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AVALIAÇÃO DAS ALTERAÇÕES CELULARES NOS OVÓCITOS DE FÊMEAS SEMI-INGURGITADAS DE CARRAPATOS ***Rhipicephalus sanguineus*** (ACARI: IXODIDAE) DECORRENTES DA EXPOSIÇÃO AO ÓLEO DE ANDIROBA

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2012

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“A alegria não chega apenas no encontro do achado, mas faz parte do processo de busca.”

(FREIRE, P. 2007, P. 142)

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RESUMO DO PROJETO

RESUMO DO PROJETO

Os carrapatos são considerados um dos mais importantes grupos dentre os artrópodes, por serem vetores de agentes patogênicos, que podem atacar animais, bem como o próprio homem. Dentre as diversas espécies de carrapato destaca-se a do *Rhipicephalus sanguineus*, pertencente à família Ixodidae e com ampla distribuição em todos os continentes. Atualmente esta espécie é considerada uma praga urbana, de grande importância médico-veterinária, que parasita principalmente o cão doméstico.

Os ovários de carrapatos são considerados órgãos vitais para o sucesso biológico deste grupo de animais. Neste sentido, desenvolver pesquisas para se conhecer a ação de produtos naturais com reconhecida ação repelente ou acaricida, sobre o sistema reprodutor feminino de carrapatos, traria importantes informações para um melhor entendimento dos efeitos da ação destes produtos nos ovócitos desses ectoparasitas, contribuindo principalmente para o desenvolvimento de métodos de controle alternativos menos tóxicos para os organismos não-alvo e menos poluentes para o meio ambiente.

Dessa forma, o presente estudo analisou as alterações morfofisiológicas causadas por diferentes concentrações do óleo de andiroba no ovário de fêmeas semi-ingurgitadas de carrapatos *R. sanguineus.*

RESUMO DOS RESULTADOS OBTIDOS

RESUMO DOS RESULTADOS OBTIDOS

O presente estudo traz informações sobre os efeitos do óleo da semente de andiroba (*Carapa guianensis*) no ovário de fêmeas semi-ingurgitadas de carrapatos *R. sanguineus*, visto que trabalhos sobre a ação de produtos naturais no sistema reprodutor deste grupo de animais são escassos na literatura. Os resultados mostraram que o óleo de andiroba é um potente agente natural causador de grandes alterações estruturais nos ovócitos, tais como: surgimento de extensas regiões citoplasmáticas vacuolizadas, redução na quantidade de grânulos de vitelo, alterações na forma das células, bem como comprometimento do material genético. Além disso, o epitélio do ovário mostrou severas alterações morfológicas, como extrema desorganização estrutural, com células altamente vacuolizadas e com núcleos picnóticos, formando uma massa amorfa.

Os dados obtidos também revelaram que este produto natural induz grandes alterações fisiológicas nos ovócitos em todos os estágios de desenvolvimento, como: drástica redução de proteínas, polissacarídeos e lipídios nessas células; componentes estes essenciais para a viabilidade do embrião. Além disso, observou-se que o óleo de andiroba estimula a oviposição, principalmente na concentração de 20%. Essa maior produção de ovos representa um mecanismo de defesa desenvolvido pelo organismo, a fim de assegurar o sucesso reprodutivo desta espécie, mesmo na presença do agente tóxico. Entretanto, os resultados histoquímicos mostraram que os ovos postos não são viáveis, devido às grandes alterações sofridas pelos ovócitos.

Assim, pode-se concluir que embora os danos causados aos ovócitos pelo óleo de andiroba sejam comparativamente menos severos em relação àqueles provocados pelos acaricidas sintéticos, este produto pode ser considerado um potente agente natural capaz de inviabilizar o sucesso reprodutivo da espécie *R. sanguineus*, com a vantagem de não causar impacto ambiental.

INTRODUÇÃO

1. INTRODUÇÃO

Os carrapatos constituem um dos mais importantes grupos de artrópodes dos pontos de vista médico e veterinário, visto serem causadores de lesões nos hospedeiros durante o processo de repasto sanguíneo, além de serem transmissores de agentes patogênicos para os animais, incluindo o homem. Durante seu ciclo biológico, estes ectoparasitas podem passar longos períodos fora de seus hospedeiros sem se alimentarem, abrigados entre a vegetação e fendas no solo (WALKER, 1994).

O grupo é constituído de animais pertencentes à ordem Acari, subclasse Arachnida, sendo classificados em duas principais famílias: Ixodidae e Argasidae (ANDERSON; MAGNARELLI, 2008).

Dentro da família Ixodidae encontra-se a espécie *Rhipicephalus sanguineus* (LATREILLE, 1806), a qual se encontra distribuída em todos os continentes do planeta, parasitando principalmente o cão doméstico. Os carrapatos pertencentes a esta família apresentam o corpo recoberto por uma grande placa dorsal de quitina, conhecida como escudo, que pode ter a sua superfície ornamentada por manchas, depressões e desenhos (REY, 1973) e é onde se encontram as peças bucais na região frontal do corpo (WALKER, 1994; WALL; SHEARER, 1997). Nos machos essa placa recobre praticamente toda a superfície do dorso, enquanto que nas fêmeas não ultrapassa metade ou um terço da área dorsal (WALKER, 1994; WALL; SHEARER, 1997).

Por ser um carrapato da família Ixodidae, o *R. sanguineus* apresenta 3 formas parasitárias dentro de seu ciclo de vida: larva, ninfa e adulto, sendo esta última a única com dimorfismo sexual. Em cada estágio o parasita se alimenta por alguns dias principalmente de sangue, mas também de linfa e de restos tissulares da epiderme e/ou

derme lesada do hospedeiro (LABRUNA, 2004). As fêmeas iniciam a oviposição quatro ou cinco dias após terem se alimentado, podendo ovipor até 3000 mil ovos em um período de 15 dias, os quais eclodem em cerca de 3 semanas. Em 4 ou 5 dias as larvas estarão aptas a infestarem seu primeiro hospedeiro e o ciclo de vida se completará em 2 ou 3 meses, podendo esse período ser diferente em regiões temperadas, onde pode haver hibernação das fases ninfal e/ou adulta (REY, 1973). No final de cada período parasitário, as larvas e ninfas ingurgitadas se desprendem do hospedeiro e realizam a ecdise (muda) (LABRUNA, 2004).

O sistema reprodutor feminino da espécie *R. sanguineus*, assim como dos carrapatos em geral, consiste em um ovário tubular localizado na região posterior do corpo, com um par de ovidutos, um útero, uma vagina e um par de glândulas acessórias que desembocam na abertura genital (SONENSHINE, 1991; SAID, 1992). O ovário é do tipo panoístico com ausência de células nutridoras. Este órgão possui uma parede formada por pequenas células epiteliais com núcleos esféricos onde se fixa um grande número de ovócitos, os quais passam por vários estágios de desenvolvimento até o momento da oviposição (OLIVEIRA et al., 2005). Nesta espécie de carrapato, os ovócitos são classificados em 5 estágios de desenvolvimento de acordo com o aspecto citoplasmático, localização da vesícula germinal (núcleo do ovócito), presença, quantidade e constituição dos grânulos de vitelo e presença de cório (OLIVEIRA et al., 2005).

Embora o cão seja seu principal hospedeiro (REY, 1973; WALKER, 1994), o carrapato da espécie *R. sanguineus* também pode ser encontrado em outros mamíferos, inclusive no homem, devido a sua baixa especificidade (VENZAL et al., 2003; NEBREDA-MAYORAL et al., 2004; LOULY et al., 2006; RIBEIRO et al., 2006). Dados da literatura também mostram que em diversas partes do mundo esta espécie também pode atacar búfalos, camelos, bovinos, cabras, cavalos, ovelhas, morcegos, répteis, além de aves que frequentam o solo (FLECHTMANN, 1973).

A espécie *R. sanguineus* além de causar grandes perdas de sangue em seus hospedeiros, é também o agente vetor de vírus, bactérias, bem como protozoários causadores de doenças, tais como: *Babesia canis*, protozoário que age sobre as hemácias causando a babesiose ou "nambiuvu" (FLECHTMANN, 1973), *Hepatozoon canis*, biopatógeno da hepatozoonose em cães na América do Sul (VICENT-JOHNSON et al.,

1997; O'DWYER; MASSARD, 2001) e também *Ehrlichia canis*, que ataca os leucócitos do cão (SIMPSON et al., 1991; DAVOUST, 1993). Em humanos, dados da literatura descrevem que os carrapatos *R. sanguineus* também são potenciais agentes transmissores da bactéria *Francisella tularensis*, agente biológico da tularemia (WALKER, 1994).

Com base nos dados acima descritos, o carrapato *R. sanguineus* tem assumido cada vez mais um papel relevante entre as espécies de carrapato de importância mundial. Tal fato é retratado pela grande atenção com que a indústria farmacêutica veterinária trata este ectoparasita. Até o início da década de 1980, não existia no mercado brasileiro nenhum produto comercial acaricida com indicação específica para tratamento de *R. sanguineus* em cães. Porém, atualmente existem dezenas deles com as mais diversas formulações e apresentações, representando cifras consideráveis no faturamento da linha veterinária das indústrias farmacêuticas do Brasil (LABRUNA, 2004).

O sucesso dos carrapatos como vetores de microrganismos, segundo Harwood e James (1979), se deve a algumas características biológicas, como hematofagismo em todas as fases do desenvolvimento; fixação profunda nos hospedeiros, o que dificultaria sua remoção; ingurgitamento lento, havendo tempo para inoculação de patógenos; adaptação a diferentes espécies de hospedeiros; resistência à adversidade climática, devido a grande esclerotização e longevidade nos ambientes, propiciando tempo para multiplicação dos patógenos.

Métodos para um controle efetivo de carrapatos vêm sendo continuamente pesquisados, porém, o uso de acaricidas sintéticos tem se mostrado o mais eficiente, embora este método ainda apresente inconvenientes, como alto custo com a aquisição de produtos químicos, instalações e mão-de-obra para a aplicação dos mesmos. Além disso, os acaricidas químicos também são responsáveis por causarem danos ao meio ambiente e à saúde pública, por meio da contaminação com resíduos (FREITAS et al., 2005). Outro problema oriundo do uso não controlado de produtos sintéticos seria o surgimento de mecanismos de resistência desenvolvidos pelos carrapatos como estratégias de sobrevivência. A seleção de linhagens de carrapatos resistentes se deve principalmente ao uso incorreto dos acaricidas (HÄUSERMAN et al., 1992).

Com base nos dados acima apresentados, a busca por novos produtos acaricidas que sejam mais baratos, e ao mesmo tempo apresentem menor toxicidade e impacto sobre o meio ambiente tem sido intensificada. Dentre estas alternativas, uma delas seria a utilização de compostos de origem natural, ou seja, de produtos provenientes de extratos de plantas, os quais teriam ingredientes ativos com ação controladora de pragas (GUERRA, 1985).

Várias plantas da família Meliaceae estão sendo reconhecidas como eficientes no controle de pragas, principalmente devido à ação repelente contra os Arthropoda (MARTINEZ, 2002). Nesta família encontra-se a espécie *Carapa guianensis* (Aubl.), popularmente conhecida como andiroba, a qual se encontra distribuída por todo o norte da América do Sul, América Central, Antilhas e África tropical. No Brasil é encontrada em toda a Bacia Amazônica, principalmente em regiões de várzea e áreas alagáveis (LOUREIRO et al., 1979).

Carapa guianensis é uma árvore de porte médio, com aproximadamente 20-30 m de altura e diâmetro de 50-120 cm. O caule é bastante espesso e desprende-se facilmente em lascas. As folhas de tonalidade verde-escuro medem cerca de 80-110 cm de comprimento e são do tipo compostas, sendo constituídas de 12-18 folíolos. A inflorescência se localiza principalmente no final dos ramos, medindo em média 30 cm de comprimento. O fruto é formado por uma cápsula globosa com quatro valvas, que se abrem quando ele cai no solo, liberando de 4-12 sementes. Esta espécie é encontrada principalmente em áreas de clima tropical úmido, com temperaturas que podem variar entre 17°-30°C, umidade relativa de 70%-90% e com índices pluviométricos que variam de 1800-3500 mm por ano. A andiroba tem melhor adaptação em solos úmidos, porém não encharcados, e ricos em matéria orgânica. Ela floresce duas vezes ao ano, nos meses de Agosto-Setembro e Janeiro-Fevereiro. Os frutos amadurecem em Junho-Julho e Fevereiro-Março. O óleo contido na semente é amarelo claro e extremamente amargo (REVILLA, 2001; LORENZI, 2002).

Todas as partes desta planta são usadas para fins medicinais. Comunidades tradicionais da Floresta Tropical Amazônica fazem a partir do óleo de suas sementes, um sabão medicinal que é usado no tratamento de doenças de pele, artrite, reumatismo, infecções de ouvido, bem como na cura de diversos tipos de feridas (HAMMER;

JOHNS, 1993). Essas comunidades também fazem uso da ingestão do óleo desta planta no intuito de curar tosses e convulsões (DUKE; VASQUEZ, 1994).

Atualmente, encontram-se disponíveis no mercado velas de andiroba, as quais são usadas para repelir mosquitos transmissores da dengue, bem como da malária (FERRAZ et al., 2002).

Com relação ao uso do óleo de andiroba no controle de carrapatos, existem poucos registros na literatura. Os dados disponíveis mostram que este óleo também apresenta potencial acaricida, além de causar inibição da oviposição em fêmeas de carrapatos *Boophilus microplus* e *Anocentor nitens* (FARIAS et al., 2007; 2009). Além disso, estudos recentes sobre os potenciais efeitos tóxicos do óleo de andiroba em fêmeas de ratos, mostraram que este produto não interfere na fertilidade (COSTA-SILVA et al., 2006), bem como não apresenta qualquer efeito tóxico durante a gestação (COSTA-SILVA et al., 2007).

Dessa forma, de acordo com as informações acima descritas, torna-se necessário o desenvolvimento de uma linha de pesquisa sobre a ação de produtos naturais, como por exemplo, o óleo de andiroba, nos principais sistemas de carrapatos. As informações geradas com este tipo de estudo poderão ser usadas como base na criação de metodologias de controle mais específicas e eficazes.

JUSTIFICATIVA

2. JUSTIFICATIVA

Métodos alternativos para o controle de pragas que sejam menos tóxicos e apresentem menor poder residual para o meio ambiente, tornam-se atualmente alvo de pesquisas, principalmente enfocando produtos de origem natural, visto que os métodos químicos, embora eficazes no controle de carrapatos, apresentam inúmeros inconvenientes por serem dispendiosos e causarem danos ao meio ambiente e à saúde pública. Nesse sentido, estudos sobre a ação de compostos naturais sobre o sistema reprodutor de fêmeas de carrapatos poderiam contribuir na geração de métodos alternativos de controle, especialmente para a espécie *R. sanguineus*, atualmente considerada como praga urbana devido aos sérios problemas de infestação. Dessa forma, o presente estudo pretende fornecer informações sobre a ação do óleo de andiroba nos ovócitos de fêmeas de *R. sanguineus* a fim de se avaliar a ação desse produto no sistema reprodutor desses indivíduos, revelando em respostas o que poderia sinalizar a perspectiva de descoberta de um novo método ou de um produto alternativo para o controle de carrapatos.

OBJETIVOS

3. OBJETIVOS

Com base nas informações acima descritas, o presente trabalho teve por objetivos submeter fêmeas adultas semi-ingurgitadas de *R. sanguineus* a diferentes concentrações do óleo de andiroba (5%, 10% e 20%), a fim de avaliar a eficiência reprodutiva desta espécie quando exposta a este produto natural, além de analisar se ocorreriam alterações morfo-histológicas e histoquímicas no sistema reprodutor feminino desses indivíduos devido à ação desta substância natural, visto que o sistema reprodutor é um órgão vital para a sobrevivência da espécie.

MATERIAL E MÉTODOS

4. MATERIAL E MÉTODOS

4.1. Material

4.1.1. Carrapatos

Para o presente estudo foram utilizadas fêmeas semi-ingurgitadas de carrapatos da espécie *Rhipicephalus sanguineus* que foram mantidas em condições controladas (28 ± 1ºC, 80% de umidade relativa e fotoperíodo de 12 horas) em estufa BOD (Biological Oxygen Demand). Os carrapatos foram obtidos a partir de colônias estabelecidas no Departamento de Biologia, Instituto de Biociências da UNESP, campus de Rio Claro, SP.

Todos os trabalhos relacionados à construção e fixação da câmara de alimentação nos hospedeiros (coelhos), bem como alocação dos carrapatos nas câmaras alimentadoras foram realizados segundo protocolo descrito por Bechara et al. (1995).

4.1.2. Hospedeiros

Neste estudo foram utilizados como hospedeiros fêmeas de coelhos adultos da raça New Zealand White que foram adquiridas no Biotério Central da UNESP, campus de Botucatu, SP. Os animais estavam isentos de contato prévio com o ácaro. Durante todos os experimentos, os hospedeiros foram mantidos em gaiolas de contenção e alimentados com ração apropriada e água *ad libitum* (projeto aprovado pelo Comitê de Ética em Uso Animal, CEUA, UNESP, Rio Claro, SP, Brasil, Protocolo n° 7124) **(Anexo)**.

4.1.3. Substância natural

Para o presente estudo foi utilizado o óleo da semente de andiroba (*C. guianensis*) (extrato puro), adquirido por meio da Farmácia de Manipulação, Drogaria e Homeopatia Art-Fármacos, Rio Claro, SP, Brasil.

4.2. Métodos

Para a realização do presente estudo foram utilizadas as dependências e os equipamentos disponíveis nos laboratórios de Histologia e da "Brazilian Central of Studies on Ticks Morphology (BCSTM)" do Departamento de Biologia da UNESP, campus de Rio Claro, SP, Brasil.

4.2.1. Ensaio com o óleo de andiroba

Para a realização deste trabalho o extrato puro do óleo da semente de andiroba foi transformado em sabão, uma vez que a emulsificação por agentes surfactantes como Triton, SDS e Tween, comumente usados nas diluições realizadas com óleos (CHAGAS et al., 2003; FARIAS et al., 2007; 2009), poderia ocasionar a formação de micelas e aprisionar o óleo em seu interior, impedindo o contato do mesmo com os carrapatos durante a imersão nas respectivas diluições do óleo de andiroba.

De forma geral, os agentes tensoativos ou surfactantes são substâncias que diminuem a tensão superficial da água, como Tween, Triton, SDS, dentre outros. Estes compostos são empregados como agentes capazes de emulsificar substâncias lipofílicas em água. Este processo de emulsificação ocorre devido à formação de micelas, que encapsulam a substância lipofílica, aprisionando-a em pequenas vesículas, rodeadas pelas moléculas do agente surfactante. Este encapsulamento é possível, uma vez que a molécula surfactante possui uma extremidade constituída por uma cadeia apolar que interage com a substância lipofílica e uma extremidade hidrofílica que interage com a água. Nas micelas, as extremidades polares hidrofílicas ficam voltadas para o exterior da vesícula, mantendo contato com as moléculas de água, enquanto que as extremidades apolares hidrofóbicas ficam voltadas para o interior. Assim, as moléculas da substância a ser emulsificada serão aprisionadas nas extremidades hidrofóbicas dos surfactantes, ou seja, na região central das micelas (MANIASSO, 2001; CUI et al., 2008; JIANG et al., 2011).

O processo de saponificação realizado neste trabalho promoveu a hidrólise dos ésteres presentes no óleo de andiroba, liberando os respectivos ácidos graxos que ao reagirem com a base, formaram um sal solúvel em água. Dessa forma, o uso de agentes surfactantes não foi necessário. Este processo permitiu o preparo de soluções do sabão de óleo andiroba em diferentes concentrações.

Neste trabalho, a diluição máxima conseguida para o sabão do óleo de andiroba foi de 20%, uma vez que diluições maiores formavam soluções muito viscosas o que impossibilitava a imersão dos carrapatos. Dessa forma, a partir da diluição máxima de 20%, outras duas diluições foram realizadas (5% e 10%) a fim de analisar a ação do óleo de andiroba sobre o sistema reprodutor de fêmeas de *R. sanguineus*.

Foram formados quatro grupos, com 15 fêmeas cada, sendo um dos grupos denominado de **controle** (**GI**), onde os indivíduos foram expostos apenas à água destilada e os outros três grupos (15 fêmeas cada), como grupos de **tratamento**, os quais foram expostos ao óleo de andiroba nas concentrações de 5% (**GII**), 10% (**GIII**) e 20% (**GIV**).

Para cada grupo de tratamento, as fêmeas semi-ingurgitadas (peso médio ± SD = 27 mg ± 4.4) foram higienizadas em água e secas com papel absorvente. As fêmeas do **GI** foram imersas em água destilada durante 5 minutos. Posteriormente, foram secas em papel absorvente e colocadas em placas de Petri previamente identificadas, em estufa BOD (28 ± 1ºC, 80% de umidade relativa e fotoperíodo de 12 horas) durante 7 dias. As fêmeas dos grupos de **tratamento** foram imersas em diluições de 5% (**GII**), 10% (**GIII**) e 20% (**GIV**) do óleo de andiroba, também durante 5 minutos cada. Posteriormente, foram secas em papel absorvente e colocadas em placas de Petri identificadas, em estufa BOD (28 ± 1ºC, 80% de umidade relativa e fotoperíodo de 12 horas) também durante 7 dias.

As diferentes concentrações do óleo de andiroba foram utilizadas para o acompanhamento dos possíveis efeitos deste produto natural no sistema reprodutor de fêmeas de *R. sanguineus*, visto que essas concentrações não causaram a mortalidade destes ectoparasitas.

4.2.2. Análise morfológica

4.2.2.1. Histologia

4.2.2.1.1. Inclusão dos ovários de carrapatos *R. sanguineus* em resina e coloração pela hematoxilina de Harris e eosina aquosa (JUNQUEIRA; JUNQUEIRA, 1983)

As fêmeas de carrapatos expostas e não expostas ao óleo de andiroba foram dissecadas em placas de Petri contendo solução fisiológica tamponada com fosfato-PBS (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2). Com o auxílio de estereomicroscópio, os ovários foram retirados com pinças e micro-tesouras e fixados por 24 horas em solução de paraformaldeído a 4% e NaCl a 0.9% em tampão fosfato 10% (0.1 M - pH 7.5). Posteriormente, o material, foi desidratado em soluções crescentes de etanol a 70, 80, 90 e 95% durante 15 minutos cada. Logo após, foi transferido para solução de historesina na ausência de catalisador, durante 24 horas em geladeira. Posteriormente, as amostras foram transferidas para moldes plásticos previamente preenchidos com resina contendo catalisador. Os moldes foram selados com suportes de alumínio para microtomia. Depois de polimerizados os blocos foram seccionados com o auxílio de micrótomo Leica RM 2255. Os cortes com 3.5 μm de espessura foram hidratados e recolhidos em lâminas de vidro. Depois de secas, as lâminas foram submetidas à coloração pela hematoxilina de Harris e eosina aquosa durante 10 e 5 minutos, respectivamente. Em seguida foram secas e montadas em bálsamo do Canadá. O material foi observado e documentado em fotomicroscópio Leica DM750.

4.2.3. Análise histoquímica

As técnicas histoquímicas foram aplicadas nas secções histológicas, sendo que, o material, após ser dissecado, foi submetido a diferentes fixadores específicos para preservação de proteínas, polissacarídeos e lipídios. Estes procedimentos foram realizados com o objetivo de se detectar alterações, tais como: presença ou não, frequência e distribuição dos componentes proteicos, polissacarídicos e lipídicos nos ovários de fêmeas de carrapatos *R. sanguineus* submetidas aos grupos controle e tratados com o óleo de andiroba.

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

O material foi fixado em paraformaldeído a 4% e NaCl a 0.9% em tampão fosfato 10% (0.1 M - pH 7.5) por aproximadamente 24 horas. Os cortes foram recolhidos em lâminas de vidro e corados com solução de azul de bromofenol à temperatura ambiente durante 1 hora, sendo em seguida lavados em solução aquosa de ácido acético 0.5% durante 5 minutos. Logo após as lâminas foram passadas no álcool butílico terciário por 5 minutos. Em seguida, foram secas e montadas em bálsamo do Canadá para posterior exame e documentação em fotomicroscópio Leica DM750.

4.2.3.2. Técnica do PAS/Alcian Blue para detecção de polissacarídeos ácidos e neutros (JUNQUEIRA; JUNQUEIRA, 1983)

O material foi fixado em Bouin aquoso por 6 horas. Logo após foi seccionado e corado com Alcian Blue 1% - pH 2.5 durante 30 minutos. Em seguida, as lâminas contendo as secções foram lavadas em água destilada e passadas em ácido periódico 1% durante 5 minutos. Posteriormente, foram submetidas ao reativo de Schiff no escuro por 30 minutos e lavadas em água corrente durante 10 minutos. Em seguida, foram secas e montadas em bálsamo do Canadá para serem examinadas e fotografadas em fotomicroscópio Leica DM750.

4.2.3.3. Técnica de Baker para detecção de lipídios (BAKER, 1946)

O material foi fixado em formol cálcio durante 15 horas. As lâminas contendo as secções histológicas foram tratadas com bicromato de cálcio por 18 horas. Posteriormente, o material foi lavado em água destilada e colocado em solução de hemateína durante 5 horas. Logo após, procedeu-se a última lavagem em água destilada. Após a secagem, as lâminas foram montadas com glicerina para serem examinadas e fotografadas em fotomicroscópio Leica DM750.

4.2.4. Quantificação dos índices de eficiência reprodutiva por meio de biocarrapaticidograma

Fêmeas semi-ingurgitadas de carrapatos *R. sanguineus* foram removidas dos hospedeiros após 5 dias de alimentação e individualmente pesadas (peso médio ± SD = 27 mg ± 4.4). Posteriormente, 120 fêmeas foram divididas em quatro grupos, com 30 indivíduos

cada, e imersas por 5 minutos em placas de Petri contendo diferentes concentrações do óleo de andiroba (5%, 10% e 20%). O grupo controle foi também composto por 30 fêmeas que foram imersas em água destilada pelo mesmo período de tempo. As fêmeas foram mantidas individualmente em câmaras até que o processo de oviposição estivesse finalizado. Posteriormente, as massas de ovos foram individualmente pesadas. Os seguintes parâmetros biológicos foram usados para se avaliar o desempenho reprodutivo das fêmeas de carrapatos: peso médio das fêmeas semi-ingurgitadas antes da oviposição, período médio de pré-oviposição, período médio de oviposição e peso médio das massas de ovos. Esses parâmetros foram usados para se calcular os índices de eficiência reprodutiva, como previamente descrito por Barriga et al. (1991; 1995).

As fêmeas semi-ingurgitadas foram pesadas imediatamente após serem retiradas dos hospedeiros. Já as massas de ovos foram pesadas 21 dias após a retirada dos carrapatos. O período de pré-oviposição correspondeu ao tempo decorrido entre a retirada da fêmea do hospedeiro e o início da oviposição, enquanto que o período de oviposição foi marcado do momento em que a fêmea de carrapato começou a ovipor até o momento do término da oviposição. Os índices de eficiência reprodutiva foram calculados pela divisão do peso de cada massa de ovos pelo peso da respectiva fêmea.

As médias dos parâmetros reprodutivos foram analisadas estatisticamente por meio do teste ANOVA com pós-teste de TUKEY, sendo consideradas significativas as diferenças com $p < 0.05$.

RESULTADOS

5. RESULTADOS

Os resultados obtidos no presente trabalho são apresentados na forma de artigos, os quais foram publicados em periódicos internacionais e especializados.

5.1. CAPÍTULO 1:

Cytotoxic effects of andiroba oil (*Carapa guianensis*) in reproductive system of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females.
Autores: Maria Cláudia Ramalho Vendramini, Maria Izabel Camargo Mathias, Adriano Uemura de Faria, Gervásio Henrique Bechara, Patrícia Rosa de Oliveira e Gislaine Cristina Roma.
Periódico: Parasitology Research, doi: 10.1007/s00436-012-3031-6, 2012.

5.2. CAPÍTULO 2:

Action of andiroba oil (*Carapa guianensis*) on *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females. Morphophysiological evaluation of reproductive system.
Autores: Maria Cláudia Ramalho Vendramini, Maria Izabel Camargo Mathias, Adriano Uemura de Faria, Karim Christina Scopinho Furquim, Leonardo Peres de Souza, Gervásio Henrique Bechara and Gislaine Cristina Roma.
Periódico: Microscopy Research and Technique, doi: 10.1002/jemt.22126, 2012.

CAPÍTULO 1

Cytotoxic effects of andiroba oil (Carapa guianensis) in reproductive system of Rhipicephalus sanguineus (Latreille, 1806) (Acari: Ixodidae) semi-engorged females

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ORIGINAL PAPER

Cytotoxic effects of andiroba oil (*Carapa guianensis*) in reproductive system of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females

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Abstract The present study performed an analysis about the effects of andiroba seed oil (*Carapa guianensis*) in the ovary of *Rhipicephalus sanguineus* semi-engorged females; once, there are few studies about the action of natural products on the reproductive system, a vital organ for the biological success of this animal group. The results showed that andiroba oil is a potent natural agent which causes significant structural changes in the oocytes, such as the emergence of large vacuolated cytoplasmic regions, reduction in the number of yolk granules, changes in the shape of the cells, as well as impairment of genetic material. In addition, the ovary epithelium showed severe morphological changes, such as extreme structural disorganization, with highly vacuolated cells and picnotic nuclei, forming an amorphous mass. This study showed also that oocytes (mainly in the initial stages of development) and the ovary epithelium of *R. sanguineus* females subjected to different concentrations of andiroba oil presented morphological changes which became more numerous and intense as the concentration of the product increased. Based on the results, it can be inferred that although the defense mechanisms are developed by oocytes to recover the cellular integrity (presence of autophagic vacuoles), these cells are not able to revert the damage caused by this product. Thus, it can be concluded that although the damages caused to the oocytes by andiroba oil are comparatively less severe than the ones caused by synthetic acaricides, this product can be considered a potent natural agent that reduce and/or prevent the reproduction of *R. sanguineus* females, with the advantage of not causing environmental impact such as synthetic chemical acaricides.

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Introduction

Among the arthropod, ticks constitute a group of great medical and veterinary importance for being hematophage parasites of animals, and occasionally of human being, due to its low specificity (Ribeiro et al. 2006). In the latter case, this has mainly occurred due to changes in the human population's lifestyle; once the dog has been more present in the domiciliar and peridomiciliar environment, and having more contact with the man, this allows a greater exposition to infectious agents, common to both species. These ectoparasites cause significant damages to the hosts during the feeding process, such as blood loss and dilacerations of tissues due to mechanical action of mouthparts of ticks, in addition to being vectors of several pathogens, which cause serious harms to domestic animals, agropecuary, and public health (Rey 1973; Harwood and James 1979; Walker et al. 2000; Coutinho et al. 2005; Demma et al. 2005; Dantas-Torres 2008).

Ticks are individuals with no present corporal segmentation, having fused head, thorax, and abdomen (Ruppert et al. 2005). These animals belong to the Acari subclass, being classified into two main families: Ixodidae and Argasidae. The first is characterized by the presence of a chitinous scutum, which, in the males, cover almost the entire dorsal surface, and in the females half or a third of the dorsal area. Argasidae individuals do not have this scutum (Walker 1994).

The species *Rhipicephalus sanguineus* (Latreille 1806) belongs to the Ixodidae family, popularly known as brown dog tick. It is the most widespread tick in the world and a well-recognized vector of many pathogens affecting dogs and occasionally humans. This tick can be found on dogs

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living in both urban and rural areas, being highly adapted to live within human dwellings and being active throughout the year not only in tropical and subtropical regions but also in some temperate areas (Dantas-Torres 2010). This species is the main vector and definitive host of *Hepatozoon canis* (Dantas-Torres et al. 2012). All the superior vertebrates are subject to the attack of this and other species of ticks. However, due to endodermy, the mammals are the main hosts (Rey 1973; Walker 1994).

The female reproductive system of Ixodidae is vital for the biological success of this animal group, once it has essential structures for the perpetuation of the species. It consists of an ovary, a pair of oviducts, and a muscular tube connecting the vagina to the genital opening (Sonenshine 1991; Said 1992). The ovary is a single and tubular horseshoe-shaped structure, with narrow lumen delimited by a wall formed by small epithelial cells with spherical nuclei, where a great number of oocytes are fixed through the pedicel cells, undergoing several stages of development until the oviposition phase (Oliveira et al. 2005, 2007).

One of the ways to control these animals, considered urban plagues (Paz et al 2008), is the use of synthetic acaricide substances (Labruna 2004); however, these chemical products are costly and contaminate the environment with residues harmful to the hosts and also the human being (Freitas et al. 2005). Moreover, the incorrect use of synthetic acaricides lead to the development of tick strains which are resistant to the active ingredients of these products (Häuserman et al. 1992).

Due to all these problems, the search for new methods of control has been intensified; inexpensive methods which are less toxic to the hosts and offer low environmental impact. One of the alternatives would be the utilization of natural compounds, obtained from extracts of plants with recognized acaricide or repellent action (Guerra 1985).

Carapa guianensis stands out among these plants. It belongs to Meliaceae family and is known as andiroba. This large tree is found in the north of South America, Central America, Antilles, and tropical Africa. In Brazil, it is found across the whole Amazon Basin. The oil extracted from its seeds is widely used by the local communities of the north of Brazil in the treatment of several diseases and also as an insect repellent (Hammer and Johns 1993).

Laboratorial experiments on the potential toxic effects of andiroba oil in rat females showed that this natural compound does not present toxic effect during gestation or interfere in the fertility of the hosts (Costa-Silva et al. 2008). Thus, this study aimed to analyze, through morphohistological techniques, the effects of andiroba seed oil on the ovaries of semi-engorged *R. sanguineus* females exposed to different concentrations of this natural product. These results will certainly help in the improvement or discovery of a new method to control these ectoparasites.

Material and methods

R. sanguineus ticks

R. sanguineus semi-engorged tick females weighing 27 mg in average were used throughout the experiment. The specimens were supplied by the tick colony maintained at the Brazilian Central of Studies on Ticks Morphology at the São Paulo State University, Rio Claro, SP, Brazil, under controlled conditions (28±1 °C, 80 % relative humidity, and 12 h photoperiod) in a biological oxygen demand (BOD) incubator and blood fed on New Zealand white rabbits.

Details on feeding and maintenance of *R. sanguineus* ticks on the hosts are given by Bechara et al. (1995). Briefly, the ticks were placed inside a feeding chamber consisting of a plastic tube (2.5 cm wide and 3 cm high) glued to the shaved back of the hosts with an atoxic and non-lesive preparation on the day prior to feeding. Elizabethan collars were used on the rabbits to prevent grooming. In order to avoid the escape of ticks during experiments, hosts were kept in cages placed in trays surrounded by a gutter filled with water and oil. Daily observations were performed on some biological parameters of the female ticks. This study was approved by Ethics Committee in the Animal Use, CEUA, UNESP, Rio Claro, SP, Brazil, Protocol no. 7124.

Andiroba oil

C. guianensis is a medium-sized tree, with heights ranging from 20 to 30 m and a diameter of 50 to 120 cm, and it has dense canopy. The stem is thick and gives off into plates. The leaves composed of 80 to 110 cm long, with 12 to 18 leaflets on dark green tone. The inflorescence occurs mainly at the end of branches, measuring 30 cm in length. The fruit is a capsule dehiscence site of four valves that separate when they fall to the ground, releasing 4 to 12 seeds that weigh on average 21 g. As regard to climate, andiroba occurs in areas with humid tropical climate, with rainfall between 1,800 and 3,500 mm per year. Temperatures can range from 17 to 30 °C and relative humidity of 70 to 90 %. The species grows best in soils not soaked and with plenty of organic matter. This tree blooms twice a year, in August–September and January–February. The fruits ripen in June–July and February–March. The oil contained in the seed is light yellow and extremely bitter (Revilla 2001; Lorenzi 2002) (Fig. 1a–e).

For this study, we used the oil from the seed of andiroba (*C. guianensis*) (pure extract), purchased through Farmácia de Manipulação, Drogaria e Homeopatia Art-Fármacos, Rio Claro, SP, Brazil. For this work, the pure extract from andiroba oil was transformed into soap, the emulsification by agents surfactants, such as Triton, SDS, and Tween, commonly used at the dilutions carried out with oils (Chagas et al. 2003; Farias et al. 2007, 2009), could lead to the formation of micelles and

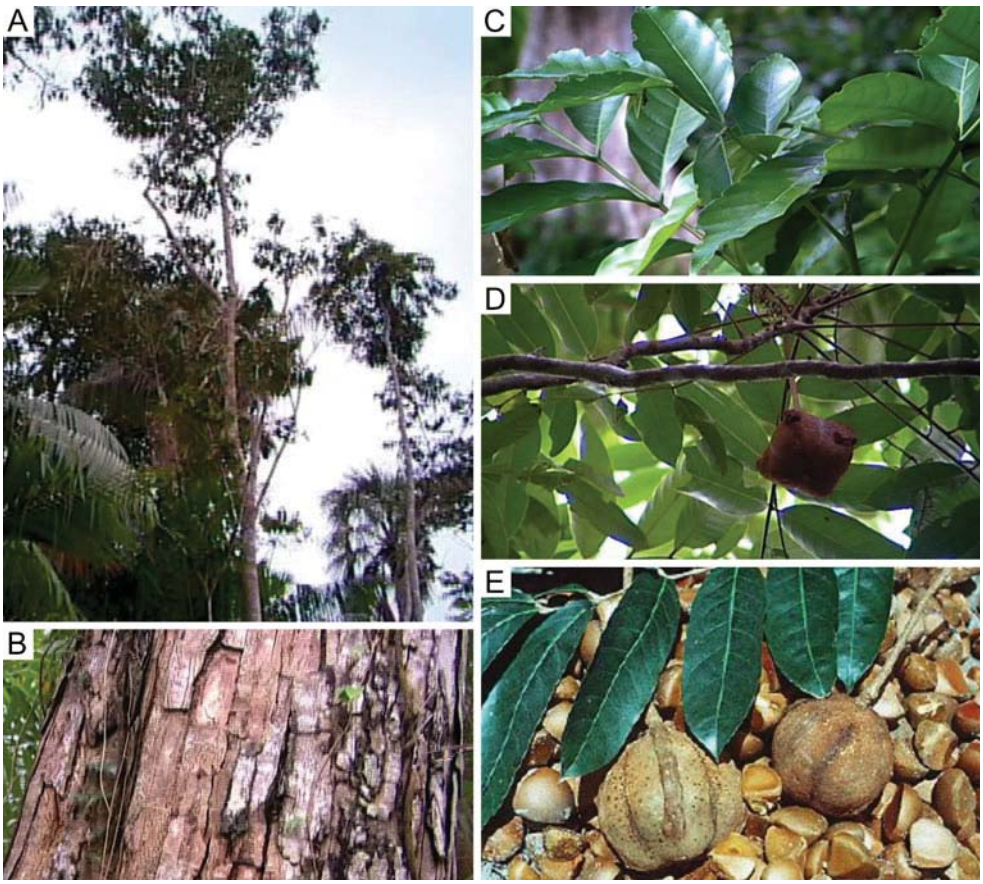

Fig. 1 *C. guianensis* tree (andiroba). **a** Andiroba tree. **b** Stem of andiroba tree. **c** Leaves of andiroba. **d** Andiroba fruit. **e** Fruits and seeds of andiroba. References: http://elizabethprovidasaudavel.blogspot.com.br/2011/11/andiroba.html; http://www.globorural.globo.com; http://www.umpedeque.com.br/site_umpedeque/arvore.php?id = 655

imprison the oil inside, preventing contact with ticks during immersion in andiroba oil dilutions.

Generally, surfactant agents are substances that reduce the surface tension of water, such as Triton, Tween, and SDS, among others. These compounds are employed as agents capable of emulsify lipophilic substances in water. This process of emulsification occurs due to formation of micelles, which encapsulate the lipophilic substance, imprisoning it in small vesicles, surrounded by molecules of surfactant agent. This encapsulation is possible since the surfactant molecule has one end which consists of a nonpolar substance that interacts with the lipophilic and hydrophilic end that interacts with the water. In the micelles, hydrophilic polar ends are facing the exterior of the vesicle, in contact with water molecules, whereas nonpolar hydrophobic ends are facing inwards. Thus, the molecules of the substance to be imprisoned in the hydrophobic ends will be emulsified of surfactants, i.e., in the central region of the micelles (Maniasso 2001; Cui et al. 2008; Jiang et al. 2011).

The saponification process carried out in this work promoted the hydrolysis of esters present in andiroba oil, releasing their fatty acids reacting with a base that formed a water soluble salt. In this way, the use of surfactants agents there was no longer needed. This process allowed the preparation of andiroba oil soap solutions in various concentrations.

In this work, the maximum dilution achieved for andiroba oil soap was 20 % since higher dilutions formed viscous solutions, preventing the immersion of ticks. In this way, from the maximum dilution of 20 %, other two dilutions were made (5 and 10 %) to analyze the action of andiroba oil on the reproductive system of *R. sanguineus* females. Any dilution of andiroba oil caused mortality in ticks analyzed here.

Dilution assays for andiroba oil

The *R. sanguineus* females, after being washed in a sieve with tap water, were dried on soft absorbent paper. Afterwards, 60 females were divided into four groups of 15 specimens each and immersed for 5 min in Petri dishes containing either of the different concentrations of andiroba oil (5, 10, and 20 %). The control group also consisting of 15 females was immersed in distilled water for the same period. Ticks were then dried on absorbent paper and placed in a BOD incubator (28±1 °C, 80 % relative humidity, and 12 h photoperiod) for 7 days. The observation period was established because frequently the effect of the acaricide is not immediate but acts slowly on the physiology.

Histology

The *R. sanguineus* females were dissected on Petri dishes containing phosphate buffered saline solution (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2). The ovaries

were removed, fixed in 4 % paraformaldehyde and 0.9 % NaCl in 10 % phosphate buffer (0.1 M; pH 7.5), dehydrated in an alcoholic series (70, 80, 90, and 95 %) for 15 min intervals. Infiltration was made with Leica resin at 4 °C and the material embedded in plastic molds at 4 °C to delay premature polymerization. The molds with material were filled and covered with Leica resin and the polymerization completed at room temperature (about 37 °C).

Sections of 3.5-μm thickness were mounted on glass slides, stained with hematoxylin–eosin (HE) and examined and photographed in a Leica DM750 photomicroscope. This device and other equipments were from the Histology Laboratory of the Biology Department at the Bioscience Institute, São Paulo State University, Rio Claro, SP, Brazil.

Results

Group I (control)

The description of the female reproductive system of *R. sanguineus* was already reported by Oliveira et al. (2005). In the present work, the same results related by these authors to the control group were obtained (Fig. 2a–f).

Group II (treated with 5 % of andiroba oil)

R. sanguineus females exposed to this concentration of andiroba oil present few oocytes with morphological changes when compared to the control group.

Oocytes I present the cytoplasm with large vacuoles located mainly next to the cell periphery and around the germ vesicle, which is rarely identified (Fig. 3a).

Oocytes II showed changes in their original shape, becoming irregular and with vacuoles distributed for the whole cell, as well as around the germ vesicle (Fig. 3b–d). In some oocytes, a large vacuole can be observed, forming a halo in the periphery of the cell (Fig. 3d). A slight decrease in the number of yolk granules in relation to control group can also be observed (Fig. 3b–d).

Oocytes III present round-shaped vacuoles distributed in all cytoplasm among the yolk granules. A slight change in the cell shape is also observed, due to the folds in the protective membranes (Fig. 3e).

No changes are observed in oocytes IV and V, except for small folds in the chorion of oocytes IV (Fig. 3f, g).

Group III (treated with 10 % of andiroba oil)

R. sanguineus females subjected to this concentration present a greater number of oocytes with morphological changes in comparison with those from treatment group II, as well as oocytes with more significant changes.

Oocytes I are rarely observed; however, those that could be identified show great changes in their original shape, becoming irregular and with large cytoplasmic vacuoles located mainly around the germ vesicle, which is dislocated to the pole of the oocyte turned to the pedicel. Ring-shaped nucleoli are also observed (Fig. 4a).

Oocytes II and III present the same characteristics described for the previous treatment; however, the vacuoles here are larger and located mainly in the periphery of the oocyte near the pedicel (Fig. 4b–d, f).

Oocytes IV do not show changes in their shape; however, there are small vacuoles located in the periphery of the cell (Fig. 4c).

Oocytes V present severe morphological changes with large empty spaces which occupy great part of the cytoplasm. Some granules appear to be ruptured and releasing part of their content into the cytoplasm. In this stage, the shape of the oocytes has irregular aspect, with folds in their protective membranes (Fig. 4e).

Changes in the ovary epithelium are also observed in this treatment group. In this epithelium, the cells lose their original cubic shape, forming a structure without a defined structure. The cells become vacuolated and with picnotic nuclei. In these regions, all the attached oocytes show severe changes and many of them could not be identified (Fig. 4a–c, f).

Group IV (treated with 20 % of andiroba oil)

The ovaries of *R. sanguineus* subjected to this concentration present several oocytes with great morphological changes when compared with those from the other treatment groups.

Oocytes in the initial development stages (I and II) are rarely visualized; however, those identified show severe changes in their shape, taking a totally irregular contour. Several vacuoles are found occupying great part of the cytoplasm, located around the germ vesicle, which is dislocated to the cell periphery near the pedicel. Several ring-shaped nucleoli can also be observed (Fig. 5a–d).

Oocytes III present vacuolated regions mainly in the cell periphery, in the opposite pole to the attachment of the oocyte to the pedicel (Fig. 5b, d).

Oocytes IV present vacuoles distributed in the whole cytoplasm and among the yolk granules. As for their shape, these cells present signs of disorganization, showed by the presence of folds in their protective membranes (Fig. 5e).

Oocytes V undergo cytoplasmic vacuolation around the yolk granules and have their original shape changed with irregular contour. Some oocytes are ruptured and release their content in the ovary lumen (Fig. 5f).

The ovary epithelium shows more significant changes in comparison with the ones found in the previous treatment group. An extreme disorganization of this tissue can be

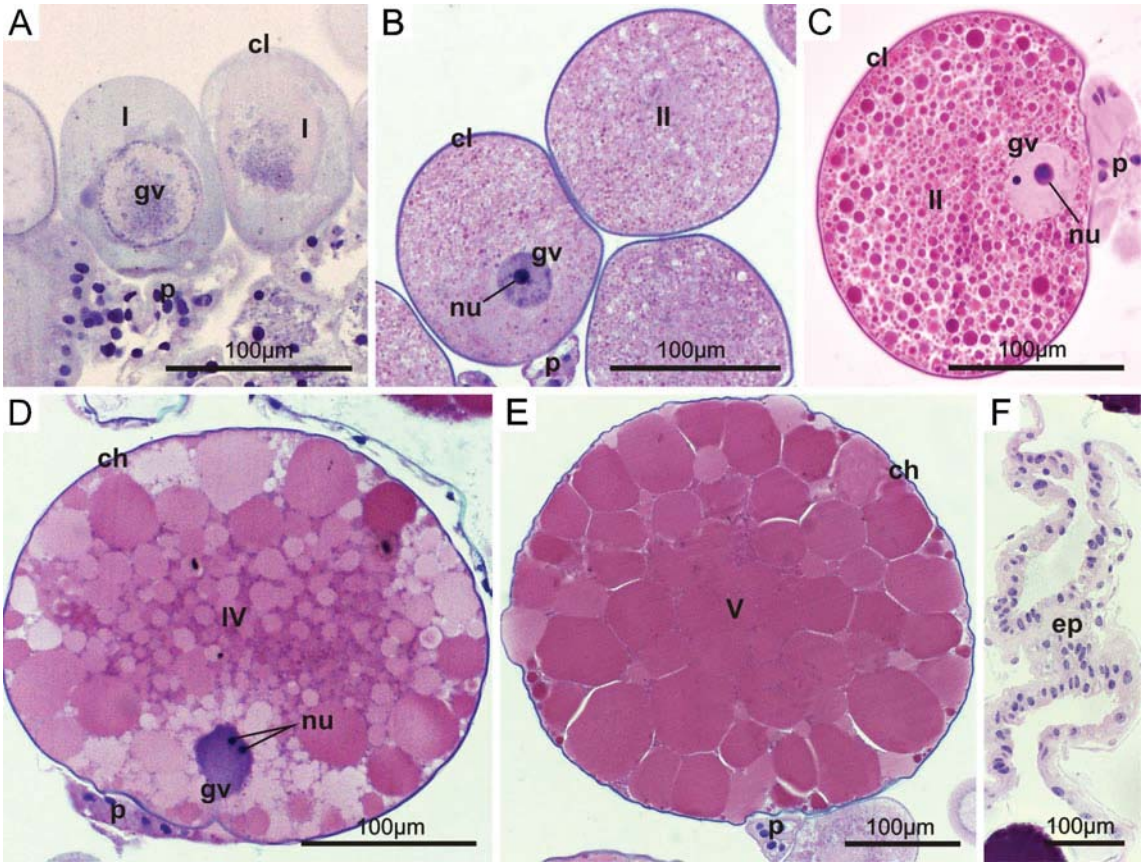

Fig. 2 Histological sections of the *R. sanguineus* ovary of the group I (control) stained with hematoxylin and eosin (HE). **a**–**e** Detail of the oocytes and **f** ovary epithelium (*ep*). *I* oocyte stage I, *II* oocyte stage II, *III* oocyte stage III, *IV* oocyte stage IV, *V* oocyte stage V, *cl* cell limit, *ch* chorion, *gv* germ vesicle, *nu* nucleolus, *p* pedicel

observed here, with severely vacuolated cells and picnotic nuclei, forming an amorphous mass (Fig. 5b–d, g).

Discussion

Actually, *R. sanguineus* species has been considered an urban plague and has drawn the attention of public health management organs (Paz et al. 2008; Dantas-Torres 2010), which is confirmed by the great expansion of the veterinary pharmaceutical industry, concerned with the creation of new acaricide substances formulations (Labruna 2004). However, the application of synthetic acaricides causes several damages such as environmental contamination with residues, as well as potential risks to human health (Freitas et al. 2005). In this sense, it is of utmost importance to develop research on alternative methods to control ticks that did not offer toxicity and environmental impact.

Few studies have been developed in this line of research, such as the ones by Arnosti et al. (2010) and Denardi et al. (2010) who reported that natural compounds such as ricinoleic acid esters from castor oil (*Ricinus communis*) and neem (*Azadirachta indica*) are able to affect the development of the female reproductive system, impairing the reproduction of *R. sanguineus*. Other studies, as those carried out by Oliveira et al. (2008, 2009), Roma et al. (2009, 2010a, b), and Nodari et al. (2011) who reported that the synthetic acaricides, when applied in concentrations much smaller than the ones commercially sold, are also able to affect the development of the main systems of the ticks, as the reproductive and glandular.

In this research line, Roma et al. (2012a, b) performed morphophysiological studies about the central nervous system (synganglion) of *R. sanguineus* ticks (larvae, nymphs, and adults) since this system is characterized as the target of several acaricide with neurotoxic action. Thus, this study serves as a basis for researches which need this information to better understand the cellular organization of this tissue as well as its physiology in order to improve or find new methods of control. In additional, Furquim et al. (2011) 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. The results revealed that the resistance acquired by hosts through

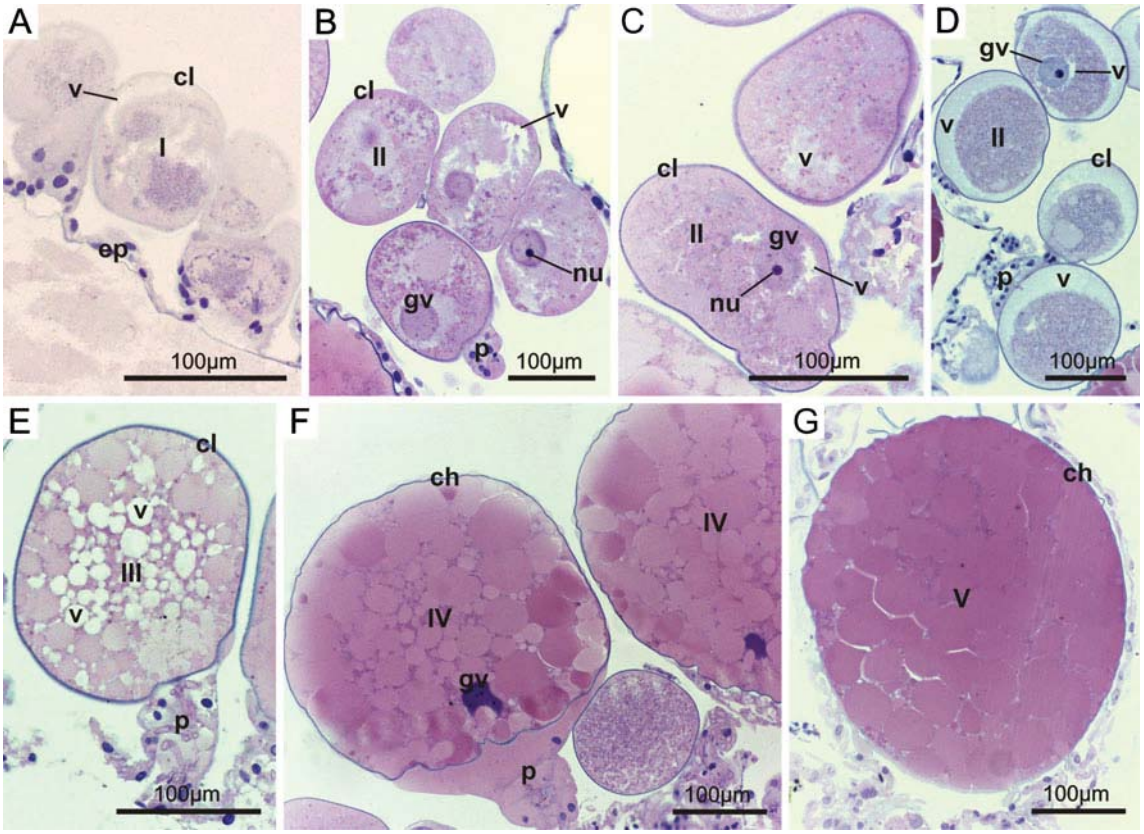

Fig. 3 Histological sections of the *R. sanguineus* ovary of the group II (treated with 5 % of andiroba oil) stained with hematoxylin and eosin (HE). **a**–**g** Detail of the oocytes. Observe that only oocytes in stages I to III present changes for this concentration of andiroba oil. The oocytes stage IV and V remain unaltered in relation to control group. *I* oocyte stage I, *II* oocyte stage II, *III* oocyte stage III, *IV* oocyte stage IV, *V* oocyte stage V, *cl* cell limit, *ch* chorion, *gv* germ vesicle, *nu* nucleolus, *p* pedicel, *v* vacuoles, *ep* ovary epithelium

immunization with extracts had affected differently the secretory activity of the glandular cells, which is an important piece of information in the search for a way to control these ectoparasites.

Thus, considering the data described above, this study performs for the first time the analysis of the effects of andiroba seed oil (*C. guianensis*) on the ovaries of semi-engorged *R. sanguineus* females exposed to different concentrations of this natural product, which according to Farias et al. (2007, 2009) have high acaricide potential and cause reduction in the fertility through the inhibition of the tick's oviposition.

R. sanguineus females subjected to the concentrations of 5, 10, and 20 % of andiroba oil showed changes in the germ cells and in the ovary epithelium when compared with the ones from control group. The main changes were presence, size and location of vacuoles in the oocytes cytoplasm, number of yolk granules, changes in the shape of the oocytes, and vacuolated ovary epithelium cells with picnotic nuclei.

The morphophysiology of the reproductive system of *R. sanguineus* females has been previously described by Oliveira et al. (2005), who showed that the ovary of these ectoparasites is panoistic, constituted by a wall formed by an epithelium, with small cells with spherical nuclei, delimiting a lumen and where a large number of oocytes attach through the pedicel cells. According to Oliveira et al. (2007) and Sanches et al. (2010), this structure in addition to the function of oocytes attachment (Diehl 1970; Briton and Oliver 1971) is related to the processes of vitellogenesis.

Oliveira et al. (2005) classified the oocytes of *R. sanguineus* in five stages of development according to morphological characteristics: cytoplasmic aspect, location of germ vesicle, presence, number and constitution of yolk granules, and presence or absence of chorion. This study showed that the oocytes (mainly in the initial stages of development) and the ovary epithelium of *R. sanguineus* females subjected to different concentrations of andiroba oil presented morphological changes which became more numerous and intense as the concentration of the product increased. These data corroborate with the studies of Oliveira et al. (2008, 2009) and Roma et al. (2010a, b) for this same species when exposed to synthetic products fipronil and permethrin, respectively.

Oocytes I of females exposed to 5 % of andiroba oil underwent changes, which are demonstrated by the presence of vacuoles in the cytoplasm of these cells. Similar data were reported by Roma et al. (2010a) for this same species when

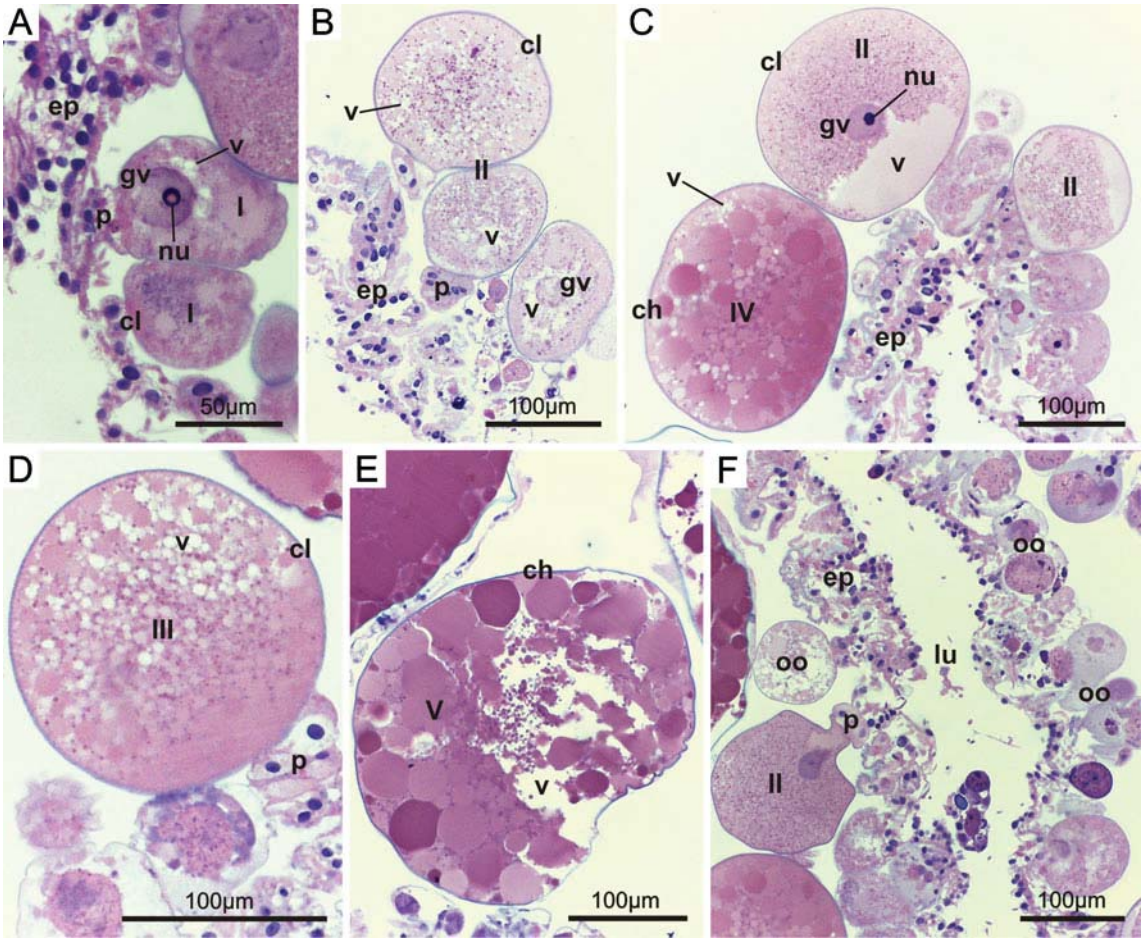

Fig. 4 Histological sections of the *R. sanguineus* ovary of the group II (treated with 10 % of andiroba oil) stained with hematoxylin and eosin (HE). **a**–**f** Detail of the oocytes. For this concentration, all oocytes stages show changes. Observe the ovary epithelium (*ep*) with changes such as picnotic nuclei and vacuoles. *I* oocyte stage I, *II* oocyte stage II, *III* oocyte stage III, *IV* oocyte stage IV, *V* oocyte stage V, *cl* cell limit, *ch* chorion, *gv* germ vesicle, *nu* nucleolus, *p* pedicel, *v* vacuoles, *lu* lumen of ovary, *oo* indeterminate oocytes

subjected to permethrin acaricide. Moreover, the emergence of the first vacuoles in the cytoplasm of these oocytes suggests that this natural product, even in low concentration, is able to cause structural damages in *R. sanguineus* germ cells. In addition, in these oocytes, the germ vesicle was of difficult visualization, which according to Denardi et al. (2010) occurred due to its fragmentation because of cytoplasmic vacuoles.

The vacuoles observed in the oocytes of *R. sanguineus* females would probably be autophagic and responsible for the degradation of cells organelles damaged by andiroba oil, as well as for the recycling of portions of cytoplasm affected by the action of this product, which justify the increase in the number of vacuoles in the oocytes exposed to higher concentration, consequently causing more damage to the cells. Similar data were reported by Roma et al. (2010a) for the oocytes of this same species exposed to a synthetic product.

Oocytes I exposed to higher concentrations of andiroba oil (10 and 20 %) were rarely observed; however, those which could be identified showed great changes, such as irregular shape and presence of cytoplasmic vacuoles, suggesting that in these concentrations the andiroba oil have more action on the reproductive system. In oocytes in initial stages of development and exposed to andiroba oil, the germ vesicle was dislocated to the cell periphery, which occurred due to accumulation of vacuoles occupying great part of the cytoplasm, changing the position of the germ vesicle, and consequently causing damages to the structure of the cell.

In higher concentrations of andiroba oil, some oocytes I and II presented nucleoli with the shape of small rings, which suggest the occurrence of degeneration processes in the nuclear material. These data were also reported by Furquim et al. (2008) during the processes of degeneration of the salivary glands and by Roma et al. (2010b) for the oocytes of *R. sanguineus* exposed to permethrin. Considering these data, the results obtained here confirmed that andiroba oil is able to cause irreversible damages to germ cells, mainly in their genetic material, once these changes affect the physiology of the cell.

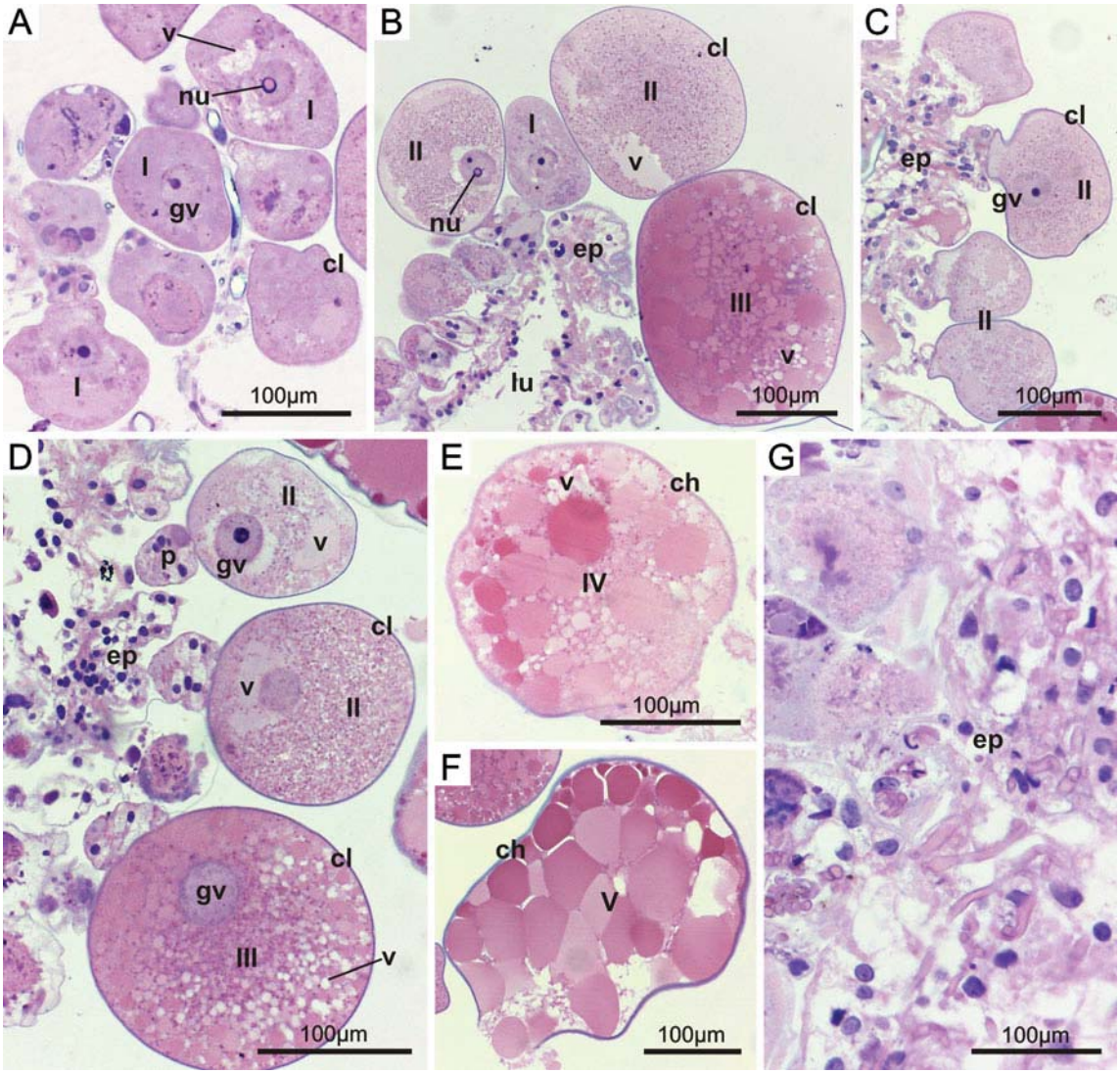

Fig. 5 Histological sections of the *R. sanguineus* ovary of the group II (treated with 20 % of andiroba oil) stained with hematoxylin and eosin (HE). **a**–**f** Detail of the oocytes. For this concentration, all oocytes stages show changes. Observe the ovary epithelium (*ep*) with changes such as picnotic nuclei and vacuoles. *I* oocyte stage I, *II* oocyte stage II, *III* oocyte stage III, *IV* oocyte stage IV, *V* oocyte stage V, *cl* cell limit, *ch* chorion, *gv* germ vesicle, *nu* nucleolus, *p* pedicel, *v* vacuoles, *lu* lumen of ovary

Oocytes II subjected to 5 and 10 % of andiroba oil showed changes in their original shape, becoming irregular, with vacuolated cytoplasm, and also showed a decrease in the number of yolk granules. According to Friesen et al. (2003) and Friesen and Kaufman (2003), in studies on the ovaries of *Amblyomma hebraeum* tick females exposed to avermectin and cypermethrin acaricides, respectively, this occurs due to decrease in the production of yolk elements by the oocytes and by a decrease in the synthesis of ecdysteroids, which reduce the production and released of vitellogenin (main yolk protein) to the hemolymph and consequent capture by the oocytes.

The changes observed in the oocytes II subjected to 20 % of andiroba oil were more severe, indicating that the higher the concentration of this natural product, the more significant were the changes undergone by germ cells, which lead to cell death by autophagy. Similar results were obtained by Oliveira et al. (2008) and Roma et al. (2010a) in studies on the reproductive system of *R. sanguineus* females exposed to synthetic products. These data become important when analyzed under the point of view of controlling the tick; by blocking the reproduction, the biological success of this group of animals is also affected.

Thus, based on the results obtained here, it was observed that oocytes I and II were the most affected by the action of andiroba oil, mainly in higher concentrations, which caused severe changes in these cells. According to Oliveira et al. (2009), the vulnerability of these cells in initial stages is explained by the absence of chorion; a membrane responsible for the protection of the eggs (Hilton 1982) and that

could function as an extra barrier to protect the cell, decreasing the entry rate of the chemical compound in the oocytes.

In oocytes III, the changes observed were also more intense in higher concentrations of the product, and more vacuolated regions were observed. This suggests that the cells would be trying to inactivate the action of the toxic product, through the recycling and/or degradation of cellular structures already affected by andiroba oil, in order to maintain the cell integrity and ensure the viability of the oocyte.

For these oocytes, as well as for the other stages of development, it was observed that the most significant cell changes, such as vacuolation, occurred more frequently and with more intensity in the pole region of the oocyte turned to the pedicel, suggesting that many of the substances captured from the hemolymph are transferred to the interior of the oocyte via pedicel cells, and this is the route of the andiroba oil, which have direct action on the reproductive system. These data corroborate Oliveira et al. (2007) who reported that the pedicel cells, in addition to the mechanical function, are directly involved in the process of capturing and producing yolk elements.

Oocytes IV and V did not present significant morphological changes when exposed to the concentration of 5 % of andiroba oil, probably due to the presence of chorion, membrane responsible for the protection of the oocyte, which in addition to promoting gaseous exchanges (Hilton 1982) is responsible for preventing the absorption of external elements from the hemolymph. However, in higher concentrations of andiroba oil, these cells presented significant changes, suggesting that for these doses, the chorion is not able to prevent the total absorption of the product due to the damages caused to its structure, which can be confirmed by the presence of folds and, in some cases, rupture of the membranes of these cells. Thus, the chorion loses its original protective function, allowing the endocytosis of acaricide by the oocytes and consequent harmful action in the cells. Thus, these oocytes are not able to complete their development or, even if this occurred, the eggs laid are not viable, due to the impairment caused in the development of the embryo.

In the concentration of 10 % of andiroba oil, some oocytes V presented empty spaces (loss of yolk granules) which occupy a great part of the cytoplasm, in addition, the yolk granules appear to be ruptured and releasing part of their content into the cytoplasm, corroborating Oliveira et al. (2008) in studies on *R. sanguineus* females exposed to fipronil chemical agent.

These results suggest that andiroba oil is able to cause changes in the oocytes in any development stage, suggesting that even if the oocytes reach stage III in unaltered state, they could be affected by harmful action of this product in later stages, which affect the structural organization of these cells, making them unviable to generate a new individual.

The ovary epithelium of *R. sanguineus* females exposed to andiroba oil showed severe morphological changes, with extreme structural disorganization in this tissue, with highly vacuolated cells and with picnotic nuclei forming an amorphous mass. According to Furquim et al. (2008), the presence of picnotic nuclei characterize the occurrence of cell death by apoptosis, which certainly is occurring here based on the morphological characteristics observed. According to Oliveira et al. (2005), the ovary epithelium, in addition to the function of oocytes attachment, is involved in the transportation of the yolk elements from the hemolymph to the oocytes and also in the synthesis of some ones. Thus, it is suggested that the changes caused by andiroba oil in the ovary epithelium interfere in the metabolism of this tissue, impairing the development of oocytes.

Based on the data described above, it can be inferred that although the defense mechanisms are developed by oocytes to recover the cellular integrity (presence of autophagic vacuoles), these cells are not able to revert the damage caused by this product. Thus, it can be concluded that although the damages caused to the oocytes by andiroba oil are comparatively less severe than the ones caused by synthetic acaricides (Oliveira et al. 2008, 2009; Roma et al. 2010a, b), this product can be considered a potent natural agent that reduce and/or prevent the reproduction of *R. sanguineus* females, with the advantage of not causing environmental impact.

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CAPÍTULO 2

Action of andiroba oil (Carapa guianensis) on Rhipicephalus sanguineus (Latreille, 1806) (Acari: Ixodidae) semi-engorged females. Morphophysiological evaluation of reproductive system.

Action of Andiroba Oil (*Carapa guianensis*) on *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) Semi-engorged Females: Morphophysiological Evaluation of Reproductive System

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KEY WORDS ticks; natural product; germ cells; cytotoxicity; reproductive efficiency index

ABSTRACT Because of the increasing medical-veterinary importance of ticks, the development of alternative control methods, less aggressive to the host and the environment has become the target of several researches. In this sense, the present study analyzed the action of different concentrations (5, 10, and 20%) of andiroba seed oil (*Carapa guianensis*) on the reproductive system of *Rhipicephalus sanguineus* females, through histochemical techniques and the quantification of the reproductive efficiency index. The results showed that andiroba oil is a potent natural agent, able to cause several changes in the oocytes of this species, impairing the reproductive success, once this natural product induces great physiological changes in the oocytes in all development stages, such as drastic reduction in proteins, polysaccharides, and lipids in these cells, and these components are essential for the viability of the embryo. In addition, it was observed that this product stimulate the oviposition, mainly at the concentration of 20%. This higher production of eggs represents a defense mechanism developed by the organism in order to ensure the reproductive success of the species, even in the presence of the toxic agent. However, the results obtained suggested that the laid eggs would not be viable, due to the great changes undergone by the oocytes. Thus, the present study showed that the use of this vegetal product would be an alternative way to control the ticks, bringing benefits similar to the ones obtained through the use of synthetic acaricides; however, with less damage to nontarget organisms and the environment as well. *Microsc. Res. Tech. 00:000–000, 2012.*

INTRODUCTION

The ticks constitute one of the most important groups of arthropods from the medical and veterinary points of view, once they cause lesions on the hosts during the process of blood feeding, in addition to being vectors of pathogenic agents, affecting animals, including the human being (Walker, 1994).

The group comprises animals belonging to Acari order, subclass Arachnida, being classified in three families: Ixodidae, Argasidae, and Nutalliellidae (Anderson and Magnarelli, 2008). *R. sanguineus* (Latreille, 1806) species is found within Ixodidae family, which is distributed throughout all the continents of the planet, mainly parasitizing the domestic dog.

In addition to causing significant blood loss in the hosts, this species is also a vector of viruses, bacteria, and protozoa, which cause diseases such as *Babesia canis*, protozoa that acts on the erythrocytes causing babesiosis or "nambiuvu" (Flechtmann, 1973), *Hepatozoon canis*, biopathogen of hepatozoonosis in dogs in Latin America (O'dwyer and Massard, 2001; Vicent-Johnson et al., 1997) and also *Ehrlichia canis*, which attack the dog leukocytes (Davoust, 1993; Simpson et al., 1991). Data from literature describe that *R. sanguineus*, due to their low specificity, are also potential vectors of the bacteria *Francisella tularensis*, etiologic agent of tularemia to the human being (Walker, 1994).

The females reproductive system of *R. sanguineus*, as for ticks in general, is essential for the development and perpetuation of the species, consisting of a tubular ovary located in the posterior region of the body, with a pair of oviducts, an uterus, a vagina, and a pair of accessory glands ending in the genital opening (Said, 1992; Sonenshine, 1991). This organ contains a wall formed by small epithelial cells with spherical nuclei, where a great number of oocytes attach; undergoing several stages of development until the oviposition phase (Oliveira et al., 2005).

Methods for an effective control of ticks have continuously been researched, as well as new acaricide

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products, which are economically viable and, at the same time, present low toxicity and impact to the environment. Among the alternatives is the use of natural compounds; i.e., products from plant extracts, which contain active ingredients with controlling action over the plagues (Guerra, 1985). In this sense, plants from Meliaceae family have been recognized as efficient in the control of plagues, mainly due to their repellent action against Arthropoda (Martinez, 2002). In this family *Carapa guianensis* species is found, popularly known as andiroba (Loureiro et al., 1979).

Few records are found in literature about the use of andiroba oil for ticks' control. The available data show that this product presents acaricide potential, in addition to inhibiting the oviposition of *Boophilus microplus*, *R. sanguineus*, and *Anocentor nitens* females (Farias et al., 2007, 2009).

According to data above described, it is necessary to develop a research line about the action of natural products on the main systems of the ticks. The information obtained through this study could be used as a basis to create more specific and efficient methodologies to control ectoparasites without inducing resistance to them, in addition to being harmless to the environment.

Thus, the present study aimed to evaluate the action of different concentrations of andiroba seed oil on the oocytes of *R. sanguineus* semi-engorged females, quantifying the reproductive efficiency index through immersion test, in addition to histochemical techniques in order to analyze whether this natural product would be able to impair the reproductive success of this species.

MATERIAL AND METHODS

Rhipicephalus Sanguineus Ticks

Rhipicephalus sanguineus tick semi-engorged females were used throughout the experiment. The specimens were supplied by the tick colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) at the São Paulo State University, Rio Claro, SP, Brazil, under controlled conditions (28 ± 1°C, 80% relative humidity and 12 h photoperiod) in a biological oxygen demand (BOD) incubator and blood fed on New Zealand white rabbits.

Details on feeding and maintenance of *R. sanguineus* ticks on the hosts are given by Bechara et al. (1995). Briefly, the ticks were placed inside a feeding chamber consisting of a plastic tube (2.5 cm wide and 3 cm high) glued to the shaved back of the hosts with an atoxic and nonlesion preparation on the day prior to feeding. Elizabethan collars were used on the rabbits to prevent grooming. To avoid the escape of ticks during experiments, hosts were kept in cages placed in trays surrounded by a gutter filled with water and oil. Daily observations were performed on some biological parameters of the female ticks, such as distribution of ticks (males and females) in the chamber, copulation, tick's attachment in the host, and engorgement.

This study was approved by Ethics Committee in the Animal Use, CEUA, UNESP, Rio Claro, SP, Brazil, Protocol n° 7124.

Andiroba Oil

For this study was used the oil from the seed of andiroba (*C. guianensis*) (pure extract), purchased through Farmácia de Manipulação, Drogaria e Homeopatia Art-Fármacos, Rio Claro, SP, Brazil.

The pure extract from andiroba oil was transformed into soap, once the emulsification by agents surfactants, such as Triton, SDS, and Tween, commonly used at the dilutions carried out with oils (Chagas et al., 2003; Farias et al., 2007, 2009), could lead to the formation of micelles and imprison the oil inside (Cui et al., 2008; Jiang et al., 2011; Maniasso, 2001), preventing contact with ticks during immersion in andiroba oil dilutions.

The saponification process carried out in this work promoted the hydrolysis of esters present in andiroba oil, releasing their fatty acids reacting with a base that formed a water soluble salt. In this way, the use of surfactants agents there was no need. This process allowed the preparation of andiroba oil soap solutions in various concentrations.

In this work, the maximum dilution achieved for andiroba oil soap was 20%, since higher dilutions formed viscous solutions, preventing the immersion of ticks. In this way, from the maximum dilution of 20%, other two dilutions were made (5% and 10%) to analyze the action of andiroba oil on the reproductive system of *R. sanguineus* females.

Dilution Assays for Andiroba Oil

The *R. sanguineus* females, after being washed in a sieve with tap water, were dried on soft absorbent paper. Afterwards, 60 semi-engorged females were divided into four groups of 15 specimens each and immersed for 5 min in Petri dishes containing either the above different concentrations of andiroba oil (5, 10, and 20%). The control group also consisting of 15 females was immersed in distilled water for the same period. Ticks were then dried on absorbent paper and placed in a BOD incubator (28 ± 1°C, 80% relative humidity and 12 h photoperiod) for 7 days.

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

Histochemistry

The *R. sanguineus* semi-engorged females were dissected on Petri dishes containing phosphate buffered saline-PBS solution (NaCl 0.13*M*, Na_2HPO_4 0.017*M*, KH_2PO_4 0.02*M*, pH 7.2). The ovaries were removed and fixed for 24 h in 4% paraformaldehyde and 0.9% NaCl in 10% phosphate buffer (0.1*M* – pH 7.5) (for proteins detection), Bouin's solution (for polysaccharides detection) and calcium formol (for lipids detection). The material was dehydrated in an alcoholic series (70, 80, 90, and 95%) at 15 min intervals. Infiltration was made with Leica resin and the material embedded in plastic molds at 4°C to delay prepolymerization. The molds with material were filled and covered with Leica resin and the polymerization completed at room temperature (about 37°C).

Sections with 3.5 μm thickness were mounted on glass slides. After they were air dried before staining with bromophenol blue (protein) (Pearse, 1985), PAS/

Alcian Blue (acid and neutral polysaccharides) (Junqueira and Junqueira, 1983) and Baker (lipids) (Baker, 1946) and examined and photographed in a Leica DM750 photomicroscope.

Reproductive Efficiency Index Through Semiengorged Female Bioassay

Ticks semi-engorged females were removed from host after 5 days of engorgement and individually weighed. Afterwards, 120 females were divided into four groups of 30 specimens each and immersed for 5 min in Petri dishes containing either the above different concentrations of andiroba oil (5, 10, and 20%). The control group also consisting of 30 females was immersed in distilled water for the same period. These females were individually maintained in a tick chamber (small closed container with holes for ventilation) until oviposition was completed. Egg clusters were weighed and maintained until hatching was achieved. The following biological parameters related to female tick reproductive performances were determined: semi-engorged female weight, preoviposition period, oviposition period, and egg mass weight. These parameters were used to calculate reproductive efficiency index as previously described (Barriga et al., 1991, 1995). Females' weight was measured immediately after removal. Egg masses were weighed 21 days after tick detachment. The preoviposition period was the time that elapsed from the detachment of the female tick until the beginning of oviposition. The oviposition period was the time that ticks started laying eggs until they finished. The reproductive efficiency index was calculated by dividing the weight of each egg mass by the weight of the respective female.

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

RESULTS

In the present study, any dilution of andiroba oil caused mortality in ticks analyzed here.

Histochemistry

The detection test for proteins, polysaccharides and lipids in oocytes of *R. sanguineus* semi-engorged females was previously described by Oliveira et al. (2005). In the present work were obtained the same results related by these authors to the control group (Figs. 1A–1E, 2A–2E, and 3A–3E).

Protein Detection. *Group I (Control).* **Oocytes I** present few protein elements, once the cytoplasm is weakly positive. The germ vesicle shows moderate positivity, with strongly positive nucleolus (Fig. 1A).

Oocytes II have fine cytoplasm granulation, moderately positive and uniformly distributed throughout the cytoplasm. As in the previous stage, the germ vesicle has moderate positivity for proteins, while the nucleolus is strongly positive (Fig. 1B).

Oocytes III have strongly positive granules. Among them others are moderately positive. The germ vesicle and the nucleolus, respectively present moderate and strong positivity (Fig. 1C).

Oocytes IV present yolk granules with strong or moderate positivity. The germ vesicle and the nucleolus have the same characteristics described for the previous stages (Fig. 1D).

Oocytes V have a great number of strongly positive yolk granules throughout the cytoplasm. As described for the other stages, the germ vesicle and the nucleolus show moderate and strong positivity, respectively (Fig. 1E).

Groups II, III, and IV (Treated with 5, 10, and 20% of Andiroba Oil, Respectively). The **oocytes I–V** subjected to different concentrations of andiroba oil present, in general, the same histochemical characteristics for the proteins described for the control group (Figs. 1F–FS). However, some changes are observed, such as:

- The **oocytes II** of all the groups treated with andiroba oil present strongly positive protein granules, while in the control group they are moderately positive (Figs. 1B, 1G, 1K, and 1P).
- The **oocytes IV** subjected to andiroba oil present granules with weak or moderate positivity, while in the control group the granules are strongly or moderately positive for protein (Figs. 1D, 1I, 1M, and 1R).
- The **oocytes V** of the group subjected to 10% of andiroba oil have most granules with weak or moderate positivity, while in the control group they are strongly positive (Figs. 1E and 1N).

Polysaccharides Detection. In all *R. sanguineus* oocytes only neutral polysaccharides are observed. The acid polysaccharides are not detected in the germ cells of control group and neither in the groups subjected to andiroba oil (Figs. 2A–2T).

Group I (Control). **Oocytes I** have weak cytoplasm positivity for polysaccharides (Fig. 2A).

Oocytes II present the cytoplasm with a strongly positive, fine and homogeneous granulation (Fig. 2B), evidencing the beginning of the synthesis and/or incorporation of polysaccharides.

Oocytes III present strongly positive cytoplasmic granules. Some of them show moderate positivity (Fig. 2C).

Oocytes IV present strongly positive yolk granules of different sizes (Fig. 2D).

Oocytes V present strongly positive yolk granules throughout the cytoplasm (Fig. 2E).

The nuclei of germ cells are not observed, once this technique is not specific for the demonstration of the nucleus.

Groups II, III, and IV (Treated with 5, 10, and 20% of Andiroba Oil, Respectively). Thus, as observed for proteins detection, the histochemical characteristics for polysaccharides of germ cells of the groups subjected to andiroba oil are the same found for control group (Figs. 2F–2T); however, some changes are observed, such as:

- The **oocytes II** of all the treatment groups' present drastically reduced polysaccharide granules; mainly in the concentration of 20% of andiroba oil (Figs. 2B, 2G, 2L, and 2Q).
- The **oocytes III** subjected to the concentration of 10% of andiroba oil also show significant reduction in polysaccharides, once the larger granules are no longer observed; only the smaller, which are strongly positive (Fig. 2M).

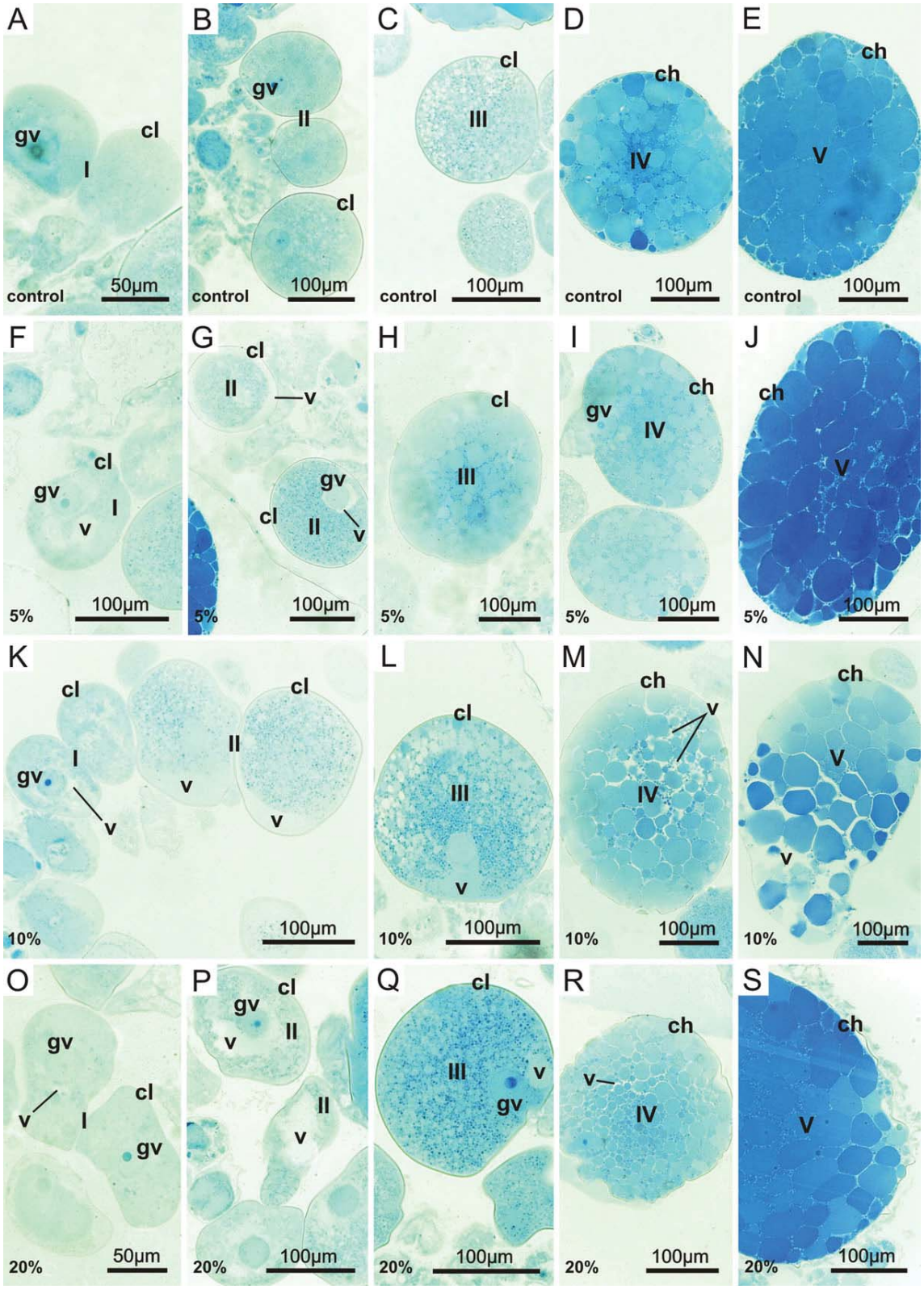

Fig. 1. Histological sections of the *Rhipicephalus sanguineus* ovary stained with bromophenol blue. (**A**–**E**) Group I (control group), (**F**–**J**) Group II (treated with 5% of andiroba oil), (**K**–**N**) Group III (treated with 10% of andiroba oil) and (**O**–**S**) Group IV (treated with 20% of andiroba oil). I, oocyte stage I; II, oocyte stage II; III, oocyte stage III; IV, oocyte stage IV; V, oocyte stage V; cl, cell limit; ch, chorion; gv, germ vesicle; v, vacuoles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

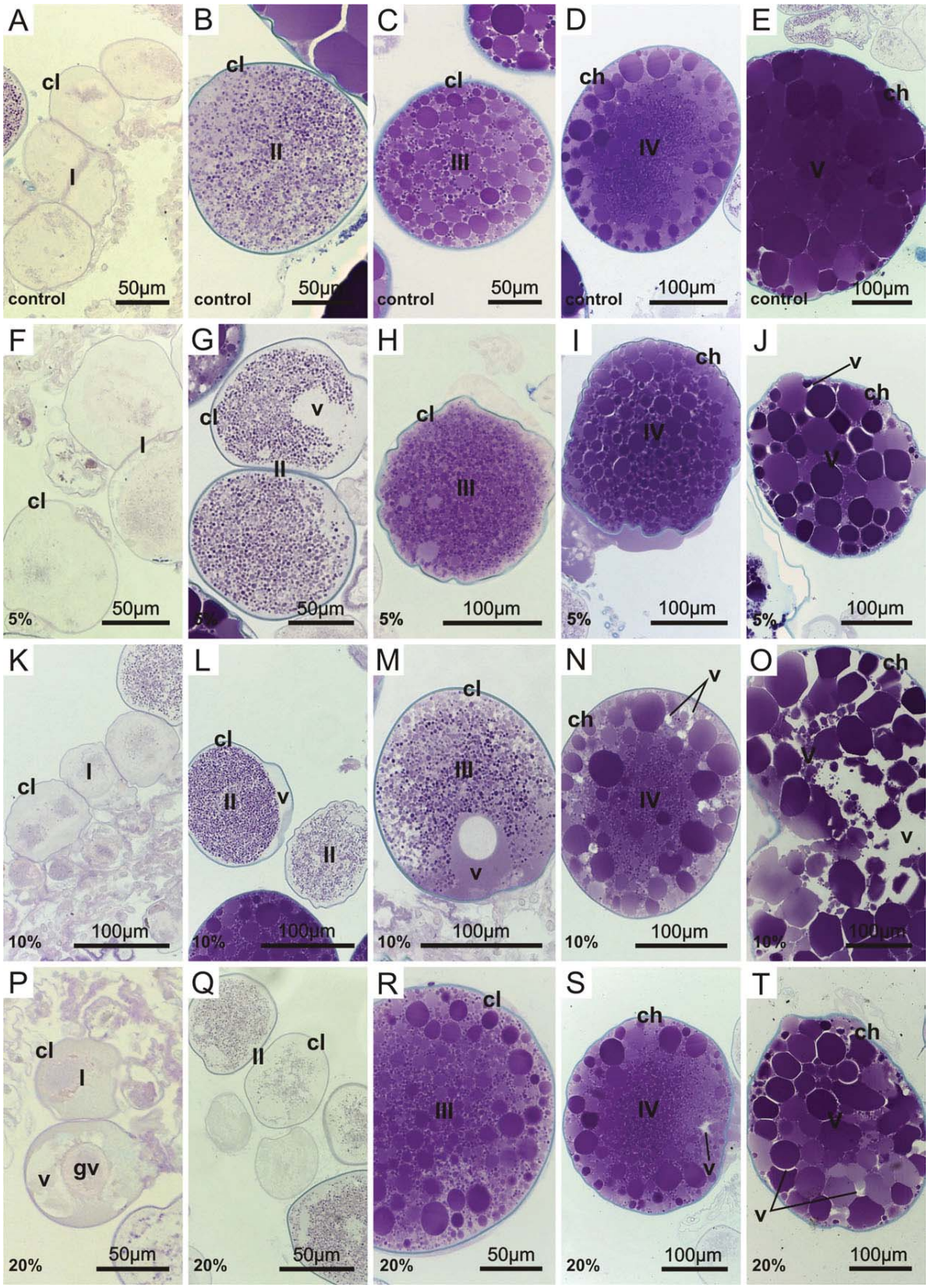

Fig. 2. Histological sections of the *Rhipicephalus sanguineus* ovary stained with PAS/Alcian Blue. (**A**–**E**) Group I (control group), (**F**–**J**) Group II (treated with 5% of andiroba oil), (**K**–**O**) Group III (treated with 10% of andiroba oil) and (**P**–**T**) Group IV (treated with 20% of andiroba oil). I, oocyte stage I; II, oocyte stage II; III, oocyte stage III; IV, oocyte stage IV; V, oocyte stage V; cl, cell limit; ch, chorion; gv, germ vesicle; v, vacuoles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

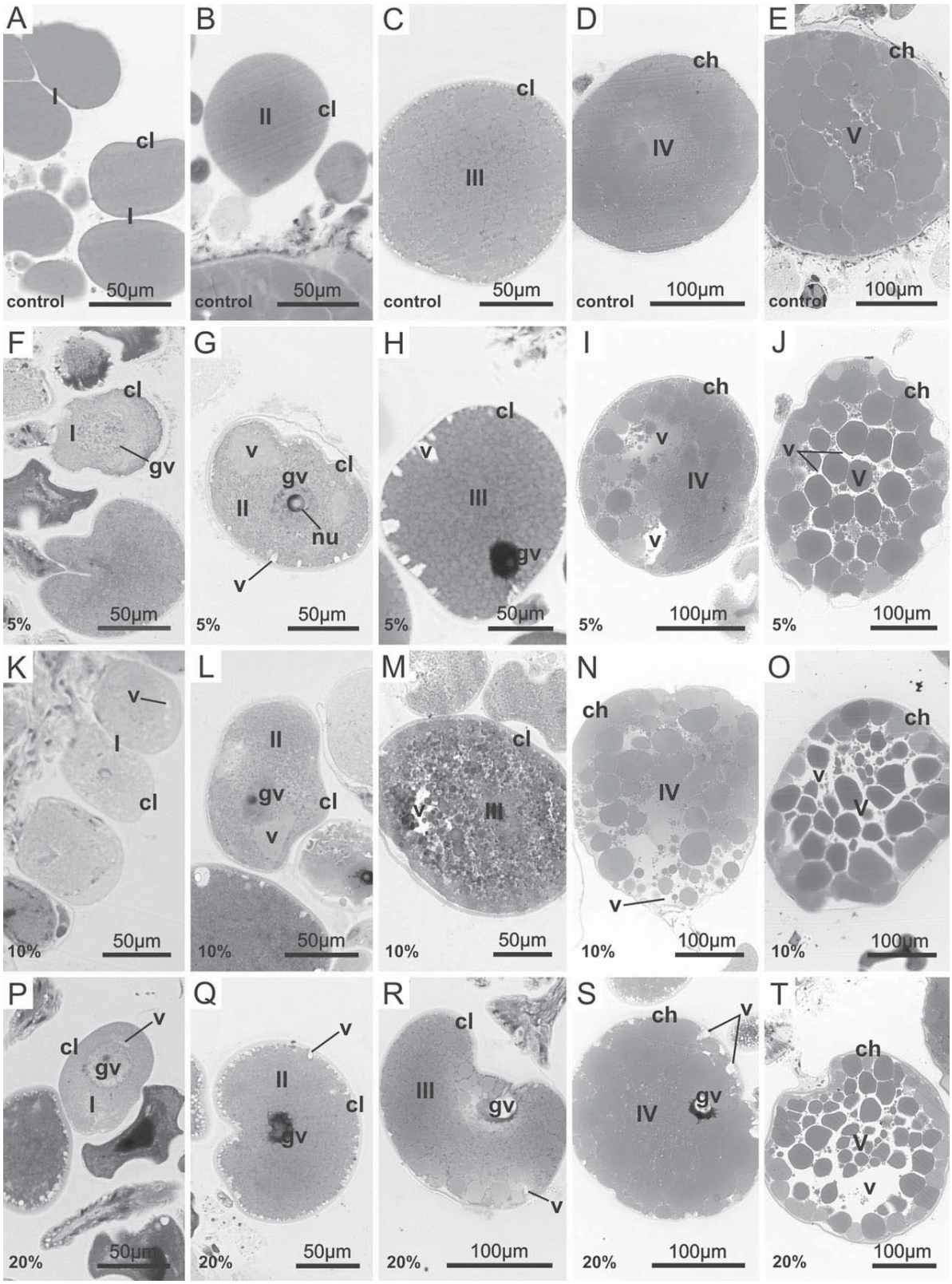

Fig. 3. Histological sections of the *Rhipicephalus sanguineus* ovary stained with Baker. (**A**–**E**) Group I (control group), (**F**–**J**) Group II (treated with 5% of andiroba oil), (**K**–**O**) Group III (treated with 10% of andiroba oil) and (**P**–**T**) Group IV (treated with 20% of andiroba oil). I, oocyte stage I; II, oocyte stage II; III, oocyte stage III; IV, oocyte stage IV; V, oocyte stage V; cl, cell limit; ch, chorion; gv, germ vesicle; nu, nucleolus; v, vacuoles.

TABLE I. Comparison of the reproductive parameters of R. sanguineus ticks females from a control group and groups treated with andiroba oil

Groups	Reproductive parameters				
	Semi-engorged female weight (g) before oviposition	Preoviposition period (days)	Oviposition period (days)	Egg mass weight (g)	Reproductive efficiency index (%)
Group I: control	0.207 ± 0.069	4.273 ± 0.467	7.273 ± 0.094[a]	0.122 ± 0.040	59.20 ± 8.95[b]
Group II: 5% of andiroba oil	0.211 ± 0.067	4.636 ± 0.924	8.818 ± 2.272	0.136 ± 0.043	66.69 ± 15.26
Grupo III: 10% of andiroba oil	0.204 ± 0.076	4.182 ± 0.404	7.800 ± 0.918	0.149 ± 0.086	70.27 ± 19.36
Grupo IV: 20% of andiroba oil	0.194 ± 0.062	4.455 ± 0.934	9.273 ± 1.104[a]	0.151 ± 0.054	80.17 ± 11.24[b]
ANOVA test	$P = 0.945$	$P = 0.476$	$P = 0.008$	$P = 0.629$	$P = 0.016$

The values are provided as average ± standard deviation.
[a,b]Significant difference ($P < 0.05$).

- The **oocytes V** subjected to 10% of andiroba oil show drastic reduction in polysaccharides, due to the great number of vacuoles present in these cells in relation to control group (Fig. 2O).

Lipids Detection. *Group I (Control).* **Oocytes I** present homogeneous cytoplasm, strongly positive for lipid (Fig. 3A).

Oocytes II have fine and strongly positive granulation, homogeneously distributed throughout the cytoplasm (Fig. 3B).

Oocytes III show larger granules occupying the peripheral region of the cytoplasm and smaller granules in the center, all of them strongly positive for lipid (Fig. 3C).

Oocytes IV most granules are strongly positive; however, some present moderate positivity, mainly in the periphery of these cells (Fig. 3D).

Oocytes V show the cytoplasm completely full of strongly positive granules for lipids (Fig. 3E).

The nuclei of germ cells are evident due to the use of hematein, a solution obtained from the oxidation of hematoxylin (Figs. 3A–3E).

Groups II, III, and IV (Treated with 5, 10, and 20% of Andiroba Oil, Respectively). The oocytes **I–V** subjected to different concentrations of andiroba oil present, in general, the same histochemical characteristics for lipid described for control group (Figs. 3F–3T). However; some changes are observed, such as:

- The **oocytes I** and **II** of females subjected to andiroba oil show weak positivity for lipid, while in the control group these cells are strongly positive (Figs. 3A, 3B, 3F–3G, 3K–3L, 3P–3Q).
- The **oocytes IV** subjected to andiroba oil show a reduction in the lipid content, once most granules are moderately positive, while in the control group strongly positive granules are observed (Figs. 3D, 3I, 3N, and 3S).
- The **oocytes V** of the females subjected to andiroba oil show drastic reduction in lipid, considering the presence of vacuoles in these cells in relation to control group (Figs. 3E, 3J, 3O, and 3T).

Reproductive Efficiency Index Through Semiengorged Female Bioassay

The results show that there is no significant difference between the parameters: weight of females before oviposition ($P = 0.945$), preoviposition period ($P = 0.476$) and egg mass weight ($P = 0.629$) between the control groups and the groups treated with andiroba oil. However, for the parameters oviposition period and reproductive efficiency index there is a significant difference between the control group and those subjected to 20% of andiroba oil ($P = 0.008$ and $P = 0.016$, respectively). No significant statistic differences concerning these latter parameters were observed in the other groups (Table I, Fig. 4).

DISCUSSION

The control of ticks is a subject that has aroused the researchers' interest and drawn the attention of public organs because of the serious damage caused by these ectoparasites, such as lesions in the hosts caused during blood feeding, transmission of pathogens affecting stock breeding, domestic animals, and public health in general (Harwood and James, 1979, Rey, 1973); high costs with the acquisition of products, facilities and workforce for the application of acaricides; concern about the environmental contamination by chemicals and potential risks to human health (Freitas et al., 2005).

Considering this, there has been a lot of research on alternative ways to control the ticks, aiming to find efficient methods; less toxic to hosts, offering low environmental impact and lower costs. One of the alternatives would be the use of synthetic compounds at lower concentrations or the use of natural products obtained from plant extracts with recognized acaricide or repellent action (Guerra, 1985).

Considering this, research has been carried out aiming to analyze the effects of synthetic and natural products on the reproductive system of the ticks (Arnosti et al., 2010; Denardi et al., 2010; Oliveira et al., 2008, 2009; Roma et al., 2010, b), once the changes in this organ (vital for the perpetuation of the species) impair the reproductive success of these ectoparasites. Thus, the present study describes the histochemical changes caused by andiroba seed oil in germ cells of semiengorged *R. sanguineus* female ticks, and analyzes the reproductive efficiency index of the species when treated to this natural product.

Data here obtained showed that the oocytes of *R. sanguineus* female from the control group presented the same morphohistochemical characteristics already described by Oliveira et al. (2005) for this same species of tick. Thus, in this study, the chemical composition of the oocytes of the control group will not be described, but only used as a reference to demonstrate the changes in germ cells subjected to the action of andiroba oil.

The oocytes of *R. sanguineus* from the control group and the ones subjected to andiroba oil are mainly constituted by proteins, polysaccharides and lipids,

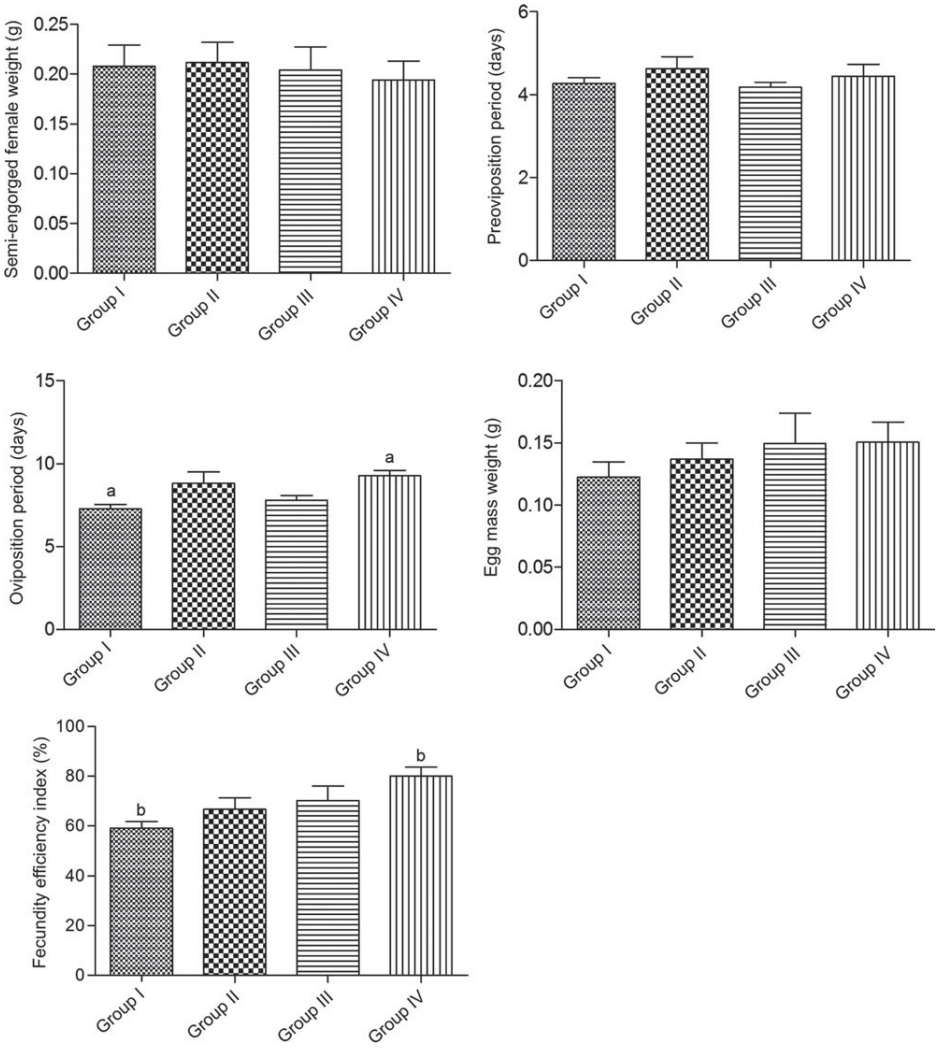

Fig. 4. Reproductive parameters of *Rhipicephalus sanguineus* ticks females from a control group and groups treated with andiroba oil. Group I = control, Group II = treated with 5% of andiroba oil, Group III = treated with 10% of andiroba oil, Group IV = treated with 20% of andiroba oil.

corroborating the analyses by Denardi et al. (2004) and Oliveira et al. (2005) for *Amblyomma cajennense* and *R. sanguineus* females, respectively. These data reveal that andiroba oil, despite causing severe morphological changes in germ cells, such as emergence of vacuoles in the cytoplasm of the oocytes, reduction in the number of yolk granules, changes in the shape of oocytes, impairment of genetic material, and vacuolation of the ovarian epithelium cells (Vendramini et al., 2012), is not able to change the chemical nature of yolk granules synthetized and/or incorporated by these cells. Similar data were also reported by Roma et al. (2011) when analyzing the action of permethrin (synthetic pyrethroid) on the oocytes of the females from the same species.

In control group, the oocytes presented a small amount of protein; however, in the groups subjected to andiroba oil these cells were strongly positive. According to Oliveira et al. (2005) the synthesis and/or incorporation of protein elements in the oocytes in the early stages of development occur at low level; however, Roma et al. (2011) reported that the increase in protein synthesis in the oocytes (initial stages of development) of females subjected to acaricide products is probably not for the production of yolk, but rather for the production of enzymes to neutralize the toxic substance, in the attempt to maintain the integrity of the cell, which certainly is occurring in the present study.

As for oocytes IV and V of the females exposed to andiroba oil, weakly or moderately positive protein granules were observed, while in the control group they were strongly positive. These results suggest that in the later stages of the oocytes development the action of andiroba oil is different from the one observed in earlier stages; i.e., in this case this product acts as an inhibitor of the synthesis and/or incorporation of protein. In addition, the presence of vacuoles among and around the yolk granules contribute for the

reduction of the protein content of these oocytes, mainly in higher concentrations of andiroba oil, at which the cytoplasmic vacuolation occurred more intensely. According to Oliveira et al. (2008) and Roma et al. (2010a, b, 2011) in studies about the action of synthetic products in the ovaries of ticks, this occurs due to the need the germ cells have to recycle cytoplasm portions impaired by the toxic substance or even eliminate the entire cell, preventing damage to the tissue and reusing the still intact material through a turnover. However, this defense mechanism also contributes for the elimination of compounds, which are essential for the cells, such as yolk granules. The reduction in the protein content in the oocytes of *R. sanguineus* females subjected to andiroba oil lead to the impairment of the embryonic development, once this element is essential to ensure the viability of the embryo.

The results obtained in the present study show the presence of only neutral polysaccharides in the oocytes of control group and treated with andiroba oil, corroborating Denardi et al. (2004) and Oliveira et al. (2005) for *A. cajennense* and *R. sanguineus*, respectively.

A severe reduction in the polysaccharides content was observed in the oocytes of the females subjected to andiroba oil, mainly at higher concentrations of this product, and also in the earlier stages of development. According to Oliveira et al. (2005), the polysaccharides are transported to germ cells through the hemolymph and directly absorbed by the plasma membrane or via pedicel cells. Considering this information, it can be suggested that andiroba oil affect the absorption route of the polysaccharide elements, and consequently contributing for the loss of functional integrity of the cell, once the polysaccharides, which in normal conditions are used in the processes of vitellogenesis, are used now as energy source for the metabolic processes of detoxification of substances, in this case, the andiroba oil.

As for the histochemical test for lipid detection, the results showed that oocytes I and II of females subjected to andiroba oil present few lipids, while in the group control these cells present a large amount of this element. These results suggest that andiroba oil, in addition to reducing the number of proteins and polysaccharides, also is able to interfere in the synthesis (endogenous production) and/or incorporation (exogenous production) of lipids. In addition, the presence of vacuoles in the cytoplasm of these cells contribute for the reduction of these elements, once these cells are involved in the elimination of a large number of organelles and portions of the cytoplasm damaged by the action of andiroba oil.

This study also showed that the increase in the concentration of andiroba oil caused a gradual reduction in the number of yolk granules in the oocytes in relation to the control group, suggesting the rupture and release of content of many of these granules, corroborating Oliveira et al. (2008) in studies on *R. sanguineus* females exposed to fipronil chemical agent.

The results here obtained also showed that the most significant histochemical changes occurred in the oocytes in the earlier stages of development (I–III). According to Oliveira et al. (2008), the vulnerability of the cells is explained by the absence of chorion, membrane responsible for the protection of the eggs (Hinton, 1982), which serve as an extra barrier for the protection of the cell, decreasing the interiorization rate of the toxic compound in the oocyte. However, in later stages (IV e V) severe histochemical changes in the germ cells were also observed, suggesting that the andiroba oil is able to damage the chorion, which would have its protection functions reduced (Hinton, 1982), permitting and facilitating the entrance of the toxic product in the oocytes. This cause damages to the cytoplasm of these oocytes, such as extended vacuolated regions mainly in the cell periphery. These results were also reported by Oliveira et al. (2008, 2009) and Roma et al. (2010a, b) in studies on the action of the synthetic products fipronil and permethrin on the oocytes of *R. sanguineus*, respectively.

The analysis of the data here obtained showed that the most significant changes induced by andiroba oil occurred at the intermediate concentration of the product (10%), suggesting that this is the concentration, which have more action on the ovary of *R. sanguineus* females. This occurs due to the physiology of the reproductive system, which naturally react preventing the entrance of part of the toxic product in the cells, which not occur at lower doses, once the physiologic system not recognize the product as toxic to the organism.

As the analysis of the reproductive parameters through immersion test it was observed that there is no significant difference between the parameters: female weight before oviposition, preoviposition period and egg mass weight comparing control groups and those subjected to andiroba oil. However, for parameters oviposition period and reproductive efficiency index was observed a significant difference between control group and group subjected to 20% of andiroba oil, demonstrating that this product act stimulating oviposition. Considering these data, it can be suggested that the higher production of eggs represent a defense mechanism developed by the organism, in order to ensure the reproductive success of the species, even in the presence of the toxic agent. However, the results obtained suggested that the eggs laid would not be viable, due to the great changes undergone by the oocytes, such as great reduction in the yolk content (essential elements for the full development of the embryo).

These data oppose Farias et al. (2007, 2009), who reported that this product (diluted in tween 80) causes the total inhibition of oviposition in *A. nitens*, *B. microplus* and *R. sanguineus* females, even at the lowest concentration tested (10%). In addition, these authors reported the occurrence of 100% of mortality for the females from all the treatment groups (10, 25, 30, 50, and 100%) after 4 days of exposure to the product. However, in this study mortality was not observed for any specimen during treatment with andiroba oil. Accordingly, Silva et al. (2007) described that the vegetal extracts of neem (*Azadirachta indica*) and lemon grass (*Cymbopogon citratus*) reduced the periods of preoviposition, as well as the total production of eggs, interfering in the oviposition and fertilization of *R. sanguineus* and *B. microplus* females.

This difference in the results obtained in the present study and those performed by Farias et al. (2007, 2009), would probably be occurring because of the use of surfactants, such as tween 80, which would maximize the effect of andiroba oil, which did not occur in

this study, once only the oil extract was used, without the presence of any dispersant.

Thus, considering the data here obtained it can be concluded that andiroba seed oil is able to cause severe changes in the reproductive system of *R. sanguineus* females, impairing their reproductive success, once this natural product induces serious changes in the oocytes, mainly in earlier stages of development, inhibiting the development until stage V, and, even whether this occurs, the andiroba oil also is able to induce morphophysiological changes in later stages.

According to Chungsamarnyart et al. (1991), the natural compounds obtained from plants not present toxic potential for mammals, due to their fast biological degradation and slow development of resistance by the ticks, characteristics that make the bioacaricides commercially appealing, permitting the control of the ticks without harming the environment.

CONCLUSIONS

The present study demonstrated that the use of andiroba oil can be an alternative way to control ticks, which will bring benefits, similar to those obtained with the use of synthetic acaricides; however, causing less damage to nontarget organisms and the environment as well.

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CONSIDERAÇÕES FINAIS

6. CONSIDERAÇÕES FINAIS

Atualmente, diversas formas de controle de carrapatos têm sido pesquisadas, no intuito de se encontrar substâncias com potencial acaricida que apresentem menor toxicidade tanto para hospedeiro quanto para o meio ambiente. Neste sentido, uma das alternativas para o emprego de uma forma de controle sustentável seria o uso de produtos naturais, com princípios ativos que auxiliem no controle destes ectoparasitas, seja através de ação repelente, acaricida ou ainda que comprometa a morfofisiologia dos principais sistemas destes animais.

Dessa forma, o presente estudo teve como objetivo avaliar morfofisiologicamente os efeitos de diferentes concentrações do óleo de andiroba; produto este com conhecida ação repelente e acaricida, sobre o aparelho reprodutor de fêmeas de carrapatos *R. sanguineus*, órgão vital para a reprodução e perpetuação da espécie.

Assim, de acordo com os resultados obtidos no presente estudo, pode-se concluir que:

- O óleo de andiroba causa alterações irreversíveis nas células germinativas e no epitélio ovariano de *R. sanguineus*.

- As alterações no sistema reprodutor feminino se tornam mais severas à medida que a concentração do produto aumenta.

- Os ovócitos I e II são os mais afetados pela ação do óleo de andiroba, principalmente nas maiores concentrações, as quais provocam intensas alterações nessas células devido à ausência de cório, membrana responsável pela proteção dos ovos e que controla a absorção de elementos vindos da hemolinfa.

- Nas células germinativas das fêmeas expostas ao óleo de andiroba, as alterações celulares mais significativas ocorrem com maior frequência e intensidade na região do pólo do ovócito voltado para o pedicelo, sugerindo que o óleo de andiroba é transferido para o interior do ovócito via células do pedicelo, tendo ação direta sobre o sistema reprodutor.

- Os ovócitos IV e V expostos a baixas concentrações do óleo de andiroba não apresentam alterações morfológicas significativas. Porém, nas maiores dosagens estas células mostram grandes alterações, uma vez que o cório não é capaz de impedir a total absorção do produto, devido aos danos causados a sua estrutura (dobras e rompimento de membrana).

- O óleo de andiroba é um agente capaz de causar alterações nos ovócitos em qualquer estágio de desenvolvimento.

- O epitélio do ovário das fêmeas de *R. sanguineus* mostra severas alterações morfológicas, como extrema desorganização estrutural deste tecido, com células altamente vacuolizadas e com núcleos picnóticos, formando uma massa amorfa, o que compromete o desenvolvimento dos ovócitos aí fixados.

- Os ovócitos II das fêmeas expostas ao óleo de andiroba possuem maior quantidade de proteínas em relação ao grupo controle. Esse aumento na produção de elementos proteicos nos ovócitos nos estágios iniciais de desenvolvimento não estaria relacionado à produção de vitelo proteico, mas sim com a síntese de enzimas que seriam ativadas para neutralizar a substância tóxica presente nesse sistema, na tentativa de manter a integridade da célula.

- Os ovócitos IV e V das fêmeas expostas ao óleo de andiroba possuem menos proteínas em comparação ao grupo controle, sugerindo que neste caso a ação do óleo de andiroba seria diferente da observada nos estágios mais iniciais, ou seja, aqui este produto agiria como um inibidor da síntese e/ou incorporação de proteínas.

- O óleo de andiroba causa severa redução de polissacarídeos nos ovócitos em relação ao grupo controle, sugerindo que esses elementos estariam sendo utilizados como fonte de energia necessária para os processos metabólicos de detoxificação de substâncias tóxicas, neste caso, o óleo de andiroba.

- Os ovócitos I e II das fêmeas submetidas ao óleo de andiroba possuem poucos elementos lipídicos em relação ao grupo controle, sugerindo que este produto é capaz de interferir na síntese (produção endógena) e/ou na incorporação (produção exógena) de lipídios.

- A análise dos parâmetros reprodutivos mostra um aumento gradual nas médias dos parâmetros: peso da massa de ovos e índice de eficiência reprodutiva dos grupos submetidos ao óleo de andiroba à medida que a concentração deste produto aumenta. Essa maior produção de ovos representa um mecanismo de defesa desenvolvido pelo organismo a fim de assegurar o sucesso reprodutivo da espécie, mesmo na presença do agente tóxico.

- Mesmo se a oviposição ocorrer, os ovos postos não são viáveis devido às grandes alterações morfofisiológicas sofridas pelos ovócitos.

- O óleo de andiroba é um potente agente natural que atua reduzindo e/ou impedindo a reprodução das fêmeas de carrapatos *R. sanguineus*, com a vantagem de não causar impacto ambiental.

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Introdução

Material e Métodos

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ANEXO

8. ANEXO

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

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

DECISÃO CEUA Nº 033/2011

Instituição: **UNESP – IB – CRC**	Departamento: Biologia
Protocolo nº: 7124	Data de Registro CEUA: 13/09/2011
Projeto de Pesquisa: "Avaliação das alterações celulares nos ovócitos de fêmeas semi-ingurgitadas de carrapatos *Rhipicephalus sanguineus* (Acari: Ixodidae) decorrentes da exposição ao óleo de andiroba (*Carapa guianensis*)".	

Pesquisador Responsável: Profa. Dra. Gislaine Cristina Roma

Orientando(a): Maria Claudia Ramalho Vendramini

Colaborador(a): Maria Izabel Camargo Mathias

Objetivo Acadêmico:	( ) TCC ( ) Mestrado ( ) Doutorado (x) Outros – (Iniciação Científica/Pesquisa)

A Comissão de Ética no Uso de Animal - CEUA do Instituto de Biociências da UNESP – Campus de Rio Claro, em sua 10ª reunião ordinária, realizada em 10/11/2011,

(x)	**Aprovou** o Projeto de Pesquisa acima citado, ratificando o parecer emitido pelo relator.
( )	**Desde** que atendidas as **pendências** apontadas na reunião (vide anexo), **aprova** o Projeto de Pesquisa acima citado (prazo máximo de 30 dias).
( )	**Referendou** o Projeto de Pesquisa acima citado, ratificando o parecer emitido pelo relator.
( )	Aprovou **retornar** ao interessado para atendimento das pendências encontradas (prazo máximo de 30 dias).
( )	**Não** Aprovou.
( )	**Retirou**, devido à permanência das pendências.

Rio Claro, 21 de novembro de 2011

Prof. Dr. Célio Fernando Baptista Haddad
Coordenador da CEUA