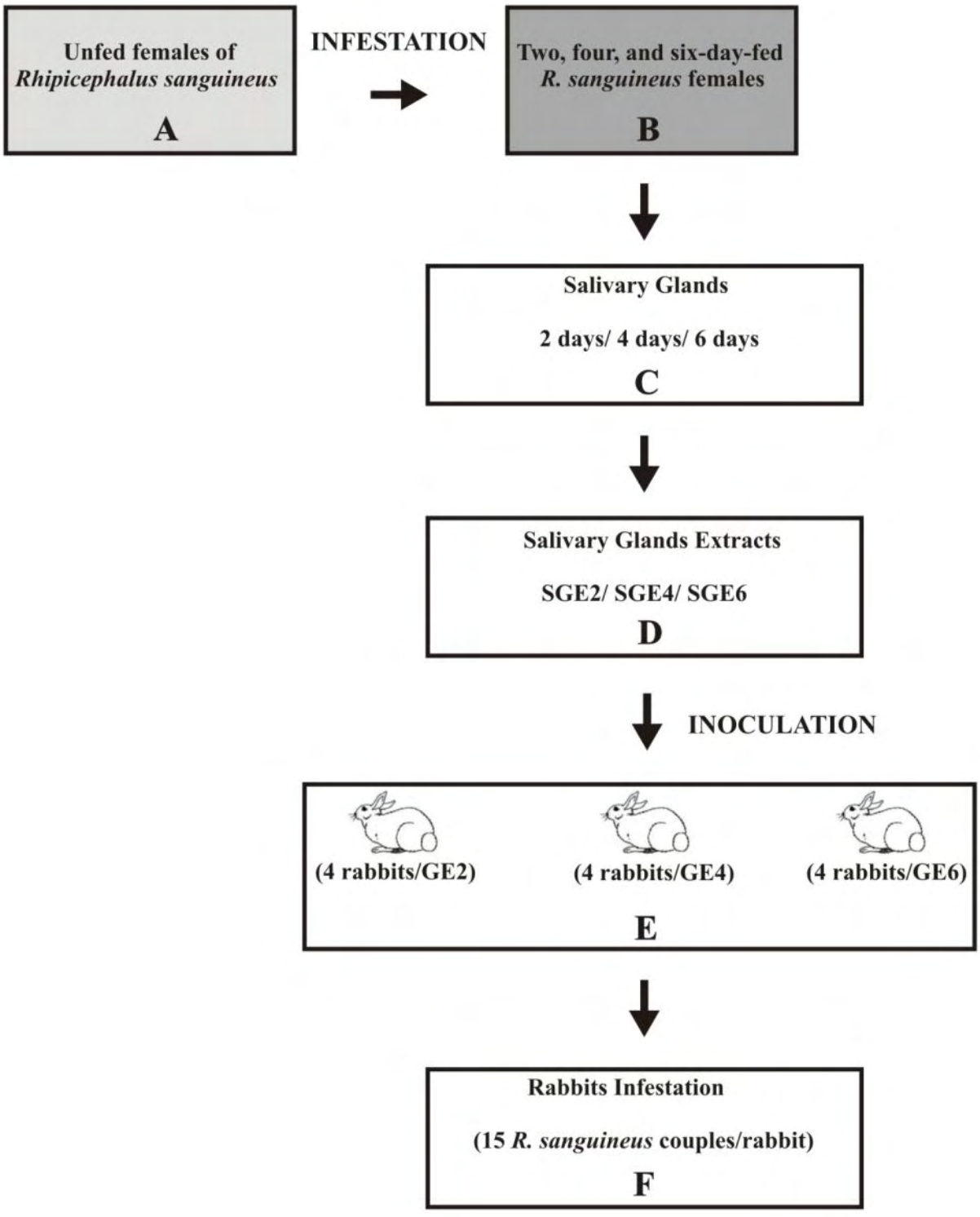

Unfed females of
Rhipicephalus sanguineus
A
INFESTATION
Two, four, and six-day-fed
R. sanguineus females
B
Salivary Glands
2 days/ 4 days/ 6 days
C
Salivary Glands Extracts
SGE2/ SGE4/ SGE6
D
INOCULATION
(4 rabbits/GE2)
(4 rabbits/GE4)
(4 rabbits/GE6)
E
Rabbits Infestation
(15 *R. sanguineus* couples/rabbit)
F

Figure 2

Schematic representation of the procedure to remove the salivary glands of two-day-fed *Rhpicephalus sanguineus* females.

or: organs; **sg**: salivary glands.

A and **B**. Half section of the ticks (dotted line);

C. Removing organs;

D. Exposing salivary glands;

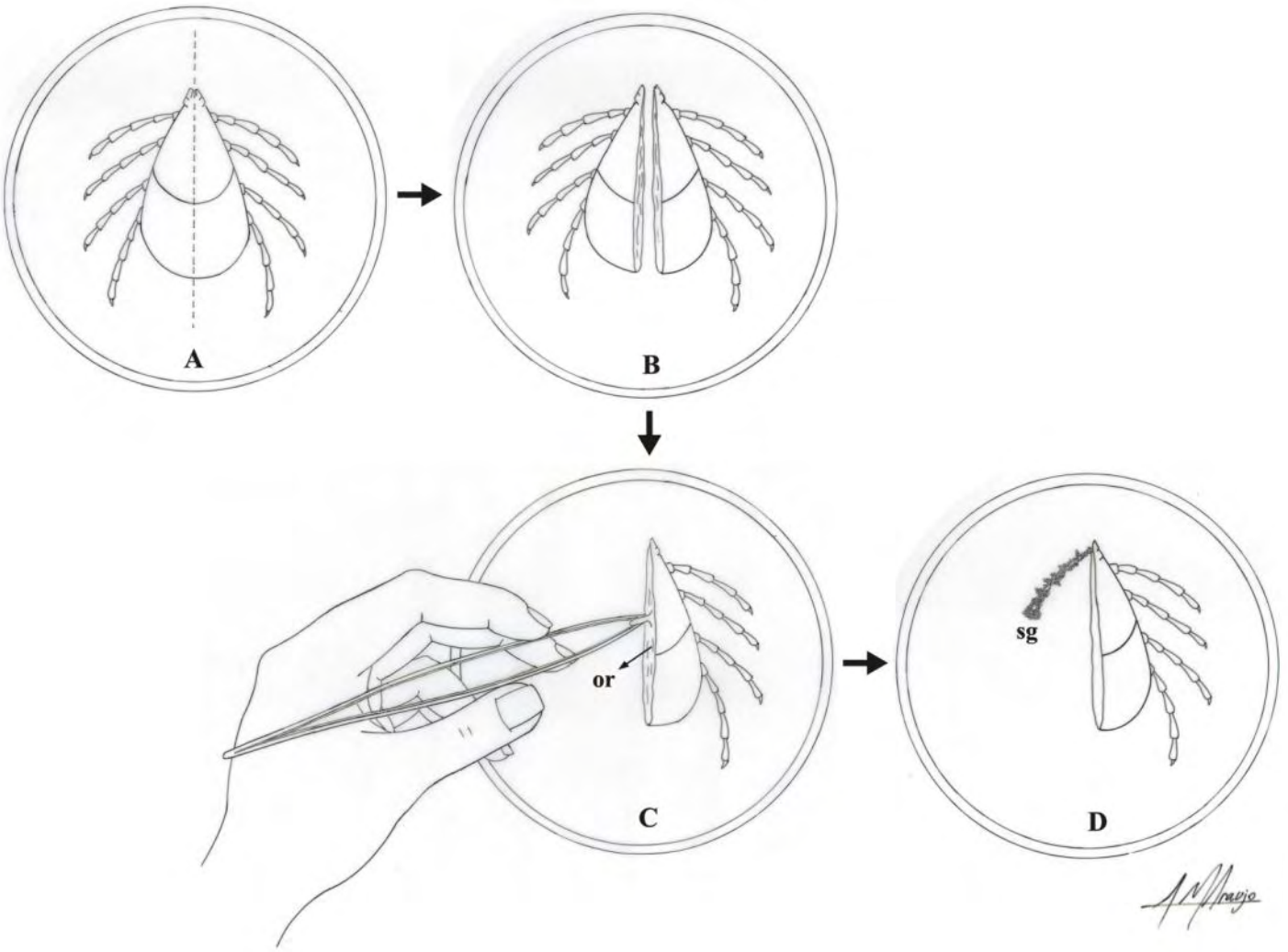

A
B
C
D
or
sg

Figure 3

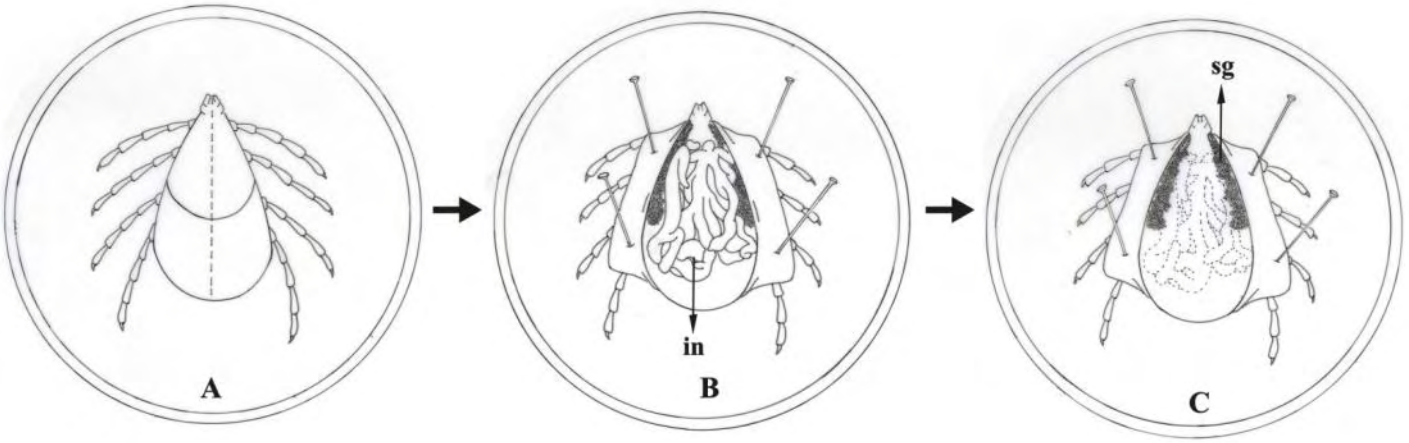
Schematic representation of the procedure remove the salivary glands of four and six-day-fed *Rhipicephalus sanguineus* females.

in: intestine; **sg**: salivary glands.

A. Incision in the central dorsal ticks region, from the posterior to the anterior portion of the body (dotted line);

B. Removal of the intestine (dotted illustration);

C. Exposed salivary glands;


A
in
B
sg
C

Discussion

The protocol developed in this study to obtain salivary gland extracts of two, four, and six-day-fed *R. sanguineus* females provide a methodology and important quantitative information on an efficient protein concentrate to induce an immune response in inoculated hosts, without inducing pathological conditions.

Many studies available in the literature have focused on the inoculation of extracts obtained from whole or parts of ticks (Wikel, 1981; Ferreira et al., 1996; Jittapalapong et al., 2000, 2008), including salivary glands. This study is the first to present information on how to obtain and inoculate salivary gland extracts of female ticks of *R. sanguineus* at specific feeding times (two, four, and six days).

The process of developing resistance in animal hosts has been studied. The failure to develop resistance to some tick species (Brumpt and Chabaud, 1947; Randolph, 1979) is still poorly understood. The development of a protocol to obtain and inoculate salivary gland extracts allows the construction of a study model to examine the process of acquired resistance by hosts as well as its implications.

An interesting example is the relationship between *R. sanguineus* and its natural host, the domestic dog, which does not develop resistance after repeated infestations (Chabaud, 1950; Ferreira and Bechara, 1995; Szabó et al., 1995; Bechara, 2006) or the inoculation of extracts of unfed adult ticks, followed by tick infestation (Bechara et al., 1994). When the domestic dog is inoculated with tick saliva or salivary gland extract, resistance is developed (Jittapalapong et al., 2000). In addition, in hosts inoculated with salivary gland extracts of three and five-day-fed ticks (Jittapalapong et al., 2000), the host immune response strongly affects the physiology of the ectoparasite, influencing the feeding process, the physiology of the salivary glands (Walker et al., 1985), and consequently reproduction (Jittapalapong et al., 2000).

This protocol describes procedures to obtain salivary gland extract of two, four, and six-day-fed female ticks. These feeding periods were chosen based on a histological and histochemical study conducted by Furquim et al. (submitted paper) on the salivary glands of *R. sanguineus* females. These authors reported that different gland cells of acini II and III become active or inactive especially during this feeding interval. Since the salivary secretion

consists of glycoproteins, these cells do not participate in the formation of the cement cone, as the latter is composed of lipoproteins (Binnington, 1987), indicating that these cells have different roles than other secretory cells, which remain active during the entire gland cycle. Consequently, this suggests a specific and localized effect on the host immune-inflammatory response. Turni et al. (2002) showed that much can be learned about the acquired resistance to a tick species by sensitized hosts, as immunosuppressive molecules synthesized by salivary glands are differently expressed during the feeding period.

In the present study, in addition to different expressed molecules, different concentrations were found in the corresponding glandular tissues during the feeding periods examined (two, four, and six days). Based on the protein concentration obtained, as the feeding process advanced in *R. sanguineus* females, the amount of secretion increased, and consequently the quantities of expressed proteins. The protein content found in the salivary glands of two, four, and six-day-fed females differed, therefore different quantities of glands were necessary for each specific extract. Unlike the obtained in this study, the procedure described by Jittapalapong et al. (2000) used 50 salivary glands in the preparation of gland extracts (15 glands from females and 10 glands from males) from three and five-day-fed individuals. These results support those obtained by Jaworski et al. (1990) and Inokuma et al. (1994), which reported that the salivary glands of different species contain different antigens, as well different quantities and concentrations, resulting in a different number of salivary glands needed in the protocol.

Another important aspect of this protocol was the use of New Zealand White rabbits instead of dogs (Jittapalapong et al., 2000), as *R. sanguineus* can induce resistance in non-habitual hosts, such as rabbits (Chabaud, 1950) and guinea pigs when exposed to successive infestations or to vaccination with extracts of unfed adult ticks (Bechara et al., 1994; Szabó et al., 1995; Bechara, 2006). Since the main goal of this protocol was to obtain gland extracts capable of inducing an immune response in hosts, were used a host-model that could develop resistance to better understand how the host responds to the pre-immunization with saliva extracts of *R. sanguineus* produced in different moments of the gland cycle and that can affect the host defense system.

Therefore, the protocol presented in this study provide detailed information on the setup and how to conduct a simple, safe, and efficient experiment to obtain different salivary gland extracts. This procedure with non-habitual hosts (rabbits) of *R. sanguineus* may be used

to answer important questions for future studies on the acquisition and expression of resistance processes.

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References

Bechara, G.H., 2006. Imunopatologia da interação carrapato-hospedeiro. In: Barros-Battesti, D.M., Arzua, M., Bechara, G.H. (Eds), Carrapatos de importância médico-veterinária da região neotropical: Um guia ilustrado para identificação de espécies. Vox/ICTTD-3/Butantan, São Paulo.

Bechara, G.H., Szabó, M.P.J., Ferreira, B.R., Garcia, M.V., 1995. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Rev. Bras. Parasitol. Vet. 4 (2), 61-66.

Bechara, G.H., Szabo, M.P.J., Mukai, L.S., Rosa, P.C.S., 1994. Immunization of dogs, hamsters and guinea pigs against the tick *Rhipicephalus sanguineus* using crude unfed adult tick extracts. Vet. Parasitol. 52, 79-90.

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

Brumpt, E., Chabaud, A.G., 1947. L'infestation par des ixodinés provoque-t-elle une immunité chez l'hôte. 1. Note préliminaire. Ann. Parasit. 22, 348-56.

Caperucci, D., Camargo-Mathias, M.I., Bechara, G.H., 2009. Histopathology and ultrastructure features of the midgut of adult females of the tick *Amblyomma cajennense* Fabricius, 1787 (Acari: Ixodidae) in various feeding stages and submitted to three infestations. Ultrastruct. Pathol. 33, 249–259.

Caperucci, D., Camargo-Mathias, M.I., Bechara, G.H., in press Ultrastructure features of the midgut of the female adult *Amblyomma cajennense* ticks Fabricius, 1787 (Acari: Ixodidae) in several feeding stages and subjected to three infestations. Micron (doi: 10.1016/j.micron.2010.05.015).

Chabaud, A.G., 1950. L'infestation par des ixodinés provoque-t-elle une immunité chez l'hôte (2me. Note). Ann. Parasit. 25, 474-479.

Ferreira, B.R., Bechara, G.H., 1995. Imunidade a carrapatos *Rhipicephalus sanguineus* (Acarina:Ixodidae) em cachorro-do-mato *Cerdocyon thous* (Linnaeus) e no cão doméstico. Braz. J. Vet. Res. Anim. Sci. 32 (4), 232-237.

Ferreira, B.R., Machado, R.Z., Bechara, G.H., 1996. Western blot analysis of tick antigens from a *Rhipicephalus sanguineus* unfed larval extract and identification of antigenic sites in tick sections using immunohistochemistry. A comparative study between resistant and susceptible host species. Vet. Parasitol. 62, 161-174.

Inokuma, H., Kemp, D.H., Willadsen, P., 1994. Prostaglandin E_2 production by the cattle tick (*Boophilus microplus*) into feeding sites and its effect on the response of bovine mononuclear cells to mitogen. Vet. Parasitol. 53, 293-299.

Jaworski, D.C., Muller, M.T., Simmen, F.A., Needham, G.R., 1990. *Amblyomma americanum*: identification of tick salivary gland antigens from unfed and early feeding females with comparasions to *Ixodes dammini* and *Dermacentor variabilis*. Exp. Parasitol. 70, 217-226.

Jittapalapong, S., Phichitrasilp, T., Chanphao, H., Rerkamnuychoke, W., Stich, R., 2008. Immunization with tick salivary gland extracts. Impact on salivary gland ultrastructure in *Rhipicephalus (Boophilus) microplus* collected from immunized naturally infested cattle. Ann. N. Y. Acad. Sci. 1149, 200-204.

Jittapalapong, S., Stich, R.W., Gordon, J.C., Wittum, T.E., Barriga, O.O., 2000. Performance of female *Rhipicephalus sanguineus* (Acari: Ixodidae) fed dogs exposed to multiple infestations or immunization with tick salivary gland or midgut tissues. J. Med. Entomol. 37 (4), 601-611.

Monteiro, G.E.R., Bechara, G.H., Franzin, A.M., de Miranda Santos, I.K.F., 2010. Antigen-presenting cells in draining lymph nodes of goats repeatedly infested by the cayenne tick *Amblyomma cajennense* nymphs. Exp. Appl. Acarol. (doi 10.1007/s10493-010-9380-x).

Monteiro, G.E.R., Bechara, G.H., 2008. Cutaneous basophilia in the resistance of goasts to *Amblyomma cajennense* nymphs after repeated infestations. Ann. N. Y. Acad. Sci. 1149, 221-225.

Nunes, P.H., Bechara, G.H., Camargo-Mathias, M.I., in press. Secretory process of salivary glands of female *Amblyomma cajennense* ticks fed on resistance rabbits. Exp. Appl. Acarol. (doi 10.1007/s10493-010-9388-2).

Randolph, S.E., 1979. Population regulation in ticks: the role of acquired resistance in natural and unnatural hosts. Parasitology. 79, 141-56.

Sauer, J.R., Essenberg, R.C., Bowman, A.S., 2000. Salivary glands in ixodid ticks: control and mechanism of secretion. J. Insect Physiol. 46, 1069-1078.

Sedmak, J.J., Grossberg, S.E., 1977. A rapid sensitive and versatile assay for protein using Coomassie Brilliant Blue G-250. Anal. Biochem. 79, 544-552.

Shapiro S.Z., Buscher. G., Dobbelaere. D., 1987. Acquired resistance to *Rhipicephalus appendiculatus* (Acarina: Ixodidae): identification of an antigen eliciting resistance in rabbits. J. Med. Entomol. 24, 47-54.
Sonenshine, D.E., 1991. Biology of ticks. Oxford University Press, New York.

Szabó, M.P.J., Mukai, L.S., Rosa, P.C.S., Bechara, G.H., 1995. Differences in the acquired resistance of dogs, hamsters and guinea pigs to infestations with adult ticks *Rhipicephalus sanguineus* (Acarina: Ixodidae). Braz. J. Vet. Res. Anim. Sci. 32 (1), 43-50.

Trager, W., 1939. Further observations on acquired immunity to the tick *Dermacentor variabilis*. J. Parasitol. 25 (2), 137-139.

Turni, C., Lee, R.P., Jackson, L.A., 2002. Effect of salivary gland extracts from the tick, *Boophilus microplus*, on leucocytes from Brahman and Hereford cattle. Parasite Immunol. 24, 355-361.

Veronez, V.A., de Castro, M.B., Bechara, G.H., Szabó, P.J., 2010. Histopatology of *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks fed on resistant hosts. Exp. Appl. Acarol. 50, 151-161.

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

Wikel, S.K., 1981. The induction of host resistance to tick infestation with a salivary gland antigen. Am. J. Trop. Med. Hyg. 30 (1), 284-288.

Capítulo II

INOCULATION OF SALIVARY GLAND EXTRACT OBTAINED FROM FEMALE OF *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI, IXODIDAE) WITH 2, 4 AND 6 DAYS OF FEEDING IN RABBIT: I. HISTOPATHOLOGY OF THE FEEDING LESION

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Resumo

Este estudo analisou histopatologicamente a pele de coelhos previamente imunizados com extratos glandulares **EGS2**, **EGS4** e **EGS6** preparados a partir de glândulas salivares de fêmeas de *Rhipicephalus sanguineus* com 2, 4 e 6 dias de alimentação, na região da lesão de alimentação de fêmeas de *R. sanguineus* aos 2, 4 e 6 dias após a fixação. Neste trabalho, coelhos New Zealand White virgens de infestação foram inoculados com extratos (grupo teste=**GT**) ou com uma mistura de tampão fosfato e adjuvante completo de Freund (grupo controle 2=**CG2**). Cada coelho inoculado (**GT** e **CG2**) e não inoculado (**CG1**) foi subsequentemente infestado com *R. sanguineus.* Biópsias de pele foram coletadas dos coelhos na região da lesão de fixação aos 2, 4 e 6 dias de alimentação. Os resultados revelaram que a imunização de coelhos com os extratos glandulares induziu a aquisição de resistência contra testa espécie. Verificou-se ainda que o extrato **EGS4** foi o mais eficaz no desenvolvimento da resposta imuno-inflamatória contra a infestação por esses ectoparasitas, caracterizando o processo pela presença de precoce e intenso infiltrado inflamatório. Por outro lado, o extrato **EGS6** provocou o aparecimento de resistência mais tardia, com menor ocorrência de infiltrado e intensas áreas de edema no local da lesão de alimentação. Já o processo inflamatório decorrente da inoculação do extrato **EGS2** foi o menos intenso. Os resultados obtidos permitiram concluir que a imunização com diferentes extratos obtidos a partir de glândulas salivares de fêmeas de *R. sanguineus* com 2, 4 e 6 dias de alimentação não alterou as características presentes no processo inflamatório, porém tornou-as precoce ou tardiamente mais intensas e dependentes do extrato inoculado.

Palavras-chaves: Imunização, carrapato, *Rhipicephalus sanguineus*, glândula salivar, histopatologia.

Abstract

This study analyzed the histopathology of rabbit skin, previously immunized with **EGS2, EGS4 and EGS6** gland extracts prepared from salivary glands of *Rhipicephalus sanguineus* female with 2, 4 and 6 days of feeding, at the region of the *R. sanguineus* female feeding lesion at 2, 4 and 6 days after tick attachment. In this work, infestation-naïve New Zealand White rabbits were inoculated either with the extracts (test group=**GT**) or with phosphate buffer and complete Freund's adjuvant mixture (control group 2= **GC2**). Each extract-inoculated (**GT** e **GC2**) and non-inoculated (**GC1**) rabbit was subsequently infested with *R. sanguineus*. Skin biopsies were collected from the rabbit at the tick feeding lesion at 2, 4 and 6 days of feeding. Results revealed that rabbit immunization with gland extracts induced acquisition of resistance against this species. It should be stated that the **EGS4** extract was the most effective in developing an immune-inflammatory response against ectoparasites, being this process characterized by the presence of an early and intense inflammatory cell infiltrate. On the other hand, **EGS6** extract caused a later appearance of resistance with less infiltrate occurrence and intense edema at the feeding lesion site. As to the inflammatory process deriving from **EGS2** extract inoculation, it was the less intense. It was concluded that immunization with different extracts from *R. sanguineus* female salivary glands did not change microscope features of the inflammatory process, although an earlier or more intense and later response, which was also dependent on the inoculate extract, was noticed.

Keywords: Immunization, tick, *Rhipicephalus sanguineus*, salivary glands, histopathology.

Introduction

Rhipicephalus sanguineus tick or "dog tick" has the dog as its preferable host (Walker, 1994), although it can also be found parasitizing other mammals, including men.

In addition to the spoliatory action on the host during hematophagy, these ticks also cause injury to the tissues. This fact makes ticks important to public health as they are involved in transmission of infectious agents (Barros-Batestti, 2006; Guglielmone et al., 2006).

Tick feeding occurs due to the combined action of mouth structures with saliva, a secretion produced by salivary glands (Balashov, 1972). In *Ixodidae*, salivary glands are organs essential for their biological success, because they produce a mixture of bioactive components that acts on the ectoparasite attachment to the host, as well as on its immune response management, thus ensuring the success of the feeding process (Sonenshine, 1991; Sauer et al. 1995; Bowman and Sauer, 2004).

Several studies have been conducted to understand the development mechanism of the host resistance to tick infections. Induction of resistance by effector mechanisms would be able to promote high-specificity protection against these infections (Wikel, 1981).

Immune response to the presence of the parasite can be expressed in the tick feeding lesion on the host. Brossard and Wikel (1997) reported presence of neutrophils and basophils in skin samples from resistant hosts. Likewise, Monteiro and Bechara (2008) showed presence of basophils in feeding lesions of *Amblyomma cajennense* tick in goats subjected to several infections. In addition, Veronez et al. (2009) showed the presence of inflammatory cells from resistant guinea pigs in bowel lumen of infecting *R. sanguineus* ticks.

Records provide studies that discuss the action of extracts obtained from full ticks (Szabó et al.; 1995) or even from their salivary glands only (Allen et al., 1979; Ramachandra e Wikel, 1981; Mejri et al., 2001; Turni et al., 2002; Zhou et al., 2006) on host immune response.

By studying the immunogenic action of proteins obtained from *R. haemaphysaloides* salivary glands, Zhou et al. (2006) verified that these proteins were capable of immunizing rabbits and that the development of this defense strategy compromised the attachment and feeding processes of ticks that were subsequently attached to the hosts.

While working with proteins obtained from *Hyalomma anatolicum anatolicum* salivary glands, Gill et al. (1986) verified that these proteins are expressed in a quantitative

and qualitative different way throughout the tick feeding process. In addition, Furquim et al. (submitted paper) showed that, once *R. sanguineus* tick feeding process starts, gland changes occur, such as activation of certain cells in specific periods of this process. This information supports the existence of a production mechanism of different molecules throughout the whole gland secretion cycle of this ectoparasite. In this sense, different molecules can cause different immune-inflammatory reactions in the host skin region close to the feeding lesion (Gill et al., 1986).

Considering the information presented above, this study intended to verify the immunogenic action of salivary gland extracts from *R. sanguineus* (**EGS2, EGS4, EGS6**) on the immune-inflammatory response in rabbits by histopathological analysis of skin from these rabbits in tick feeding lesion site.

Material e Methods

Material

In order to perform this study, skin samples from New Zealand White rabbits previously immunized with gland extracts (**EGS2, EGS4, EGS6**) were analyzed in the *R. sanguineus* tick feeding lesion site with 2, 4 and 6 days. For such, male and female *R. sanguineus* ticks derived from a colony maintained in a BOD incubator, under controlled conditions (28^{o}C ± 1ºC, 80% humidity and 12-hour photoperiod), located at Biotério do Departamento de Biologia da UNESP *campus* de Rio Claro (SP), were used in **A, B,** and **C** infections in rabbits, according to the procedure described by Bechara et al. (1995).

- **A infestation:** Infestation-naïve rabbits were subsequently infested with 25 *R. sanguineus* tick couples/host for acquisition of females with 2 (55 subjects), 4 (36 subjects) and 6 (25 subjects) days of feeding. Salivary glands were removed from these females in order to obtain the extracts: **EGS2=** female gland extract with 2 days, **EGS4=** with 4 and **EGS6**= with 6 days.

Determination of selected feeding periods (2, 4 and 6 days) was based on salivary gland secretion cycle, because fasting subjects have glands containing **a, c1, c3, d,** and **e** active cells, those with two days have **b, c2, c4, c5, c6,** and **f** active cells, those with 4 days have **c5** and **f** inactive cells, while only **a, b, c1-c4,** and **c6** cells remain active, and finally subjects with 6 days of feeding have **c6** inactive cells (Furquim, 2007).

After being processed, extracts were inoculated into the hosts subjected to **B** infestation.

- B infestation (test group)

12 rabbits were sensitized, being 4 inoculated with **EGS2** extract, 4 with **EGS4** and 4 rabbits with **EGS6**. They were subsequently subjected to challenge infestation with 15 *R. sanguineus* tick couples/host.

- C infestation (control group)

4 infestation-naïve rabbits were inoculated with complete Freund's adjuvant and phosphate buffer mixture (control group 2) and 4 were not inoculated (control group 1). All 8 animals were subsequently subjected to a challenge infestation with 15 *R. sanguineus* tick couples/host.

This project was submitted to the Ethics Committee in Animal Research UNIARARAS, Fundação Hermínio Ometto, Araras, São Paulo, Brazil and was approved under protocol # 008/2009.

Methods

Preparation and inoculation of gland extracts

Female *R. sanguineus* with 2, 4 and 6 days of feeding were collected and dissected in saline solution (7.5 g NaCl, 2.38 g Na_2HPO_4, 2.72 g KH_2PO_4 and 1000 mL distilled water) to remove their salivary glands.

At facilities from Laboratório de Biologia Molecular do Departamento de Biologia da UNESP *campus* de Rio Claro (SP), Brazil, glands were placed separately by study status (obtained from females with 2, 4 and 6 days of feeding) in eppendorf tubes containing 200-µL 7.4-pH phosphate buffer. Then, glands were macerated, centrifuged by 30 minutes at 10,000 × g, supernatant was collected and processed for protein content analysis according to methodology described by Sedmark and Grossberg (1977) (Bradford method) and concentration to be obtained could not be lower than 0.2 µg/µL.

Following protein content analysis of each sample, extracts were filtered separately by study status with support of 0.22 µm sterile filtering units (JBR610303, Millex GV disposable filtering unit, durapore, PVDF, Millipore, MilliUni membrane) with 13 mm diameter, coupled to hypodermic syringes, inside a pre-sterile vertical laminar flow hood. After this, extracts were aliquoted in volumes of 50 µL and stored inside a freezer at -20^{o} C. Only at the moment of the inoculations, each extract was mixed (50 µL of extract/host) with 50 µL complete Freund's adjuvant (reference # F 5881, Sigma-Aldrich), as well as with the mixture of Freund's adjuvant with 7.4 pH phosphate buffer (AF+PBS). These procedures were also performed in pre-sterile vertical laminar flow.

Rabbits from **GT** and **CG2** groups had their dorsolateral right region sheared, same site where subcutaneous inoculation occurred (**EGS2**, **EGS4** and **EGS6)**, as well as with AF+PBS, via hypodermic syringe, three times at 21-day intervals. Only after 15 days from last inoculation, all hosts from **GT** and **GC** groups were subjected to a challenge infestation with 15 *R. sanguineus* tick couples/host.

Histopathological Analysis

Skin samples were collected from rabbits in tick feeding sites at 2, 4 and 6 days, by a "punch" with 0.3 cm in diameter. Upon collection, the ectoparasite attached to the host was cut at the head region, remaining only the hypostome attached to the rabbit skin. With the "punch" placed at the site and with circular moves, a piece of skin was removed. Obtained pieces were fixed in paraformaldehyde 4% at 4^{o}C, were dehydrated in a series of increasing concentrations of ethanol (70%, 80%, 90% and 95%), embedded in resin (Leica), and sectioned at 3 μm thickness. Sections were mounted on glass slides and stained with hematoxylin-eosin. Slides were mounted with Canada balsam and examined and photographed under Motic BA 300 light microscope.

Results

1. Control Group 1 (GC1)

Analysis of skin pieces showed similar changes when compared to collection times of 2, 4 and 6 days. Presence of a moderate epithelium stratification (Fig: 1A, 1C, 1E), formation of edema (Fig. 1B, 1D, 1F), presence of few inflammatory cells in connective tissue and moderately dilated capillaries (Fig. 1A, 1D, 1F) were noticed.

Results of the analyses of skin pieces obtained from animals in control group 2 (**GC2**) were very similar to those obtained from **GC1**.

2. Test Group 1 (EGS2)

In subjects from this group, feeding lesion showed mild changes in relation to those from control group subjects. These changes included less epithelium stratification with some disorganized areas (Fig. 2A, 2C, 2E) and consequent filling with connective tissue (Fig. 2C, 2E) in addition to the presence of edema (Fig. 2F) and moderately dilated capillaries and with inflammatory cells inside them (Fig. 2B, 2D).

3. Test Group 2 (EGS4)

Histopathological test of skin pieces obtained from subjects under this situation showed that this extract was the one that caused the most significant changes when compared to those from other studied groups. Epithelium showed a marked increase in stratification with keratin layer loss and presence of cells with pyknotic nuclei reflecting cell changes in this tissue (Fig.3A, 3B). Development of epidermal vesicles under the parasite attachment site was noticed after the forth day of the feeding process (Fig. 3C). Underlying connective tissue presented large regions of edema, as well as inflammatory cells, which increased in number due to the feeding process (Fig. 3B, 3D, 3F, 3G). The presence of cells with acidophilic cytoplasmic granulation and hemorrhagic areas (Fig. 3D, 3E, 3G) was also noticed. Vasodilation could be noticed during the whole process.

4. Test Group 3 (EGS6)

Area with poorly preserved epithelium was noticed in the skin of hosts in this group (Fig.4A, 4C), as well as regions where same finding was not identified, especially in attachment site surroundings, due to the lesion (Fig. 4A). In these regions, invasion of inflammatory cells, vasodilation (Fig. 1A) and formation of edema (Fig. 1B, 1D) were markedly noticed.

At the region of tick feeding lesion, after 6 days of feeding, the presence of large areas of disorganized epithelium with formation of epidermal vesicles was noticed (Fig. 4E, 4F).

Figure 1.

Rabbit skin histological sections of the *Rhipicephalus sanguineus*-infested control group at different feeding periods. **A-B.** 2 days of feeding. **A.** Moderate epithelium stratification (**ep**). **B.** Presence of edema in dermis (**dr**). Dermal papilla (**dp**). **C-D.** 4 days of feeding. **C.** Moderate epithelium stratification (**ep**). Presence of cement (**ce**). **D.** Dilated blood vessel (**bv**) with leucocytes inside it (**arrow**). Regions of edema in dermis (**dr**). **E-F.** 6 days of feeding. Presence of edema regions in dermis (**dr**). Cement (**ce**). Epithelium (**ep**).

Bars: 10µm.

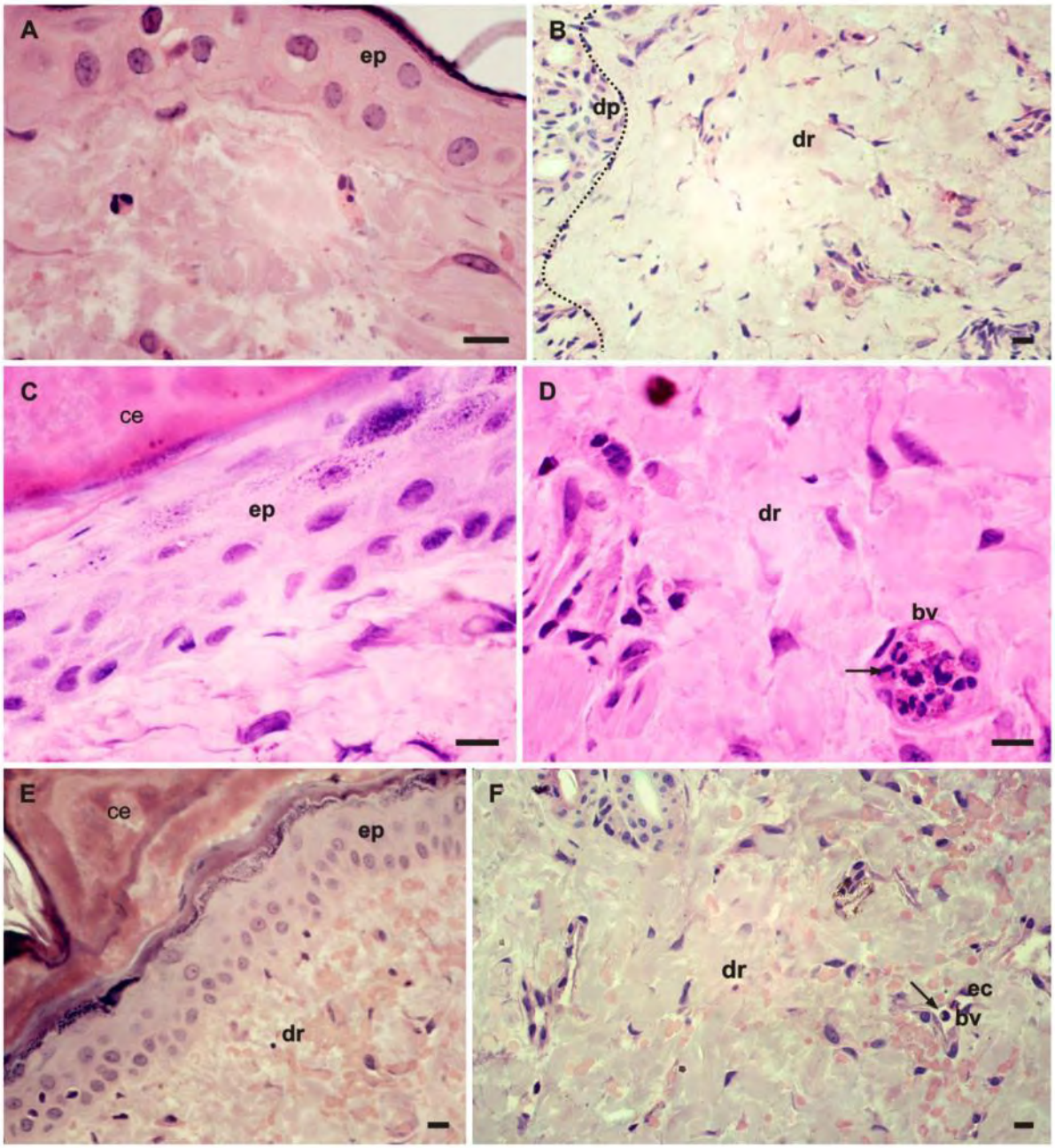
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Figure 2.

Rabbit skin histological sections of the *R. sanguineus*-infested **GT (EGS2)** group with 2, 4 and 6 days of feeding. **A-B.** 2 days of feeding. **A**. Moderate epithelium stratification (**ep**). **B**. Vasodilation (**bv**) with presence of leucocytes inside it (**arrow**). **C-D.** 4 days of feeding. **C.** Epithelium disorganization (**dotted**). **D**. Edema and vasodilation region (**bv**) with presence of leucocytes margination (**arrow**) in blood vessel lumen (**lu**). **E-F.** 6 days. **E.** Epithelium disorganization (**dotted**). **F**. Edema region (**er**).

Bars: 10µm.

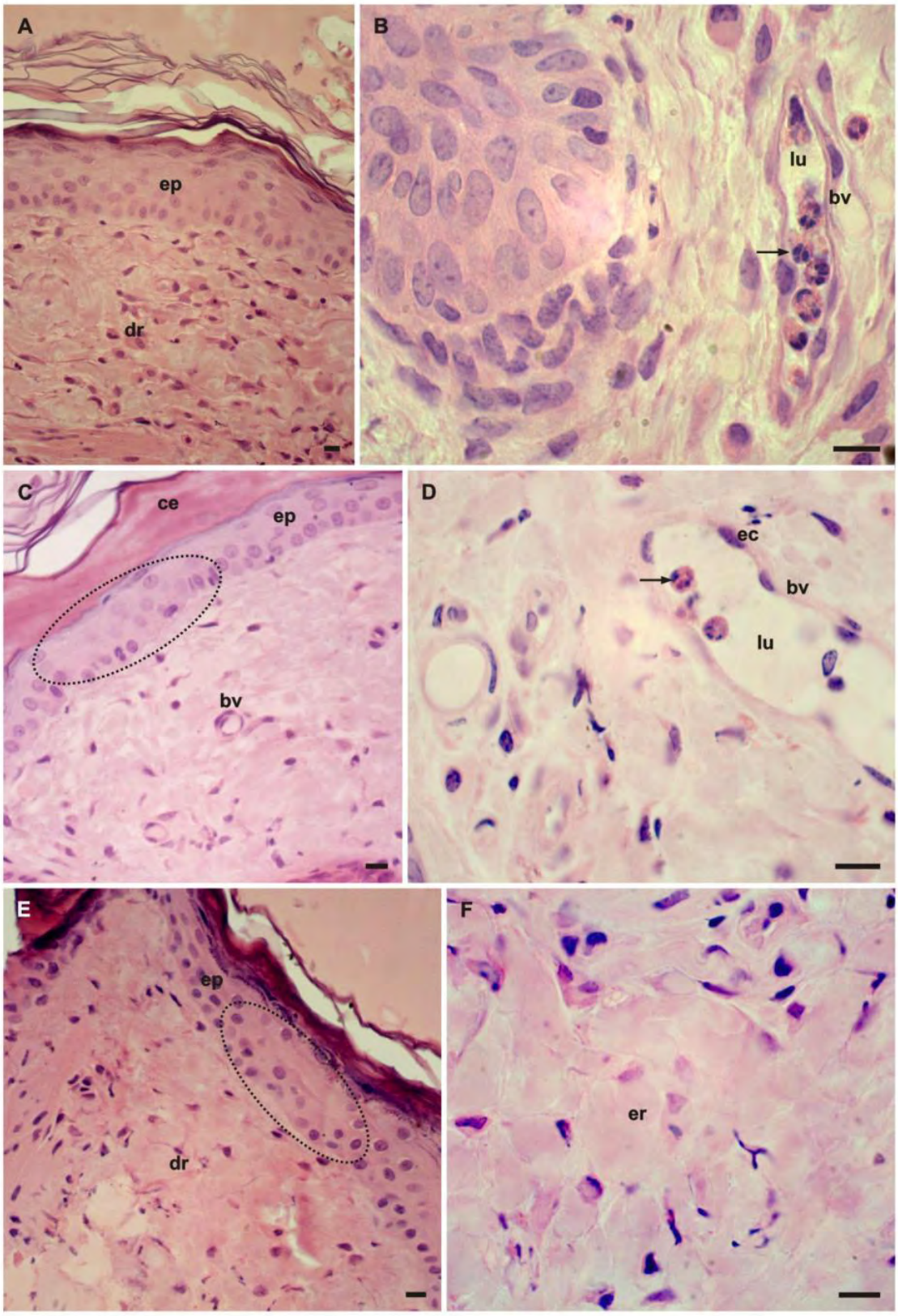
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Figure 3.

Rabbit skin histological sections of the *R. sanguineus*-infested **GT (EGS4)** group. **A-B.** 2 days of feeding. **A.** Small epithelium stratification (**ep**) with keratin layer loss (**dotted**) and presence of cells with pyknotic nuclei (arrow). **B.** Presence of edema region (er). **C-E.** 4 days of feeding. **C.** Epithelium stratification (**between arrows**). Epidermal vesicle (**ev**). **D.** Presence of inflammatory cells (**arrow**) and edema. **E.** Presence of hemorrhage area (**ha**). **F-G.** 6 days of feeding. **F.** Presence of edema and epidermal vesicle (**ev**). **G.** Hemorrhage area (**ha**).

Bars: 10µm.

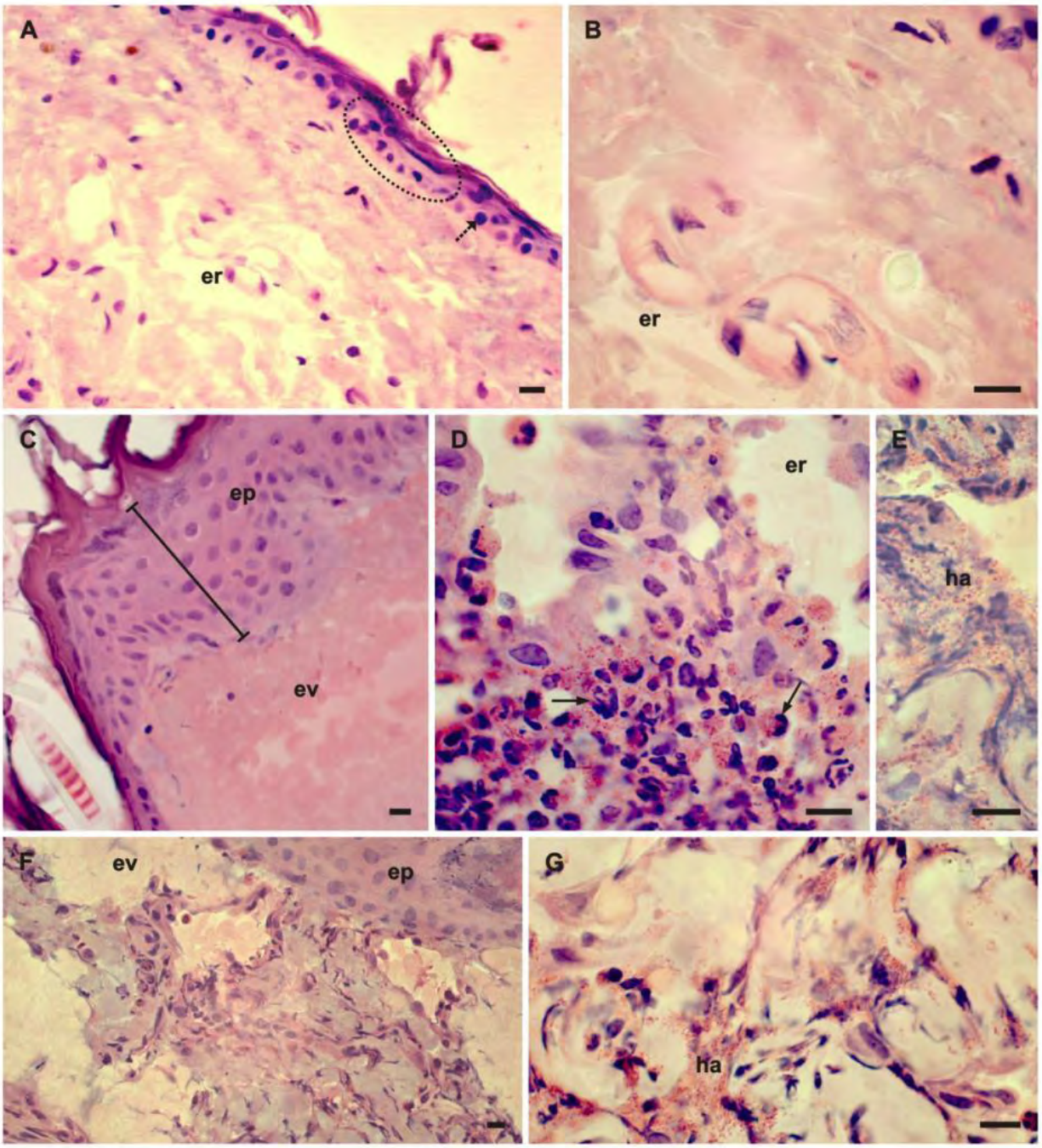
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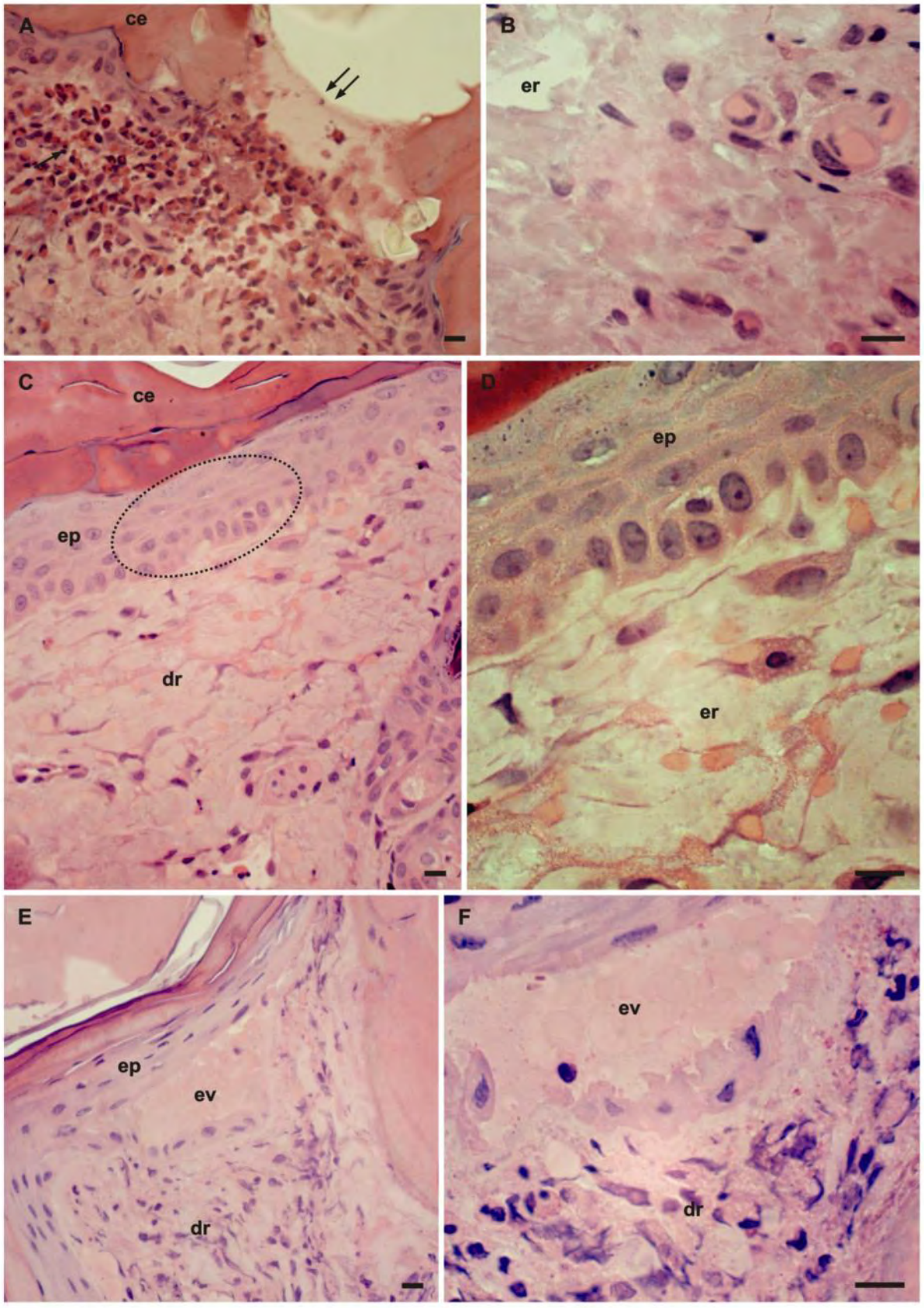
Figure 4.

Rabbit skin histological sections of the *R. sanguineus*-infested **GT (EGS6)** group. **A-B.** 2 days of feeding. **A.** Accumulation of inflammatory cells, epithelium disorganization in feeding lesion (**arrows**). Presence of cement (**ce**). **B.** Presence of edema area. **C-D.** 4 days of feeding. Dermis region (**dr**). **C.** Presence of poorly preserved epithelium (**dotted**). **D.** Presence of edema region. **E-F.** 6 days of feeding. Epidermal vesicle (**ev**).

Bars: 10µm.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Discussion

Histopathological analysis results of rabbit skin previously subjected to immunization with gland extracts (**EGS2-EGS6)**, collected in different *Rhipicephalus sanguineus* tick infestation time points (2, 4 and 6 days), were presented in this study.

Results showed that different salivary gland extracts affected and increased the local immune-inflammatory response. These data support the ones obtained by Monteiro and Bechara (2008) that reported inflammatory response increase at the feeding lesion in goats sensitized by successive infestations with *Amblyomma cajennense* tick.

In ticks, salivary glands are important antigen production sites (Wikel et al., 1978; Gill et al., 1986; Almeida et al., 1994; Ferreira et al., 1996; Szabó and Bechara, 1997; Jittapalapong et al., 2000a; Nunes et al., in press) and, for this reason, following first contact of these ectoparasites with some hosts, such hosts may or may not develop resistance (Wikel et al., 1978; Gill et al., 1986; Jittapalapong et al., 2000a; Zhou et al., 2006).

Regarding the efficacy of salivary gland extracts on the process of ability to mount an immune-inflammatory response against tick infestations, **EGS4** presented the most satisfactory result when compared with the two other extracts (**EGS2** and **EGS6**). This occurred due to the inflammatory response developed by the **GT** (**EGS4**) group hosts while presenting a significant inflammatory infiltrate. This result supported the one obtained by the challenge test and showed that ticks fed in **GT (EGS4)** group rabbits presented lower index of feed efficiency and decreased reproductive parameters in relation to those from other groups (Furquim et al., submitted paper). In addition, according to Monteiro and Bechara (2008), presence of intensive basophils in cells involved with the inflammatory process would indicate host development of resistance against tick infestations. Yet, presence of epidermal vesicles, present in hosts inoculated with this extract, as well as in the one described by Wikel and Allen (1977), would indicate involvement of complement system in animals that developed this resistance, thus supporting Allen et al. (1979) that detected the presence of complement system proteins inside these vesicles.

On the other hand, rabbits of **GT (EGS6)** group rabbits, developed less intense immune-inflammatory response, although large edema areas and formation of epidermal vesicles were noticed. In **GT (EGS2)** hosts, inflammatory response was generally less intense, which reflected challenge test data that revealed that ticks fed in **GT (EGS2)** group presented a lower index of feed efficiency in relation to **GT (EGS6)**. In those ticks fed in **GT**

(EGS6) group rabbits, opposite results were obtained, i.e., there was an increase on the index of feed efficiency, as well as on reproductive parameters, showing that, although inflammatory response on **GT (EGS6)** group hosts was more intense than in **GT (EGS2)** hosts, an intense edema with accumulation of liquid and cell debris in **GT (EGS6)** hosts positively changed feeding in infesting ticks, providing it with benefits (Furquim et al., submitted paper).

Local inflammatory response developed by **GT (EGS4)** group hosts not only presented more inflammatory infiltrate, but also was developed earlier than in **GT (EGS6)** group hosts. In **GT (EGS6)**, a more intense response was noticed in subjects that had attached ticks for up to 6 days of feeding. This information supports even more the fact that the **EGS4** extract presented higher efficacy on controlling this infestation.

Differences in local inflammatory responses between **GT (EGS2, 4 and 6)** groups were also noticed. This probably occurred due to the fact that salivary glands of female *R. sanguineus* with 4 days of feeding used in preparation of the extract are much more active than those with 2 and 6 days, showing that this extract **(EGS4)** contains the largest quantity of immunogenic molecules, which would actually present a significant role in the immune-inflammatory response of hosts.

In this study, it was also verified that, despite antigens (**EGS2, EGS4 or EGS6**) used for immunizing hosts, the intensity of inflammatory response increased in relation to the infestation period, i.e., it gradually increased according to the course of the feeding process, showing that the host immune-inflammatory system was gradually activated by antigen molecules released by ticks, synthesis and secretion of which were increased and changed throughout the process. According to Turni et al. (2002), several information about resistance acquired by hosts sensitized to different tick species or even with the same species could be obtained because immune-suppressive molecules synthesized by salivary glands would be differently expressed during the process of tick feeding.

In this study, it was clear that the presence of significant changes on rabbit skin, such as: epithelium stratification, local edema, increased inflammatory infiltrate and vascular dilation and congestion, would characterize the local inflammatory process. However, these inflammatory changes did not vary according to immunization with different extracts; it was the process intensity that varied according to the **GT (EGS2), (EGS4)** or **(EGS6)** studied group. Thus, it was clear that the developed inflammation intended to inactivate damaging agents, i.e., antigens derived from the tick saliva, as well as to isolate them, in an attempt to

eliminate the origin of the aggression. In this sense, immunization with **EGS4** extract was the most successful one, as it was capable of neutralizing *R. sanguineus* tick saliva antigen most efficiently, thus modulating immune-inflammatory response in hosts.

Considering all histopathological characteristics noticed on the host skin in each of the **GC** and **GT (EGS2-EGS6)** groups, it was verified that the immunization with **EGS4** extract was the most effective in immune-inflammatory response development, showing that molecules present in this extract could have higher immunogenicity, causing inactivation of *R. sanguineus* saliva immunomodulation action. However, the effects observed in hosts immunized with the extracts and **EGS2** and **EGS6** could have been less intense because of the evasion mechanisms of immune-inflammatory response of the tick still working in the feeding lesion.

References

Allen, JR, Khalil, HM, Graham, J (1979) The location of tick salivary antigens, complement and immunoglobulin in the skin of guinea-pigs infested with *Dermacentor andersoni* larvae. Immunology 38: 467-472.

Almeida, APG, Bechara, GH, Varma, RMG (1994) Cross-reactivity between hard ticks antigens. Braz J Med Biol. Res 27: 617-707.

Balashov, YS (1972) Bloodsucking ticks (Ixodoidea) -Vectors of diseases of man and animals. Misc. Publ. Entomol. Soc Am 8: 160-376.

Bechara, GH, Szabó, MPJ, Ferreira, BR, Garcia, MV (1995) *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Braz J Med Biol Res 4: 61-66.

Barros-Battesti, DM (2006) Introdução. In: Barros-Battesti, DM, Arzua, M, Bechara, GH (Eds.) Carrapatos de importância médico-veterinária da região neotropical. Um guia ilustrado para a identificação de espécies. Butantan, São Paulo, pp. 1-3.

Bowman, AS, Sauer, JR (2004) Tick salivary glands: function, physiology and future. Parasitology 129: s67-s81.

Brossard, M, Wikel, SK (1997) Immunology of interactions between ticks and hosts. Med Vet Entomol 11: 270-276.

Ferreira, BR, Machado, RZ, Bechara, GH (1996) Westernblot analysis of tick antigen from *Rhipicephalus sanguineus* unfed larval extract and identification of antigenic sites in tick section using immunohistochemistry. A comparative study between resistant and susceptible host species.Vet Parasitol 62: 161-174.

Jittapalapong, S, Stich, RW, Gordon, JC, Wittum, TE, Barriga, OO (2000) Performance of female *Rhipicephalus sangineus* (Acari: Ixodidae) fed on dogs exposed to multiple infestation or immunization with tock salivary gland or midgut tissues. J Med Entomol 37: 601-611.

Gill, HS, Boid, R, Ross, CA (1986) Isolation and characterization of salivary antigens from *Hyalomma anatolicum anatolicum.* Parasite Immunol 8: 11-25.

Guglielmone, A, Szabó, MJP, Martins, JRS, Estrada-Pena, A (2006) Diversidade e importância de carrapatos na sanidade animal. In: Barros-Battesti, DM, Arzua, M, Bechara, GH (Eds.) Carrapatos de importância médico-veterinária da região neotropical. Um guia ilustrado para a identificação de espécies. Butantan, São Paulo, pp. 115-138.

Mejri, N, Rutti, B, Brossard, M (2002) Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. Parasitol Res 88: 192-197.

Monteiro, GER, Bechara, GH (2008) Cutaneus basophilia in the resistance of goats to *Amblyomma cajennense* nymphs after repeated infestations. Animal Biodiversity and Emerging Diseases: Ann N.Y. Acad Sci 1149: 221–225.

Nunes, PH, Bechara, GH, Camargo Mathias, MI, in press. Secretory process of salivary glands of female *Amblyomma cajennense* Fabricius, 1787, (Acari: Ixodidae) ticks fed on resistant rabbits. Exp Appl Acarol. doi: 10.1007/s10493-010-9388-2.

Ramachandra, RN; Wikel, SK (1992) Modulation of host-immune response by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production. J Med Entomol 29: 818-826.

Sauer, JR, McSwain, AS, Bowman, AS, Essenberg, RC (1995) Tick salivary gland physiology. Ann Rev Entomol 40: 245-267.

Sedmark, JJ, Grossberg, SE (1977) A rapid sensitive and versatile assay for protein using Coomassie Brilliant Blue G-250. Anal Biochem 79: 544-552.

Sonnenshine, DE (Ed.) (1991) Biology of ticks. Oxford University Press, New York, v. 1, 447 pp.

Szabó, MPJ, Arantes, GJ, Bechara, GH (1995) Immunological characterization of adult tick *Rhipicephalus sanguineus* (Latreille, 1806) antigens by Western blot analysis using sera from infested or vaccinated dogs and guinea pigs. Braz J Vet Parasitol 4: 79-83.

Turni, C, Lee, RP, Jackson, LA (2002) Effect of salivary gland extracts from the tick, *Boophilus microplus,* on leucocytes from Brahman and Hereford cattle. Parasite Immunology 24: 355-361.

Veronez, VA, Castro, MB, Bechara, GH, Szabó, MPJ (2009) Histopathology of *Rhipicephalus sanguineus* (Acari: Ixadidae) ticks fed on resistant hosts. Exp Appl Acarol. doi: 10.1007/s10493-009-9286-7.

Walker, A (1994) Arthropods of domestic animals. A guide to preliminary identification. Chapman and Hall, London, 213 pp.

Wikel, SK, Allen, JR (1977) Acquired resistance to ticks. III. Cobra venom factor and the resistance response. Immunology 32: 457-465.

Wikel, SK, Graham, JE, Allen, JR (1978) Acquired resistance to ticks. IV- Skin reactivity and in vitro lymphocyte responsiveness to salivary gland antigen. Immunology 34: 257-263.

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

Wikel, SK (1996) Host immunity to ticks. Ann Rev Entomol 41: 1-22.

Zaia, DAM, Zaia, CTBV, Lichtig, J (1998) Determinação de proteínas totais via espectrofometria: vantagens e desvantagens dos métodos existentes. Quim Nova 21: 787-793.

Zhou, J, Gong. H, Zhou, Y, Xuan, X, Fujisaki, K (2006) Identification of a glycina-rich protein from the tick *Rhipicephalus haemophysaloides* and evaluation of its vaccine potential against tick feeding. Parasitol Res 100: 77-84.

Capítulo III

INOCULATION OF GLANDULAR EXTRACTS OF *Rhipicephalus sanguineus* FEMALES (LATREILLE, 1806) (ACARI, IXODIDAE) WITH 2, 4 AND 6 DAYS OF FEEDING IN RABBITS. II. INFLAMMATORY CELLS IN THE FEEDING LESION

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Resumo

O presente trabalho analisou a presença de células envolvidas na reação imuno-inflamatória na pele de coelhos na região da lesão de fixação de fêmeas de *Rhipicephalus sanguineus* aos 2, 4 e 6 dias, previamente imunizados com extratos glandulares **EGS2, EGS4 e EGS6** obtidos a partir do processamento de glândulas salivares de fêmeas de *R. sanguineus* com 2, 4 e 6 dias de alimentação. Neste trabalho, coelhos New Zealand White virgens de infestação, foram inoculados ou com os extratos (grupo teste= **GT**), ou com mistura de tampão fosfato e adjuvante completo de Freund (grupo controle 2= **GC2**). Cada coelho inoculado com extrato (**GT** e **GC2**) e não inoculado (**GC1**) foi posteriormente infestado com 15 casais de *R. sanguineus* em jejum. Da região da lesão de alimentação dos carrapatos na pele dos coelhos, foram coletados fragmentos nos períodos de alimentação de 2, 4 e 6 dias. Os resultados revelaram a presença precoce de neutrófilos, eosinófilos e basófilos na região da lesão de fixação. Verificou-se ainda que o extrato **EGS4** foi o mais eficaz no desenvolvimento de uma reação imuno-inflamatória no local da lesão de alimentação caracterizada pela presença destas células. Por outro lado, o extrato **EGS6** também estimulou o desenvolvimento de aporte dessas células inflamatórias porem em quantidade intermediária. Já o processo inflamatório decorrente da inoculação do extrato **EGS2** foi o menos intenso. Os resultados obtidos permitiram concluir que os extratos obtidos a partir de glândulas salivares de fêmeas de *Rhipicephalus sanguineus* foram eficientes em permitir que o hospedeiro pudesse desenvolver uma resposta imuno-inflamatória caracterizada pela presença de neutrófilos, eosinófilos e basófilos na região da lesão de alimentação.

Palavras-chaves: Imunização, resistência, carrapato, *Rhipicephalus sanguineus*, glândulas salivares, inflamação, leucócitos.

Abstract

This study examined the presence of cells involved in immuno-inflammatory reaction of rabbit skin in the feeding lesion area caused by *Rhipicephalus sanguineus* females at 2, 4 and 6 days, previously immunized with glandular extracts **EGS2**, **EGS4** and **EGS6** obtained from *R. sanguineus* females salivary glands with 2, 4 and 6 days of feeding. New Zealand White naïve rabbits were inoculated with extracts (test group = **TG**) or with a mixture of phosphate buffer and complete Freund's adjuvant (control group 2 = **CG2**). Each rabbit inoculated and non-inoculated was subsequently infested with couples of *R. sanguineus*. From the tick feeding lesion area on rabbit skin, samples were collected during ticks feeding periods of 2, 4 and 6 days. The results revealed the early presence of neutrophils, eosinophils and basophils in these areas. It was also found that the extract **EGS4** was the most effective in the development of an immuno-inflammatory reaction in the feeding lesion characterized by the presence of these cells. On the other hand, the extract **EGS6** also stimulated the input of these inflammatory cells however in an intermediate amount. The inflammatory process arising from the inoculation of extract **EGS2** was less intense. The results showed that extracts obtained from the salivary glands of *R. sanguineus* female were effective in allowing the development of immuno-inflammatory response by the host characterized by the presence of neutrophils, eosinophils and basophils in the feeding lesion area.

Keywords: Immunization, resistance, tick, *Rhipicephalus sanguineus*, salivary glands, inflammation, leukocytes.

Introduction

Ticks represent one of the most important groups of ectoparasites affecting animal health. Some species colonize large regions may be associated with specific hosts, as is the case of *Rhipicephalus sanguineus*, known as "brown dog tick" because the dog is its preferential host. They are vectors of pathogens and this role is facilitated by its life cycle and for their expertise and ability to adapt to urban hosts, it becomes a serious problem for public health (GUGLIELMONE, 2006). The characteristics of the ticks' life cycle should increase the chances of developing an inflammatory response by the host (CUPP, 1991). However, the parasite develops strategies for evading this response, modulating, through the saliva secretion, regional and local immunity of the host (FERREIRA and SILVA, 1998; CAVASSANI et al., 2005).

The ticks' saliva contains multiple components with immunosuppressive property. In addition, it also features an anti-hemostatic and vasoactive action enabling the consumption of blood by the tick (CUPP, 1991; BROSSARD; WIKEL, 1997; GILLESPIE et al., 2000; BECHARA, 2006).

Accordingly, the development of immunological reaction by the host against this ectoparasite has been advocated as part of an effective strategy for its control (GOSH et al., 2007), since the indiscriminate use of acaricides and insecticides has led to a disruption in effectiveness (FERNANDES, 2000; DANTAS-TORRES, 2008).

Previous studies have demonstrated the ability of immunogenic proteins obtained from ticks' salivary glands to develop a immune-inflamatory reaction. In this sense, Zhou et al. (2006), by immunizing rabbits with a specific salivary gland protein of *R. haemaphysaloides*, demonstrated a reaction that has undertaken the process of fixation and feeding of ticks later infested. Gill and Walker (1987) demonstrated that glandular proteins of *Hyalomma anatolicum anatolicum* would be expressed differently throughout the feeding process of ectoparasites. Adding to this, Furquim et al. (submitted paper) reported glandular changes that occurred throughout the feeding process of *R. sanguineus*, as the activation of certain cells in specific periods. It is therefore possible to suppose that host immunization with immunogenic molecules synthesized by tick glandular tissue at different stages of secretory cycle would stimulate the development of different immuno-inflammatory reactions in the hosts' skin in the ticks feeding lesion area (GILL et al, 1986).

Askenase et al. (1982), associated host resistance with the presence of basophils in the feeding lesion area. Likewise, Girardin and Brossard (1989) demonstrated the role of these cells (and T lymphocytes and mast cells) in skin reaction to *Ixodes ricinus* by the use of immunosuppressive drugs in rabbits subjected to reinfestation. Similarly, Szabó and Bechara (1997) showed inflammation in the feeding lesion area of *R. sanguineus* ticks maintained in hosts subjected to reinfestation that was marked by basophilia and neutrophilia. Monteiro and Bechara (2008) demonstrated that basophils have an important role in the acquisition of resistance to *Amblyomma cajennense* in goats subjected to repeated infestations. In addition, Wada et al. (2010) showed in mice the role of basophils in acquired immunity against ticks. According to the authors, the removal of basophils in these animals would jeopardize the development of resistance to a second infestation of *Haemaphysalis longicornis*.

Considering the information above, this study aimed to verify the action of immunogenic salivary gland extracts of *R. sanguineus* females (**EGS2, EGS4, EGS6**) in immuno-inflammatory response in rabbits, through the presence of inflammatory cells at the feeding lesion area.

Material and Methods

Material

In order to perform this study, skin samples from New Zealand White rabbits previously immunized with gland extracts (EGS2, EGS4, EGS6) were analyzed in the *R. sanguineus* tick feeding lesion site with 2, 4 and 6 days. For such, male and female *R. sanguineus* ticks derived from a colony maintained in a BOD incubator, under controlled conditions (28º C ± 1ºC , 80% humidity and 12-hour photoperiod), located at Biotério do Departamento de Biologia da UNESP campus de Rio Claro (SP), were used in **A**, **B**, and **C** infestations in rabbits, according to the procedure described by Bechara et al. (1995).

- A infestation

Infestation-naïve rabbits were subsequently infested with 25 *R. sanguineus* tick couples/chamber for acquisition of females with 2 (55 individuals), 4 (36 individuals) and 6 (25 individuals) days of feeding. Salivary glands were removed from these females in order to obtain the extracts: **EGS2**= female gland extract with 2 days, **EGS4**= with 4 and **EGS6**= with 6 days.

Determination of selected feeding periods (2, 4 and 6 days) was based on salivary gland secretion cycle, because unfed individuals have glands containing **a**, **c1**, **c3**, **d**, and **e** active cells, those with two days have **b**, **c2**, **c4**, **c5**, **c6**, and **f** active cells, those with 4 days have **c5** and **f** inactive cells, while only **a**, **b**, **c1**-**c4**, and **c6** cells remain active, and finally individuals with 6 days of feeding have c6 inactive cells (Furquim, subjected paper).

After being processed, extracts were inoculated into the hosts subjected to **B** infestation.

- B infestation (test group)

12 rabbits were sensitized, being 4 inoculated with **EGS2** extract, 4 with **EGS4** and 4 rabbits with **EGS6**. They were subsequently subjected to challenge infestation with 15 *R. sanguineus* tick couples/chamber.

- C infestation (control group)

4 infestation-naïve rabbits were inoculated with complete Freund's adjuvant and phosphate buffer mixture (control group 2) and 4 were not inoculated (control group 1). All 8 animals were subsequently subjected to a challenge infestation with 15 *R. sanguineus* tick couples/chamber.

Methods

Preparation and inoculation of gland extracts

Female of *R. sanguineus* with 2, 4 and 6 days of feeding were collected and dissected in saline solution (7.5 g NaCl, 2.38 g Na_2HPO_4, 2.72 g KH_2PO_4 and 1000 mL distilled water) to remove their salivary glands.

At facilities from Laboratório de Biologia Molecular do Departamento de Biologia da UNESP *campus* de Rio Claro (SP), Brazil, glands were placed separately by study status (obtained from females with 2, 4 and 6 days of feeding) in eppendorf tubes containing 200 µL 7.4 pH phosphate buffer. Then, glands were macerated, centrifuged by 30 minutes at 10,000 × g, supernatant was collected and processed for protein content analysis according to methodology described by Sedmark and Grossberg (1977) (Bradford method) and concentration to be obtained was 0.2 µg/µL.

Following protein content analysis of each sample, extracts were filtered separately by study status with support of 0.22 µm sterile filtering units (JBR610303, Millex GV disposable filtering unit, durapore, PVDF, Millipore, MilliUni membrane) with 13-mm diameter, coupled to hypodermic syringes, inside a pre-sterile vertical laminar flow hood. After this, extracts were aliquoted in volumes of 50 µL and stored inside a freezer at -20^{o} C. Only at the moment of the inoculations, each extract was mixed (50 µL of extract/host) with 50 µL complete Freund's adjuvant (reference # F 5881, Sigma-Aldrich), as well as with the mixture of Freund's adjuvant with 7.4 pH phosphate buffer (AF+PBS). These procedures were also performed in pre-sterile vertical laminar flow.

Rabbits from **TG** and **CG2** groups had their dorsolateral right region shaved, same site where subcutaneous inoculation occurred (**EGS2**, **EGS4** and **EGS6)**, as well as with AF+PBS, via hypodermic syringe, three times at 21-day intervals. Only after 15 days from last inoculation, all hosts from **TG** and **CG** groups were subjected to a challenge infestation with 15 *R. sanguineus* tick couples/chamber.

Rabbits from **TG** and **CG2** groups had their dorsolateral right region shaved, same site where subcutaneous inoculation occurred (**EGS2**, **EGS4** and **EGS6)**, as well as with AF+PBS, via hypodermic syringe, three times at 21-day intervals. Only after 15 days from last inoculation, all hosts from **TG** and **CG** groups were subjected to a challenge infestation with 15 *R. sanguineus* tick couples/chamber.

Histopathological Analysis

Skin samples were collected from rabbits in tick feeding sites at 2, 4 and 6 days, by a "punch" with 0.3 cm in diameter. Upon collection, the ectoparasite attached to the host was cut at the head region, remaining only the hypostome attached to the rabbit skin. With the "punch" placed at the site and with circular moves, a piece of skin was removed. Pieces were fixed in paraformaldehyde 4% at 4^{o}C, were dehydrated in a series of increasing concentrations of ethanol (70%, 80%, 90% and 95%), embedded in resin (Leica), and sectioned at 3 μm thickness. Sections were mounted on glass slides and stained with Giemsa technique. Slides were mounted with Canada balsam and examined and photographed under Motic BA 300 light microscope.

Results

1- Control Group 1

In skin samples collected from hosts in the control group (**CG**), an inflammatory reaction was noted. It develops during the feeding process of the ectoparasite. However, there is later and little intense reaction characterized by the presence of few inflammatory cells at the feeding lesion area, especially by the absence of basophils and few active fibroblasts.

The control group (**CG2**) presents characteristics similar to those found in the **CG1**.

2- Test Group 1 (EGS2)

In skin samples collected from hosts of this group was observed more evident development of inflammatory response than in the control group (**CG**), although with the presence of few basophils, neutrophils and eosinophils in the feeding damaged area of these skin animals. There is, throughout the feeding process of the ectoparasites, increase in the number of active fibroblasts.

3- Test Group 2 (EGS4)

The skin samples collected from the hosts of this group are those which present more inflammatory infiltrate during the feeding process. This infiltrate is markedly neutrophilic, eosinophilic and basophilic. The presence of basophils is observed at the outset of the infestation while the number of neutrophils and eosinophils increases on the fourth day of the ticks feeding process. The number of active fibroblasts in the lesion increases during the ectoparasites feeding process.

4- Test Group 3 (EGS6)

The skin samples of the hosts of this group showed intermediate inflammatory reaction as compared to animals in the test group (**EGS2**) and (**EGS4**) and marked by a basophilic, neutrophilic and eosinophilic infiltrate. This infiltrate, as in the previous group, shows more intense at fourth day of the ticks feeding process. In addition, the formation of

vesicles in the epidermis of samples was observed in this group at the end of the feeding process.

Figure 1.

Rabbit skin histological sections of the *Rhipicephalus sanguineus*-infested groups at different feeding periods. **A-B.** 2 days of feeding in control group. Epithelium (**ep**), presence of fibroblast (**f**). **C.** 4 days of feeding in control group. Epithelium (**ep**). Presence of blood vessels (**bv**). **D.** 6 days of feeding in control group. Presence of activated fibroblast (**af**) in dermal region (**dr**). **E.** 2 days of feeding in **EGS2** group. Dermal region (**dr**). Epithelium (**ep**). **F.** 4 days of feeding in **EGS2** group. Presence of basophils (**b**) and fibroblast (**f**). **G-H.** 6 days of feeding in **EGS2** group. Epithelium (**ep**). Dermal region (**dr**).

Bars: 10 µm.

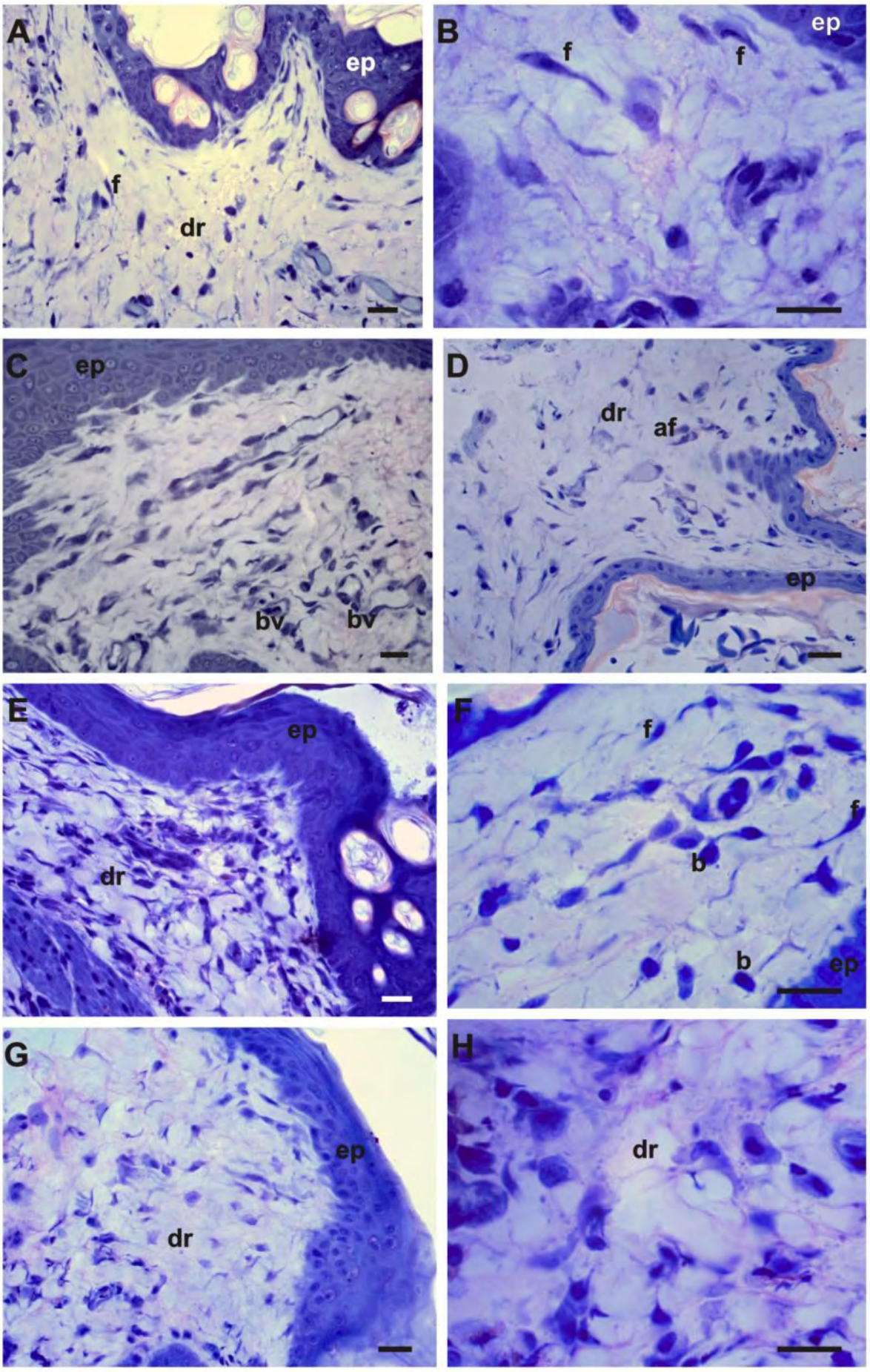
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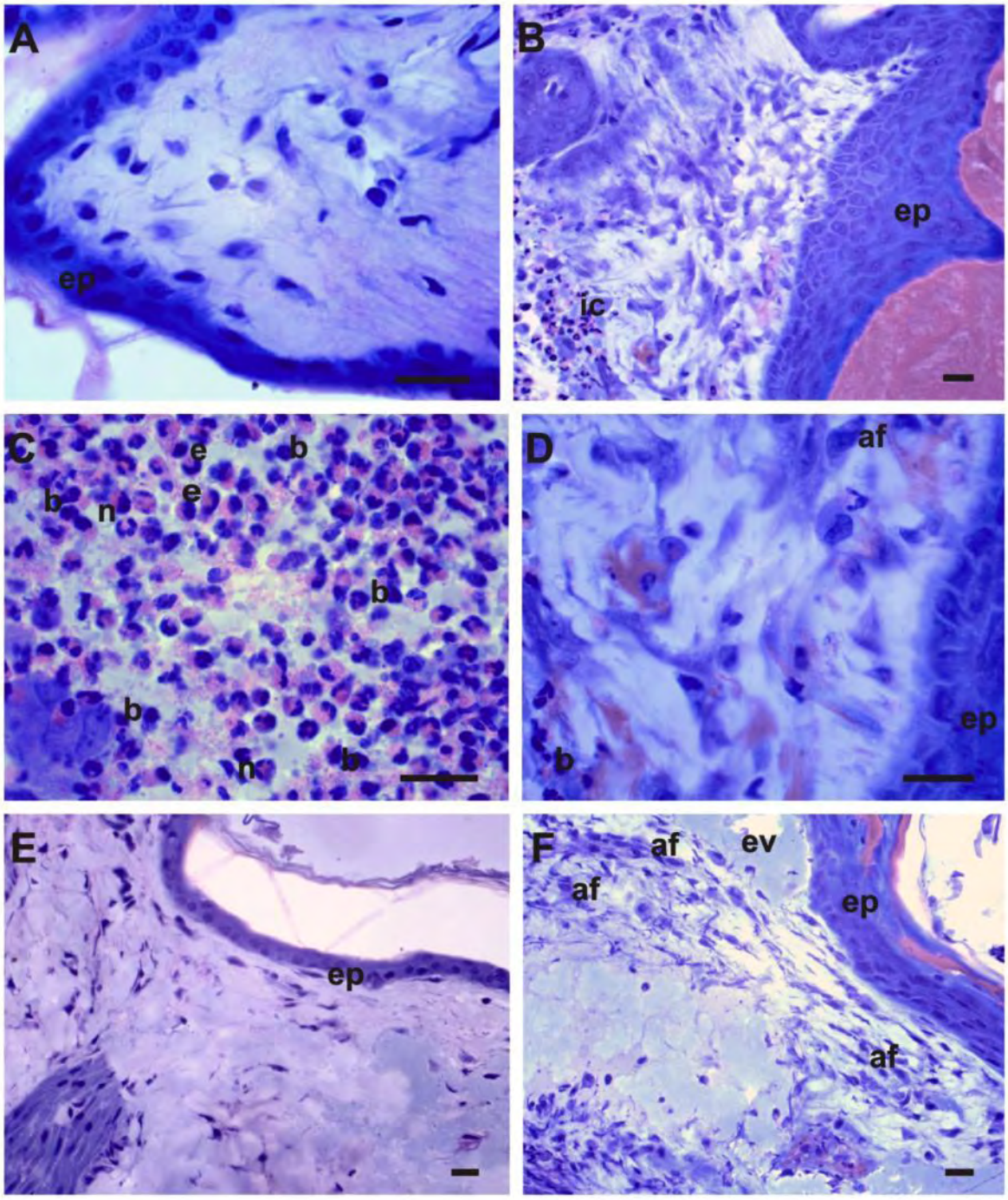
Figure 2.

Rabbit skin histological sections of the *R. sanguineus*-infested **GT** (**EGS4**) and **GT** (**EGS6**) groups with 2, 4 and 6 days of feeding. **A.** 2 days of feeding in (**EGS4**) group. **B.** 4 days of feeding in (**EGS4**) group. Presence of inflammatory cells (**ic**). Epithelium (**ep**). **C**. 4 days of feeding in (**EGS4**) group. Presence of eosinophils (**e**), neutrophils (**n**) and basophils (**b**). **D.** 6 days of feeding in (**EGS4**). Presence of activated fibroblast (**af**). **E.** 2 days of feeding in (**EGS6**) group. Epithelium (**ep**). **F**. 6 days of feeding in (**EGS6**) group. Epithelium (**ep**). Activated fibroblast (**af**). Epidermal vesicle (**ev**).

Bars: 10 µm.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Discussion

In this study, biopsies of rabbits skin previously inoculated with extracts of salivary glands of *R. sanguineus* females and subsequently subjected to infestation challenge were analyzed. Animals from test group that were previously inoculated with extracts of salivary glands of *R. sanguineus* females showed differences in the intensity of inflammatory reaction at the feeding lesion area at the later infestation.

The literature reports that the salivary secretion of the *R. sanguineus* tick presents different components with ability to inhibit some aspects of the host's immune response (FERREIRA and SILVA, 1998; CAVASANI *et al*, 2005). However, stimulation of this response by prior presentation of antigens derived from the saliva might enable the host to respond efficiently and early to parasites, impairing their feeding process, as observed in this study and as demonstrated by Zhou *et al.* (2006).

The results here obtained showed that the expression of resistance was more intense in the animals of test group compared to those of the control group. This result confirmed those obtained in animals that have developed resistance from repeated infestations (SZABÓ and BECHARA, 1985). In this study, in addition to the presence of basophils, eosinophils and neutrophils, expected in animals that have developed resistance to ticks, the formation of epidermal vesicles highlighted the activation of the complement system (WIKEL and ALLEN, 1977; ALLEN *et al.*, 1979). Due to the development of these vesicles in animals from group **TG** (**EGS4** and **EGS6**), it can be inferred that the activation of the complement system would be following the formation of a humoral response in these hosts.

According to literature, the local inflammatory process includes the cell-mediated immune response and humoral response. Inflammatory cells are attracted to the site of injury by the action of chemotactic components of fragments of the complement system (C3 and C5) and chemokine produced by cells at the site of injury. Wikel and Allen (1977) showed that depletion of complement system proteins compromise the immuno-inflammatory response to the presence of ticks in resistant hosts resulting in a decrease in the input of basophils in the feeding lesion.

The results here obtained on different host inflammatory responses developed in the control group and those of different test groups showed that the glandular extracts were able to promote resistance in previously immunized hosts. However, we observed that these extracts had different degrees of effectiveness in the development of inflammatory response,

once it was possible to observe a greater or lesser infiltration of inflammatory cells in the feeding lesion area.

From the qualitative point of view, there was no difference between the responses obtained in the previously inoculated hosts. However, there was quantitatively more intense inflammatory reaction with presence of increased infiltration of inflammatory cells in animals that received the extract **EGS4**. In these individuals the inflammatory reaction, besides being more intense, was also earlier as it was possible to note a greater number of inflammatory cells, particularly neutrophils, eosinophils and basophils at the feeding lesion. On the other hand, the hosts that received the extract **EGS6** presented an inflammatory reaction of medium intensity when compared to individuals of other test groups (**EGS2** and **EGS4**) since those who were inoculated with extract **EGS2** produced less inflammatory reaction.

The fact that the extract **EGS4** was more effective in promoting immuno-inflammatory response in the host indicates that it is a reflection of the metabolic activity of the ectoparasite salivary gland that at four days feeding initiates the phase of rapid consumption of blood and thus its salivary glands are in intense activity of synthesis and secretion, modulating the host response, as well as producing anticoagulants and vasodilators substances that enable feeding (FURQUIM et al., subjected paper).

On the other hand, the immuno-inflammatory response to extracts **EGS2** and **EGS6** corroborated the information that two days of feeding, the salivary glands would be starting the phase of synthesis and secretion of saliva components, and at six days, the glandular tissue would be entering degeneration which would mean that only a few glandular cells would still in activity (FURQUIM et al., subjected paper).

Among the changes related to inflammatory processes in the skin of the hosts in the fixation damaged area, there was the formation of epidermal vesicles from the fourth day of infestation challenge in animals inoculated with the extracts **EGS4** and **EGS6**, a fact that probably occurred by activation of the complement system as also shown by Wikel and Allen (1977) that, causing protein depletion from such system, jeopardized the response from resistant hosts.

At the end of the infestation challenge, all animals belonging to the test group showed a higher amount of activated fibroblasts in relation to the control group, demonstrating the development of a local repair process more efficient in those previously immunized and corroborating Kramer et al. (2008) that demonstrated suppression of fibroblast migration by the saliva in *Dermacentor variabilis*.

In this study it was found that the immune-inflammatory response of the hosts of the **TG** to tick infestation first occurred in attempting to eliminate the offending factor and only at the end of the infestation, when the components of the ectoparasite saliva ceased to produce most of its immunosuppressive properties, anti-coagulant and vasodilator is that the host response has focused on repairing the damage caused by the ticks spoliative action.

The results presented here indicated that the glandular extracts would be able to stimulate the development of host resistance in the test group and that this developed resistance would be able to change the handling capacity of the host immune response allowing it to develop local inflammatory response with an input of inflammatory cells and activation of the complement system more intense and earlier than in the **TG**. Furthermore, the **TG** animals also showed a remedial action earlier than those of **CG**.

References

Allen, J. R.; Khalil, H. M.; Graham, J. The location of tick salivary antigens, complement and immunoglobulin in the skin of guinea-pigs infested with *Dermacentor andersoni* larvae. Immunology**,** Oxford, v. 38, p. 467-472, 1979.

Askenase, P. W.; Bagnall, B. G.; Worms, M. J. (1982) Cutaneous basophil-associated resistance to ectoparasites (ticks). I- Transfer with immune serum or immune cells. Immunology**.** 45: 501-511.

Bechara, G. H. (2006) Imunopatologia da interação carrapato-hospedeiro. In Barros-Battesti, D. M.; Arzua, M.; Bechara, G. H. Carrapatos de importância médico-veterinária da região neotropical. Um guia ilustrado para a identificação de espécies. Butantan, São Paulo. P. 139-143.

Brossard, M.; Wikel, S. K. (1997) Immunology of interactions between ticks and hosts. Medical and Veterinary Entomology. 11: 270-276.

Cavassani KA, Aliberti JC, Dias ARV, Ferreira BR (2005) Tick saliva inhibits differentiation, maturation and function of murine bone-marrow-derived dendritic cells. Immunology 114:235–245

Cupp, E. W. (1991) Biology of ticks. The veterinary clinics of North America. 21: 1-26.

Dantas-Torres, F. (2008) The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): From taxonomy to control. Veterinary Parasitology. 152: 173–185.

Fernandes, F. F. (2000) Atividade in vitro de permetrina, cipermetrina e deltametrina sobre larvas de *Rhipicephalus sanguineus* (Latreille, 1806) (Acari, Ixodidae). Arquivo Brasileiro de Medicina Veterinária e Zootecnia. V. 5: 6. doi: 10.1590/S0102-09352000000600012

Ferreira BR, Silva JS (1998) Saliva of *Rhipicephalus sanguineus* tick impairs T cell proliferation and IFN-*c*induced macrophage microbicidal activity. Vet Immunol Immunopathol 64:279–293

Gill, HS; Walker, AR. (1987) The salivary glands of *Hyalomma anatolicum anatolicum:* nature of salivary gland components and their role in tick attachment and feeding. International Journal for Parasitology. 18 (1): 83-93.

Gillespie, R. D.; Mbow, M. L.; Titus, R. G. (2000) The immunomodulatory factors of bloodfeeding arthropod saliva. Parasite Immunology. 22: 319-331

Girardin, P.; Brossard, M. Effects of cyclosporin A on humoral immunity to ticks and on cutaneous immediate and delayed hypersensitivity reactions to *Ixodes ricinus* L. salivary-gland antigens in re-infested rabbits. Parasitology Research. 1989. v. 75. p. 657-662.

Gosh, S.; Azhahianambi, P.; Yadav, M. P. (2007) Upcoming and future strategies of tick control: a review**.** Journal of Vector Borne Diseases. 44: 79-89.

Guglielmone, A, Szabó, MJP, Martins, JRS, Estrada-Pena, A (2006) Diversidade e importância de carrapatos na sanidade animal. In: Barros-Battesti, DM, Arzua, M, Bechara, GH (Eds.) Carrapatos de importância médico-veterinária da região neotropical. Um guia ilustrado para a identificação de espécies. Butantan, São Paulo, pp. 115-138.

Kramer, C.; Nahmias, Z.; Norman, D. D.; Mulvihill, T. A.; Coons, L. B.; Cole, J. A. (2008) *Dermacentor variabilis*: regulation of fibroblast migration by tick salivary gland extract and saliva. Experimental Parasitology. 119: 391-397.

Monteiro, G.; Bechara, G. H. (2008) Cutaneous basophilia in the resistance of goats to *Amblyomma cajennense* nymphs after repeated infestations. Annals of the New York Academy of Sciences. 1149: 221-225.

Sedmark, J.J.; Grossberg, S.E. (1977) A rapid sensitive and versatile assay for protein using Coomassie Brilliant Blue G-250. Analytical Biochemistry, v. 79, p. 544-552.

Szabó, M. P. J.; Bechara, G. H. (1985) Sequencial histopathology at *Rhipicephalus sanguineus* tick feeding site on dogs and guineas pigs. Experimental and Applied Acarology. v. 23, n. 11, p. 915-928.

Szabó, M. P. J.; Bechara, G. H. (1997) Immunization of dogs and guinea pigs against *Rhipicephalus sanguineus* ticks using gut extracts. Veterinary Parasitology, v. 68, p. 283-294.

Wada, T.; Ishiwata, K.; Koseki, H.; Ishikura, T.; Ugajin, T.; Ohnuma, N.; Obata, K.; Ishikawa, R.; Yoshikawa, S.; Mukai, K.; Kawano, Y.; Minegishi, Y.; Yokozeki, H.; Watanabe, N.; Karasuyama, H. (2010) Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. The Journal of Clinical, Investigation. v. 120, n. 8. p. 2867-2875.

Wikel, S. K.; Allen, J. R. (1977) Acquired resistance to ticks. III- Cobra venom factor and the resistance response. Immunology. 32: 457-465.

Capitulo IV

Rhipicephalus sanguineus (LATREILLE, 1806) (ACARI, IXODIDAE) FEMALES FED ON RABBITS IMMUNIZED WITH EXTRACTS OF SALIVARY GLANDS IN DIFFERENT PERIODS OF THE SECRETORY CYCLE. ANALYSIS OF THE FEEDING AND REPRODUCTIVE PARAMETERS.

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Resumo

Este estudo mostrou a eficácia de extratos de glândulas salivares de fêmeas de *Rhipicephalus sanguineus* com 2, 4 e 6 dias de alimentação na imunização de coelhos contra infestação por esses carrapatos. Para tanto, foram analisados os parâmetros alimentar e reprodutivo de fêmeas da infestação desafio. Glândulas salivares de fêmeas alimentadas por 2, 4 e 6 dias foram processadas para a obtenção de extratos **SGE2**, **SGE4** e **SGE6,** respectivamente. Coelhos New Zealand White, virgens de infestação, foram inoculados com os extratos (grupo teste = **TG**) ou com uma mistura de tampão fosfato e adjuvante completo de Freund (grupo controle 2 = **CG2**). Cada coelho inoculado (**TG** ou **CG2**) e não inoculado (grupo controle 1 = **CG1**) recebeu uma infestação desafio com 15 casais de *R. sanguineus* em jejum que foram posteriormente coletados e analisados para os seguintes parâmetros: a) porcentagem de fêmeas recuperadas em relação ao número inicial, b) tempo médio para o completo ingurgitamento das fêmeas, c) avaliação do peso médio das fêmeas completamente ingurgitadas, d) taxa de eficiência alimentar (FER), e) tempo médio de oviposição, f) peso médio da massa de ovos, g) porcentagem de viabilidade de ovos. Considerando os resultados de teste desafio, as médias obtidas para os parâmetros alimentar e reprodutivo das fêmeas da infestação desafio, as diferenças apresentadas não foram significativas, exceto para o peso médio de ingurgitamento **TG** (**SGE6**) ($p < 0.05$) em relação ao **CG1**. Essas diferenças, causadas pela resposta imune-inflamatória dos hospedeiros, como foi observado nas análises histopatológicas anteriores, não foram estatisticamente significativas, porém, representam tendências que precisam ser consideradas biologicamente. Os resultados obtidos demonstraram que as fêmeas alimentadas nos hospedeiros do **TG (SGE2)** (**TG** inoculados com extrato **SGE2**) mostraram tendência a: 1) menor porcentagem de recuperação (96,67%), este grupo apresentou maior mortalidade em relação ao **TG (SGE6)** e **CG2**, 2) maior tempo de ingurgitamento (9.1034 days, ± 1.462) em relação ao **TG (SGE4** e **SGE6)** e **CG1**, 3) menor peso ao ingurgitamento (0.1763 g, ± 0.042) e taxa de eficiência alimentar (2.0%, ± 0.679) em relação ao **TG (SGE6)**, **CG1** e **CG2**, 4) maior tempo de oviposição (9.9310 dias, ± 0.413) em relação ao **TG (SGE4)** e **CG1**, 5) menor peso de massa de ovos (0.0959 g, ± 0.028) em relação ao **TG (SGE6)** e **CG1** e **CG2** e 6) menor viabilidade de ovos (95.3257%, ± 15.117). As fêmeas coletadas dos hospedeiros do **TG (SGE4)** tiveram uma tendência a: 1) apresentar menor porcentagem de recuperação de fêmeas (93.33%), 2) maior tempo de

ingurgitamento (9.0892 dias, ± 1.443) em relação ao **TG (SGE6)** e **CG1**, 3) menor peso de ingurgitamento (0.1741 g, ± 0.030), 4) menor taxa de eficiência alimentar (1.96%, ± 0.435), 5) menor tempo de oviposição (9.9636 dias, ± 0.188), 6) menor peso da massa de ovos (0.0976g, ± 0.024) em relação ao **TG (SGE6)** e **CG1** e **CG2** and 7) menor viabilidade dos ovos (98.0494%, ± 6.999) em relação ao **TG (SGE6)** e **CG2**. Nas fêmeas do **TG (SGE6)** houve tendência a: 1) menor mortalidade de indivíduos (98.33% de recuperação das fêmeas infestantes), 2) menor tempo de ingurgitamento (8.4576 dias ± 1.087), 3) maior taxa de eficiência alimentar (2.2%, ± 0.640), 4) maior tempo de oviposição (9.9322 dias , ± 0.520) em relação ao **TG (SGE4)** e **CG1**, 5) maior peso da massa de ovos (0.1028 g, ± 0.024) em relação ao **TG (SGE2** e **SGE4)** e **CG1,** 6) maior viabilidade de ovos (97.8721%, ± 6.870) em relação ao **TG (SGE2** e **SGE4)** e **CG1**, além de apresentarem maior peso de ingurgitamento (0.1848 g, ± 0.038). Assim, ficou aqui demonstrado que os extratos glandulares **SGE2** e **SGE4** foram eficientes na imunização de hospedeiros contra carrapatos *R. sanguineus*, embora cada um deles tenha apresentado um ação específica. O **SGE2** mostrou tendência a controlar a infestação reduzindo o número de descendentes (ação na reprodução) e o **SGE4** reduziu o número de carrapatos que ingurgitaram completamente (ação na alimentação). Além disso, a resistência adquirida por meio dos extratos **SGE2** e **SGE4** levaram a uma diminuição no consumo de sangue pelos ectoparasitas. Por outro lado, o **SGE6** não foi eficiente na imunização contra a infestação por carrapatos, ao contrário, induziu uma resposta nos hospedeiros que beneficiou o processo de alimentação das fêmeas quando comparadas aos resultados do **CG1**.

Palavras-chaves: *Rhipicephalus sanguineus*, inoculação de extratos glandulares, imunização, infestação desafio, alimentação, oviposição.

Abstract

The present study showed the efficacy of the extracts of salivary glands of *Rhipicephalus sanguineus* females with 2, 4 and 6 days of feeding in the immunization of rabbits against ticks infestation. For this, feeding and reproductive parameters of these females were analyzed. Salivary glands of females fed for 2, 4 and 6 days were processed to obtain **SGE2**, **SGE4** and **SGE6** extracts respectively. After, New Zealand White naive rabbits were inoculated with the extracts (test group = **TG**) or with a mixture of phosphate buffer and complete Freund's adjuvant (control group 2 = **CG2)**. Each inoculated (**TG** and **CG2**) and non-inoculated (control 1= **CG1**) rabbit was posteriorly subjected to challenge infestation with 15 *R. sanguineus* fasting couples that, after collected, were analyzed according to the following parameters: a) percentage of female recovery in relation to the initial number, b) average time for the females to complete engorgement, c) evaluation of the average weight of completely engorged females, d) feeding efficiency rate (FER), e) average time of oviposition, f) average total weight of egg e g) percentage of eggs feasibility. Concerning the results of the challenge test, the averages obtained for the feeding and reproductive parameters of the females from the challenge infestation did not present significant differences, except for the average weight of engorgement for **TG (SGE6)** ($p < 0.05$) in relation to **CG1**. These differences, caused by the imune-inflamatory responses developed by the hosts, as was observed in the previous histopathological analysis of their skin, were not statistically significant. Thus, these variations represent trends that need to be biologically considered. The results obtained demonstrated that the females fed on hosts of **TG (SGE2)** (**TG** inoculated with **SGE2** extract) showed a tendency to: 1) lower percentage of recovery (96.67%), i.e., this group presented higher mortality in relation to **TG (SGE6)** and **CG2**, 2) longer engorgement time (9.1034 days, ± 1.462) in relation to **TG (SGE4** and **6)** and **CG1**, 3) lower engorgement weight (0.1763 g, ± 0.042) and feeding efficiency rate (2.0%, ± 0.679) in relation to **TG (SGE6)** and **CG1** and **2**, 4) longer oviposition time (9.9310 days, ± 0.413) in relation to **TG (SGE4)** and **CG1**, 5) lower egg weight (0.0959 g, ± 0.028) in relation to **TG (SGE6)** and **CG1** and **2** and 6) lower egg feasibility (95.3257%, ± 15.117). The females collected from hosts of **TG (SGE4)** had a tendency to: 1) present lower percentage of recovery of the infesting females (93.33%), 2) longer engorgement time (9.0892 days, ± 1.443) in relation to **TG (SGE6)** and **CG1**, 3) lower engorgement weight (0.1741 g, ± 0.030), 4) lower feeding efficiency rate (1.96%, ± 0.435), 5) shorter oviposition time (9.9636 days, ±

0.188), 6) lower egg weight (0.0976 g, ± 0.024) in relation to **TG (SGE6)** and **CG1** and **2** and 7) lower eggs feasibility (98.0494%, ± 6.999) in relation to **TG (SGE6)** and **CG2**. In the females of **TG (SGE6)** there was a tendency to: 1) lower mortality of individuals (98.33% of recovery of the infesting females), 2) shorter engorgement time (8.4576 days ± 1.087), 3) higher feeding efficiency rate (2.2%, ± 0.640), 4) longer oviposition time (9.9322 days , ± 0.520) in relation to **TG (SGE4)** and **CG1**, 5) higher egg weight (0.1028 g, ± 0.024) in relation to **TG (SGE2** and **4)** and **CG1** and 6) higher eggs feasibility (97.8721%, ± 6.870) in relation to **TG (SGE2** and **4)** and **CG1**, in addition to presenting higher engorgement weight (0.1848 g, ± 0.038). Thus, it was here demonstrated that the glandular extracts **SGE2** and **SGE4** were efficient in the immunization of the hosts against *R. sanguineus* ticks, although each of them have behaved in a specific way. **SGE2** tended to control infestation due to the reduction of descendents (action on reproduction) and **SGE4** tended to reduce the number of ticks that engorged completely (action on the feeding). In addition, the resistance acquired to **SGE2** and **SGE4** extracts caused a reduction in the blood consumption by the parasites. On the other hand, **SGE6** was not efficient in the immunization against infestation by ticks, instead, induced the hosts to a response that significantly benefited the progression of females' feeding, even more than what was observed in **CG1**.

Keywords: *Rhipicephalus sanguineus*, inoculation of glandular extracts, immunization, challenge infestation, feeding, oviposition.

Introduction

Ticks are hematophage ectoparasites which are harmful for the hosts and because of this are economically and veterinary important. In addition to their own spoliatory action, which impact milk and meat production (Jittapalapong et al. 2008), they are also pathogen transmitters (Balashov 1983).

All the success of these ectoparasites in spoliating the hosts and transmitting pathogens is provided by the saliva, a complex mixture (Sonenshine 1991) produced by the salivary glands which contain immunosuppressing, antiinflammatory, anticoagulant and vasodilating substances (Sauer et al. 2000), among others. The action of the saliva – complex misture produced by the salivary glands that contains immunosuppressant, anti-inflammatory, anticoagulant and vasodilating substances - is responsible for all the success of these ectoparasites in the spoliation of the hosts, as well as for the transmission of pathogens.

Some hosts develop resistance after the first contact with the ticks because the saliva contains immunogenic substances. Because of this many studies are carried out approaching successive infestations (Nunes et al. in press; Monteiro et al. 2008, 2010; Veronez et al. 2010; Caperucci et al. 2009, 2010; Jittapalapong et al. 2000a, b), inoculating extracts of ticks (or part of them) (Wikel 1980; Ferreira et al. 1996; Szabó and Bechara 1997; Jittapalapong et al. 2000a, b, 2008) to verify how resistant against the tick the host becomes and to what extent this resistance interferes in the infestation by these ectoparasites (Jittapalapong et al. 2000a; Szabó and Bechara 1997). In addition, many studies using molecular biology and immunology techniques have tried to provide useful information to develop vaccines by identifying the antigens that provide such resistance (Gill et al. 1986; Almeida et al. 1994; Ferreira et al. 1996; Willadsen et al. 1997).

Data from literature have showed that two ticks' organs are the main focus of study to develop vaccines: the salivary glands (Wikel et al. 1978; Gill et al. 1986; Almeida et al. 1994; Ferreira et al. 1996; Szabó and Bechara 1997; Jittapalapong et al. 2000a; Nunes et al. in press) and the intestine (Szabó and Bechara 1997; Jittapalapong et al. 2000a; Caperucci et al. 2009, 2010; Veronez et al. 2010).

According to Jittapalapong et al. (2000a) the immunization with extracts of salivary and intestinal glands would stimulate the development of resistance by the host, which would influence the infestation by ticks. However, they would act in a specific and more or less efficient way in the physiology of certain systems of the ectoparasite (Jittapalapong et al.

2000a). The immunization with antigens from the salivary glands would bring an immunologic response that would affect directly the salivary glands themselves, reducing their efficiency during the feeding process of the ectoparasite and probably reducing the transmission of pathogens (Jittapalapong et al. 2000a, 2008). On the other hand, the immunization with antigens from the intestine would act more efficiently in the reproductive process of female ticks, reducing the number of eggs laid as well as their feasibility (Jittapalapong et al. 2000a).

Still concerning the immunization with ticks tissues, the study by Jittapalapong et al. (2000a) used antigens from salivary glands of *R. sanguineus* ticks fed for only 3 and 5 days. However, histological and histochemical studies by Furquim et al. (submitted paper) on salivary glands of *R. sanguineus* females have showed that different secretory cells of acini II and III would become active or instead would stop present activity mainly in females fed for 2, 4 and 6 days, showing that some of them would have assynchronic secretory cycle in relation to the rest of the glandular cells, which would remain active during the whole process, allowing the consideration for the specific action of the secretion synthesized by these cells also in the manipulation of the immune-inflammatory of the host.

Considering that the periods of 2, 4, and 6 days of feeding would be the most important moments in the secretory cycles of the salivary glands of *R. sanguineus* females, the present study had the objective to evaluate the influence of the resistance acquired by the immunization of the hosts with antigens obtained from the salivary glands, specifically during these periods, analyzing the feeding and reproductive parameters of *R. sanguineus* females from the challenge infestation.

Material and Methods

Material

To perform this study, fasting individuals (males and females of *Rhipicephalus sanguineus*) from a colony kept in BOD incubator in controlled conditions (28ºC ± 1ºC, 80% of humidity and photoperiod of 12 hours), in the Biotério of the Departamento de Patologia Veterinária da UNESP *campus* of Jaboticabal (SP), Brazil were used in **B** and **C** infestations on New Zealand White rabbits according to procedures described by Bechara et al. (1995).

- **Infestation A**

Performed in the Biotério of the Departamento de Biologia of UNESP *campus* of Rio Claro (SP), Brazil, in naive rabbits, using 25 *R. sanguineus* couples/host for the acquisition of tick females fed for 2 (55 individuals), 4 (36 individuals) and 6 (25 individuals) days, to obtain glandular extracts: **SGE2**= glandular extract of females fed for 2 days, **SGE4**= females fed for 4 days and **SGE6**= females fed for 6 days.

The chosen feeding periods (2, 4 and 6 days) were determined based on the salivary glands' secretion cycle, once the fasting individuals have glands which contain active **a**, **c1**, **c3**, **d** and **e** cells, those fed for 2 days contain active **b**, **c2**, **c4**, **c5**, **c6** and **f** cells those fed for 4 days present inactive **c5** and **f** cells and **a**, **b**, **c1**-**c4** and **c6** active cells and finally individuals fed for 6 days present inactivation of **c6** cells (Furquim et al., submitted paper).

The extracts were processed and inoculated in the hosts subjected to infestation **B**.

- Infestation B (test group= TG)

Performed in 12 sensibilized rabbits, 4 of them inoculated with **SGE2** extract, 4 with **SGE4** extract and 4 with **SGE6**, which were subjected to challenge infestation with 15 couples of *R. sanguineus* adult ticks/host.

- **Infestation C (control group= CG)**

Performed in 8 naive rabbits, 4 of them were not inoculated (**CG1**) and 4 were inoculated with a mixture of complete Freund's adjuvant and sodium phosphate buffer (**CG2**) and after subjected to challenge infestation with 15 couples of *R. sanguineus* adult ticks/host.

This experiment was approved by the Comitê de Ética em Pesquisa e Mérito Científico UNIARARAS, Protocol nº 021/2009.

Methods

Preparation and inoculation of glandular extracts

R. sanguineus females fed for 2, 4 and 6 days were collected and dissected in saline solution (7.5 g de NaCl, 2.38 g of Na_2HPO_4, 2.72 g of KH_2PO_4 and 1000 mL of distilled water), for the removal of the salivary glands.

In the Laboratory of Molecular Biology of the Biology Department of UNESP *campus* Rio Claro (SP), Brazil, the salivary glands were put in eppendorf tubes containing 200 µL of phosphate buffer pH 7.4 separately by situation of study (females fed for 2, 4 and 6 days). They were then labeled and centrifuged by 30 minutes at 10.000 xg. The supernatant was collected and subjected to protein dosage according to the methodology described by Sedmark and Grossberg (1977) (Bradford method) (0.2 µg/µL).

After the determination of the protein content of each sample, the extracts were filtered – separately by situation of study – with the help of sterile filtering units (JBR610303, disposable filtering unit Millex GV, durapore membrane PVDF, Millipore, MilliUni) of 0.22 µm and diameter of 13mm, attached to hypodermic syringes, in pre-sterile vertical laminar flow. The extracts were aliquoted in volumes of 50 µL and stored in freezer at -20 ^{o}C. Only at the moment of the inoculation each of the extracts was mixed (50 µL of extract/host) with 50 µL of complete Freund's adjuvant (reference # F 5881, Sigma-Aldrich), as well as the mixture of Freund's adjuvant with phosphate buffer (FA+PBS), procedures also performed in pre-sterile vertical laminar flow.

Rabbits from **TG (SGE2, 4 and 6)** and **CG2** had their right dorsal side sheared and were subcutaneously inoculated with **SGE2**, **SGE4** and **SGE6** extracts, and with FA+PBS, via hypodermic syringe for three times in intervals of 21 days. Only after 15 days after the last inoculation all the hosts from groups **TG (SGE2, 4 e 6)** e **CG1** and **2** were subjected to challenge infestation with 15 couples of *R. sanguineus*/host.

Challenge test with evaluation of the feeding and reproductive performance of the females

This test was performed in the Biotery of Biology Department of UNESP Rio Claro (SP) Brazil, using the females that were deposited on rabbits from group **TG (SGE2, 4** and **6)** (infestation **B**) and **CG1** and **2** (infestation **C**). For the evaluation of the effect of the immunizations the following parameters were considered: a) percentage of recovery of the females in relation to the released number, b) average time required by the females to complete engorgement c) evaluation of the average weight of the completely engorged females d) feeding efficiency rate (average weight of the fully engorged females/average time for the female to complete engorgement) (FER) e) average time of oviposition, f) average weight of eggs mass and g) percentage of eggs feasibility.

The averages of feeding and reproductive parameters were statistically analyzed by ANOVA test with TUKEY post-test, differences with $p < 0.05$ were considered significant.

Results

The results obtained by challenge test revealed that only the parameter average weight of engorgement of the females from group **TG (SGE6)** presented statistically significant difference ($p < 0.05$) in relation to **CG1.** However, although these small variations are not statistically significant, they represent trends that must be taken into consideration.

1. **Females recovery rate**

The results here obtained show that the percentage of recovery for the females of group **CG1** and **CG2** is of 90% (n= 54) and 96.67% (n= 58), respectively (Fig. 1).

For the females of group **TG (SGE2)** (females fed on hosts from **TG** immunized with glandular extract **SGE2**) the recovery percentage is of 96.67% (n= 58), for the ones from **TG (SGE4)** of 93.33% (n= 56) and those from **TG (SGE6)** of 98.33% (n= 59) (Fig. 1).

2. Average time of complete engorgement

The average engorgement time went from 8.8703 days (± 1.89) in the females from group **CG1** to 9.2931 days (± 1.44) in **CG2**, 9.1034 (± 1.46) in those from group **TG (SGE2)** and 9.0892 (± 1.44) days in those from group **TG (SGE4)**. Conversely, it decreased (8.4576 days, ± 1.08) in the females from group **TG (SGE6)** (Fig. 2A).

3. Average weight of complete engorgement

The results for this parameter in the females from groups **CG1** are of 0.1828 g (with deviation of ± 0.047, ± 0.028) (Fig 2B).

There is a reduction in the engorgement weight for the females from group **TG (SGE2)** and **TG (SGE4)**, which correspond to 0.1763 g (± 0.042) and 0.1741 (± 0.030), respectively (Fig. 2B).

In those from group **TG (SGE6)** the average engorgement is of 0.1848 g (± 0.038), respectively (Fig 2B).

4. Feeding efficiency rate (FER)

There is a reduction of 2.15% (± 0.731) in the females from group **CG1** to 2.01% (± 0. 417) in those from group **CG2**, 2% (± 0.679) in those from **TG (SGE2)** and 1.96% (± 0.435) in those from **TG (SGE4)** (Fig. 2C).

In the females from group **TG (SGE6)** this parameter is of 2.2% (± 0.640) (Fig. 2C).

5. Average time of oviposition

In the females from **CG1** this parameter is of 10 days (± 0) with reduction in the females from **CG2**, i.e., of 9.9827 (± 0.131) (Fig. 3A).

The average oviposition time for the females from **TG (SGE2)** is of 9.9310 days (± 0.413), for the ones from **TG (SGE4)** of 9.9636 days (± 0.188) and for the ones from **TG (SGE6)** of 9.9322 days (± 0.520) (Fig. 3A).

6. Average weight of egg

In the females from **CG1** this parameter is of 0.1014 g (± 0.030) and in those from **CG2** of 0.1027 g (± 0.019) (Fig. 3B).

In those from **TG (SGE2)** the egg weight is of 0.0959 g (± 0.028), in those from **TG (SGE4)** of 0.0976 g (± 0.024) and in those from **TG (SGE6)** of 0.1028 g (± 0.024) (Fig. 3B).

7. Egg feasibility rate

An increase in the egg feasibility from 97.9461% (± 6.012) in the females from **CG1** to 98.2829% (± 8.902) in those from **CG2** was observed (Fig. 3C).

This parameter for the females from **TG (SGE2)** is of 95.3257% (± 15.117), 98.0494% (± 6.999) for those from **TG (SGE4)** of 97.8721% (± 6.870) for **TG (SGE6)** (Fig. 3C).

Table 1: Comparison of the feeding and reproductive parameters results of *R. sanguineus* females from challenge infestation on hosts from groups **TG (SGE2**, **4** and **6)** in relation to the groups **CG1** and **2**.

	Study Groups				
Parameters	**CG1**	**CG2**	**TG (SGE2)**	**TG (SGE4)**	**TG (SGE6)**
FR (%)	90	96.67	96.67	93.33	98.33
ET (days)*	8.8703 ± 1.893	9.2931 ± 1.446	9.1034 ± 1.462	9.0892 ± 1.443	8.4576 ± 1.087
CEW (g)*	0.1828 ± 0.047	0.1828 ± 0.028	0.1763 ± 0.042	0.1741 ± 0.030	**0.1848 ± 0.038**[a]
FER (%)*	2.15 ± 0.731	2.01 ± 0.417	2.0 ± 0.679	1.96 ± 0.435	2.2 ± 0.640
OT (days)*	10 ± 0	9.9827 ± 0.131	9.9310 ± 0.413	9.9636 ± 0.188	9.9322 ± 0.520
EW (g)*	0.1014 ± 0.030	0.1027 ± 0.019	0.0959 ± 0.028	0.0976 ± 0.024	0.1028 ± 0.024
EF (%)*	97.9461 ± 6.012	98.2829 ± 8.902	95.3257 ± 15.117	98.0494 ± 6.999	97.8721 ± 6.870

***:** values provided as average ± standard deviation; **FR:** females recovery in relation to the number released; **ET:** necessary time to complete engorgement; **CEW:** weight of completely engorged females; **FER:** feeding efficiency rate; **OT:** oviposition time; **EW:** egg mass weight; **EF**: eggs feasibility; **[a]**: significant difference ($p < 0.05$).

Figure 1

Recovery rate in relation to the released number of *Rhipicephalus sanguineus* females fed on hosts immunized with glandular extracts of 2= **TG (SGE2)**, 4= **TG (SGE4)** and 6 days = **TG (SGE6)** and non-immunized = **CG1** e **CG2**.

n= absolute frequency of recovered females.

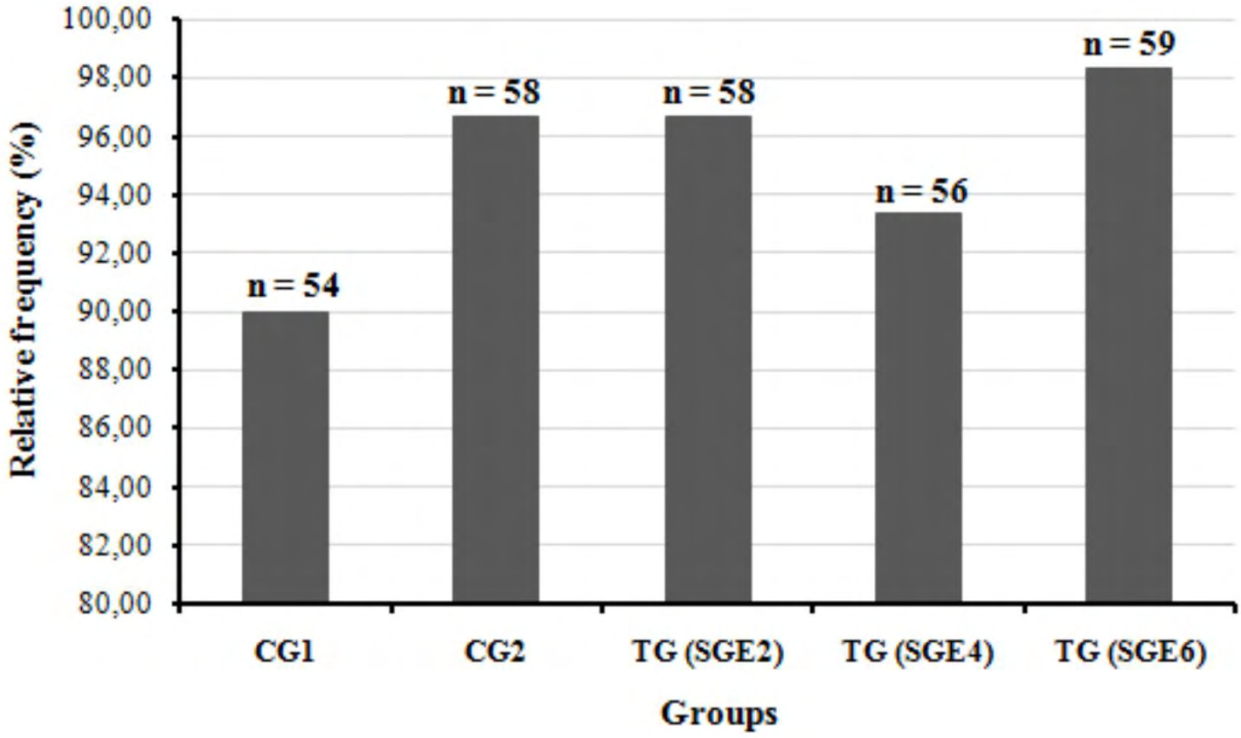

100,00
98,00
96,00
94,00
92,00
90,00
88,00
86,00
84,00
82,00
80,00
Relative frequency (%)
n = 54
n = 58
n = 58
n = 56
n = 59
CG1
CG2
TG (SGE2)
TG (SGE4)
TG (SGE6)
Groups

Figure 2

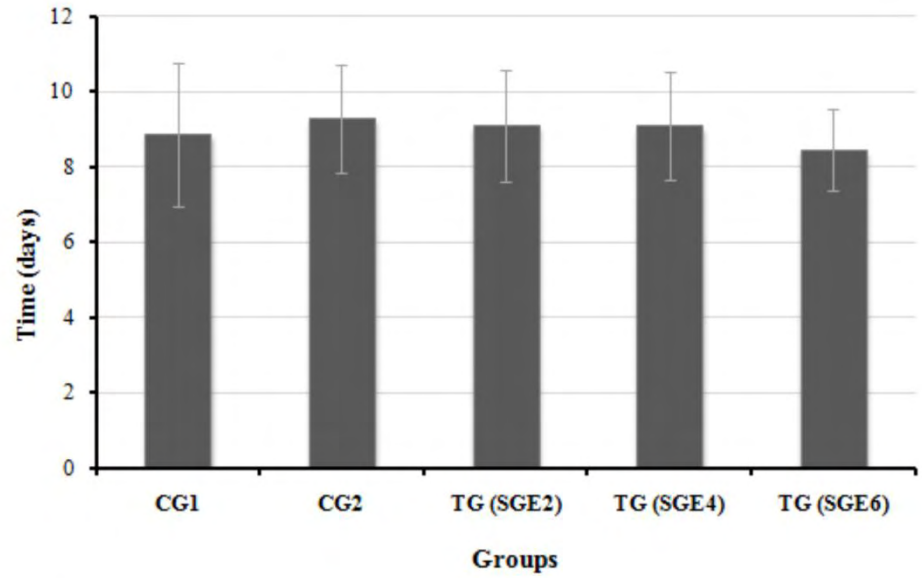
Feeding parameters of *Rhipicephalus sanguineus* females fed on hosts immunized with glandular extracts of 2= **TG (SGE2)**, 4= **TG (SGE4)** and 6 days = **TG (SGE6)** and non-immunized = **CG1** e **CG2**.

A. Feeding time.

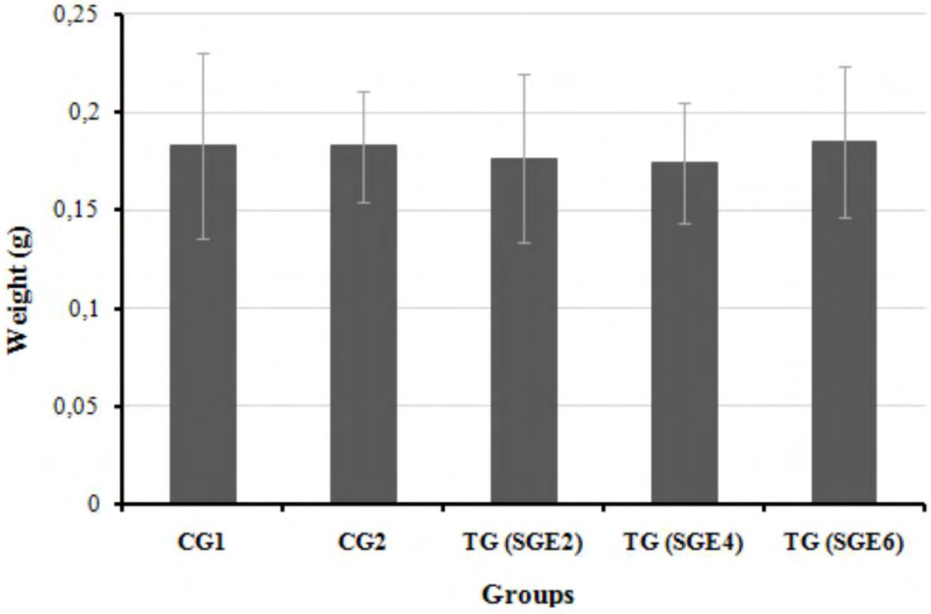
B. Engorgement weight.

C. Feeding efficiency rate (FER).

A


12
10
8
6
4
2
0
Time (days)
CG1
CG2
TG (SGE2)
TG (SGE4)
TG (SGE6)
Groups

B


0,25
0,2
0,15
0,1
0,05
0
Weight (g)
CG1
CG2
TG (SGE2)
TG (SGE4)
TG (SGE6)
Groups

C

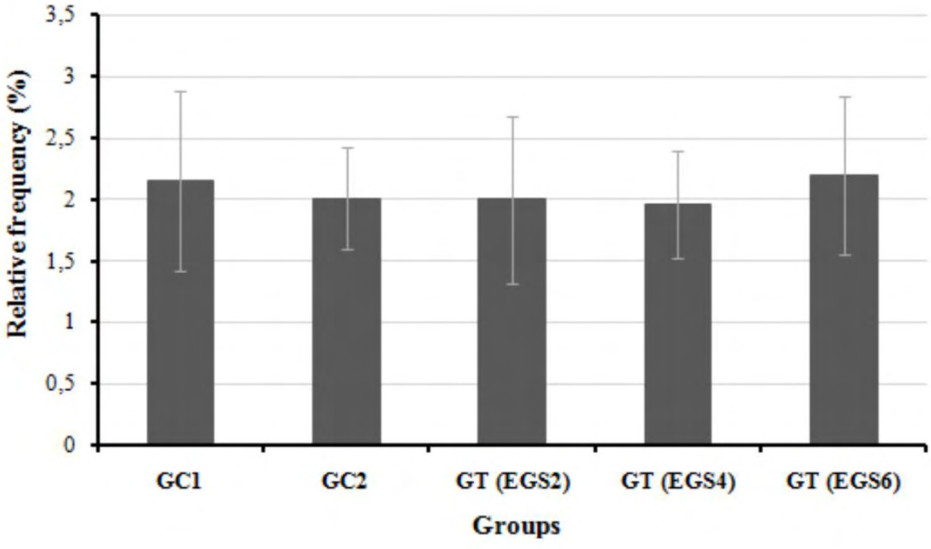

3,5
3
2,5
2
1,5
1
0,5
0
Relative frequency (%)
GC1
GC2
GT (EGS2)
GT (EGS4)
GT (EGS6)
Groups

Figure 3

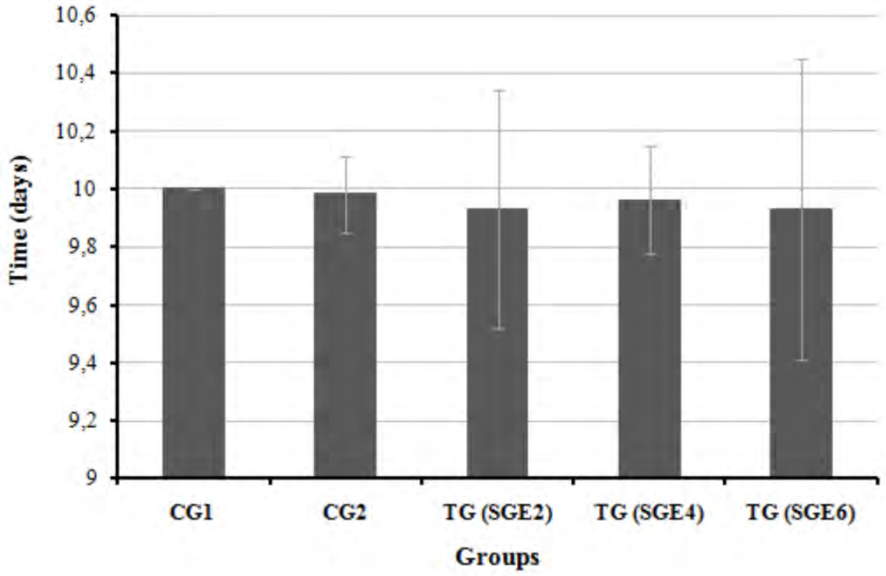
Reproductive parameters of *Rhipicephalus sanguineus* females fed on hosts immunized with glandular extracts of 2= **TG (SGE2)**, 4= **TG (SGE4)** and 6 days = **TG (EGS6)** and non-immunized = **CG1** and **CG2**.

A. Oviposition time.

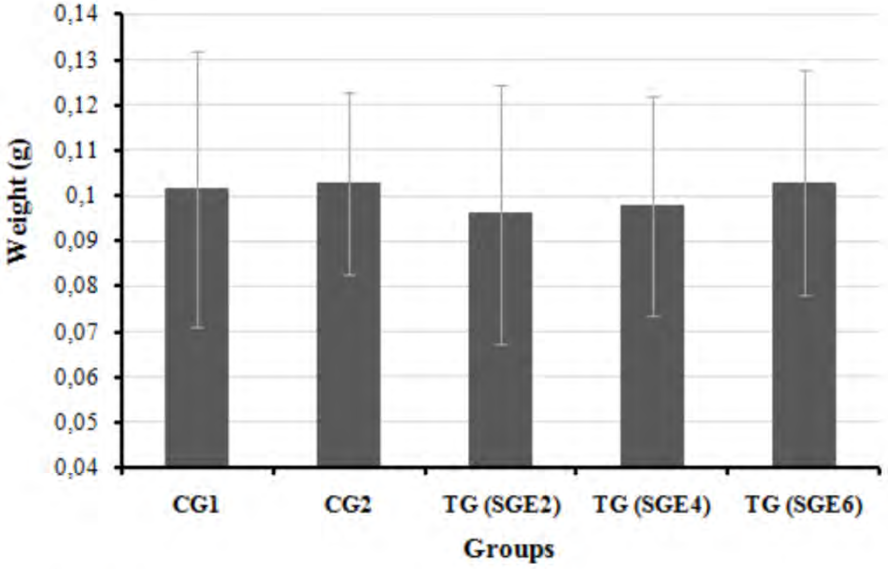
B. Egg mass weight.

C. Eggs feasibility.

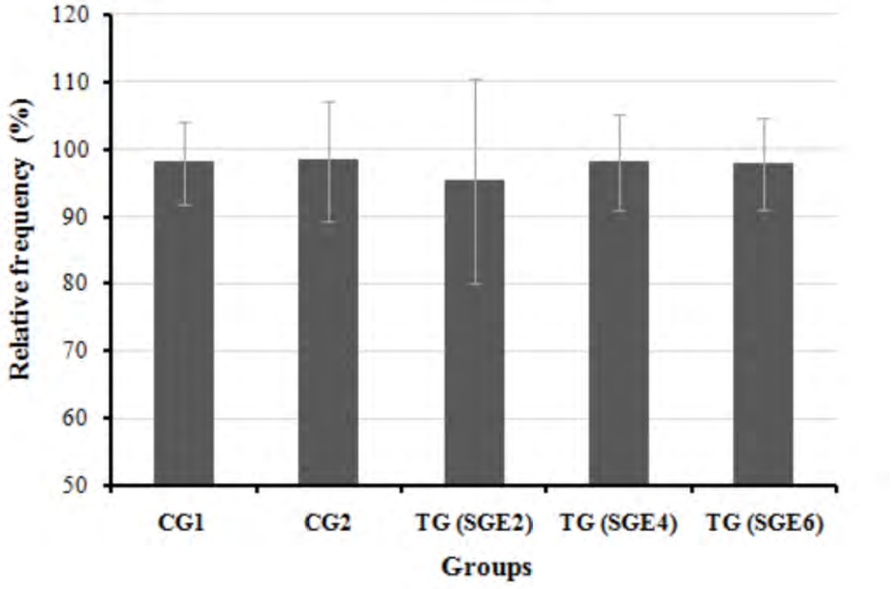
A


10,6
10,4
10,2
10
9,8
9,6
9,4
9,2
9
Time (days)
CG1
CG2
TG (SGE2)
TG (SGE4)
TG (SGE6)
Groups

B


0,14
0,13
0,12
0,11
0,1
0,09
0,08
0,07
0,06
0,05
0,04
Weight (g)
CG1
CG2
TG (SGE2)
TG (SGE4)
TG (SGE6)
Groups

C


120
110
100
90
80
70
60
50
Relative frequency (%)
CG1
CG2
TG (SGE2)
TG (SGE4)
TG (SGE6)
Groups

Discussion

The present study showed the potential of glandular extracts of *R. sanguineus* ticks to induce resistance by analyzing the feeding and reproductive parameters of females obtained from challenge infestation. In this sense, many studies have been performed to verify the ability of different extracts - such as extract of salivary glands, intestine or even whole ticks - to induce resistance (Wikel 1980; Ferreira et al. 1996; Szabó and Bechara 1997; Jittapalapong et al. 2000a, b, 2008).

According to Jittapalapong et al. (2000a) both the immunization of the hosts with ticks' extracts and the performance of successive infestations are ways to stimulate the development of resistance in *R. sanguineus* ticks' hosts. However, according to the authors, the immunization with glandular extract would represent a great impact in the feeding parameters, while the resistance acquired by successive infestations would cause less alteration in the feeding and reproductive performance of the ticks after the third infestation.

The averages obtained from the values of feeding and reproductive parameters for the females form the challenge infestation did not present significant differences, except for the average engorgement weight for group **TG (SGE6)** ($p < 0.05$) in relation to **CG1.** However, even not being significantly different under the biological point of view, these small variations in the averages occurred due to the pronounced differences in the inflammatory responses developed by the hosts from the different groups (Hebling et al. submitted paper).

In the present study, differently from other experiments (Wikel 1981; Szabó and Bechara 1997; Jittapalapong et al. 2000a), the occurrence of non significant differences in the feeding and reproductive parameters in ticks from group **TG (SGE2-SGE6)** in relation to **CG1** could be explained by the fact that the expression of resistance in immunized animals would be different among the different hosts species (Szabó and Bechara 1997), once the mechanism of resistance development would be varied and dependent on the host-ectoparasite interaction involved (Willadsen 1980; Oberem 1984).

The feeding efficiency rate in group **CG2** was reduced in comparison to **CG1**. On the other hand, the oviposition time, egg mass weight and feasibility in **CG2** were higher than **CG1**, making it clear that the inoculation of Freund's adjuvant stimulated a

king of response by the host that reduced the ticks' blood consumption what, however, was compensated by a better reproductive performance of the ticks in group **CG2**. The fact that the glandular extracts had been inoculated along with the adjuvant did not affect the response to the host to each extract, because the results of the feeding and reproductive parameters obtained in group **TG** presented differences which would have been caused by the different antigens of **SGE2-SGE6** extracts, once all of them were inoculated along with the adjuvant.

Szabó and Bechara (1997) have concluded in their study on extracts of salivary glands and intestine of partially engorged *R. sanguineus* females that the use of Freund's adjuvant along with antigens from the intestine would stimulate the development of non-specific cellular immunity by the dog. Thus, the authors have suggested that in the development of resistance by the host, an adequate mechanism to induce immunity would be as important as the obtainance of the correct antigen and that both factors would act in synergy against the tick, which would justify the need of the use of adjuvant along with antigens for the inoculation of the host.

According to the results here obtained, although with some few differences, **SGE2** and **SGE4** extracts induced resistance in the hosts, which tended to affect the feeding and reproductive parameters negatively, including the rates of feeding efficiency, which were the most reduced, data that corroborate those obtained by other authors concerning the development of resistance as a consequence of immunization of hosts with glandular extracts (Wikel 1981; Needham et al. 1986; Szabó and Bechara 1997; Jittapalapong et al. 2000a).

Furthermore, data presented above would be in accordance to those provided by Willadsen (1980), Brown et al. (1984) and Brown (1985), who have reported that the most common ways of manifestation of resistance would be the reduction of the number of ticks that would attach, engorge and undergo ecdise in any stage, the death of the ectoparasite, the enlargement of the engorgement period, reduction of weight of the females fed and reduction of egg mass, as well as decrease in the egg feasibility rate.

On the other hand, **SGE6** extract did not present the ability to immunize the hosts against the ticks, once this group presented the highest feeding efficiency rate among the analyzed groups. On the contrary, this extract contributed to the feeding and reproductive success of the females from group **TG (SGE6)**, which presented the higher

percentage of females recovery. In a short period of time (shorter observed time) they reached the highest engorgement weight and their feasibility and egg mass were the highest indexes in comparison to other test groups and **CG1** itself.

Considering data here obtained by the performance of the challenge test in females from **TG (SGE6)**, it was concluded that **SGE6** extract would present immunogenic molecules which would stimulate an inflammatory response which would benefit the ticks' consumption of blood. Such fact is supported by Hebbling et al. (submitted paper), who reported that the response of the hosts from group **TG (SGE6)** to infestation by *R. sanguineus* would be characterized by the early occurrence of intense areas of edema and later development of inflammatory infiltrate. This demonstrated that **SGE6** could not be considered a good antigen choice, corroborating Wikel (1976), who reported that whole ticks' extract would contain substances which would reduce the immune response of the host to infestation by such ectoparasites. Therefore, data here obtained indicate that antigen molecules present in **SGE6** extract presented a way of action on the host similar to those found by Wikel (1976).

The information provided by this study confirms the choice of 2, 4 and 6 days of feeding for the obtainance of glandular extracts (**SGE2**, **SGE4** and **SGE6**, respectively). According to Furquim et al. (submitted paper), in the salivary glands of *R. sanguineus* females fed for 2 days cells *b*, *c2*, *c4*, *c5*, *c6* and *f* would be active, in females fed for 4 days cells *c5* and *f* would become inactive, remaining active only cells *a*, *b*, *c1*-*c4* and *c6* and finally in those fed for 6 days there would be inactivation of cells *c6*, showing that the actuation of cells *c5*, *c6* and *f* would be crucial for the feeding process and permanence of the ticks on the host, and would also act in a specific way to modulate the immune-inflammatory response.

According to the results here obtained, **SGE4** extract tended to decrease the number of individuals that reached complete engorgement (reduction of the % parameter of recovery), i.e., acted on the ticks' feeding process and caused the death of many. On the other hand, although **SGE2** has also acted on the feeding process, the reduction of eggs feasibility rate was more significant, i.e., it acted directly on reproduction. Thus, although with few differences, the antigens of **SGE4** extract would control the infestation reducing the number of infesting individuals and **SGE2** would control via reduction of the number of descendents, data that corroborate Turni et al.

(2002); who reported that different information could be obtained about the resistance acquired by hosts sensibilized to one or more tick species as the immunosupressant molecules synthesized by the salivary glands would be differently expressed during their feeding process.

In addition, Jittapalapong et al. (2000a) have concluded that the immunization with different antigens from salivary and intestinal glands of *R. sanguineus* females would act differently in the reduction of infestation, once the glandular extract would affect the period of feeding and engorgement weight, while the immunization with intestinal extract would reduce the parameters related to the ticks fecundity, including periods of pre-oviposition, oviposition and incubation of eggs, and would also reduce the production of eggs and their feasibility. Thus, it is proposed here that the antigen molecules which are present in **SGE2** extract would act similarly to those present in the intestinal extract in the experiment by Jittapalapong et al. (2000a).

The present study also demonstrated that both **SGE2** and **SGE4** extracts reduced the infestation by *R. sanguineus* ticks, although the different immunogenic molecules from both have stimulated different responses, i.e., an extract acted on the feeding process and the other on the reproduction of the ticks, showing that a possible combination of them would be an efficient way to control these ectoparasites controlling the transmission of pathogens. In addition, this study showed that it is important to understand the reaction of the hosts to prescribed antigens because the developed response can, opposite to what was is expected, contribute to the tick's feeding success, data observed during the inoculation by **SGE6** extract.

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References

Almeida APG, Bechara GH, Varma RMG (1994) Cross-reactivity between hard tick antigens. Braz J Med Biol Res 27: 697-707

Balashov YuS (1983) An Atlas of Ixodid Tick Ultrastructure. In: Raikhel AS, Hoogstraal II (ed) Entomological Society of America (Special Publication), Lanham, pp 99-128

Bechara GH, Szabó MPJ, Ferreira BR, Garcia MV (1995) *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Rev Bras Parasitol Vet 4(2): 61-66

Brown SJ (1985) Immunology of acquired resistance to ticks. Parasitol Today 1: 166-171

Brown SJ, Barker RW, Askenase PW (1984) Bovine resistance to *Amblyomma americanum* ticks: an acquired immune response characterized by cutaneous basophils infiltrates. Vet Parasitol 16: 147-165

Caperucci D, Bechara GH, Camargo-Mathias MI (2009) Histopathology and ultrastructure features of the midgut of adult females of the tick *Amblyomma cajennense* Fabricius, 1787 (Acari: Ixodidae) in various feeding stages and submitted to three infestations. Ultrastruct Pathol 33:249–259

Caperucci D, Bechara GH, Camargo-Mathias MI (2010) Ultrastructure features of the midgut of the female adult *Amblyomma cajennense* ticks Fabricius,1787 (Acari: Ixodidae) in several feeding stages and subjected to three infestations. Micron. doi: 10.1016/j.micron.2010.05.015

Ferreira BR, Machado RZ, Bechara GH (1996) Western blot analysis of tick antigens from a *Rhipicephalus sanguineus* unfed larval extract and identification of antigenic

sites in tick sections using immunohistochemistry. A comparative study between resistant and susceptible host species. Vet Parasitol 62: 161-174

Gill HS, Boid R, Ross CA (1986) Isolation and characterization of salivary antigens from *Hyalomma anatolicum anatolicum*. Parasite Immunol 8: 11-25

Jittapalapong S, Stich RW, Gordon JC, Wittum TE, Barriga OO (2000a) Performance of female *Rhipicephalus sanguineus* (Acari: Ixodidae) fed dogs exposed to multiple infestations or immunization with tick salivary gland or midgut tissues. J Med Entomol 37(4): 601-611

Jittapalapong S, Stich RW, Gordon JC, Bremer CA, Barriga OO (2000b) Humoral Immune Response of Dogs Immunized with Salivary Gland, Midgut, or Repeated Infestations with *Rhipicephalus sanguineus*. Ann N Y Acad Sci 916: 283–288

Jittapalapong S, Phichitrasilp T, Chanphao H, Rerkamnuychoke W, Stich RW (2008) Immunization with tick salivary gland extracts. Ann N Y Acad Sci 1149: 200-204

Monteiro GER, Bechara GH (2008) Cutaneus basophilia in the resistance of goats to *Amblyomma cajennense* nymphs after repeated infestations. Ann N Y Acad Sci 1149: 221-225

Monteiro GER, Bechara GH, Franzin AM, IKF de Miranda Santos (2010) Antigen-presenting in draining lymph nodes of goads repeatedly infested by the Cayenne tick *Amblyomma cajennense* nymphs. Exp Appl Acarol. doi: 10.1007/s10493-010-9380-x

Needham GR, Jaworski D, Pemberton B, Muller MT (1986) Resistance expressed by hosts to feeding by adult lone start ticks. In: Borovsky D, Apielman A (ed) Host Regulated Developmental Mechanisms in Vector Arthropods, University of Florida-IFAS, Vero Beach, pp. 168-174

Nunes PH, Bechara GH, Camargo-Mathias MI (in press) Secretory process of salivary glands of female *Amblyomma cajennense* Fabricius, 1787, (Acari: Ixodidae) ticks fed on resistant rabbits. Exp Appl Acarol. doi: 10.1007/s10493-010-9388-2

Oberem PT (1984) The immunological basis of host resistance to ticks. J S Af Vet Med Assoc 55: 215-217

Sauer JR, Essenberg RC, Bowman AS (2000) Salivary glands in ixodid ticks: control and mechanism of secretion. J Insect Physiol 46: 1069-1078

Sonenshine DE (1991) Biology of ticks. Oxford University Press, New York

Szabó MPJ, Bechara GH (1997) Immunisation of dogs and guinea pigs against *Rhipicephalus sanguineus* ticks using gut extract. Vet Parasitol 68: 283-294

Turni C, Lee RP, Jackson LA (2002) Effect of salivary gland extracts from the tick, Boophilus microplus, on leucocytes from Brahman and Hereford cattle. Parasite Immunol 24: 355-361

Veronez VA, de Castro MB, Bechara GH, Szabó MPJ (2010) Histopathology of *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks fed on resistant hosts. Exp Appl Acarol 50: 151-161

Wikel SK (1976) Acquired resistance in guinea pigs to the ixodid tick, *Dermacentor andersoni* (Stiles, 1908). Ph.D Thesis, University of Saskatchewan
Wikel SK (1981) The induction of host resistance to tick infestation with salivary gland antigen. Am J Trop Med Hyg 30(1): 284-288

Wikel SK, Graham JE, Allen JR (1978) Acquired resistance to ticks. IV. Skin reactivity and *in vitro* lymphocyte responsiveness to salivary gland antigen. Immunol 34: 257-263

Willadsen P (1980) Immunity to ticks. Adv Parasitol 18: 293-313

Willadsen P (1997) Novel vaccines for ectoparasites. Vet Parasitol

Capitulo V

TICKS' RESPONSE TO FEEDING ON HOST IMMUNIZED WITH GLANDULAR EXTRACTS OF *Rhipicephalus sanguineus* FEMALES FED FOR 2, 4 AND 6 DAYS. I. INACTIVITY OR EARLY DEGENERATION OF SALIVARY GLANDS?

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Resumo

O presente estudo analisou histologicamente as glândulas salivares de fêmeas de *Rhipicephalus sanguineus* alimentadas por 2, 4 e 6 dias em hospedeiros previamente imunizados com extratos glandulares obtidos de fêmeas da mesma espécie em diferentes períodos de alimentação tendo como principal objetivo verificar a ação destes extratos no ciclo secretor dessas glândulas. Para tanto, extrato glandular de fêmeas alimentadas por 2 dias (**SGE2)**, extrato glandular de fêmeas alimentadas por 4 dias (**SGE4),** e extrato glandular de fêmeas alimentadas por 6 dias (**SGE6)** foram obtidos a partir de glândulas salivares de fêmeas de *R. sanguineus* alimentadas por 2, 4 e 6 dias, respectivamente. Coelhos New Zealand White virgens de infestação foram inoculados com os extratos (grupo teste = **TG**), ou com uma mistura de tampão fosfato e adjuvante completo de Freund (grupo controle 2 = **CG2**). Cada coelho inoculado **(TG** e **CG2)** e não inoculado (grupo controle 1= **CG1**) foi posteriormente infestado com 15 casais de *R. sanguineus.* Dessas fêmeas foram coletadas as glândulas salivares aos 2, 4 e 6 dias de alimentação. Os resultados revelaram que a resistência adquirida por imunização com os extratos afetou diferentemente a atividade secretora das células glandulares. Verificou-se que a resistência adquirida por meio dos extratos **SGE2** e **SGE4** agiu nas células dos ácinos II e III, sendo **c1** e **c5** do ácino II e **d** do III inativadas devido a ação do **SGE2** e **c1** e **c4** do II e **f** do III inativadas por ação do **SGE4**. A resistência ao **SGE6** mostrou efeito somente sobre as células do ácino II (**c1**, **c3** e **c4**), as quais foram inativadas. Assim, a resistência aos extratos **SGE2-SGE6** determinou maior precocidade ao processo degenerativo em comparação ao **CG1.** Por outro lado, a resistência aos extratos não influenciou as características do processo degenerativo normalmente encontrado nas glândulas salivares. O assincronismo do processo degenerativo foi mantido – o ácino III foi sempre o mais afetado e o ácino I o menos afetado. As alterações estruturais celulares, como a vacuolização citoplasmática, alterações nucleares e formação de corpos apoptóticos, que caracterizam a ocorrência de apoptose atípica, também foram mantidas nas glândulas dos indivíduos do **TG** tornando claro que a imunização dos hospedeiros com os extratos **SGE2-SGE6** influenciaram a fisiologia glandular do *R. sanguineus,* sendo uma importante informação na busca de um método de controle desse ectoparasita.

Palavras-chaves: Imunização, resistência**,** *Rhipicephalus sanguineus*, glândulas salivares, ciclo secretor, degeneração.

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ORIGINAL PAPER

Ticks' response to feeding on host immunized with glandular extracts of *Rhipicephalus sanguineus* females fed for 2, 4, and 6 days. I. Inactivity or early degeneration of salivary glands?

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Abstract The present study histologically analyzed the salivary glands of *Rhipicephalus sanguineus* females fed for 2, 4, and 6 days in hosts which had been previously immunized with glandular extracts obtained from females from this same species in different periods of feeding, having as main objective verify the action of these extracts in the secretor cycle of these glands. For this, glandular extract of females fed for 2 days (*SGE2*), glandular extract of females fed for 4 days (*SGE4*), and glandular extract of females fed for 6 days (*SGE6*) extracts were obtained from salivary glands of *R. sanguineus* females fed for 2, 4, and 6 days respectively. Then, New Zealand White naive rabbits were inoculated either with extracts (test group= TG), or with a mixture of phosphate buffer and Freund's complete adjuvant (control group 2=CG2). Each inoculated rabbit (TG and CG2) and non-inoculated (control group 1= CG1) was posteriorly infested with 15 couples of fasting *R. sanguineus* from which the salivary glands had been collected from females fed for 2, 4, and 6 days. The results revealed that the resistance the hosts had acquired by the immunization with the extracts affected differently the secretory activity of the glandular cells. It was verified that the resistance to SGE2 and SGE4 extracts acted in the cells of acini II and III, being *c1* and *c5* from II and *d* from III inactivated due to the action of SGE2 and *c1* and *c4* from II and *f* from III inactivated by the action of SGE4. As for the resistance to SGE6 the effect was only on cells of acini II (*c1*, *c3* e *c4*), which were also inactivated. In addition, the hosts' resistance to SGE2–SGE6 extracts made the degenerative process earlier in comparison to CG1. On the other hand, the resistance to the extracts did not influence the characteristics of the degenerative process normally found in salivary glands. The assynchronism of the degenerative process was maintained—acini III were always the most affected and I the less affected. The structural cell alterations, such as cytoplasmic vacuolation, nuclear alterations and formation of apoptotic bodies which characterize the occurrence of atypical apoptosis were also maintained in the glands of individuals from TG making it clear that the immunization of the hosts with glandular extracts SGE2–SGE6 had influenced the glandular physiology of *R. sanguineus,* which is an important piece of information in the search for a way to control these ectoparasites.

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Introduction

The pharmacological and immunological properties of ticks' saliva provide them with a very successful spoliative action on different species of animals (Ribeiro et al. 1985; Wikel 1999). The saliva is a complex and efficient mixture which acts in the modulation of immune-inflammatory modulation of the host, allowing feeding and the permanence on the host (Ribeiro et al. 1985; Wikel 1999; Sauer et al. 2000). In addition, the fact that the saliva immunosuppresses

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the host makes the ticks distinguished transmitters of pathogenic agents which are veiculated by the saliva itself (Wikel 1999).

The ticks' saliva is produced by salivary glands, organs that present great morphofunctional complexity (Till 1961; Binnington 1978; Balashov 1983; Walker et al. 1985; Fawcett et al. 1986; Gill and Walker 1987; Sonenshine 1991; Marzouk and Darwish 1994). In the females they are composed by types I, II, and III acini (Binnington 1978; Walker et al. 1985; Gill and Walker 1987; Sonenshine 1991). Considering the importance of acini II and III for the feeding and attachment of the ticks (Binnington 1978; Walker et al. 1985), as well as in the release of pathogens, these have to be deeply studied (Jittapalapong et al. 2008).

In the ticks, acini II are constituted by *a*, *b*, *c1*, *c2*, *c3* e *c4* cells (Binnington 1978) and in *Rhipicephalus sanguineus* females *c5* and *c6* (Furquim et al., submitted paper) were additionally found. It is known that *a* cells are involved with cement secretion for the construction of the fixation cone (Binnington 1978; Walker et al. 1985; Fawcett et al. 1986; Gill and Walker 1987), and *b* and *c* with several functions that have been attributed to the saliva in the modulation of the host's immune-inflammatory response (Binnington 1978; Walker et al. 1985). Acini III are formed by *d*, *e* and *f* cells (Binnington 1978; Walker et al. 1985; Gill and Walker 1987). *d* and *e* secrete components of the cement (Binnington 1978; Walker et al. 1985; Gill and Walker 1987) and *f* have two functions: secretory and osmoregulative (Binnington 1978; Walker et al. 1985; Gill and Walker 1987).

In the ticks, the salivary glands (Wikel et al. 1978; Gill et al. 1986; Almeida et al. 1994; Ferreira et al. 1996; Szabó and Bechara 1997; Jittapalapong et al. 2000a; Nunes et al. 2010) and the intestine (Szabó and Bechara 1997; Jittapalapong et al. 2000a; Caperucci et al. 2009, 2010; Veronez et al. 2010) are important sites of antigens production. Because of this, after the first contact of these ectoparasites with some hosts, the latter develop resistance (Wikel et al. 1978; Gill et al. 1986; Jittapalapong et al. 2000a; Zhou et al. 2006). In this sense, several studies are carried out to verify the acquisition of resistance by the hosts when these are immunized by successive infestations (Nunes et al. 2010; Monteiro and Bechara 2008; Monteiro et al. 2010; Veronez et al. 2010; Caperucci et al. 2009, 2010; Jittapalapong et al. 2000a, b) or by the inoculation of extracts of whole ticks or part of them (Wikel 1981; Ferreira et al. 1996; Szabó and Bechara 1997; Jittapalapong et al. 2000a, b, 2008).

The acquisition of resistance by the hosts is measured by the analysis of feeding and reproductive parameters of infesting ticks of previously immunized animals (Wikel 1981; Szabó and Bechara 1997; Jittapalapong et al. 2000b), as well as by the analysis of the impact of this resistance in the salivary glands (Sanders et al. 1996; Jittapalapong et al. 2008; Nunes et al. 2010) and intestines (Veronez et al. 2010; Caperucci et al. 2009, 2010) of different species.

According to Wikel (1981) and Jittapalapong et al. (2000b, 2008) the immunization from antigens originated in the salivary glands would stimulate the immunological response of the hosts which would affect the ticks of subsequent infestations. This would be caused by the direct action of the resistance acquired by the host in the salivary glands' secretory cycle reducing the efficiency of the feeding process and the transmission of pathogens by the ticks (Jittapalapong et al. 2008).

There are no studies which address the immunization of hosts with extracts of salivary glands of *R. sanguineus* females in different periods of the glandular cycle, which would provide important information to understand how the host's organism would respond to pre-immunization with the different glandular components of this species. The interest in studies of this nature would be justified by the fact that *R. sanguineus* species is considered cosmopolite and the most predominant of Ixodidae family (Pegram et al. 1987a, b). In addition to being an important transmitter of diseases like botounneuse fever for the men and babesiosis and erchilliosis for dogs (Walker et al. 2000). Furthermore, the ability of *R. sanguineus* to transmit *Rickettsia rickettsii*—the causing agent of Rocky Mountain spotted fever to the human being (Demma et al. 2005) and canine visceral leishmaniosis (Coutinho et al. 2005; Dantas-Torres 2008).

Considering the information above, the present study had the objective to histologically analyze the salivary glands of *R. sanguineus* females with 2, 4, and 6 days of feeding subjected to the infestation on hosts previously immunized with glandular extracts.

Material and methods

Material

To perform this study, salivary glands of adult *R. sanguineus* females fed for 2, 4, and 6 days subjected to infestation on New Zealand White rabbits immunized with glandular extracts were used. For this, fasting individuals (males and females) from a colony kept in BOD incubator, in controlled conditions (29°C, 80% of humidity and photoperiod of 12 h), in the Biotery of the Biology Department of UNESP *campus* Rio Claro, SP were used in the infestations **A**, **B** and **C** made in rabbits according to the procedure described by Bechara et al. (1995).

– Infestation A: made in naive rabbits using 25 couples of *R. sanguineus* couples/host, for the acquisition of females of ticks fed for 2 (55 individuals), 4 (36 individuals), and 6 (25 individuals) days, for the

obtainance of glandular extracts: *SGE2*=glandular extract of females fed for 2 days, *SGE4*=4 days, and *SGE6*=6 days.

The chosen periods of feeding (2, 4, and 6 days of feeding) were determined based on the cycle of salivary gland secretion, once fasting individuals have glands containing *a*, *c1*, *c3*, *d*, and *e* active; those fed for 2 days presented activation of *b*, *c2*, *c4*, *c5*, *c6*, and *f* cells, in those fed for 4 days cells *c5* and *f* became inactive remaining alive only cells *a*, *b*, *c1–c4*, and *c6* and finally individuals fed for 6 days had inactivation of *c6* cells (Furquim et al., submitted paper).

The extracts were processed and inoculated in the hosts subjected to infestation B.

- Infestation B (test group=TG): was made in 12 sensitized rabbits, four of them inoculated with SGE2 extract, four with SGE4, and four with SGE6, which were subjected to challenge infestation with 15 couples of adult *R. sanguineus* ticks/host.
- Infestation C (control group=CG): made in eight naive rabbits, considering that four of these animals were not inoculated (control group 1=CG1) and four were inoculated with a mixture of the Freund's complete adjuvant and phosphate buffer (control group 2=CG2) and then these animals were subjected to challenge infestation with 15 couples of adult *R. sanguineus* ticks/host.

This experiment was approved by the Ethics Committee in Research and Scientific Merit–UNIARARAS, Protocol n° 021/2009.

Methods

In the Laboratory of the Department of Molecular Biology of UNESP Rio Claro, SP, Brazil, the salivary glands were put separately (glands of females fed for 2, 4, and 6 days) in Eppendorfs tubes containing 200 μL of phosphate buffer pH 7.4. They were then macerated, centrifuged for 30 min at 10,000×*g*, the supernatant was collected and was put for proteins dosage, which occurred according to the methodology described by Sedmak and Grossberg (1977) (Bradford method), which should be of at least 0.2 μg/μL.

After the determination of the protein content of each sample, the extracts were filtered, separately by situation of study, with the help of sterile filtering units (JBR610303, disposable filtering unit Millex GV, durapore membrane PVDF, Millipore, MilliUni), of 0.22 μm and 13 mm of diameter, attached to hypodermic syringes in the interior of a pre-sterile vertical laminar flow. The extracts were then divided in volumes of 50 μL and kept in freezer at −20°C. Only at the moment of inoculation the extracts were mixed (50 μL of extract/host) with 50 μL of complete Freund's complete adjuvant (reference # F 5881, Sigma-Aldrich), as well as the mixture of Freund's complete adjuvant with phosphate buffer pH 7.4 (FA+PBS), procedures that are also made in pre-sterile vertical laminar flow.

After, rabbits from TG and CG2 had the right dorsal side sheared and were subcutaneously inoculated with SGE2, SGE4, and SGE6 extracts, as well as with FA+PBS, via hypodermic syringe, for three times in intervals of 21 days. Only after 15 days from the last inoculation, all the hosts from TG and CG were subjected to infestation challenge with 15 couples of *R. sanguineus*/host.

In the sequence, *R. sanguineus* females fed for 2, 4, and 6 days were removed from the rabbits inoculated with SGE2, SGE4 e SGE6 (GT) extracts, with the mixture FA+PBS (GC2), as well as from those not inoculated (CGI) and subjected to histological analysis.

Histological analysis

In the Histology Laboratory of the Biology Department of UNESP *campus* Rio Claro, SP, Brazil, salivary glands of *R. sanguineus* females of each group (TG e CG1 e 2) fed for 2, 4, and 6 days were removed in buffered saline solution (7.5 g NaCl+2.38 g Na_2HPO_4+2.72 g KH_2PO_4 in 1,000 mL distilled water) and fixed in 4% paraformaldehyde at 4°C. After fixation, the material was dehydrated in a series of increasing concentrations of ethanol (70%, 80%, 90%, and 95%), embedded in resin (Leica), and sectioned at 3 μm thickness. Sections were mounted on glass slides and stained with hematoxylin-eosin. Slides were mounted with Canada balsam and examined and photographed under Motic BA 300 light microscope.

Results

Control group 1

- Females fed for 2 days

  Salivary glands are intact (Fig. 1a–c).
  Acini I present dilated lumen (Fig. 1a).
  Acini II present reduced lumen and contain cells *a*, *c1*, *c2*, *c3*, *c4* and *c5* all of them full of secretion (Fig. 1a–b).
  Acini III present reduced lumen and contain cells *d*, *e*, and *f*, all of them full of secretion (Fig. 1c).

- Females fed for 4 days

  Salivary glands are intact (Fig. 1d–g).
  Acini I with reduced (Fig. 1d) or dilated (Fig. 1e) lumen.

Acini II present slight increase in the diameter of the lumen (Fig. 1e–f) and cells *a*, *c2*, *c3*, *c4*, and *c5* are observed, all of them full of secretion granules (Fig. 1e–f).
Acini III present reduced lumen (Fig. 1d) or a slight increase in the diameter (Fig. 1g), in addition, cells *d*, *e* and *f* are also observed, *d* and *e* full of secretion granules (Fig. d, g).

– Females fed for 6 days

In these, acini I, II, and III and Indeterminate are present, the latter scarce and this way named due to the advanced stage of degeneration which made their identification difficult and countless apoptotic bodies (Fig. 1h–n). Acini II and III also present degenerative characteristics, more intense in the latter (Fig. 1j–l).
Acini I present very dilated lumen (Fig.1h).
Acini II present reduced lumen, intact (Fig. 1i) or in degeneration (beginning of vacuolation; Fig. 1j), the latter being more numerous. Secretory cells *a*, *c1, c2*, *c3*, and *c4* are also observed, full of secretion granules (Fig. 1i–j).
As for acini III, these can present reduced (Fig. 1k) or dilated (Fig. 1l) lumen. In addition, they present *d*, *e*, and *f* cells, only *d* and *e* contain secretion granules and *f*, cytoplasmic vacuolation (Fig. 1k–l).

Control group

The salivary glands of females fed for 2, 4, and 6 days on the hosts of this group presented similar characteristics to the females from CG1 fed for 2, 4, and 6 days, respectively.

Test group

Inoculation of glandular extract of 2 days

– Females fed for 2 days

These salivary glands were intact (Fig. 2a–e).
Acini I presented reduced (Fig. 2a) or dilated (Fig. 2b) lumen.
Acini II presented reduced lumen (Fig. 2b–d). Cells *a*, *c2, c3*, *c4* and *c5* are present, all of them full of secretion (Fig. 2b–d).
Acini III present reduced lumen and in them there are *d*, *e*, and *f* cells, all of them full of secretion (Fig. 2e).

– Females fed for 4 days

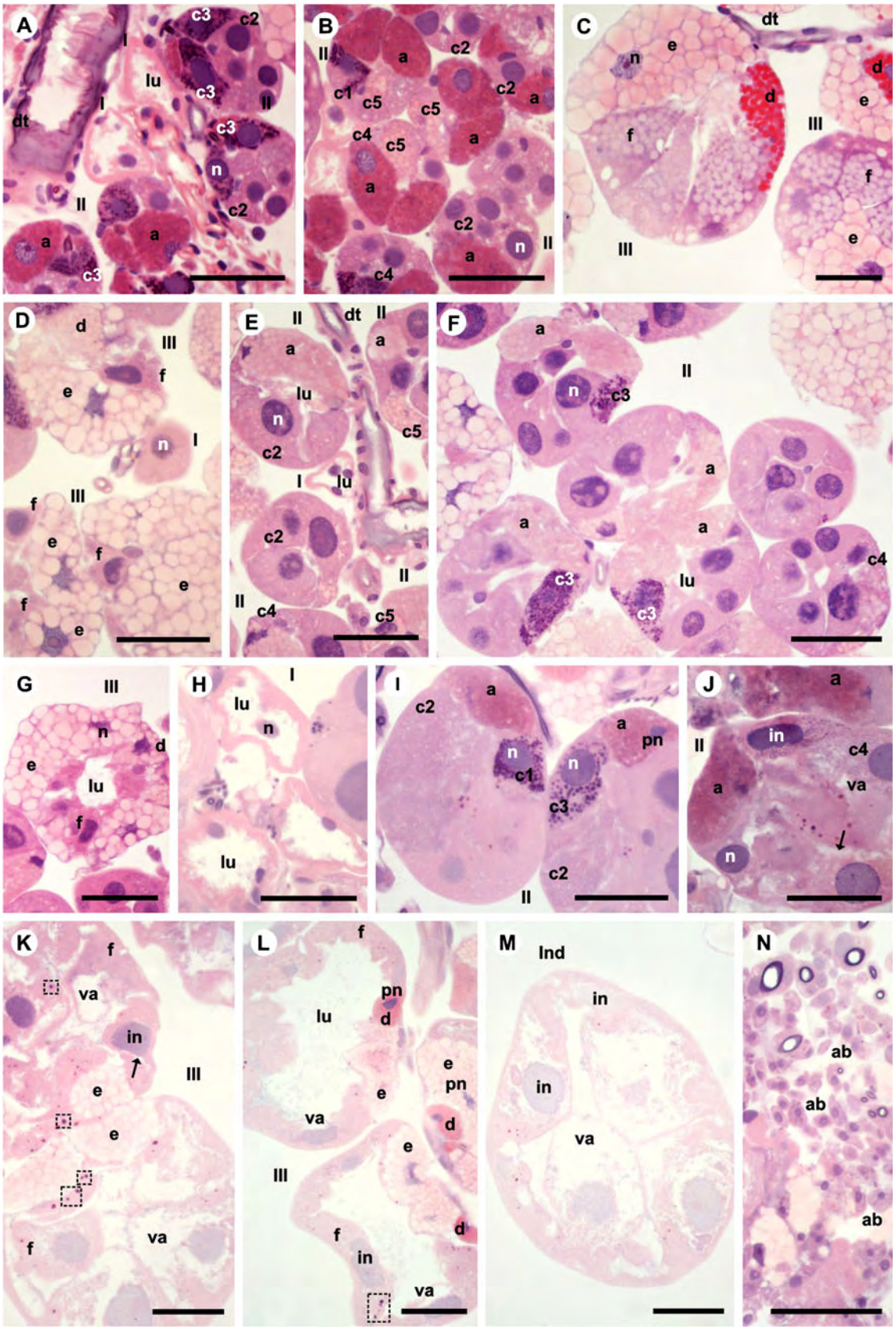
These glands present signs of degeneration such as beginning of cytoplasmic vacuolation (Fig. 2h–j) and the presence of apoptotic bodies (Fig. 2k).

Fig. 1 Histological sections of salivary glands of *Rhipicephalus sanguineus* females used as control group 1 (CG1). **a–c** Two days of feeding presenting the glandular tissue intact, synthesis activity and secretion and cells onset **a** (*a*), *c1*, *c2*, *c3*, *c4*, and *c5* of acini II (*II*) (**a–b**) and *d*, *e*, and *f* of III (*III*) (**c**) full of secretion. **d–g** Four days of feeding with intact glands and intense secretory activity. In acini II (*II*) there are cells *a*, *c2*, *c3*, *c4*, and *c5* (**e**, **f**) and in III cells *d* (**d**), *e* and *f* (*III*) (**d**, **g**), the latter without secretion. Acini II (*II*) present dilated lumen (*lu*) (**e–f**). Acini III (*III*) present reduced (**d**) or dilated (**g**) lumen (*lu*). **h–n** Six days of feeding with acini II (*II*) (**j**), III (*III*) (**k–l**) and indeterminate (*Ind*) (**m**) presenting signs of degeneration. Acini II (*II*) are intact or in degeneration (**j**). Acini III (*III*) present reduced (**k**) or dilated (**l**) lumen (*lu*) and their cells *f* (**f**) (**k–l**) present intense cytoplasmic vacuolation (*va*). Acini in degeneration present loss of cellular contact (*arrow*) (**j–k**), cytoplasmic vacuolation (*va*) (**j–m**), irregular (*in*) (**j**, **k**, **m**), picnotic (*pn*) (**l**), and fragmented (*dotted rectangle*) (**k**) nuclei. There are also apoptotic bodies (*ab*) (**n**). *I* acinus type I, *lu* acinus lumen, *n* nucleus; *a* cell a, *c1* cell c1, *c2* cell c2, *c3* cell c3, *c4* cell c4, *d* cell d, *e* cell e. *Bars* 50 μm

Acini I are intact and can present reduced (Fig. 2f) or dilated (Fig. 2g) lumen.
Acini II are starting to degenerate, present increase size in relation to those from females fed for 2 days and subjected to the extract of 2 days and reduced lumen (Fig. 2h). In addition, many acini II present cells without secretion granules (Fig. 2h).
In acini II cells *a*, *c2*, *c3*, and *c4* are observed, all of them full of secretion granules (Fig. 2h).
Acini III are in process of degeneration and can present reduced (Fig. 2i) or slightly dilated (Fig. 2j) lumen, being the former more numerous.
In acini III cells *d*, *e*, and *f* are found (Fig. 2i–j). Cells *d* and *e* are full of secretion granules and in *f*, the cytoplasm does not contain secretion granules in addition to being disorganized due to the beginning of vacuolation(Fig. 2i–j).

– Females fed for 6 days

Glands are in degeneration (Fig. 2l–p), beginning cytoplasmic vauolation (Fig. 2m–p), which is more intense than that observed in the females fed for 4 days and subjected to glandular extract of days as well as the presence of apoptotic bodies (Fig. 2q).
All kinds of acini with degenerative characteristics are found (Fig. 2l–p).
Acini I are in degeneration and present very dilated lumen (Fig. 2l).
Acini II are in degeneration, with reduced lumen and with cells *a*, *c2*, *c3*, and *c4*, all full of secretion granules (Fig. 2m). In addition, some acini with cells free of secretion granules were found (Fig. 2m).
Acini III are in degeneration, therefore presenting irregular shape, cytoplasmic vacuolation, with reduced (Fig. 2n) or dilated (Fig. 2o) lumen, being the latter more numerous.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In acini III only cells *e* are found, full of secretion granules, and *f*, free of secretion and presenting cytoplasmic vacuolation (Fig. 2n–o).

Inoculation of glandular extract of 4 days

– Females fed for 2 days

Glands are intact (Fig. 3a–f).
Acini I present dilated lumen and with irregular shape (Fig. 3a).
Acini II present reduced (more numerous) lumen (Fig. 3b) or slight increase in the diameter (Fig. 3c–d). Cells *a*, *c2*, *c3*, *c4*, and *c5*, contain secretion (Fig. 3b–d).
Acini III present reduced (Fig. 3e) or dilated lumen (Fig. 3f), being the former more numerous. Cells *d*, *e* and *f* are all full of secretion (Fig. 3e–f).

– Females fed for 4 days

Degenerative signs are found in acini II (Fig. 3h–j) and III (Fig. 3k–j) being more numerous in the latter. In addition, apoptotic bodies are also found (Fig. 3m).
Acini I are intact and present dilated lumen (Fig. 3g).
Acini II are little developed, starting cytoplasmic vacuolation (Fig. 3h–i) and some with dilated lumen (Fig. 3i), while others present reduced lumen (Fig. 3h, j). Cells *a*, *c1*, *c2*, *c3*, e *c5* are found (Fig. 3h–j).
Acini III present reduced (Fig. 3k) or dilated lumen (Fig. 3l), the latter more numerous.
In acini III cells *d*, *e* (Fig. 3k–l), and *f* (Fig. 3k) are present, the latter presenting degenerative signs, such as cytoplasmic vacuolation, more significant.

– Females fed for 6 days

These glands present degenerative signs such as vacuolation of glandular cells (Fig. 3n–q), nuclear fragmentation (Fig. 3p) and presence of apoptotic bodies (Fig. 3r), signs that are more intense than those detected in the salivary glands of females fed for 4 days subjected to glandular extract of 4 days.
In these glands acini I, II, and III were found (Fig. 3n–p), with degenerative characteristics such as cytoplasmic vacuolation and nuclear fragmentation. In addition, acini in advanced degenerative stage were found, and as they could not be identified were named indeterminate (Fig. 3q).
Acini I present reduced or dilated lumen (Fig. 3n).
Acini II present degeneration signs, reduced lumen and cells *a*, *c1*, *c2*, *c3*, and *c4*, contain secretion (Fig. 3o).
All acini III present degenerative characteristics and reduced lumen. In addition, cells *d* and *e* are full of secretion granules (Fig. 3p).

Fig. 2 Histological sections of salivary glands of *Rhipicephalus sanguineus* females used as test group TG (SGE2). **a–e** Two days of feeding with intact glandular tissue, in the beginning of synthesis activity an secretion with the cells **a** *a*, *c2*, *c3*, *c4*, and *c5* of acini II (*II*) (**b–d**) and *d* (**d**), *e* (**e**), and *f* (**f**) of acini III (*III*) (**e**) full of secretion. **f–k** Four days of feeding with acini II (*II*) (**h**) and III (*III*) (**i–j**) presenting degeneration signs. Acini III (*III*) present reduced (**i**) or dilated (**j**) lumen (*lu*) and their cells *f* (**f**) present intense cytoplasmic vacuolation (*va*). Acini in degeneration present loss of cellular contact (*arrow*) (**h**), cytoplasmic vacuolation (*va*) (**h–i**), irregular (in) (**h–j**) and picnotic (*pn*) (**h–i**) nuclei. There are also apoptotic bodies (**ab**) (**k**). **l–q** Six days of feeding with acini I (**l**), II (**m**), III (**n–o**) and indeterminate (Ind) (**p**) in degeneration. Acini III present reduced (**n**) or dilated (**o**) lumen (lu) and their cells *f* (**f**) present intense cytoplasmic vacuolation (*va*). Acini in degeneration present loss of cellular contact (*arrow*) (**m**), cytoplasmic vacuolation (*va*) (**m–p**), irregular nuclei (in) (**o–p**), picnotic nuclei (pn) (**l–n**) and nuclei with chromatic marginalization (*dotted circle*) (**m**). There are also apoptotic bodies (*ab*) (**q**). *I* acinus type I, *dt* duct, *n* nucleus, *v* valve, *lu* acinus lumen, *asterisk* cell without secretion granules, *a* cell a, *c2* cell c2, *c3* cell c3, *c4* cell c4, *d* cell d, *e* cell e, *f* cell *f*. *Bars* 50 μm

Inoculation of glandular extract of 6 days

– Females fed for 2 days

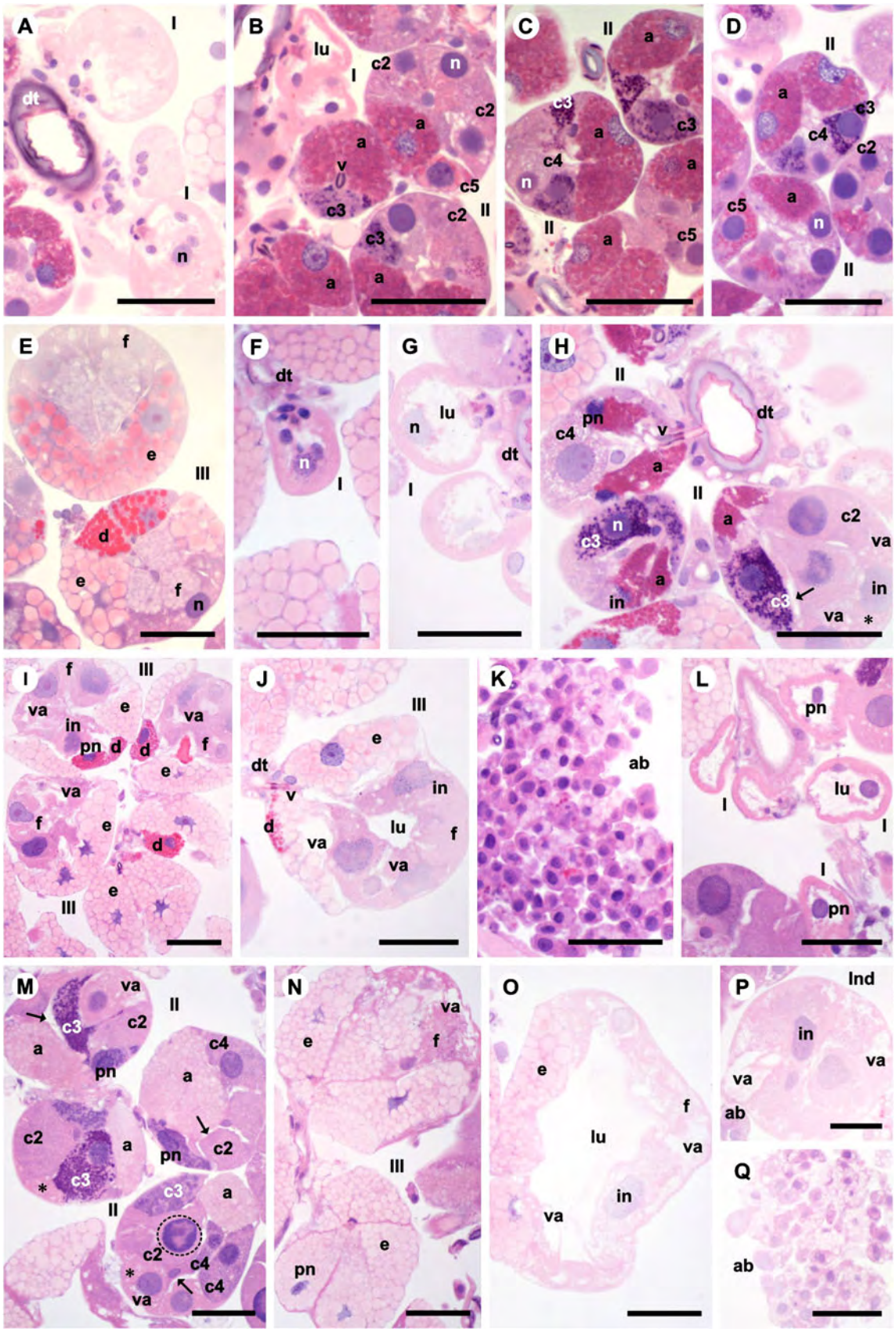
Salivary glands are intact (Fig. 4a–d).
Acini I present reduced or dilated lumen (Fig. 4a), the former more numerous and irregular-shaped.
Acini II present reduced lumen and cells *a*, *c2*, *c3*, *c4*, and *c5* are full of secretion (Fig. 4b–c).
Acini III present reduced lumen and cells *d*, e and *f*, contain secretion (Fig. 4d).

– Females fed for 4 days

The glandular tissue presents signs of degeneration in all types of acini I, II, and III (Fig. 4f–j). Apoptotic bodies were also found (Fig. 4k).
Acini I are intact (Fig. 4e) or in degeneration (Fig. 4f) with reduced lumen.
Acini II are in degeneration, present reduced lumen and cells *a*, c1, *c2*, and *c5* contain secretion (Fig. 4g–h).
Acini III are in degeneration and are found with reduced or slightly (Fig. 4i) or very dilated (Fig. 4j) lumen. Those with reduced lumen are more numerous and those that have more dilated lumen are more scarce.
Cells *d*, *e*, and *f* are present and *f* do not contain secretion granules presenting slight signs of degeneration (Fig. 4i–j).

– Females fed for 6 days

The glands present degenerative signs (with numerous cytoplasmic vacuoles, nuclear fragmentation, and presence of apoptotic bodies; Fig. 4m–p) that is more intense than those observed in the females fed for 4 days and subjected to the same glandular extract.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Acini I, II, III and indeterminate, all of them are with degenerative characteristics (Fig. 4l–p).
Acini I present dilated lumen (Fig. 4l).
Acini II present reduced lumen and cells *a*, *c1*, *c2*, *c3*, and *c4* contain secretion granules (Fig. 4m–n).
Acini III present reduced lumen and cells *d*, *e*, and *f* (Fig. 4O); *f* present intense vacuolation in the cytoplasm without secretion granules (Fig. 4o).
The results of the glandular alterations found in females fed for 2, 4, and 6 days form groups CG1 and TG are summarized in Tables 1, 2, 3, 4, and 5.

Fig. 3 Histological sections of the salivary glands of *Rhipicephalus sanguineus* femalesused as test group TG (SGE4). **a–f** Two days of feeding with intact cells, in the beginning of the secretory activity and cells *a*, *c2*, *c3*, *c4*, and *c5* of acini II (*II*) (**b–d**) and *d* (**d**), *e* (**e**), e *f* (**f**) of acini III (*III*) (**e–f**) full of secretion. Acini II (*II*) and III (*III*) present reduced (**b**, **e**) or dilated (**c–d**, **f**) lumen (lu). **g–m** Four days of feeding with degenerative signs in acini II (*II*) (**h–j**) and III (*III*) (**k–l**). Acini II (*II*) and III (*III*) present reduced (**h**, **j–k**) or dilated (**i**, **l**) lumen (lu). Acini in degeneration present loss of cellular contact (*arrow*) (**h**, **j**), cytoplasmic vacuolation (*va*) (**h–i**, **k–l**), irregular nuclei (*in*) (**h**, **k–l**), picnotic nuclei (*pn*) (**h–i**) and nuclei with chromatic marginalization (*dotted circle*) (**i–j**). There are also apoptotic bodies (*ab*) (**m**). **n–r** Six days of feeding with acini I (*I*) (**n**), II (*II*) (**o**), III (*III*) (**p**) and indeterminate (*Ind*) (**q**) in degeneration. In acini III (*III*) the cells *f* (**f**) present intense cytoplasmic vacuolation (*va*). Acini in degeneration present loss of cellular contact (*arrow*) (**o**), cytoplasmic vacuolation (*va*) (**n–q**), irregular (*in*) (**n–p**), picnotic (*dotted circle*) (**n–p**) and fragmented (*dotted rectangle*) (**p**) nuclei. Apoptotic bodies (*ab*) (**r**) are also observed. *I* acinus type I, *lu* acinus lumen, *n* nucleus, *v* valve, *dt* duct, *a* cell a, *c1* cell c1, *c2* cell c2, *c3* cell c3, *c4* cell c4, *c5* cell c5, *d* cell d, *e* cell e, *f* cell *f*. *Bars* 50 μm

Discussion

The present study provided histological results of salivary glands of *R. sanguineus* females fed for 2, 4, and 6 days in rabbits which were previously immunized with glandular extracts SGE2–SGE6. It is clear that the resistance of the host influenced in the secretory cycle of the infesting females glands, acting both in the secretory activity of the glandular cells (making them inactive) and leading the glands to early degeneration. Nunes et al. (2010) and Jittapalapong et al. (2008) have reported that the resistance acquired by the hosts studied by them induced the salivary glands of *Amblyomma cajennense* and *R.* (*Boophilus*) *microplus* females, respectively, to necrosis. On the other hand, the present study showed that the resistance of the host led to the loss of function (inactivity) of some secretory cells as well as to the early degeneration of the salivary glands due to the occurrence of apoptosis. However, it is known that different species of ticks respond differently to the resistance acquired by the host (Nunes et al. 2010).

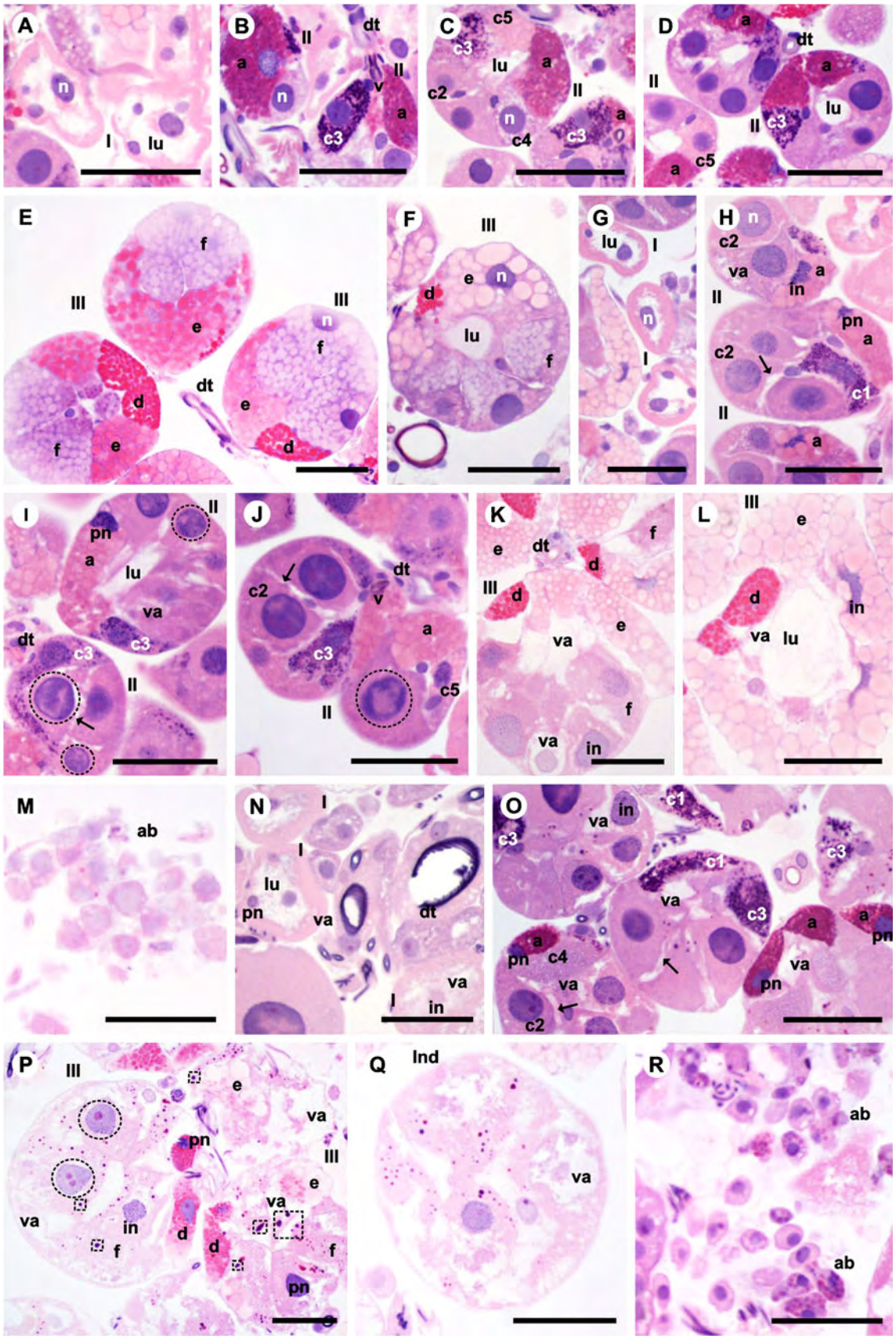
The results here obtained showed that under the histological point of view, the glands of females fed for 2, 4, and 6 days from CG1 (control) are similar to those from CG2, demonstrating that Freund's adjuvant, used with glandular extracts did not influence the glandular physiology. As for the glandular extracts, it is clear that they would induce the development of resistance by the hosts, which affected the glandular physiology, i.e., the glands had the secretory activity reduced, becoming less efficient when compared to individuals from CG1, data that corroborate Jittapalapong et al. (2008), who reported that the immunization of the host with glandular extracts would affect directly the salivary glands of the infesting ticks, which would make the feeding process less efficient and would possibly reduce the transmission of pathogens.

The results here presented showed that the resistance acquired by the hosts immunized with SGE2, SGE4, and SGE6 extracts affected much more acini II and III cells than those of acini I. This has probably occurred because these cells present secretory function, while those of acini I act on the osmoregulation and in the hydric balance of the ectoparasite (Binnington 1978; Walker et al. 1985). Thus, cells of acini II and III are responsible for the secretion of antigenic molecules and shape the immune-inflammatory system of the host, corroborating Jittapalapong et al. (2008) and Nunes et al. (2010) who also demonstrated that acini II and III cells were more affected by the resistance of the host.

Adding to this, Ferreira et al. (1996) demonstrated that acini II and III of *R. sanguineus* ticks would be the ones that synthesize more and secrete antigenic molecules which would cause the development of elevated immunologic response of the hosts. Gill et al. (1986) reported the occurrence of intense immunological response to some glandular antigens of *Hyalomma anatolicum anatolicum* tick, such as glycoproteins, acid phosphatase, esterase and aminopetidase, molecules synthesized mainly by the glandular cells of acini II and III (Binnington 1978; Walker et al. 1985).

Other important point considered in the present study was the resistance of the hosts of each of the GT groups affected the salivary glands of females fed for 2, 4, and 6 days. In those fed on hosts immunized with SGE2 and SGE4, there were alterations in cells of acini II and III, while in those subjected to SGE6 only those of II were affected, demonstrating that the immune system of the hosts responded in a different and specific way to the immunization with different extracts.

The resistance to extract SGE2 acted on the secretory activity of cells *c1* of acini II, which were inactive for three glandular periods, *c5* of acini II, which were inactive in the glands of females fed for 4 days and in cells *d* of acini III, which would stay inactive in the glands of females fed for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6 days. As for the resistance to SGE4, it acted on cells *c1* and *c4* of acini II and *f* of acini III. Cells *c1* were inactive in glands of 2 days and started to present activity in those of 4 days and 6 days. Cells *c4* were active in glands of 2 days, lost activity in those of 4 days and became active again in glands of 6 days. As for cells *f*, they were active in glands of 2 and 4 days and became inactive in those of 6 days.

The resistance acquired with the immunization with extract **SGE6** acted only on cells *c1*, *c3*, *c4*, *c1* were inactive in the glands of 2 days and started to present activity in those of 4 and 6 days. Cells *c3* e *c4* were active in glands of 2 days, became active in those of 4 and start to present activity again in those of 6 days.

It was demonstrated here that the glandular cells that had their secretory performance more affected by the resistance of the host were *c1*. The *c4* presented intermediate alterations, followed by *c3*, *c5*, *d*, and *f*. Data here obtained about cells from group *c* and *f* corroborate those obtained by Jittapalapong et al. (2008), who showed that these cells would be very affected by the resistance of the host and the study by Nunes et al. (2010) corroborated data here obtained for cells *c1*, *c3*, and *c4*, once according to the authors the cells from group *c* would also be very affected by the action of the resistance of the host.

Here, the fact that cells *c1*, *c3*, *c4*, *c5*, *d*, and *f* were affected by the resistance indicated that the secretions produced by them would be highly immunogenic in relation to those from other types of cells present in the salivary glands. In addition, the alterations suffered by cells *c1*, *c3*, *c4*, *c5*, *d*, and *f* caused alterations in the glandular secretory process, reducing it, which caused a reduction in the feeding efficiency of *R. sanguineus* females and demonstrated that such cells would possibly play an important role in the modulation of the host. Differently from what was here demonstrated, Almeida et al. (1994) reported that the molecules from cells *a*, *d*, and *e* would be the most immunogenic, while those from group *c* would not present this characteristic.

The fact that the secretions of cells *c1*, *c3*, and *c4* are related to the modulation of the host corroborates Binnington (1978) and Walker et al. (1985), who reported that the cells from group *c* would act in the manipulation of the response of the host. According to Binnington (1978), cells *c1* of acini II of the salivary glands of *Boophilus microplus* females would be responsible for the production of esterase, which, according to Geczy et al. (1971) would act in the increase of the vascular permeability and in the hydrolisis of cholesteryl ester.

From the analysis of the glands of females fed for 4 and 6 days on host immunized with extract SGE4 important information was obtained about cells *c4* concerning their participation in the modulation of the host. It was observed that these cells were inactive in glands of 4 days, impairing their glandular performance, which made the ectoparasite's feeding deficient. This can be proved by the fact that cells *f* of glands of 6 days were inactive, because in normal conditions they would be hypertrophied due to their intense osmoregulating action, which would occur due to the full consumption of blood by the tick, making it clear that the secretion produced by cells *c4* would present, in addition to others, the function of modulating the vasodilating mechanisms and coagulation of the host's blood, which would allow the great consumption of blood by the ectoparasite.

Although literature has indicated that *d* cells are involved with the fabrication of components of the cement cone during the attachment of the tick (Walker et al. 1985; Gill and Walker 1987), the results here obtained demonstrated that, in *R. sanguineus* females, the secretion produced by these cells also play a role in the modulation of the host, once they were affected by the resistance acquired by the animals immunized with SGE2 extract. Binnington (1978), studying the salivary glands of *B. microplus* females, observed that cells *d* would also synthesize and secrete acid phosphatase, enzymes that catalyze the molecules break (digestion). Thus, considering what has previously been reported, it can be inferred that the secretion released by cells *d* of *R. sanguineus* females would also be acting in the digestion of the tissues of the host's skin in order to maintain the feeding lesion open.

In addition to the ability of resistance of the hosts from group TG in making some glandular cells inactive, it is clear that this resistance affected the glandular cells *a* and *c5* of acini II and *d* and *e* of acini III in a way that they altered the composition of the synthesized secretion, alteration reflected in the histology and that varied both according to the study group and to the period of feeding of the females, allowing the assumption that the secretion synthesized by such cells would be immunogenic and would probably act in the modulation of the host.

Fig. 4 Histological sections of salivary glands of *Rhipicephalus sanguineus* females used as test group TG (SGE6). **a–d** Two days of feeding with intact glandular tissue, beginning of the secretion phase with cells *a*, *c2*, *c3*, *c4*, and *c5* dos acini II (*II*) (**b–c**) e *d* (**d**), *e* (**e**) e *f* (**f**) (**d**) of III (*III*) full of secretion. **e–k** Four days of feeding with intact (*I*) acini I (*e*) or in degeneration (*f*) and acini II (*II*) and III (*III*) in degeneration (**g–j**). Os acini III (*III*) present reduced (**i**) or dilated (**j**) lumen (*lu*). In acini in process of degeneration are observed loss of cellular contact (*arrow*) (**h–j**), cytoplasmic vacuolation (*va*) (**f**, **i–j**), irregular (*in*) (**e**, **g**, **i–j**), and picnotic (*pn*) (**g**) nuclei. Apoptotic bodies are also observed (*ab*) (**k**). **l–q** Six days of feeding with acini I (**i**) (**l**), II (*II*) (**m–n**), III (*III*) (**o**) and indeterminate (*Ind*) (**p**) in degeneration. In acini III (*III*) the cells *f* (**f**) (**o**) present intense cytoplasmic vacuolation (*va*). Acini in degeneration present loss of cellular contact (*arrow*) (**m–n**), cytoplasmic vacuolation (*va*) (**m–o**), irregular (in) (**m**, **o–p**) and picnotic (*pn*) (**l**, **o**) nuclei, with chromatic marginalization (*dotted circle*) (**n**, **p**) and fragmented nuclei (*dotted rectangle*) (**o**). Apoptotic bodies are also observed (*ab*) (**q**). *I* type I acinus, *lu* acinus lumen, *n* nucleus, *dt* duct, *v* valve, *a* cell a, *c1* cell c1, *c2* cell c2, *c3* cell c3, *c4* cell c4, *c5* cell c5, *d* cell *d*, *e* cell e, *f* cell f. *Bars* 50 μm

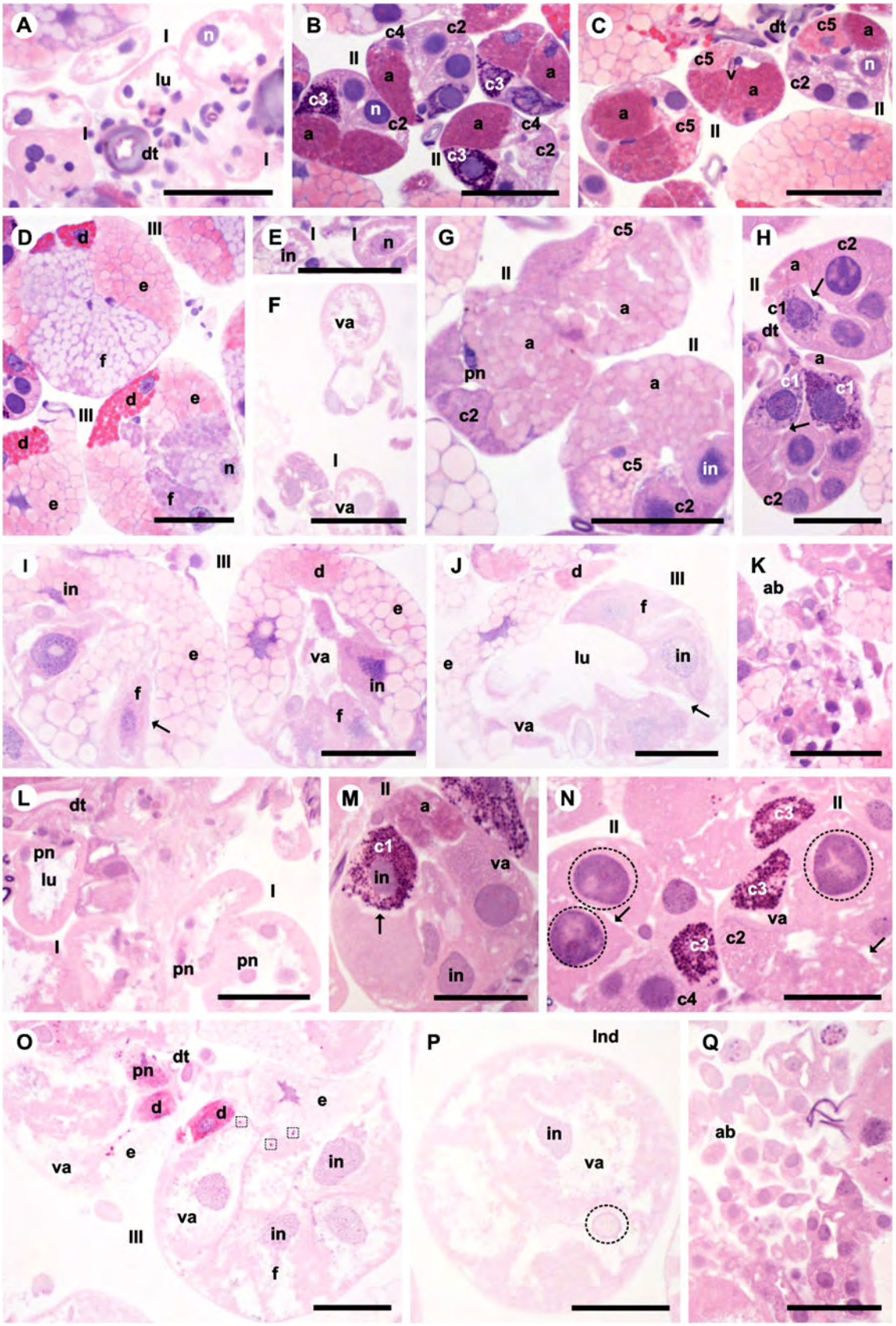
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Table 1 Comparison of the histological results of the salivary glands of *R. sanguineus* females from control group (CG1) and test group (TG) with 2 days of feeding

Acini	Study groups			
	CG1	TG		
		SG2	SGE4	SGE6
I	Intact (Fig. 1a)	Intact (Fig. 2a–b)	Intact (Fig. 3a)	Intact (Fig. 4a)
	Lumen: dilated (Fig. 1a)	Reduced (Fig. 2a) and dilated (Fig. 2b)	Dilated (Fig. 3a)	Reduced and dilated (Fig. 4a)
II	Intact (Fig. 1b)	Intact (Fig. 2b–c)	Intact (Fig. 3b,d)	Intact (Fig. 4b,c)
	Lumen: reduced (Fig. 1b)	Reduced (Fig. 2b,c)	Reduced (Fig. 3b) and dilated (Fig. 3c–d)	Reduced (Fig. 4b–c)
	Cells: *a*, *c1*, *c2*, *c3*, *c4*, *c5* (Fig. 1b)	Cells: *a*, *c2*, *c3*, *c4*, *c5* (Fig. 2b–c)	*a*, *c2*, *c3*, *c4*, *c5* (Fig. 3b–d)	*a*, *c2*, *c3*, *c4*, *c5* (Fig. 4b–c)
III	Intact (Fig. 1c)	Intact (Fig. 2e)	Intact (Fig. 3e–f)	Degeneration (Fig. 4i–j)
	Lumen: reduced (Fig. 1c)	Reduced (Fig. 2e)	Reduced (Fig. 3e) and dilated (Fig. 3f)	Reduced (Fig. 4d)
	Cells: *d*, *e*, *f* (Fig. 1c)	*d*, *e*, *f* (Fig. 2e)	*d*, *e*, *f* (Fig. 3e–f)	*d*, *e*, *f* (Fig. 4d)
Indeterminate	Absent	Absent	Absent	Absent

According to Binnington (1978), Walker et al. (1985), Fawcett et al. (1986), and Gill and Walker (1987), the secretions produced by cells *a*, *d*, and *e* (lipoprotein nature) would participate in the formation of the cement cone; however, it is known that the present other molecules in their composition such as acid phosphatase, catechol oxidase (cells *a*), and aminopeptidase (cells *d* and *e*; Binnington 1978; Walker et al. 1985). Walker et al. (1985) discussed the possibility of aminopeptidase acting on the facilitation of the infiltration of the cement in the feeding lesion, acting directly in the organism of the host. Thus, the fact that the secretions produced by cells *d* and *e* play a role in the immunogenicity can be explained.

In the present study, it was also verified that the resistance of the host, induced by glandular extracts SGE2–SGE6, led the glands of females fed for 4 days to degeneration, which would be precocious in relation to group CG1, where this alteration only took place in the glandular tissue of 6-day fed females. These data corroborate those by Jittapalapong et al. (2008) who demonstrated

Table 2 Comparison of the histological results of the salivary glands of *R. sanguineus* females from control group (CG1) and test group (TG) with 4 days of feeding

Acini	Study groups			
	CG1	TG		
		SG2	SGE4	SGE6
I	Intact (Fig. 1d–e)	Intact (Fig. 2f–g)	Intact (Fig. 3g)	Intact (Fig. 4e) and in degeneration (Fig. 4f)
	Reduced (Fig. 1d) and dilated lumen (Fig. 1e)	Reduced (Fig. 2f) and dilated (Fig. 2g)	Dilated (Fig. 3g)	Reduced (Fig. 4e–f)
II	Intact (Fig. 1e–f)	Beginning of degeneration (Fig. 2h)	Degeneration (Fig. 3f–j)	Degeneration (Fig. 4g–h)
	Lumen: dilated (Fig. 1e–f)	Reduced (Fig. 2h)	Reduced (Fig. 3h, j) and dilated (Fig. 3i)	Reduced (Fig. 4g–h)
	Cells: *a*, *c2*, *c3*, *c4*, *c5* (Fig. 1e–f)	*a*, *c2*, *c3*, *c4* (Fig. 2h)	*a*, *c1*, *c2*, *c3*, *c5* (Fig. 3h–j)	*a*, *c1*, *c2*, *c5* (Fig. 4g–h)
III	Intact (Fig. 1d,g)	Degeneration (Fig. 2i–j)	Degeneration (Fig. 3k–l)	Degeneration (Fig. 4i–j)
	Lumen: reduced (Fig. 1d) and dilated (Fig. 1g)	Reduced (Fig. 2i) and dilated (Fig. 2j)	Reduced (Fig. 3k) and dilated (Fig. 3l)	Reduced (Fig. 4i) and dilated (Fig. 4j)
	Cells: *d*, *e*, *f*[a] (Fig. 1d,g)	*d*, *e*, *f*[a] (Fig. 2i–j)	*d*, *e*, *f*[a] (Fig. 3k–l)	*d*, *e*, *f*[a] (Fig. 4i–j)
Indeterminate	Absent	Absent	Absent	Absent

[a] Cell without secretion granules

Table 3 Comparison of the histological results of the salivary glands of *R. sanguineus* females from control group (CG1) and test group (TG) with 6 days of feeding

Acini	Study groups			
	CG1	TG		
		SG2	SGE4	SGE6
I	Intact (Fig. 1h)	Degeneration (Fig. 2l)	Degeneration (Fig. 3n)	Degeneration (Fig. 4l)
	Lumen: dilated (Fig. 1h)	Dilated (Fig. 2l)	Dilated (Fig. 3n)	Dilated (Fig. 4l)
II	Intact (Fig. 1i) and in degeneration (Fig. 1j)	Degeneration (Fig. 2m)	Degeneration (Fig. 3o)	Degeneration (Fig. 4m–n)
	Lumen: reduced (Fig. 1i–j)	Reduced (Fig. 2m)	Reduced (Fig. 3o)	Reduced (Fig. 4m–n)
	Cells: *a*, *c1*, *c2*, *c3*, *c4* (Fig. 1i–j)	*a*, *c2*, *c3*, *c4* (Fig. 2m)	*a*, *c1*, *c2*, *c3*, *c5* (Fig. 3o)	*a*, *c1*, *c2*, *c3*, *c4* (Fig. 4m–n)
III	Degeneration (Fig. 1k–l)	Degeneration (Fig. 2n–o)	Degeneration (Fig. 3p)	Degeneration (Fig. 4o)
	Lumen: reduced (Fig. 1k) and dilated (Fig. 1l)	Reduced (Fig. 2n) and dilated (Fig. 2o)	Reduced (Fig. 3p)	Reduced (Fig. 4o)
	Cells: *d*, *e*, *f*[a] (Fig. 1k–l)	*e*, *f*[a] (Fig. 2n–o)	*d*, *e* (Fig. 3p)	*d*, *e*, *f*[a] (Fig. 4o)
Indeterminate	Scarce (Fig. 1m)	Scarce (Fig. 2p)	Numerous (Fig. 3q)	Numerous (Fig. 4p)

[a] Cell without secretion granules

that the immunization of the hosts with glandular extracts would precociously induce the degeneration of the salivary glands of *R.* (*Boophilus*) *microplus* females. In addition, Nunes et al. (2010) also reported that the resistance acquired by successive infestations would also induce salivary glands of *A. cajennense* females to early degeneration.

A possible explanation for the early induction of degeneration would be the mechanism which controls the synthesis of ecdisteroids, hormone which stimulates the glandular degeneration (Mao et al. 1995; Lomas et al. 1998; Weiss and Kaufman 2001), which was intensively synthesized in females from group TG (SGE2 and GS24), fed for 4 days, a precocious synthesis in relation to what was obtained in those exposed to normal conditions (occurring in the end of the feeding process; Lomas 1993).

The information above is probably explained by the acquisition of resistance by the immunized hosts, which reduced the infesting ectoparasites' blood consumption by making it more difficult (Furquim et al., submitted paper). Even with the reduction of blood ingestion these females would reach the "critical weight" (increase in ten times the weight of a fasting tick) which would trigger the initial production of ecdysteroid, as would happen in normal conditions (Weiss and Kaufman 2001). However, upon reaching 4 days of feeding they would not be able to reach the stage of rapid engorgement (consumption of a large volume of blood in a short period of time), which was

Table 4 Comparison of the result of eosin staining of secretion granules of cells *a* and *c5* of acini II and *d* and *e* of III of the salivary glands of females from CG1 and TG fed for 2, 4, and 6 days

Acini	Period of feeding	Study groups			
		CG1	TG		
			SGE2	SGE4	SGE6
II	2 Days	*a* (**5**) (Fig. 1a–b)	*a* (**5**) (Fig. 2b–d)	*a* (**5**) (Fig. 3b–d)	*a* (**6**) (Fig. 4b–c)
		c5 (**3**) (Fig. 1a–b)	*a* (**4**) (Fig. 2b–d)	*c5* (**3**) (Fig. 3**b**–d)	*c5* (**4**) (Fig. 4c)
	4 Days	*a* (**3**) (Fig. 1e–f)	*a* (**6**) (Fig. 2h)	*a* (**4**) (Fig. 3h–j)	*a* (**7**) (Fig. 4g–h)
		c5 (**2**) (Fig. 1e)	*c5*[a]	*c5* (**2**) (Fig. 3j)	*c5* (**3**) (Fig. 4g)
	6 Days	*a* (**3**) (Fig. 1i–j)	*a* (**7**) (Fig. 2m)	*a* (**5**) (Fig. 3o)	*a* (**7**) (Fig. 4m)
		c5[a]	*c5*[a]	*c5*[a]	*c5*[a]
III	2 Days	*d* (**5**) (Fig. 1c)	*d* (**5**) (Fig. 2e)	*d* (**5**) (Fig. 3e–f)	*d* (**5**) (Fig. 4d)
		e (**3**) (Fig. 1c)	*e* (**3**) (Fig. 2e)	*e* (**4**) (Fig. 3e–f)	*e* (**4**) (Fig. 4d)
	4 Days	*d* (**3**) (Fig. 1d, g)	*d* (**5**) (Fig. 2i–j)	*d* (**5**) (Fig. 3k–l)	*d* (**4**) (Fig. 4i–j)
		e (**3**) (Fig. 1d, g)	*e* (**2**) (Fig. 2i–j)	*e* (**5**) (Fig. 3k–l)	*e* (**3**) (Fig. 4i–j)
	6 Days	*d* (**3**) (Fig. 1l)	*d*[a]	*d* (**5**) (Fig. 3p)	*d* (**4**) (Fig. 4o)
		e (**0**) (Fig. 1k–l)	*e* (**1**) (Fig. 2n–o)	*e* (**6**) (Fig. 3p)	*e* (**4**) (Fig. 4o)

(***0***) no staining, (*7*) strongly positive staining

[a] Cell not observed

Table 5 Comparison of the intensity of the degenerative process in the salivary glands of *R. sanguineus* females from groups CG1 and TG fed for 2, 4, and 6 days

Salivary glands	Study groups		Intensity of glandular degeneration
2 Days	CG1		**0** (Fig. 1a–c)
	TG	SGE2	**0** (Fig. 2a–e)
		SGE4	**0** (Fig. 3a–e)
		SGE6	**0** (Fig. 4a–e)
4 Days	CG1		**0** (Fig. 1d–g)
	TG	SGE2	**1** (Fig. 2f–l)
		SGE4	**2** (Fig. 3g–m)
		SGE6	**3** (Fig. 4e–k)
6 Days	CG1		**4** (Fig. 1h–n)
	TG	SGE2	**5** (Fig. 2l–q)
		SGE4	**6** (Fig. 3n–r)
		SGE6	**7** (Fig. 4l–q)

(***0***) absence of degenerative characteristics, (*7*) more intense degenerative characteristics

proved by the need of a longer time to reach complete engorgement (Furquim et al., submitted paper).

Because of the fact that the females had not started the phase of rapid engorgement, the rest of the feeding process was impaired, and upon arriving on the sixth day of feeding did not present intense consumption of blood (Furquim et al., submitted paper) demonstrated by: (a) the weight of the completely engorged females subjected to extracts SGE2 and SGE4 was inferior to those from group CG (Furquim et al., submitted paper) and (b) the histology of the glandular cells of these females showed acini III with less dilated lumen in relation to those from CG1, a result of the less intense actuation in the osmoregulating process of the ticks (Fawcett et al. 1986; Sonenshine 1991; Sauer et al. 2000).

The fact that the females did not present intense consumption of blood showed that their feeding process was not sufficiently able to fulfill all the needs, such as provide the necessary conditions to make the vitellogenic process successful (Rosell-Davies and Coons 1989), fact that was proven by data obtained by the reduction of the eggs mass as well as by the minor feasibility of the eggs in comparison to those from CG1 (Furquim et al., submitted paper). Thus, the nervous system of the females would be activated and the synthesis of ecdysteroid already initiated, would be intensified, increasing the concentration of this hormone in the hemolymph and stimulating the glandular degeneration (Lomas 1993; Weiss and Kaufman 2001).

Possibly, the physiological mechanism which led the salivary glands of *R. sanguineus* females subjected to SGE2 and SGE4 extracts to degenerate earlier in comparison to those from CG1 is similar to the one of *Amblyomma hebraeum* females, where the females were removed from the host after having reached "critical weight", meaning that they did not present intense blood consumption, which resulted in a power body weight, continuous increase in the synthesis of ecdysteroid, leading the salivary glands to early degeneration in addition to reducing oviposition (Weiss and Kaufman 2001).

On the other hand, the stimulus for the glandular degeneration of females fed on hosts immunized with SGE6 were more significant, i.e., it was even earlier than in females subjected to extracts SGE2 and SGE4, suggesting that the occurrence of a mechanism completely opposite to the one described above. The fact that these females presented high consumption of blood and consequent increase in weight in a short period of time (Furquim et al., submitted paper), stimulated the early production of ecdysteroids, which made the process of glandular degeneration also precocious.

Thus, according to the results obtained here, the immune-inflammatory responses developed by the hosts immunized by the different glandular extracts made the occurrence of the glandular degeneration process precocious in relation to the control group (CG1). However, such responses of the hosts do not alter other characteristics observed in *R. sanguineus* females in normal conditions (Furquim et al. 2008). In this sense, it was verified that in the glandular tissue of the females from **TG** that the degenerative assynchronism was maintained, where acini II and III were the most affected in relation to acini I, data which corroborate Nunes et al. (2010). According to these authors, the glandular degeneration induced by the resistance of the host would maintain the same pattern.

In addition, in the salivary gland of females from TG the most affected cells of acini II and III were those from groups *c* and *f*, which presented intense cytoplasmic vacuolation, corroborating Jittapalapong et al. (2008), who reported that cells with more intense degenerative characteristics were *c* (agranular) and *f*.

Other degenerative glandular characteristics maintained in the glands of GT in comparison to CG1 were: loss of cellular contact, cytoplasmic vacuolation, nuclear changes (picnosis and fragmentation), as well as formation of apoptotic bodies characterizing the occurrence of atypical apoptosis, according to what was previously suggested by Furquim et al. (2008) for completely engorged *R. sanguineus* females 3 days after engorgement in normal conditions. Although Jittapalapong et al. (2008) and Nunes et al. (2010) have observed in their studies the occurrence of degenerative characteristics in the glandular cells similar to those here verified, they suggested that the acquisition of resistance by the hosts, either by immunization by glandular extracts (Jittapalapong et al. 2008) or successive infestations (Nunes

et al. 2010), led the glandular cells of *R.* (*Boophilus*) *microplus* and *A. cajennense* females to necrosis.

Thus, it was here established that the immunization of hosts with SGE2 and SGE4 glandular extracts of *R. sanguineus* females have influenced negatively in the secretory cycle of salivary glands, reducing their secretory ability and consequently reducing the ectoparasites feeding (making it less efficient) important information for the better understanding and creation of alternatives to control these ectoparasites.

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References

Almeida APG, Bechara GH, Varma RMG (1994) Cross-reactivity between hard tick antigens. Braz J Med Biol Res 27:697–707

Balashov YuS (1983) An atlas of ixodid tick ultrastructure. In: Raikhel AS, Hoogstraal II (eds) Entomological Society of America. Special Publication, Lanham, pp 99–128

Bechara GH, Szabó MPJ, Ferreira BR, Garcia MV (1995) *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. Rev Bras Parasitol Vet 4(2):61–66

Binnington KC (1978) Sequential changes in salivary gland structure during attachment and feeding of the cattle tick *Boophilus microplus*. Int J Parasitol 8:97–115

Caperucci D, Bechara GH, Camargo-Mathias MI (2009) Histopathology and ultrastructure features of the midgut of adult females of the tick *Amblyomma cajennense* Fabricius, 1787 (Acari: Ixodidae) in various feeding stages and submitted to three infestations. Ultrastruct Pathol 33:249–259

Caperucci D, Bechara GH, Camargo-Mathias MI (2010) Ultrastructure features of the midgut of the female adult Amblyomma cajennense ticks Fabricius, 1787 (Acari: Ixodidae) in several feeding stages and subjected to three infestations. Micron. doi:10.1016/j.micron.2010.05.015

Coutinho MTZ, Bueno LL, Sterzik A, Fujiwara RT, Botelho JR, de Maria M, Genaro O, Linardi PM (2005) Participation of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the epidemiology of canine leishmaniasis. Vet Parasitol 128:149–155

Dantas-Torres F (2008) Canine vector-borne deseases in Brazil. Parasit Vectors 1(25):1–17

Demma LJ, Traeger MS, Nicholson WL, Paddock CD, Blau DM, Eremeeva ME, Dasch GA, Levin ML, Singleton J, Zaki SR, Cheek JE, Swerflow DL, McQuiston JH (2005) Rocky mountain spotted fever from an unexpected tick vector in Arizona. N Engl J Med 353(6):587–594

Fawcett DW, Binnington KC, Voight WR (1986) The cell biology of the ixodid tick salivary gland. In: Sauer JR, Hair JA (eds) Morphology, physiology and behavioral biology of ticks. Ellis Horwood, Chichester, pp 22–45

Ferreira BR, Machado RZ, Bechara GH (1996) Western blot analysis of tick antigens from a *Rhipicephalus sanguineus* unfed larval extract and identification of antigenic sites in tick sections using immunohistochemistry. A comparative study between resistant and susceptible host species. Vet Parasitol 62:161–174

Furquim KCS, Bechara GH, Camargo-Mathias MI (2008) Death by apoptosis in salivary glands of females of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari, Ixodidae). Exp Parasitol 119:152–163

Geczy F, Nayghton MA, Clegg JB, Heweton RW (1971) Esterases and a carbohydrate-spliting enzyme in the saliva of the cattle tick, *Boophilus microplus*. J Parasitol 57:437–438

Gill HS, Walker AR (1987) The salivary glands of *Hyalomma anatolicum anatolicum*: nature of salivary gland components and their role in tick attachment and feeding. Int J Parasitol 18 (1):83–93

Gill HS, Boid R, Ross CA (1986) Isolation and characterization of salivary antigens from *Hyalomma anatolicum anatolicum*. Parasite Immunol 8:11–25

Jittapalapong S, Stich RW, Gordon JC, Wittum TE, Barriga OO (2000a) Performance of female *Rhipicephalus sanguineus* (Acari: Ixodidae) fed dogs exposed to multiple infestations or immunization with tick salivary gland or midgut tissues. J Med Entomol 37(4):601–611

Jittapalapong S, Stich RW, Gordon JC, Bremer CA, Barriga OO (2000b) Humoral immune response of dogs immunized with salivary gland, midgut, or repeated infestations with *Rhipicephalus sanguineus*. Ann N Y Acad Sci 916:283–288

Jittapalapong S, Phichitrasilp T, Chanphao H, Rerkamnuychoke W, Stich RW (2008) Immunization with tick salivary gland extracts. Ann N Y Acad Sci 1149:200–204

Lomas LO (1993) The influence of a male-derived protein factor on salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum* Koch. Ph.D Thesis, University of Alberta

Lomas LO, Gelman D, Kaufman WR (1998) Ecdysteroid regulation of salivary gland degeneration in the ixodid tick *Amblyomma hebraeum*: a reconciliation of in vivo and in vitro observations. Gen Comp Endocrinol 109:200–211

Mao H, Macblain WA, Kaufman WR (1995) Some properties of the ecdysteroid receptor in the salivary gland of the ixodid tick, *Amblyomma hebraeum*. Gen Comp Endocrinol 99:340–348

Marzouk AS, Darwish ZEA (1994) Changes in the salivary glands of female *Hyalomma (Hyalomma) dromedarii* during and after feeding. J Egypt Soc Parasitol 24(1):39–57

Monteiro GER, Bechara GH (2008) Cutaneus basophilia in the resistance of goats to *Amblyomma cajennense* nymphs after repeated infestations. Ann N Y Acad Sci 1149:221–225

Monteiro GER, Bechara GH, Franzin AM, de Miranda Santos IKF (2010) Antigen-presenting in draining lymph nodes of goads repeatedly infested by the Cayenne tick *Amblyomma cajennense* nymphs. Exp Appl Acarol. doi:10.1007/s10493-010-9380-x

Nunes PH, Bechara GH, Camargo-Mathias MI (2010) Secretory process of salivary glands of female *Amblyomma cajennense* Fabricius, 1787 (Acari: Ixodidae) ticks fed on resistant rabbits. Exp Appl Acarol. doi:10.1007/s10493-010-9388-2

Pegram RG, Clifford CM, Walker JB, Keirans JE (1987a) Clarification of the *Rhipicephalus sanguineus* group (Acari, Ixodoidea, Ixodidae). I. *R. sulcatus* Neuman 1908 and *R. turanicus* Pomerantsev 1936. Syst Parasitol 10:3–26

Pegram RG, Keirans JE, Clifford CM, Walker JB (1987b) Clarification of the *Rhipicephalus sanguineus* group (Acari, Ixodoidea, Ixodidae). II. *R. sanguineus* (Latreille, 1806) and related species. Syst Parasitol 10:27–44

Ribeiro JMC, Makoul GT, Levine J, Robinson DK, Spilman A (1985) Antihaemostatic, antiinflammatory and immunosupres-

sive properties of the saliva of a tick, *Ixodes dammini*. J Exp Med 161:332–344

Rosell-Davies R, Coons LB (1989) Relationship between feeding mating, vitellogenin production and vitellogenesis in the tick *Dermacentor variabilis*. Exp Appl Acarol 7:95–105

Sanders ML, Scott AL, Glass GE, Schwartz BS (1996) Salivary gland changes and host antibody responses associated with feeding of male lone star ticks (Acari: Ixodidae). J Med Entomol 33(4):628–634

Sauer JR, Essenberg RC, Bowman AS (2000) Salivary glands in ixodid ticks: control and mechanism of secretion. J Insect Physiol 46:1069–1078

Sedmak JJ, Grossberg SE (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal Biochem 79:544–552

Sonenshine DE (1991) Biology of ticks. Oxford University Press, New York

Szabó MPJ, Bechara GH (1997) Immunisation of dogs and guinea pigs against *Rhipicephalus sanguineus* ticks using gut extract. Vet Parasitol 68:283–294

Till WM (1961) A contribution to the anatomy and histology of the brown ear tick *Rhipicephalus appendiculatus* Neumann, vol 6. Memoirs of the Entomological Society of Southern Africa, Pretoria

Veronez VA, de Castro MB, Bechara GH, Szabó MPJ (2010) Histopathology of *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks fed on resistant hosts. Exp Appl Acarol 50:151–161

Walker A, Fletcher JD, Gill HS (1985) Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. Int J Parasitol 15(1):81–100

Walker JB, Keirans JE, Horak IG (2000) The genus Rhipicephalus (Acari, Ixodidae): a guide to the brown ticks of the world. Cambridge University Press, Cambridge

Weiss BL, Kaufman WR (2001) The relationship between 'critical weight' and 20-hydroxyecdysone in the female ixodid tick *Amblyomma hebraeum*. J Insect Physiol 47:1261–1267

Wikel SK (1981) The induction of host resistance to tick infestation with salivary gland antigen. Am J Trop Med Hyg 30(1):284–288

Wikel SK (1999) Tick modulation of host immunity: an important factor in pathogen transmission. Int J Parasitol 29:851–859

Wikel SK, Graham JE, Allen JR (1978) Acquired resistance to ticks. IV. Skin reactivity and in vitro lymphocyte responsiveness to salivary gland antigen. Immunology 34:257–263

Zhou J, Gong H, Zhou Y, Xuan X, Fujisaki K (2006) Identification of a glycine-rich protein from the tick *Rhipicephalus haemaphysaloides* and evaluation of its vaccine potential against tick feeding. Parasitol Res 100:77–84

Discussão Geral

5.Discussão Geral

O presente estudo avaliou o potencial de indução de resistência de extratos glandulares (**EGS2-EGS6**) em coelhos que foram posteriormente desafiados com carrapatos *Rhipicephalus sanguineus*. Esta investigação deu-se principalmente por meio da análise da resposta imune-inflamatória desenvolvida pelos hospedeiros na região da lesão de fixação dos carrapatos desafiados. Foram também analisados os dados alimentar e reprodutivo obtidos das fêmeas de carrapatos oriundas da infestação desafio, bem como o estudo histológico das glândulas salivares aos 2, 4 e 6 dias de alimentação destas fêmeas. Neste sentido, muitos trabalhos têm sido realizados para se verificar a capacidade de indução de resistência por diferentes extratos de carrapatos, como de glândulas salivares, intestino ou mesmo de indivíduos inteiros (WIKEL, 1981; FERREIRA et al., 1996; SZABÓ; BECHARA, 1997; JITTAPALAPONG et al., 2000a, b, 2008).

No presente trabalho ficou claro que o potencial de imunização dos extratos **EGS2-EGS6** refletiu diretamente nas respostas imune-inflamatórias desenvolvidas pelos hospedeiros posteriormente desafiados, o que afetou fisiologicamente de forma negativa ou positiva nos processos alimentar e reprodutivo das fêmeas de carrapatos utilizadas na infestação desafio. A literatura tem mostrado que a imunização dos hospedeiros com extratos glandulares faz com que estes desenvolvam resposta imune-inflamatória que afeta drasticamente os carrapatos neles alimentados, influenciando de forma negativa os parâmetros dos processos alimentar e reprodutivo (JITTAPALAPONG et al., 2000a).

Os dados sobre os parâmetros alimentar e reprodutivo obtidos durante a realização do teste de desafio (WIKEL, 1980; SZABÓ, BECHARA, 1997; JITTAPALAPONG et al., 2000a) mostraram aqui que as médias ± desvio padrão não apresentaram diferenças significativas, com exceção do peso médio de ingurgitamento das fêmeas do grupo **GT (EGS6)** ($p < 0.05$) quando comparadas às do **GC1**. Contudo, considerou-se que mesmo não sendo estatisticamente significativas, estas diferenças nas médias ocorreram em virtude das respostas imune-inflamatórias locais desenvolvidas pelos hospedeiros dos diferentes grupos, dado que foi comprovado pela análise histopatológica da pele destes. Assim, biologicamente estas variações nos valores dos parâmetros alimentar e reprodutivo representaram tendências que deveriam ser consideradas como resultado da imunização dos hospedeiros por meio dos extratos de glândulas salivares.

O presente estudo mostrou que a imunização dos hospedeiros com os extratos glandulares influenciou o ciclo secretor das glândulas salivares das fêmeas oriundas da infestação desafio, inativando as células glandulares e levando as glândulas a degeneração precoce.

A literatura pertinente traz muitos trabalhos que abordam aspectos da imunização de hospedeiros por meio da inoculação de extratos obtidos a partir de carrapatos ou partes destes (WIKEL, 1981; JITTAPALAPONG et al., 2000a; TURNI et al., 2002; MEJRI et al., 2002; PECHOVÁ et al., 2004), inclusive aqueles de glândulas salivares. No entanto, o presente trabalho é o primeiro a considerar a aquisição de resistência por parte de coelhos contra a infestação pelo *R. sanguineus* frente à imunização com extratos de glândulas salivares de fêmeas em períodos específicos de alimentação (2, 4 e 6 dias).

Para a realização deste trabalho foi necessária a adequação de técnicas disponíveis na literatura (WIKEL, 1981; JITTAPALAPONG et al., 2000a; TURNI et al., 2002; MEJRI et al., 2002; PECHOVÁ et al., 2004) para as condições experimentais aqui consideradas, o que tornou possível o desenvolvimento de uma metodologia para obtenção e inoculação dos extratos de glândulas salivares de fêmeas de *R. sanguineus* com 2, 4 e 6 dias de alimentação. Assim, estabeleceu-se um protocolo que trouxe a metodologia e informações quantitativas importantes para se conseguir um concentrado protéico que fosse eficaz no desenvolvimento da resposta imune nos hospedeiros inoculados, porém que não induzisse nestes o desenvolvimento de patologias.

Outro ponto importante deste protocolo foi em relação à utilização de glândulas de fêmeas de *R. sanguineus* em diferentes etapas do processo de alimentação (com 2, 4 e 6 dias), pois o conteúdo protéico encontrado no tecido glandular destas seria ideal dos pontos de vista tanto qualitativo quanto quantitativo para obtenção de extratos glandulares. De acordo com a dosagem protéica aqui obtida, ficou claro que quanto mais adiantado o processo de alimentação das fêmeas de *R. sanguineus* maior quantidade de secreção glandular é produzida com conseqüente expressão maior de proteínas. Os teores protéicos encontrados nas glândulas salivares das fêmeas com 2, 4 e 6 dias de alimentação foram diferentes, o que impediu o estabelecimento de um mesmo número (padrão) de glândulas salivares para obtenção dos diferentes extratos. Por este motivo, foi necessária a utilização de quantidades diferentes de glândulas para preparação de cada extrato específico, tendo sido utilizadas 55 fêmeas (110 glândulas salivares) com 2 dias, 36 (72 glândulas salivares) com 4 dias e 25 (50 glândulas)

com 6 dias de alimentação para obtenção dos extratos **EGS2**= glândulas com 2 dias, **EGS4**= com 4 dias e **EGS6**= com 6 dias, respectivamente.

Diferentemente do obtido no presente estudo, a metodologia descrita por Jittapalapong et al. (2000), relatou que 50 glândulas salivares seriam suficientes para preparação de extratos glandulares (15 glândulas de fêmeas e 10 de machos) a partir de indivíduos com 3 e 5 dias de alimentação. No entanto, os dados aqui apresentados corroboraram aqueles de Jaworski et al. (1990) e Inokuma et al. (1994), que relataram que glândulas salivares de espécies diferentes apresentariam diferentes antígenos, também em quantidades e concentrações diferentes, fato este que justificaria a utilização de número diferente de glândulas salivares que seria necessário para o estabelecimento deste protocolo.

Os resultados aqui obtidos mostraram que do ponto de vista histológico e histopatológico os fragmentos de pele dos hospedeiros provenientes do grupo **GC1** (controle) se assemelharam àquelas do grupo **GC2**, demonstrando que o adjuvante completo de Freund, administrado juntamente com os extratos glandulares, não influenciou a resposta imune-inflamatória local desenvolvida pelos hospedeiros.

Quanto aos resultados obtidos no teste de desafio, verificou-se que o índice de eficiência alimentar do grupo **GC2** foi reduzido em relação ao **GC1**. Por outro lado, o tempo de oviposição, o peso da massa de ovos e a viabilidade destes no **GC2** foram maiores que os do **GC1**, dados que deixaram claro que a inoculação do adjuvante de Freund afetou de alguma forma a resposta do hospedeiro refletindo na redução no consumo de sangue por parte dos carrapatos, o que foi compensado com a melhora do desempenho reprodutivo nas fêmeas do grupo **GC2**.

Szabó e Bechara (1997) verificaram que a utilização do adjuvante de Freund, juntamente com antígenos do intestino de fêmeas de *R. sanguineus*, estimulariam o desenvolvimento de imunidade celular não específica pelo cão. Então, os autores sugeriram que no estabelecimento de resistência pelo hospedeiro, um mecanismo adequado indutor de imunidade seria tão importante quanto à apresentação do antígeno correto e, ambos os fatores, atuariam em sinergia contra o carrapato, justificando a necessidade da utilização de um adjuvante juntamente com os antígenos para inoculação no hospedeiro.

O fato dos extratos glandulares terem sido inoculados juntamente com o adjuvante de Freund não afetou a resposta específica do hospedeiro para cada extrato inoculado, pois os resultados dos parâmetros alimentar e reprodutivo obtidos no grupo **GT** apresentaram

diferenças entre si, que só poderiam ser provocadas pelos diferentes antígenos presentes nos diferentes extratos **EGS2-EGS6**, pois os três foram misturados a adjuvante.

Quanto aos extratos glandulares, ficou claro que eles induziram o desenvolvimento de resistência pelos hospedeiros, manifestada por meio do aumento da resposta imune-inflamatória local, a qual influenciou os parâmetros alimentares e reprodutivos aqui considerados, dados que corroboraram aqueles disponíveis na literatura mostrando que a imunização do hospedeiro com extratos glandulares afetaria de forma negativa a alimentação e reprodução destes (JITTAPALAPONG et al., 2000a). Além disso, os dados aqui apresentados corroboraram Monteiro e Bechara (2008) que relataram aumento da resposta inflamatória na lesão de alimentação de cabras sensibilizadas por meio de infestações sucessivas com ninfas de *Amblyomma cajennense*.

Independentemente dos antígenos (**EGS2**, **EGS4** ou **EGS6**) utilizados para imunizar os hospedeiros, a intensidade da resposta foi crescente em relação ao período de infestação, ou seja, a resposta aumentou gradualmente em função do decorrer do processo de alimentação, já que foram analisadas amostras da região da lesão de fixação de fêmeas de *R. sanguineus* obtidas aos 2, 4 e 6 dias de alimentação durante a infestação desafio. Isto demonstrou que o sistema imune-inflamatório do hospedeiro respondeu gradualmente à presença das moléculas antigênicas liberadas pelos carrapatos, cujas síntese e secreção foram não só aumentando, porém também modificando-se com o decorrer do processo de alimentação. Segundo Turni et al. (2002) diversas informações seriam obtidas sobre a resistência adquirida por hospedeiros sensibilizados a diferentes espécies de carrapato ou mesmo a uma mesma espécie, visto que as moléculas imunossupressoras sintetizadas pelas glândulas salivares seriam diferentemente expressas durante a alimentação dos carrapatos.

As alterações inflamatórias locais aqui detectadas não variaram em relação à imunização com os diferentes extratos, sendo sempre observadas as mesmas características. De acordo com o grupo estudado **GT (EGS2)**, **(EGS4)** e **(EGS6)** não houve mudança nas características, mas sim na intensidade com que elas ocorreram. Além disso, ficou claro que o processo inflamatório agiu como diluidor e isolante dos agentes lesivos (antígenos oriundos da saliva), tentativa do hospedeiro de eliminar a origem da agressão. De forma geral, os extratos **EGS2** e **EGS4** foram os mais eficazes na imunização dos hospedeiros por eles sensibilizados. Contudo, considerando as metodologias aqui aplicadas, ficou claro que a imunização com o extrato **EGS4** foi a mais eficiente, sinalizando que as moléculas presentes

neste extrato apresentariam maior imunogenicidade, permitindo que a resposta do hospedeiro reduzisse a ação imunomoduladora da saliva do *R. sanguineus*.

A resposta inflamatória local desenvolvida pelos hospedeiros do grupo **GT (EGS4)** além de apresentar maior infiltrado inflamatório, foi também desenvolvida mais precocemente que a dos hospedeiros dos grupos **GT (EGS6)**. Por outro lado, nos hospedeiros do grupo **GT (EGS2)** a resposta inflamatória, de forma geral, foi menos intensa. Isto refletiu nos dados do teste desafio, que revelaram que os carrapatos alimentados nos hospedeiros do grupo **GT (EGS2)** apresentariam menor índice de eficiência alimentar em relação ao **GT (EGS4)**.

De acordo com os resultados aqui obtidos, ficou claro que mesmo com a ocorrência de diferenças pouco significativas, a resistência adquirida aos extratos **EGS2** e **EGS4** afetaria de forma negativa a alimentação e a reprodução das fêmeas do carrapato, incluindo os índices de eficiência alimentar os quais foram os mais reduzidos, corroborando dados obtidos por outros autores quanto ao desenvolvimento de resistência em função da imunização de hospedeiros com extratos glandulares (WIKEL, 1981; NEEDHAM et al., 1986; SZABÓ, BECHARA, 1997; JITTAPALAPONG et al., 2000a).

Além disso, segundo Willadsen (1980), Brown et al. (1984) e Brown (1985) as formas mais comuns de manifestação de resistência seriam a redução no número de carrapatos que se fixariam, ingurgitariam e sofreriam ecdise (em qualquer estágio), a morte dos ectoparasitas, o prolongamento do período de ingurgitamento, a redução no peso das fêmeas alimentadas e da massa de ovos, bem como a queda na viabilidade dos ovos.

A pequena diferença notada entre os parâmetros alimentar e reprodutivo dos carrapatos do grupo **GT (EGS2 e EGS4)** em relação àqueles de outros experimentos (WIKEL, 1981; SZABÓ, BECHARA, 1997; JITTAPALAPONG et al., 2000a) poderia ser explicada pelo fato de que a expressão de resistência em animais imunizados diferiria com a espécie de hospedeiro considerada (SZABÓ, BECHARA, 1997), pois o mecanismo de desenvolvimento de resistência aos carrapatos sofreria variação que dependeria da interação hospedeiro-ectoparasita (WILLADSEN, 1980; OBEREM, 1984).

Nos indivíduos do grupo **GT (EGS4)** a maior redução notada no índice de eficiência alimentar das fêmeas deste grupo, embora estatisticamente não significativa, foi decorrente do infiltrado inflamatório que foi bastante intenso em relação aos outros grupos. Além disso, segundo Monteiro e Bechara (2008) a presença de intensa basofilia (células envolvidas no processo inflamatório) indicaria o desenvolvimento de resistência dos hospedeiros contra a infestação por carrapatos.

De acordo com os resultados aqui obtidos, o extrato **EGS4** agiu no controle dos carrapatos principalmente diminuindo o número de indivíduos que atingiram o completo ingurgitamento, devido à morte (redução do parâmetro percentual de recuperação), ou seja, agiu diretamente sobre o processo de alimentação. Por outro lado, embora o **EGS2** também tenha agido sobre a alimentação, sua maior ação foi reduzindo a viabilidade dos ovos, e assim indiretamente, inibindo a reprodução. Assim, embora com poucas diferenças, os antígenos presentes no extrato **EGS4** controlaram a infestação por carrapatos reduzindo o número de indivíduos infestantes e o **EGS2** controlou a infestação reduzindo o número de descendentes, dados que corroboraram Turni et al. (2002).

Jittapalapong et al. (2000a) concluíram em seu trabalho que a imunização com diferentes antígenos oriundos das glândulas salivares e do intestino de fêmeas de *R. sanguineus* agiriam de forma diferente na redução da infestação por carrapatos. De acordo com esses autores, o extrato glandular afetaria grandemente o período de alimentação e o peso de ingurgitamento, enquanto que a imunização com extrato de intestino reduziria os parâmetros relacionados à fecundidade, incluindo períodos de pré-oviposição, oviposição e incubação dos ovos, bem como reduziria a produção de ovos e a viabilidade destes. Desta forma, poder-se-ia propor que as moléculas antigênicas presentes no extrato intestinal agiriam de forma semelhante àquelas presentes no extrato **EGS2** utilizado neste estudo.

Em relação ao extrato **EGS6**, verificou-se que este provocou nos hospedeiros o desenvolvimento de resposta imune-inflamatória local menos intensa (com menor infiltrado inflamatório) e mais tardia do que aquela observada nos indivíduos do grupo **GT (EGS4)**. Somando-se a isso, verificou-se de acordo com o teste de desafio que o extrato **EGS6** não apresentou capacidade de imunização dos hospedeiros contra os carrapatos, pelo contrário, contribuiu para o sucesso alimentar e reprodutivo das fêmeas deste grupo, onde a maior porcentagem de recuperação das fêmeas, o maior índice de eficiência alimentar em um período muito curto de tempo (menor tempo para o ingurgitamento observado entre os **GT**), maior peso de ingurgitamento, bem como redução da massa de ovos e da viabilidade destes, foram os maiores valores obtidos quando comparados aos valores dos outros extratos e ao **GC1**, mostrando mais uma vez que, embora, a resposta inflamatória nos hospedeiros do grupo **GT (EGS6)** tivesse sido a segunda mais intensa do experimento, o fato de haver intensas áreas de edema nos primeiros (regiões com acúmulo de líquidos e debris celulares), permitiu maior sucesso na alimentação dos carrapatos infestantes.

Considerando-se as características apresentadas pelas fêmeas do grupo **GT (EGS6)**, concluiu-se que o extrato **EGS6** apresentou moléculas imunogênicas que estimularam a resposta inflamatória caracterizada pela presença precoce de intensas áreas de edema próximas à lesão de alimentação, porém com tardia formação de infiltrado inflamatório, o que beneficiaria o consumo de sangue pelos carrapatos, demonstrando assim que o **EGS6** não seria um bom extrato imunogênico, corroborando Wikel (1976), que relatou que extratos de carrapatos inteiros conteriam substâncias que reduziriam a resposta imune do hospedeiro à infestação.

Estas informações aqui obtidas vêm então mais uma vez confirmar a proposta de Furquim et al. (artigo submetido), confirmando os períodos de alimentação que foram escolhidos para a extração das glândulas utilizadas no presente trabalho, ou seja, 2, 4 e 6 dias para obtenção dos extratos glandulares (**EGS2**, **EGS4** e **EGS6**, respectivamente), uma vez que já tinha sido verificado anteriormente que diferentes células dos ácinos II e III tornar-se-iam ativas ou deixariam de apresentar atividade principalmente nestes intervalos. Uma vez que a secreção salivar teria natureza glicoprotéica, estas células não participariam da formação do cone de cemento em virtude da sua natureza lipoprotéica (BINNINGTON, 1987) e indicaria que as células em questão apresentariam funções diferenciadas do restante das células secretoras (as quais permaneceriam ativas durante todo ciclo glandular) o que sinalizaria ação específica e pontual destas na manipulação da resposta imune-inflamatória do hospedeiro.

Além disso, as diferenças nas respostas inflamatórias locais dos hospedeiros dos grupos **GT (EGS2, EGS4 e EGS6)** foram possivelmente decorrentes da grande variedade de moléculas antigênicas (que de fato apresentaram papel na resposta imune-inflamatória dos hospedeiros) presentes nas glândulas salivares das fêmeas de *R. sanguineus* com 4 dias de alimentação, glândulas estas utilizadas para preparação do extrato **EGS4** as quais estariam muito mais ativas do que aquelas retiradas de fêmeas com 2 e 6 dias (FURQUIM et al., artigo submetido).

Ficou, portanto, aqui demonstrado que tanto o extrato glandular **EGS2** quanto o **EGS4** seriam eficazes na redução da infestação por carrapatos *R. sanguineus*, embora as diferentes moléculas imunogênicas presentes em ambos teriam estimulado diferentes respostas imune-inflamatórias, agindo diferentemente no controle da infestação, ou seja, o **EGS4** agiria reduzindo o período de alimentação e o **EGS2** inibiria a reprodução dos carrapatos, demonstrando que ambos poderiam ser eficazes no controle destes ectoparasitas. Além disso,

fica o alerta para o fato de que não basta imunizar os hospedeiros para que estes desenvolvessem intensa resposta inflamatória frente à infestação por carrapatos; dever-se-ia primeiramente conhecer como seria a resposta aos antígenos administrados, uma vez que aqui demonstrou-se que a resposta do hospedeiro poderia, ao contrário do esperado, contribuir com o sucesso alimentar do carrapato, como foi observado quando da inoculação do extrato **EGS6** no presente trabalho.

Conclusões

6.Conclusões

1) Os extratos de glândulas salivares de fêmeas de *R. sanguineus* sensibilizaram os coelhos hospedeiros com eles inoculados intensificando a resposta imune-inflamatória dos animais do grupo **GT** (**EGS2**, **EGS4** e **EGS6**), desenvolvida precocemente quando comparada àquela dos animais do grupo controle (**GC1** e **GC2**).

2) Os coelhos imunizados com os extratos apresentaram resposta imune-inflamatória com diferentes graus de precocidade e intensidade, sendo que os do grupo **GT** (**EGS4**) apresentaram maior precocidade e intensidade sugerindo que os diferentes extratos apresentariam moléculas com diferentes potenciais imunogênicos.

3) Os extratos **EGS2** e **EGS4** reduziram a infestação por carrapatos *R. sanguineus* sendo que **EGS4** agiu na alimentação desses carrapatos reduzindo o número de indivíduos que ingurgitaram por completo e o **EGS2** agiu na reprodução dos carrapatos reduzindo o número de descendentes.

4) A inoculação com o extrato **EGS6** no hospedeiro, ao contrário do esperado, estimulou o processo alimentar do carrapato, incluindo até a degeneração precoce das glândulas salivares, não sendo, portanto, o extrato **EGS6** indicado como fonte de potencial imunogênico para hospedeiros.

5) A imunização dos hospedeiros com os extratos **EGS2** e **EGS4** alterou o ciclo secretor das células glandulares das fêmeas de *R. sanguineus* neles alimentadas, tornando o processo alimentar destes ectoparasitas menos eficiente.

Referências

7 Referências

BALASHOV, Y. S. Bloodsucking ticks (Ixodidae)- vectors of diseases of man and animals. **Miscellaneous Publications of the Entomological Society of America**, Lanham, v.8, p. 161-376, 1972.

BECHARA, G. H.; SZABÓ, M. P. J.; FERREIRA, B. R.; GARCIA, M. V. *Rhipicephalus sanguineus* tick in Brazil: feeding and reproductive aspects under laboratorial conditions. **Brazilian Journal of Veterinary Parasitology**, Jaboticabal, v. 4, n.2, p. 61-66, 1995.

BECHARA, G.H. Imunopatologia da interação carrapato-hospedeiro. In: BARROS-BATTESTI, D.M., ARZUA, M., BECHARA, G.H. (eds), **Carrapatos de importância médico-veterinária da região neotropical: Um guia ilustrado para identificação de espécies**. São Paulo: Vox/ICTTD-3/Butantan, p. 139-143, 2006.

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

BROSSARD, M.; WIKEL, S. K. Immunology of interactions between ticks and hosts. **Medical and Veterinary Entomology**, Lanham, v. 11, p. 270-276, 1997.

BROWN, S.J. Immunology of acquired resistance to ticks. **Parasitology Today**, [S.I.] v.1, p.166-71, 1985.

BROWN, S.J., BARKER, R.W., ASKENASE, P.W. Bovine resistance to *Amblyomma americanum* ticks: an acquired immune response characterized by cutaneous basophils infiltrates. **Veterinary Parasitology**, [S.I.] v.16, p.147-65, 1984.

BRUMPT, E.; CHABAUD, A.G. L' infestation par des ixodinés provoque-t-elle une immunité chez l'hôte. 1. Note préliminaire. **Annales de Parasitologie**, [S.I.] v.22, p.348-56, 1947.

CHABAUD, A.G. L' infestation par des ixodinés provoque-t-elle une immunité chez l'hôte (2me. Note). **Annales de Parasitologie**, [S.I.] v.25, p.474-9, 1950.

DANTAS-TORRES, F. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): From taxonomy to control. **Veterinary parasitology**, [S.I.] 152: 173-185, 2008.

FAWCETT, D. W.; BINNINGTON, K. C.; VOIGHT, W. R. The cell biology of the ixodid tick salivary gland. In: Sauer, J. R.; Hair, J. A. (ed.). **Morphology, Physiology and Behavioral Biology of Ticks**. Chichester: Ellis Horwood, 1986, p. 22-45.

FERREIRA, B.R.; BECHARA, G.H. Imunidade a carrapatos *Rhipicephalus sanguineus* (Acarina:Ixodidae) em cachorro-do-mato *Cerdocyon thous* (Linnaeus) e no cão doméstico. **Brazilian Journal of Veterinary Research and Animal Science**, São Paulo, v.32, n.4, p.232-237, 1995.

FERREIRA, B.R., MACHADO, R.Z., BECHARA, G.H. Western blot analysis of tick antigens from a *Rhipicephalus sanguineus* unfed larval extract and identification of antigenic sites in tick sections using immunohistochemistry. A comparative study between resistant and susceptible host species. **Veterinary Parasitology**, [S.I.] v. 62, p. 161-174, 1996.

GILL, H. S.; WALKER, A. R. The salivary glands of *Hyalomma anatolicum anatolicum*: nature of salivary gland components and their role in tick attachment and feeding. **International Journal for Parasitology**, Oxford, v. 18, n. 1, p. 83-93, 1987.

GILLESPIE, R. D.; DOLAN, M. C.; PIESMAN, J.; TITUS, R. G. Identification of an IL-2 Binding Protein in the Saliva of the Lyme Disease Vector Tick, *Ixodes scapularis*. **Journal of Immunology**, Bethesda, 166;4319-4326, 2001.

INOKUMA, H.; KEMP, D.H.; WILLADSEN, P. Prostaglandin E2 production by the cattle tick (*Boophilus microplus*) into feeding sites and its effect on the response of bovine mononuclear cells to mitogen. **Veterinary Parasitology**, [S.I.] v. 53, p. 293-299, 1994.

JAWORSKI, D.C.; MULLER, M.T.; SIMMEN, F.A.; NEEDHAM, G.R. *Amblyomma americanum*: identification of tick salivary gland antigens from unfed and early feeding females with comparasions to *Ixodes dammini* and *Dermacentor variabilis*. **Experimental Parasitology**, [S.I.] v. 70, p. 217-226, 1990.

JITTAPALAPONG, S., STICH, R.W., GORDON, J.C., WITTUM, T.E., BARRIGA, O.O. Performance of female *Rhipicephalus sanguineus* (Acari: Ixodidae) fed dogs exposed to multiple infestations or immunization with tick salivary gland or midgut tissues. **Journal of Medical Entomology**, Lanham, v. 37, n. 4, p. 601-611, 2000a.

JITTAPALAPONG, S., STICH, R.W., GORDON, J.C., BREMER, C. A., BARRIGA, O.O. Humoral Immune Response of Dogs Immunized with Salivary Gland, Midgut, or Repeated Infestations with *Rhipicephalus sanguineus*. **Annals of the New York Academy of Sciences**, v. 916, p. 283–288, 2000b.

KAUFMAN, W. R.; SAUER, J. R. Ion and water balance in feeding ticks: mechanism of tick excretion. In: OBENCHAIN, F. D., GALUN, R. (eds.), **Physiology of Ticks**. Oxford: Pergamon Press, 1982. p. 213-243.

LABRUNA, M.B.; PEREIRA, M.C. Carrapato em Cães no Brasil. **Clínica Veterinária**, São Paulo, v. 30, n. 1, p. 24-32, 2001.

MEJRI, N., RUTTI, B., BROSSARD, M. Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of Balb/c mice. **Parasitology Research**, [S.I.] v. 88, n. 3, p. 192-197, 2002

MONTEIRO, G.E.R., BECHARA, G.H. Cutaneous basophilia in the resistance of goats to *Amblyomma cajennense* nymphs after repeated infestations. **Annals of the New York Academy of Sciences**, v. 1149, p. 221-225, 2008.

NAZARIO, S.; DAS, S.; de SILVA, A. M.; DEPONTE, K.; MARCANTONIO, N.; ANDERSON, J. F.; FISH, D.; FIKRIG, E.; KANTOR, F. S. Prevention of *Borrelia*

burgdorferi transmission in guinea pigs by tick immunity. **American Journal of Tropical Medicine Hygiene**, Stanford, v. 58(6), p. 780–785, 1998.

NEEDHAM, G.R., JAWORSKI, D., PEMBERTON, B., MULLER, M.T. Resistance expressed by hosts to feeding by adult lone start ticks. In: Borovsky, D., Apielman, A. (ed.). **Host Regulated Developmental Mechanisms in Vector Arthropods**. v. 2, Vero Beach, University of Florida-IFAS, 1986, p. 168-174.

NUTTAL, P. A.; TRIMNELL, A. R.; KAZIMIROVA, M.; LABUDA, M. Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. **Parasite immunology**. Brentwood, v. 28, p. 155-163, 2006.

OBEREM, P.T. The immunological basis of host resistance to ticks. **Journal of the South African Veterinary Association**, Pretoria, v. 55, p. 215-7, 1984.

PAZ, E. G.; LEITE, R. C.; OLIVEIRA, P. O. Controle de *Rhipicephalus sanguineus* (LATREILLE, 1806) (ACARI: IXODIDAE) no canil da escola de veterinária da UFMG, Belo Horizonte, Minas Gerais, Brasil. **Revista Brasileira de Parasitologia Veterinária**, São Carlos, v. 17, p. 41-44, 2008.

PECHOVÁ, J.; STEPANOVÁ, G.; KOVÁR, L.; KOPECKY, J. Tick salivary gland extract-activated transmission of *Borrelia afzelii* spirochaetes. **Folia Parasitologica**. Ceske Budejovice, v. 49, p. 153-159, 2002.

RANDOLPH, S.E. Population regulation in ticks: the role of acquired resistance in natural and unnatural hosts. **Parasitology**, Cambridge, v.79, p.141-56, 1979.

REY, L. **Parasitologia**. Rio de Janeiro: Guanabara Koogan, 1973. p. 633-641.

ROSELL-DAVIS, R., COONS, L.B. Relationship between feeding mating, vitellogenin production and vitellogenesis in the tick *Dermacentor variabilis*. **Experimental and Applied Acarology**, [S.I.], v. 7, p. 95-105, 1989.

SAITO, K.C., BECHARA, G.H., NUNES, E.T., OLIVEIRA, P.R., DENARDI, S.E., CAMARGO-MATHIAS, M.I. Morphological, histological, and ultrastructural studies of the ovary of the cattle-tick *Boophilus microplus* (CANESTRINI, 1887) (Acari: Ixodidade). **Veterinary Parasitology**, [S.I.] v. 129, p. 299-311, 2005.

SAUER, J. R, ESSENBERG, R. C., BOWMAN, A. S. Salivary glands in ixodid ticks: control and mechanism of secretion. **Journal of Insect Physiology**, [S.I.], v. 46, p. 1069-1078, 2000.

SONENSHINE, D. E. **Biology of ticks**. New York: Oxford University Press, 1991. p. 141-158.

SZABÓ, M.P.J., MUKAI, L.S., ROSA, P.C.S., BECHARA, G.H. Differences in the acquired resistance of dogs, hamsters and guinea pigs to infestations with adult ticks *Rhipicephalus sanguineus* (Acarina: Ixodidae). **Brazilian Journal of Veterinary Research and Animal Science**, São Paulo, v. 32, n. 1, p.43-50, 1995.

SZABÓ, M.P.J., BECHARA, G.H. Immunization of dogs and guinea pigs against *Rhipicephalus sanguineus* ticks using gut extract. **Veterinary Parasitology**, [S.I.] v. 68, p. 283-294, 1997.

TELLAM, R.L.; SMITH, D.; KEMP, D.H. Vaccination against ticks. In: Yong, W.K. (ed.). **Animal parasite control using biotechnology**. Boca Raton: CRC Press, 1992. p. 303-331.

TURNI, C.; LEE, R.P.; JACKSON, L.A. Effect of salivary gland extracts from the tick, *Boophilus microplus*, on leucocytes from Brahman and Hereford cattle. **Parasite Immunology**, Brentwood, v. 24, p. 355-361, 2002.

VERONEZ, V. A.; CASTRO, M. B.; BECHARA, G. H.; SZABO, M. P. J. Histopathology of *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks fed on resistant hosts. **Experimental and Applied Acarology**, [S.I.] v. 50, p. 151-161, 2010.

WALKER, A.; FLETCHER, J. D.; GILL, H. S. Structural and histochemical changes in the salivary glands of *Rhipicephalus appendiculatus* during feeding. **International Journal for Parasitology**, Oxford, v. 15, n. 1, p. 81-100, 1985.

WALKER, A. **Arthropods of domestic animals. A guide to preliminary identification**. London: Chapman and Hall, p. 25-29, 1994.

WIKEL, S.K. The induction of host resistance to tick infestation with salivary gland antigen. **The American Society of Tropical Medicine and Hygiene**, Stanford, v. 30, n. 1, p. 284-288, 1981.

WIKEL, S.K. Host immunity to ticks. **Annual Reviews in Entomology**, Palo Alto, v. 41, p. 1-22, 1996.

WIKEL, S. K.; ALLEN, J. R. Acquired resistance to ticks. III. Cobra venom factor and the resistance response. **Immunology,** Oxford, v. 32, p. 457-465, 1977.

WILLADSEN, P. Immunity to ticks. **Advances in Parasitology**, [S.I.] v. 18, p. 293-313, 1980.

WILLADSEN, P. Novel vaccines for ectoparasites. **Veterinary Parasitology**, [S.I.] v. 71, p. 209- 22, 1997.

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