ISAAC FILIPE MOREIRA KONIG

ASSESSMENT OF SUBLETHAL EFFECTS OF ACETYLCARVACROL IN THE OOCYTE MORPHOLOGY OF *Rhipicephalus microplus* (CANESTRINI, 1888) AND *Rhipicephalus sanguineus* sensu lato (LATREILLE, 1806) TICKS (ACARI: IXODIDAE)

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências da Saúde para a obtenção do título de Mestre.

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AVALIAÇÃO DOS EFEITOS SUBLETAIS DO ACETILCARVACROL NA MORFOLOGIA DOS OVÓCITOS DE CARRAPATOS *Rhipicephalus microplus* (CANESTRINI, 1888) E *Rhipicephalus sanguineus* sensu lato (LATREILLE, 1806) (ACARI: IXODIDAE)

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências da Saúde para a obtenção do título de Mestre.

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RESUMO

Os carrapatos do gênero *Rhipicephalus* possuem dois representantes com ampla distribuição no Brasil, sendo eles *R. sanguineus* sensu lato (s. l.) e *R. microplus*. Esses carrapatos apresentam grande importância médico-veterinária por transmitirem patógenos ao homem e ao cão, no caso de *R. sanguineus* (s. l.); e ao gado, no caso de *R. microplus*. Além disso, *R. microplus* é responsável por prejuízos econômicos da ordem de um bilhão de dólares por ano, apenas no Brasil. Por isso, esses parasitos são alvos de diferentes métodos de controle, como o uso de substâncias acaricidas. O carvacrol, um monoterpeno fenólico extraído de plantas, apresenta elevada ação carrapaticida. Seu derivado semi-sintético, acetilcarvacrol, tem sido estudado como nematicida, especialmente por apresentar maior eficiência, maior estabilidade e mais baixa toxicidade aos hospedeiros quando comparado ao carvacrol. Com base nestas informações, esse trabalho teve como objetivo avaliar os efeitos de concentrações subletais do acetilcarvacrol, diluído em solução de dimetilsulfóxido (DMSO) a 3%, na morfologia do ovário de carrapatos *R. sanguineus* (s. l.) e *R. microplus*. Para isso, os carrapatos foram submersos, durante cinco minutos, em diferentes concentrações de acetilcarvacrol, baseadas em experimentos prévios, conforme procedimento já estabelecido na literatura. Após um período de sete dias, os carrapatos foram dissecados para coleta dos ovários. O material foi processado e incluído em historesina, seccionado, corado e avaliado por meio de morfometria e um protocolo de análise semiquantitativa. As alterações morfológicas comuns nos ovários de *R. sanguineus* s. l. e *R. microplus* foram vacuolização citoplasmática e nucleolar, diminuição de tamanho e formato irregular das células germinativas e descolamento do córion. Para *R. sanguineus* (s. l.) houve ainda drástica alteração das células epiteliais do oviduto, inclusive com desaparecimento de células germinativas jovens. Para ambas as espécies, concentrações maiores de acetilcarvacrol causaram danos estatisticamente significativos de acordo com a análise semiquantitativa proposta. Os resultados demonstram o potencial de acetilcarvacrol como método de controle da reprodução desses parasitos a longo prazo.

Palavras- chave: Carrapato-do-boi. Carrapato-marrom-do-cão. Ovário. Toxicidade. Acetilcarvacrol.

ABSTRACT

The ticks of the genus *Rhipicephalus* have two species with wide distribution in Brazil, namely *R. sanguineus* sensu lato (s. 1) and *R. microplus*. These ticks are of great medical and veterinary importance for transmitting pathogens to humans and dogs in the case of *R. sanguineus* (s. 1); and cattle, in the case of *R. microplus*. In addition, *R. microplus* is responsible for economic losses in the order of one billion dollars per year, only in Brazil. Therefore, these parasites are targets of different control methods, such as the use of acaricidal substances. Carvacrol, a phenolic monoterpene extracted from plants, shows high acaricidal action. Its semi-synthetic derivative, acetylcarvacrol, has been studied as nematicide, especially since it presents greater efficiency, greater stability and lower toxicity to the hosts, when compared to carvacrol. Based on this information, this study aimed to evaluate the effects of sublethal concentrations of acetylcarvacrol diluted in 3% dimethyl sulfoxide solution (DMSO) in the morphology of the ovaries of *R. sanguineus* (s. 1) and *R. microplus* ticks. In order to do this, the ticks were submerged, during five minutes, in different concentrations of acetylcarvacrol, based on previous experiments, according to procedure already established in the literature. After a period of seven days, the ticks were dissected for collection of the ovaries. The material was processed and included in historesin, sectioned, stained and evaluated by means of morphometry and a semiquantitative analysis protocol proposed in this study. The common morphological alterations in the ovaries of *R. sanguineus* s. l. and *R. microplus* were cytoplasmic vacuolization, ring-shaped nucleolus, irregular size and shape of germ cells and chorion detachment. For *R. sanguineus* (s. 1.), there was still a drastic alteration of oviduct epithelial cells, including the disappearance of young germ cells. For both species, higher concentrations of acetylcarvacrol caused statistically significant damages according to the proposed semiquantitative analysis. The results demonstrate the potential of acetilcarvacrol as a method for long-term control of these parasites' reproduction.

Keywords: Southern cattle tick. Brown dog tick. Ovary. Toxicity. Acetylcarvacrol.

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PARTE I

1. INTRODUÇÃO

Os carrapatos *R. microplus*, também conhecido como carrapato-do-boi, e *R. sanguineus* sensu lato, o carrapato-marrom-do-cão, apresentam ampla distribuição global (ANDREOTTI et al., 2014) e reconhecida importância na saúde humana e veterinária (JONGEJAN; UILENBERG, 2004). A infestação por esses parasitos causa dor, prurido e desconforto aos hospedeiros, além de estar relacionada com a transmissão de uma grande variedade de patógenos (DANTAS-TORRES et al., 2006). Além disso, uma única fêmea ingurgitada desses carrapatos é capaz de fazer a postura de milhares de ovos (TROUGHTON; LEVIN; 2007), tornando necessária a utilização de diferentes métodos de controle (DE LA FUENTE, 2018).

A utilização de acaricidas sintéticos é o principal método de controle de carrapatos. Todavia, o uso prolongado ou inapropriado dessas substancias pode acelerar a seleção de parasitos resistentes, além de gerar resíduos no meio ambiente (KLAFKE et al. 2017; TSABOULA et al., 2016). Dessa forma, extratos vegetais surgem como acaricidas alternativos promissores devido ao relativo baixo custo e toxicidade, além de grande abundância (TAK; ISMAN, 2017). Acetilcarvacrol é derivado de um composto natural com atividade acaricida comprovada, o carvacrol (CETIN et al., 2010 NOVATO et al., 2015). Estudos demonstraram que a acetilação desse composto aumenta sua atividade biológica (RAMÍREZ et al., 2016), enquanto diminui a toxicidade para mamíferos (ANDRE et al., 2016). Além disso, outras atividades biológicas já foram atribuídas ao acetilcarvacrol, como efeito ansiolítico em ratos (PIRES et al., 2013), atividade bactericida (CACCIATORE et al., 2015), ação anti-inflamatória (DAMASCENO et al., 2014), atividade anti-helmíntica *in vitro* contra *Schistosoma mansoni* (MORAES et al., 2013), dentre outras.

Além da atividade acaricida, a utilização de compostos capazes de afetar a morfologia de órgãos fundamentais para a sobrevivência ou perpetuação da espécie são de particular interesse no controle de carrapatos (CAMARGO-MATHIAS, 2018). Desse modo, o presente estudo avaliou os efeitos de acetilcarvacrol na morfologia dos ovários de *R. microplus* e *R. sanguineus* s. l.. Inicialmente, avaliou-se as alterações nos ovários do carrapato do boi e propôs-se um novo método para avaliação dessas alterações morfológicas. Por fim, demonstrou-se o potencial acaricida de acetilcarvacrol e os efeitos desse composto nos ovários de *R. sanguineus* sensu lato através de técnicas histológicas e histoquímicas.

2. REFERENCIAL TEÓRICO

2.1. Carrapatos

Carrapatos são parasitos hematófagos obrigatórios em pelo menos um estágio da vida (NAVA et al., 2010). Esses parasitos apresentam ampla distribuição mundial e infestam mamíferos, aves, répteis e anfíbios (SAUER, 1995). Carrapatos pertencem à subclasse Acari e são divididos em três famílias: Argasidae, Ixodidae e Nuttalliellidae. Os carrapatos ixodídeos são também conhecidos como ‘carrapatos duros’ devido à presença de um escudo que cobre integralmente, no caso dos machos, e parcialmente, no caso das fêmeas, a parte dorsal de seus corpos (SONENSHINE; ROE, 2014). No Brasil existem aproximadamente 61 espécies de carrapatos, dentre ixodídeos e argasídeos. Os carrapatos ixodídeos pertencem aos gêneros *Amblyomma* (30 spp.), *Ixodes* (8 spp.), *Haemaphysalis* (3 spp.), *Rhipicephalus* (2 spp.) e *Dermacentor* (1 spp.) (DANTAS-TORRES et al., 2009). Para o gênero *Amblyomma*, três novas espécies foram posteriormente descritas: *A. tonelliae* n. sp., *A. interandinum* n. sp., e *A. patinoi* n. sp. (NAVA et al., 2014).

Os carrapatos ixodídeos apresentam ciclo de vida dividido em três estágios (larva, ninfa e adultos), todos com um único instar (SAUER, 1995). Esses parasitos se destacam como importantes transmissores de patógenos, como vírus, bactérias, fungos e protozoários (DANTAS-TORRES et al., 2012). Algumas características do ciclo de vida dos carrapatos ixodídeos os colocam em posição de destaque na transmissão de patógenos, a citar: o lento metabolismo, com exceção da fêmea ingurgitada; a possibilidade de permanecer longos períodos sem se alimentar; a ingestão de grande quantidade de sangue; e a produção de grande quantidade de ovos (SONENSHINE; ROE, 2014).

O gênero *Rhipicephalus* possui dois representantes com alta incidência no Brasil: *R. sanguineus* e *R. microplus*. Essas espécies se destacam por ampla distribuição no Brasil e no mundo, além de sua grande importância veterinária na transmissão de patógenos (ANDREOTTI et al., 2014).

2.1.1. *Rhipicephalus microplus*

Rhipicephalus microplus é popularmente conhecido como "carrapato-do-boi" devido a sua grande preferência em parasitar bovinos, especialmente os das raças taurinas

(ANDREOTTI et al., 2014). Todavia, podem também parasitar equinos, cães, gatos e os animais silvestres, embora com menor frequência (BARRÉ; UILENBERG, 2010). A infestação de *R. microplus* causa desconforto aos animais, além de afetar o desenvolvimento do rebanho, e a produção de carne e de leite (JONSSON, 2006). A produção de couro é também fortemente afetada pela infestação por *R. microplus*. Embora o aparelho bucal dessa espécie de carrapato seja relativamente pequena, as áreas preferidas para infestação normalmente são as de maior potencial de produção de couro (JONGEJAN; UILENBERG, 2004). Ademais, *R. microplus* é o vetor de *Babesia bovis*, *Babesia bigemina*, *Anaplasma marginale* (CLERCQ et al., 2012) e *Borrelia theileri* (SOARES et al., 2000) aos bovinos.

Devido aos fatores mencionados acima, estima-se que, no Brasil, a infestação de carrapato-do-boi representa uma perda econômica de US$ 968 milhões de dólares por ano (RODRIGUES; LEITE, 2013). Já Grisi et al. (2014), em reavaliação mais abrangente do potencial impacto econômico de diversas parasitoses em bovinos no Brasil, estimam que apenas a infestação por *R. microplus* é responsável por uma perda anual de 3,24 bilhões de dólares. Esses danos provocados pela infestação de *R. microplus* ocorrem não apenas no Brasil, mas em grande parte do mundo (MADDER et al., 2011). Essa espécie de carrapato é originária do sudeste da Ásia e se espalhou por todos os trópicos, incluindo a Austrália, a África oriental e ocidental, além da América do Sul e Central (JONGEJAN; UILENBERG, 2004). De fato, esse parasito é bem adaptado em toda a América Latina e na Austrália, sendo também reportada alarmante ‘invasão’ no oeste da África (CLERCQ et al., 2012). Madder et al. (2011) relatam que a introdução de *R. microplus* no oeste de África foi capaz de reduzir significativamente a população da espécie nativa de carrapatos, *R. decoloratus*. Os autores argumentam que *R. microplus* é uma espécie extremamente invasiva e que tem causado sérias perdas econômicas na região (MADDER et al., 2011).

Rhipicephalus microplus é um parasito monoxênico, alcançando o hospedeiro no estágio larval e permanecendo até o completo ingurgitamento (HITCHCOCK, 1954). Em condições laboratoriais, a fêmea ingurgitada apresenta um período pré-oviposição médio de quatro dias. Já o período de oviposição tem duração média de nove dias, com produção máxima no terceiro dia, totalizando aproximadamente 589.1±119.2 ovos por fêmea. Em média, uma única fêmea faz a postura de três mil ovos, permanecendo viva por dois a seis dias após a oviposição. A eclosão das larvas acontece em até 25 dias após a postura. Após alcançar o hospedeiro, as larvas se alimentam por até oito dias, realizam a ecdise para o

estágio de ninfa e posteriormente para o estágio adulto. Considerando condições ambientais ótimas e a disponibilidade de hospedeiro, cada ciclo de *R. microplus* apresenta duração de 65±8.0 dias (SENBILL et al.,2018). Hitchcock (1954), ao infestar cinco bovinos com larvas de *R. microplus,* observou a presença de fêmeas ingurgitadas 18,9 dias após a infestação. Essas fêmeas apresentaram um ritmo diurno para deixarem o hospedeiro para iniciarem a postura. Não havendo disponibilidade de hospedeiro, larvas de *R. microplus* podem permanecer vivas no ambiente por até cinco meses sem se alimentarem (UTECH et al., 1983).

Fêmeas ingurgitadas de *R. microplus* são capazes de fazer a postura de milhares de ovos, apresentam ciclo de desenvolvimento rápido, alta capacidade invasiva e de transmissão de patógenos (MADDER et al., 2011). Ademais, essa espécie de carrapato apresenta diversos mecanismos de resistência aos acaricidas comercialmente disponíveis (GUERRERO et al., 2012). Dentre os mecanismos mencionados por Guerrero et al. (2012) destacam-se os que causam modificações no local de ação dos acaricidas, aumento do metabolismo ou sequestro do acaricida, ou ainda redução na capacidade do acaricida em penetrar através das camadas protetoras do corpo do carrapato. Diante disso, a busca constante por substâncias com atividades acaricidas faz-se necessária. (CONTRERAS et al., 2017). Dentre os diversos métodos disponíveis, os métodos de controle baseados na reprodução dos parasitos se destacam por atuarem em etapa fundamental para a perpetuação da espécie (KARUNARATNE, 2017; DE LA FUENTE et al., 2007).

2.1.2. *Rhipicephalus sanguineus*

Rhipicephalus sanguineus sensu lato (s.l.), popularmente conhecido como "carrapato-marrom-do-cão", representa um grupo de aproximadamente 17 espécies de carrapatos amplamente distribuídos no mundo (DANTAS-TORRES, 2010). Anteriormente, o complexo de espécies *R. sanguineus* era referido como *R. sanguineus* sensu stricto (PEGRAM et al., 1987). Todavia, Dantas-Torres et al. (2013) demonstraram, através de estudos morfológicos e genéticos, que o complexo *R. sanguineus* possivelmente compreende espécies diferentes alocadas em um mesmo grupo, sugerindo, por este motivo, que se evite o uso do termo *R. sanguineus* sensu stricto.

Rhipicephalus sanguineus é um parasito trioxeno que alcança o hospedeiro no estágio larval, se alimenta e deixa o hospedeiro para sofrer ecdise no ambiente. O mesmo ocorre no estágio de ninfa e, posteriormente, o adulto alcança o hospedeiro pela terceira vez para

finalizar o ciclo (DANTAS-TORRES, 2010). *R. sanguineus* possui grande especificidade pelo cão, mas ocasionalmente parasita outras espécies, inclusive humanos (DANTAS-TORRES, 2008). A preferência por hospedeiro foi determinada em experimento realizado com 30 voluntários humanos, além de cães e porcos da índia, no qual *R. sanguineus* nos três estágios de desenvolvimento (larva, ninfa e adultos) foram liberados em compartimentos fixados nos braços dos voluntários e no dorso de cachorros e porcos da índia. Após 24 horas, foi observado que 3,5% das larvas, 2% das ninfas e 5% dos adultos se fixaram em humanos. Já em cães, 21,3% das larvas, 61,1% das ninfas e 31,8% dos adultos se fixaram nesse hospedeiro após 24 horas (NELSON, 1969). Trabalhos mais recentes demonstraram a infestação de *R. sanguineus* em humanos na Itália (OTRANTO et al., 2014), Uruguai (VENZAL et al., 2003), Panamá (BERMÚDEZ et al., 2012), dentre outros. No Brasil, a infestação por *R. sanguineus* em humanos já foi reportada nos estados do Rio Grande do Sul (MENTZ et al., 2016), Pernambuco (DANTAS-TORRES et al., 2006) e Goiás (LOULY et al., 2006).

Além de dor, incômodo e prurido, a picada de *R. sanguineus* está relacionada com a transmissão de mais de uma dezena de agentes infecciosos aos cães e ao homem. *R. sanguineus* é um parasito de grande importância em saúde pública por estar relacionado com a transmissão de *Rickettsia rickettsii*, agente causador da Febre Maculosa das Montanhas Rochosas, nos Estados Unidos da América e no México, e *R. conorii*, agente causador da Febre Maculosa do Mediterrâneo na Europa (DANTAS-TORRES; OTRANTO, 2015). No Brasil, *R. sanguineus* é considerado um potencial vetor da bactéria *R. rickettsii*, agente causador da Febre Maculosa Brasileira (SILVA et al., 2017). Isso se deve à identificação molecular de *R. rickettsii* em *R. sanguineus* coletados em cachorros naturalmente infestados no município de Resende, Rio de Janeiro, no ano de 2006, onde ocorreram cinco casos humanos de Febre Maculosa Brasileira (CUNHA et al., 2009). Também foi observada a presença de *R. rickettsii* em *R. sanguineus* no município de Juiz de Fora, Minas Gerais (PACHECO et al., 2011), no estado do Rio de Janeiro (GEHRKE et al., 2009), no município de Campo Grande, Mato Grosso do Sul (ALMEIDA et al., 2013), na região de Maciço de Baturité, Ceará (SILVA et al., 2017), dentre outros. Além disso, Costa et al. (2011) demonstraram a transmissão transovariana e transestadial, especialmente do estágio de ninfa para adultos, de *R. rickettsii* em carrapatos *R. sanguineus*. Dessa forma, fica evidente que existe uma chance considerável de *R. sanguineus* participar ativamente da transmissão de *R. ricketsii* no território brasileiro. Todavia, mais estudos são necessários para confirmar essa hipótese (CUNHA et al., 2009).

Rhipicephalus sanguineus é um parasito altamente adaptado para sobreviver em habitações humanas, sendo ativo durante todo o ano em regiões tropicais e subtropicais, bem como em algumas áreas temperadas (DANTAS-TORRES, 2010). Além disso, estudos demonstraram que o aumento da temperatura aumenta a preferência de *R. sanguineus* por humanos e coelhos (PAROLA et al., 2008; SOCOLOVSCHI et al., 2009). Diante disso, especula-se que o aumento de dois a três graus Celsius provocados pelo aquecimento global pode ser fator determinante para o aumento de casos de parasitoses transmitidas por esse carrapato a humanos (DANTAS-TORRES, 2010).

O ciclo de *R. sanguineus* se inicia com a oviposição da fêmea, capaz de fazer a postura de até quatro mil ovos. O período de oviposição pode perdurar por várias semanas, sendo a produção de ovos dependente do peso da fêmea e tempo de postura dos ovos (KOCH, 1982). Em condições experimentais controladas, as larvas eclodem em até 32 dias após a postura e se alimentam por quatro dias, em média. A ecdise da larva ocorre, em média, 32 dias após a alimentação. Posteriormente, a ninfa se alimenta por quatro a sete dias e sofre ecdise após uma média de 32,8 dias. Quando no estágio adulto, a fêmea se alimenta por cerca de nove dias e, sem seguida, inicia o período pré-oviposição, que varia de três dias a algumas semanas (DANTAS-TORRES et al., 2010; TROUGHTON; LEVIN, 2007). Vale ressaltar que, embora a fêmea possa iniciar a alimentação na ausência do macho, é necessário a cópula para seu completo ingurgitamento (DANTAS-TORRES, 2010). O ciclo de *R. sanguineus* se completa em média em 23 a 25 semanas sob condições laboratoriais. Na ausência de hospedeiro, adultos podem permanecer em jejum por períodos maiores que 12 meses quando armazenados de quatro a oito graus Celsius (TROUGHTON; LEVIN, 2007). Diante do exposto, fica evidente a necessidade de métodos eficientes de controle para esse parasito (DANTAS-TORRES, 2008, 2006; SILVA et al., 2017).

2.2. Controle de carrapatos

O controle de carrapatos pode ser realizado através de vacinação (CONTRERAS et al., 2017; DE LA FUENTE et al., 2007), controle biológico (WASSERMANN et al., 2016), controle químico através de acaricidas sintéticos (MILLER et al., 2011) ou extratos vegetais (TAK; ISMAN, 2017), dentre outros. Embora De La Fuente (2018) argumente que o controle de carrapatos deva ocorrer de maneira integrada, o uso de acaricidas sintéticos ainda é o método mais amplamente utilizado. Todavia, o uso frequente desses compostos pode resultar

na seleção de carrapatos resistentes, necessitando de dosagens maiores e aplicações em intervalos menores (KLAFKE et al., 2017). Além disso, o uso indiscriminado de acaricidas sintéticos pode gerar efeitos tóxicos em animais, plantas e humanos, bem como contaminar o solo e água (TSABOULA et al., 2016). Extratos vegetais e óleos essenciais se apresentam como alternativas promissoras no controle desses parasitos devido à grande variedade de espécies de plantas, baixo custo e grande abundância (BENELLI et al., 2016).

2.2.1. Controle alternativo

Óleos essenciais são compostos voláteis originários do metabolismo secundário de plantas, utilizados para atrair polinizadores, bem como proteger contra estresse térmico, microrganismos ou outros patógenos (PAVELA, 2015). Os terpenos são uma classe de compostos presentes em óleos essenciais sintetizados por plantas, a partir de unidades de isopreno, que se destacam por sua ampla atividade biológica (TAK; ISMAN, 2017). A modificação enzimática desses compostos para adição de átomos de oxigênio gera uma subclasse de terpenos, os terpenoides (BURT, 2004). De fato, uma variedade de terpenoides se destacam por suas atividades biológicas, especialmente como acaricidas, a citar: carvacrol (CRUZ et al., 2013; NOVATO et al., 2018, 2015; RAMÍREZ et al., 2016; SENRA et al., 2013), timol (DAEMON et al., 2009; JACK et al., 2006; NOVATO et al., 2015), eugenol (ARAÚJO et al., 2016; MONTEIRO et al., 2012; NOVATO et al., 2018), dentre outros. A atividade acaricida desses três terpenoides foi avaliada por Araújo et al. (2016) em larvas em jejum de *R. microplus* e *R. sanguineus*. O timol apresentou os melhores resultados em ambas as espécies com CL_{50} de 1,53 mg/mL para *R. microplus* e 2.98 mg/mL para *R. sanguineus* s.l., seguido de carvacrol com CL_{50} de 1,76 mg/mL e 3.29 mg/mL, respectivamente. *R. microplus* mostrou-se mais suscetível frente a todos os compostos quando comparado ao *R. sanguineus* s. l. (MATOS et al., 2018; ARAÚJO et al., 2016).

Extratos vegetais ou metabólitos secundários isolados de plantas atuam de maneira variada para mitigar a infestação por carrapatos, inibindo o desenvolvimento dos ovos, neutralizando os hormônios que coordenam a ecdise, ocasionando efeitos neurotóxicos, além de atuarem como repelentes (CAMARGO-MATHIAS, 2018; DE LA FUENTE, 2018; MATOS et al., 2018; ROSADO-AGUILAR et al., 2017). Dentre uma variedade de metabólitos secundários com atividade acaricida, carvacrol se destaca como uma alternativa promissora para o controle desses parasitos (SOUZA et al., 2019).

2.2.2. Carvacrol e acetilcarvacrol

Carvacrol (2-metil-5-(1-metiletil)fenol) é um monoterpeno fenólico encontrado em óleos essenciais de plantas da família Lamiaceae. É um dos constituintes majoritários dos óleos essenciais de plantas *Origanum* sp. e *Thymus* sp, assim como seu isômero, o timol (SANTORO et al., 2007). Kulisic et al. (2004), por exemplo, ao empregar aparelho de Clevenger com quantidade conhecida de n-pentano para coleta das frações do extrato aquoso de *Origanum* sp., obtiveram o óleo essencial com 2,9% de rendimento, contendo 41,1% de carvacrol e 58,9% de timol (KULISIC et al., 2004).

Ademais, o carvacrol é também uutilizado como aditivo alimentar devido às suas propriedades antimicrobianas e antioxidantes, tendo sido considerado seguro (*Generally Recognized as Safe* – GRAS) pelo órgão fiscalizador dos Estados Unidos da América (*US Food and Drug Administration* – FDA) (GUARDA et al., 2011). Além disso, foi inserido na lista de substâncias químicas aromatizantes pelo Departamento de Saúde e Segurança Alimentar do Conselho da Europa. A adição desse composto é permitida na Europa na concentração de até 25 ppm (=mg/L), no caso de aromatizantes para doces (VINCENZI et al., 2004). Experimento realizado com leitões 28 dias após o nascimento, fornecendo carvacrol nas concentrações de 500 e 2000 mg/kg da dieta, resultou no aumento da relação altura de cripta/vilosidades no intestino, aumentando a capacidade de digestão e absorção do intestino, sem alterar a composição da microbiota intestinal (MICHIELS et al., 2010). Dessa forma, Michiels et al. (2010) consideraram a adição de carvacrol na dieta dos leitões uma alternativa segura para aumento da produção.

Carvacrol apresenta comprovada ação acaricida para uma gama de espécies de carrapatos, entre elas larvas em jejum de *R. sanguineus* (ARAÚJO et al., 2016), ninfas e adultos em jejum de *Ixodes scapularis* e *Amblyomma americanum* (DOLAN et al., 2009), larvas em jejum de *A. sculptum* e *Dermacentor nitens* (NOVATO et al., 2015), dentre outras. Outros estudos avaliaram a atividade acaricida de óleos essenciais no qual carvacrol era um dos constituintes majoritários, além de avaliarem a atividade do composto isoladamente a título de comparação. Com isso, demonstraram que carvacrol é o principal responsável pela atividade acaricida contra adultos em jejum de *Hyalomma marginatum* (CETIN et al., 2010), larvas em jejum de *R. microplus* (CRUZ et al., 2013), adultos em jejum de *R. turanicus* (KOC et al., 2013), dentre outros.

Para larvas em jejum de *R. sanguineus* e *R. microplus*, carvacrol mostrou promissora ação acaricida, com concentrações letais para a metade da população (CL_{50}) de 3,29 e 1,76 mg/mL, respectivamente (ARAÚJO et al., 2016). O mecanismo pelo qual carvacrol atua, embora possivelmente não seja o principal, é através da inibição da acetilcolinesterase (ANDERSON; COATS, 2012). A inibição dessa enzima, responsável pela hidrólise do grupamento éster da acetilcolina e diminuição dos impulsos elétricos, ocasiona superestimulação dos neurônios, levando ao aumento da contração muscular, convulsões e, eventualmente, morte dos artropodes (MUKHERJEE et al., 2007). Ademais, carvacrol demonstrou ação repelente em ninfas em jejum de *Ixodes scapularis* (DIETRICH et al., 2006). Finalmente, o desenvolvimento de células germinativas de fêmeas semi-ingurgitadas de *R. sanguineus* mostrou-se drasticamente afetado quando submetido a concentrações subletais de carvacrol (SOUZA et al., 2019).

Embora muitos compostos isolados de óleos essenciais de plantas apresentem alta atividade acaricida, algumas limitações dificultam o uso desses compostos em aplicações a campo. Pavela e Benelli (2016) argumentam que fatores como a baixa estabilidade frente à oxidação dificultam o uso prático dessas substâncias. Carvacrol, por exemplo, apresenta um grupamento hidroxila em sua estrutura que pode ser facilmente oxidado (SOLOMONS et al., 2016). Diante disso, estudos passaram a utilizar compostos químicos naturais para a síntese de análogos semissintéticos, com o objetivo de aumentar sua estabilidade e atividade biológica, além de reduzir a toxicidade para organismos não-alvo (CACCIATORE et al., 2015). Dentre essas modificações destaca-se a acetilação do carvacrol (NOVATO et al., 2018).

O acetilcarvacrol apresenta um grupamento éster em sua estrutura, em substituição ao grupo hidroxila do carvacrol, o que confere maior estabilidade ao composto (SOLOMONS et al., 2016). Além disso, a acetilação demonstrou aumentar a atividade acaricida em larvas em jejum de *R. microplus*. Em comparação ao carvacrol, a taxa de mortalidade média das larvas aumentou de 35,85 para 67.83%, quando se empregou o composto acetilado (RAMÍREZ et al., 2013). Em estudo posterior também realizado por Ramírez et al. (2016), acetilcarvacrol mostrou atividade acaricida superior ao carvacrol, com CL_{50} de 5,01 e 59,72 µmol/mL, respectivamente, para larvas em jejum de *R. microplus*. Para esse teste, as larvas foram incubadas por 24h a 28 °C em envelopes previamente expostos a concentrações variando de 0,0625 a 1% (m/v) dos compostos, diluídos em uma mistura de tricloroetileno e azeite de oliva (2:1) (RAMÍREZ et al. 2016). Todavia, Novato et. al. (2018) demonstraram que

carvacrol foi mais eficiente que seu derivado acetilado, acetilcarvacrol, em teste realizado empregando etanol absoluto como solvente. Nesse trabalho, carvacrol apresentou uma CL_{50} de 0,83 mg/mL e acetilcarvacrol 2,49 mg/mL em larvas de *R. microplus*. Diante disso, Novato et al. (2018) argumentam que os resultados da atividade acaricida de carvacrol apresentados por Ramírez et al. (2013) são curiosamente mais baixos dos que os já relatados na literatura, salientando também que a atividade acaricida de compostos pode variar entre espécies e entre os estágios de desenvolvimento de uma mesma espécie. Vale ressaltar ainda que, não surpreendentemente, a utilização de solventes diferentes pode ocasionar resultados diversos dos trivialmente esperados (KARAASLAN et al., 2018). Finalmente, cumpre destacar que embora os derivados acetilados de timol, eugenol e carvacrol apresentaram atividade acaricida inferior aos compostos originários, acetilcarvacrol mostrou-se o mais eficiente dentre os demais compostos acetilados (NOVATO et al., 2018).

Por fim, André et al. (2016) demonstraram que acetilcarvacrol apresenta atividade *in vitro* e *in vivo* contra nematóides gastrointestinais de pequenos ruminantes e toxicidade reduzida para ratos, quando comparado ao carvacrol. Nesse trabalho, a dose letal por via oral para a metade dos ratos (DL_{50}) foi de 1.544,5 mg/kg para acetilcarvacrol e 919 mg/kg para carvacrol. Compostos que apresentam DL_{50} superior a 1.000 mg/kg quando administrados por via oral, são considerados seguros ou de baixa toxicidade (LEI et al., 2018). Além disso, outras atividades biológicas já foram atribuídas ao acetilcarvacrol, como efeito ansiolítico em ratos (PIRES et al., 2013), atividade bactericida (CACCIATORE et al., 2015), ação anti-inflamatória (DAMASCENO et al., 2014), atividade anti-helmíntica *in vitro* contra *Schistosoma mansoni* (MORAES et al., 2013), dentre outras.

2.3. Controle baseado na reprodução

Fêmeas de carrapatos ixodídeos utilizam grande quantidade da energia obtida durante a alimentação para a produção de ovos. Com isso, uma única fêmea é capaz de fazer a postura de milhares de ovos (DANTAS-TORRES, 2010; TROUGHTON; LEVIN, 2007). Diante disso, diversos estudos têm sido realizados com o objetivo de verificar o efeito de concentrações subletais de compostos, sobretudo os extraídos de plantas, na eficiência reprodutiva desses parasitos. Alguns parâmetros avaliados são o período pré-oviposição, a massa dos ovos, o tempo de oviposição, a eclodibilidade das larvas, dentre outros (AGNOLIN et al., 2014; PAZINATO et al., 2016). Além disso, técnicas histológicas podem ser

empregadas como ferramentas adicionais importantes para a avaliação do efeito de drogas em órgãos particularmente importantes para a sobrevivência/perpetuação dos carrapatos (CAMARGO-MATHIAS, 2018; CAMARGO-MATHIAS; FURQUIM, 2013; FONTANETTI et al., 2010).

Em geral, aberrações cromossomais e nucleares são consideradas alterações toxicológicas típicas, que podem ser encontradas em células de animais expostos a determinada droga (FONTANETTI et al., 2010). De fato, diversos estudos empregaram a histologia para analisar o efeito de compostos químicos na morfologia do ovário de carrapatos objetivando o controle da reprodução (ARNOSTI et al., 2011; BARBOSA et al., 2016; CAMARGO-MATHIAS et al., 2017; DENARDI et al, 2012, 2010; SOUZA et al., 2019; OLIVEIRA et al., 2008). Dentre as principais alterações encontradas destacam-se: a vacuolização citoplasmática e nucleolar, formato e tamanho irregular das células germinativas, diminuição do conteúdo de lipídeos e proteínas, dentre outros (OLIVEIRA et al, 2016; SOUZA et al., 2019). Ocorre que uma célula germinativa com tamanho reduzido, por exemplo, pode dar origem a um descendente com suprimento limitado de nutrientes (ARNOSTI et al., 2011). Se considerarmos que um composto também seja capaz de afetar a morfologia da glândula salivar do parasito, sua alimentação, fixação ao hospedeiro e balanço hídrico também ficarão prejudicados (CAMARGO-MATHIAS, 2018). Alterações como vacuolização citoplasmática, perda de contato celular, formato irregular dos ácinos, ruptura de grânulos secretórios, dentre outros, já foram evidenciadas por uma série de pesquisadores nas glândulas salivares de carrapatos expostos a compostos acaricidas (MATOS et al., 2018; PEREIRA et al., 2009; REMEDIO et al., 2016; VENDRAMINI et al., 2012). Finalmente, os parasitos afetados poderiam, por exemplo, chegar ao estágio adulto ingurgitado e produzir um número reduzido de ovos, visto que essa produção é dependente da massa/tamanho do carrapato (KOCH, 1982). Considerando que sua alimentação tenha sido hipoteticamente afetada pela substância química, vê-se um exemplo de controle à longo prazo no qual o descendente da terceira geração poderá ser prejudicado pela exposição de gerações anteriores a determinada droga.

2.3.1. Morfologia do ovário de carrapatos

A morfologia dos ovários de *R. microplus e R. sanguineus* foi descrita através de técnicas histológicas e ultraestruturais por Saito et al. (2005) e Oliveira et al. (2005),

respectivamente. Os ovários desses parasitos consistem em um tubo único em formato de 'ferradura', no qual uma grande quantidade de ovócitos em diferentes estágios de desenvolvimento está aderida a uma camada de células epiteliais denominada pedicelo (Figura 1). Cinco estágios de desenvolvimento estão presentes no ovário de uma fêmea ingurgitada, sendo que o desenvolvimento ocorre assincronicamente ao longo do órgão. Os ovócitos I são as menores células da linhagem germinativa, com citoplasma homogêneo e basófilo, e núcleo (vesícula germinativa) em posição central, com nucléolo evidente. A medida que o desenvolvimento avança, finas granulações são observadas nos ovócitos II. A partir do estágio III os grânulos de vitelo tornam-se maiores, e a vesícula germinativa desloca-se para o polo basal da célula (OLIVEIRA et al., 2005; SAITO et al., 2005). Nesse estágio inicia-se a deposição do córion, camada que envolve o ovócito, confere proteção e permite trocas gasosas quando o ovo alcança o exterior (SAMPIERI et al., 2012). No estágio IV o núcleo é raramente observado, os grânulos tornam-se maiores e mais acidófilos. Finalmente, no estágio V, os ovócitos são maiores, com córion completamente depositado e grânulos de vitelo também maiores. Nesse estágio, os ovócitos alcançam o oviduto e são depositados pelas fêmeas durante a postura (OLIVEIRA et al., 2005; SAITO et al., 2005). Saito et al. (2005) relataram a presença de ovócitos do tipo VI em *R. microplus*. Segundo os autores, tais ovócitos são normalmente encontrados em *R. microplus* e apresentam características morfológicas anormais, tais como: desorganização citoplasmática, formato irregular e dobramentos no córion. Todavia, a presença desse estágio de desenvolvimento em *R. microplus* não foi confirmada em trabalhos posteriores (BARBOSA et al., 2016; SREELEKHA et al., 2017).

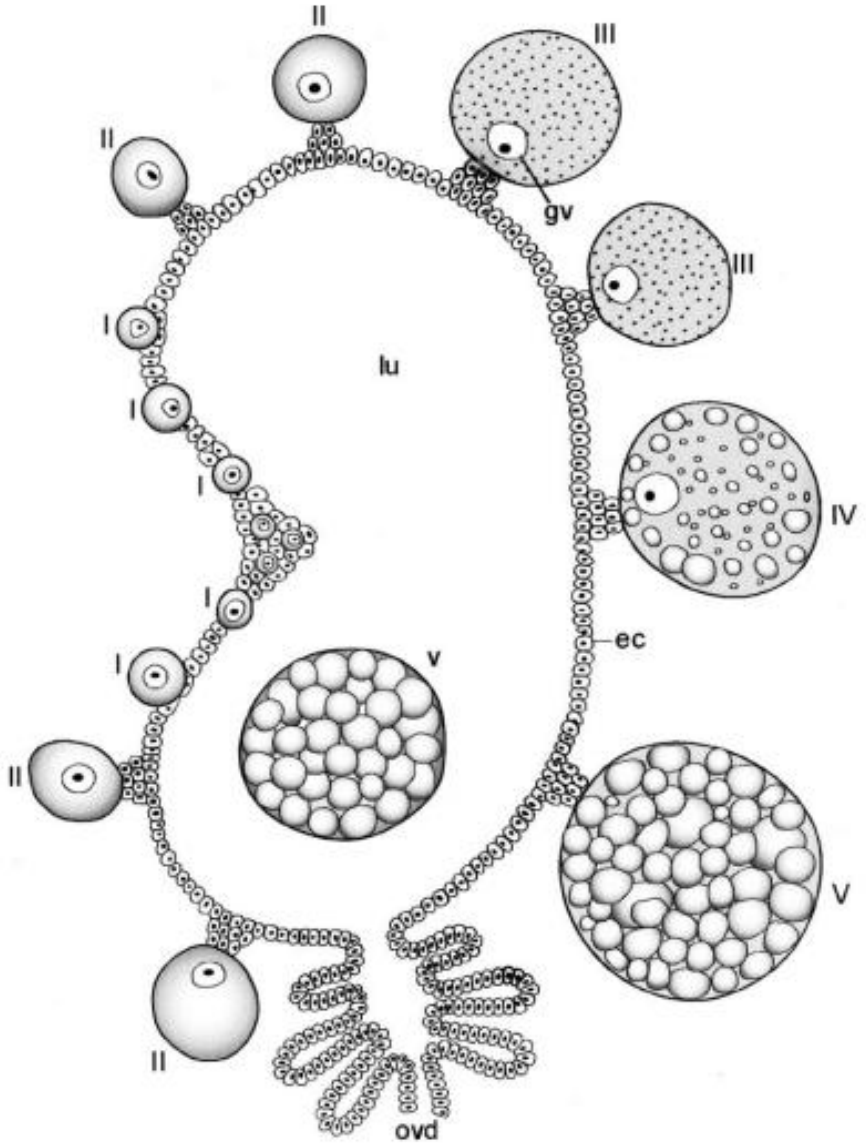

Figura 1: Estrutura do ovário de *Rhipicephalus microplus*. I – ovócito I; II – ovócito II; III – ovócito III; IV – ovócito IV; V – ovócito V; ec – epitélio; lu – lúmen; ovd – oviduto; gv – vesícula germinal. Adaptado de Saito et al. (2005).

Souza et al. (2019) demonstraram drástico prejuízo nos ovócitos de fêmeas semi-ingurgitadas de *R. sanguineus* s.l tratadas com 100 µL/mL de carvacrol, diluído em solução de etanol a 50%. Nesse tratamento, apenas ovócitos nos estágios I e II foram observados. Além disso, essas células apresentaram grandes alterações, como formato irregular, diminuição de tamanho, marginalização da cromatina e vacuolização citoplasmática. Alterações similares, porém menos intensas, foram encontradas nos tratamentos empregando concentrações mais baixas de carvacrol (50 e 25 µL/mL). Diante disso, constatou-se que

concentrações subletais de um composto extraído de plantas pode afetar a reprodução desse parasito, agindo como um método de controle a longo prazo (SOUZA et al., 2019).

Extrato hexânico de *Acmella oleraceae*, popularmente conhecido como Jambú, também apresentou resultados promissores para o controle reprodutivo a longo prazo de de fêmeas semi ingurgitadas de *R. microplus* (OLIVEIRA et al., 2016). Nesse trabalho, os carrapatos foram expostos às concentrações de 12,5 mg/mL, 25mg/mL e 50 mg/mL do extrato de *A. oleraceae* diluído em solução de etanol a 50% acrescido de DMSO a 1%. Os resultados demonstraram que, mesmo em concentrações reduzidas (12,5 mg/mL), extensas vacuolizações citoplasmáticas e nucleolares foram observadas em ovócitos I e II, além da presença de vacúolos ao redor dos grânulos de vitelo dos ovócitos IV. Já em concentrações mais elevadas (50 mg/mL), não foi possível observar a presença de ovócitos I e II devido ao dano morfológico significativo provocado pelo extrato. Os pesquisadores argumentam que a vacuolização nucleolar é um processo irreversível que irá culminar na morte celular. Já a vacuolização citoplasmática é explicada como um processo celular possivelmente reversível que visa a expulsão do composto tóxico ou a destruição de organelas afetadas pela substância (OLIVEIRA et al., 2016). Além disso, o extrato aquoso de folhas de *Azadirachta indica*, popularmente conhecido como neem, foi incapaz de causar mortalidade em fêmeas semi ingurgitadas de *R. sanguineus* na concentração de 10 e 20%. Todavia, diversas alterações morfológicas foram observadas nos ovários desses parasitos expostos ao extrato. Dentre essas alterações, destacam-se diminuição do tamanho e do conteúdo protéico dos grânulos, formato irregular e vacuolização citoplasmática dos ovócitos. Além disso, esse estudo demonstrou que os carrapatos expostos a 10% de extrato aquoso de neem apresentaram alterações morfológicas mais significativas nos ovários quando comparado àqueles expostos a 20% do extrato (DENARDI et al., 2011). Dessa forma, fica evidente o potencial de concentrações subletais de produtos de origem vegetal em causar danos nos ovócitos e auxiliar na diminuição das taxas reprodutivas de carrapatos no longo prazo.

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar o efeito de concentrações subletais de acetilcarvacrol na morfologia do ovário de fêmeas ingurgitadas de carrapatos *Rhipicephalus microplus* e *Rhipicephalus sanguineus* sensu lato.

3.2. Objetivos Específicos

1. Analisar, após a exposição a diferentes concentrações subletais de acetilcarvacrol, a morfologia do ovário de fêmeas ingurgitadas de carrapatos *R. microplus* e *R. sanguineus* s. l. por meio de técnica histológica de rotina.
2. Determinar, por meio de análise morfométrica, possíveis diferenças no tamanho de ovócitos de fêmeas ingurgitadas de carrapatos *R. microplus* e *R. sanguineus* s. l. expostos a concentrações subletais de acetilcarvacrol.
3. Classificar os possíveis efeitos tóxicos observadas no ovário dos carrapatos expostos a concentrações subletais de acetilcarvacrol a partir de um protocolo de análise semiquantitativa, baseado no grau de reversibilidade e na extensão das alterações.

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PARTE II - Artigo I

Sublethal concentrations of acetylcarvacrol strongly impact oocyte development of engorged female cattle tick *Rhipicephalus microplus* (Canestrini, 1888) (Acari: Ixodidae).

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Sublethal concentrations of acetylcarvacrol strongly impact oocyte development of engorged female cattle ticks *Rhipicephalus microplus* (Canestrini, 1888) (Acari: Ixodidae

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ABSTRACT

Rhipicephalus microplus, commonly known as southern cattle tick, causes huge economic losses in the cattle industry. Its infestation affects the production of meat and milk and causes discomfort to hosts. In addition, it is the vector of *Babesia* spp. and *Anaplasma* spp. The most frequent method used to control these parasites is through synthetic acaricides. However, their indiscriminate use can be toxic for hosts and environment as well as cause selection of resistant ticks. Plant extracts and essential oils emerge as promising alternatives to manage tick infestation. Carvacrol, an aromatic monoterpene extracted from plants, has recognized antimicrobial, antioxidant, insecticidal, repellent and acaricidal activities. Acetylation of carvacrol is believed to enhance its nematicidal and acaricidal activities and to decrease its toxicity to hosts. Thus, the aim of this study was to evaluate the effect of different concentrations of acetylcarvacrol in the morphology of ovaries of engorged *R. microplus* ticks. The most remarkable morphological alterations found in the female germ cells were irregular and thicker chorion, decreasing in size and irregular shape of female germ cells (oocytes), cytoplasmatic vacuolization as well as ring-shaped nucleoli. These alterations were analyzed through a semi-quantitative method proposed in this study for ixodid ticks. Treatment group IV, which was exposed to 4.5 µL/mL of carvacrol acetate, showed the most significant alterations, and it was also statistically different when compared to control groups. Therefore, sublethal concentrations of acetylcarvacrol demonstrated to impact the reproductive system of *R. microplus* by causing several damages in the female germ cells. This would hinder the generation of new individuals, probably contributing for a long-term control of tick infestation.

Keywords: Acetylcarvacrol; Morphology; Acaricide.

1. INTRODUCTION

Ticks are widely spread hematophagous parasites divided into three families: Ixodidae, Argasidae and Nuttalliellidae. Several members of the Ixodidae family are known for their capacity to transmit various pathogens to humans and other animals, mainly due to their feeding habits (SONENSHINE; ROE, 2014). Among over 60 species of the Brazilian Ixodidae fauna, *Rhipicephalus microplus*, known as southern cattle tick, is considered to be of great veterinary importance (DANTAS-TORRES et al., 2009; NAVA et al., 2014). *R. microplus* infestation causes discomfort to animals and affects the development of herd and the production of meat and milk. In Brazil, it is estimated that cattle tick infestation accounts for an economical loss of US$ 968 million dollars per year (RODRIGUES; LEITE, 2013). In addition, this tick transmits *Babesia* spp. and *Anaplasma* spp., which occur even simultaneously and cause the "bovine parasitic sadness" (PASCOETI et al., 2016). Severe economic losses caused by this parasite around the world as well as the increasing number of reports of resistance to acaricides currently available on the market has led to the search of cost-effective control methods (BANUMATHI et al., 2017; KLAFKE et al., 2017).

Carvacrol is a phenolic monoterpene found in plant essential oils, especially oregano and thyme (*Origanum* sp. and *Thymus* sp.) (KULISIC et al., 2004). Besides its biological activities, such as antimicrobial, antioxidant, insecticidal and antifungal properties, carvacrol has already demonstrated a recognized acaricidal activity (MECHERGUI et al., 2016; NOVATO et al., 2015, 2018). Also, chemical modification of carvacrol can enhance its biocidal effect (CACCIATORE et al., 2015; NESTERKINA et al., 2018). Acetylation, in particular, confers greater stability to carvacrol by conversion of the phenolic hydroxyl group, more susceptible to oxidation, into an ester group (SOLOMONS et al., 2016). Moreover, acetylcarvacrol, also known as carvacrol acetate, exhibited antibacterial (MYANGAR and PATEL, 2011) as well as acaricidal effect (RAMÍREZ et al., 2016), with reduced toxicity to the host when compared to carvacrol (ANDRE et al., 2016).

The ovary of *R. microplus* is composed of a single tubular structure, continuous and delimitated by a wall of small epithelial cells, to which the oocytes in different developmental stages (I - VI) are attached through a pedicel (SAITO et al., 2005). The mature oocytes are released into the ovary lumen and from there to the exterior. A single female cattle tick is able to lay up to three thousand eggs, which contributes immensely to the perpetuation of this parasite (SENBILL et al., 2018). Therefore, control methods based on the vitellogenesis stand

out as highly recommended to mitigate tick infestation.

Several studies over the last decade were based on morphological analysis to qualitatively demonstrate various alterations in the ovary of ticks, such as cytoplasmic vacuolization, irregular shape of the oocytes, folds in the cell membrane, as well as many ultrastructural alterations (CAMARGO-MATHIAS et al. 2017; DENARDI et al. 2011; OLIVEIRA et al., 2009, 2017; REMEDIO et al. 2014). Notwithstanding the great contribution of these studies in trying to find a cost-effective chemical compound to control tick infestation, they were based on qualitative analysis, which makes it difficult to compare the effectiveness of different substances evaluated. Barbosa et al. (2016) firstly introduced morphometric analysis in the study of tick's ovary by measuring cytoplasmic and germinal vesicle diameters. However, several other alterations found in their study remained as qualitative analysis.

Thus, the present study aimed to evaluate morphological changes in the ovary of *R. microplus* engorged ticks exposed to sublethal concentrations of carvacrol acetate, in order to demonstrate the interference of this compound in the reproduction of this parasite. Moreover, a novel method for semi-quantitative analysis to measure these alterations is proposed.

2. MATERIAL AND METHODS

2.1. *R. microplus* ticks

Engorged *R. microplus* females weighing 160 mg in average were manually collected on naturally infested cattle without recent acaricide treatment, in the municipality of Piau (21°32'05.9"S 43°16'40.5"W), state of Minas Gerais, in the southeastern of Brazil. The ticks were washed in a sieve with tap water, dried on soft absorbent paper and selected under a stereomicroscope (Zeiss/Stemi 2000C/AxioCamERc 5s) according to external morphological conditions, using10x eyepiece lens.

2.2. Synthesis of carvacrol acetate

Acetylcarvacrol was obtained by acetylation of carvacrol, 5-isopropyl-2-methylphenol, purchased from Sigma-Aldrich Co. (St Louis Mo, USA) in 99% purity (Fig.1). For the reaction, 5 mL of carvacrol was added to a volumetric flask containing 25 mL of 10% sodium hydroxide solution at room temperature. Subsequently, 5.5 mL of acetic anhydride was added to the flask under cooling. The reaction mixture was left under stirring for 15 minutes. The oil obtained was separated from solution and characterized according to its melting point and by infrared (IR) spectroscopy (MORAES et al., 2013; SOLOMONS et al., 2016).

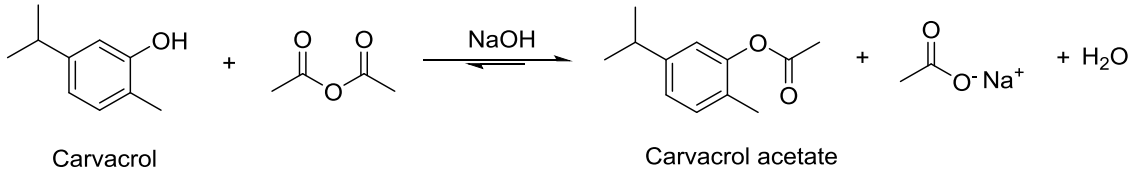

Figure 1: Synthesis of carvacrol acetate by the acetylation of carvacrol.

2.3. Adult immersion test

The adult immersion test was performed according to Drummond et al. (1973). Sixty ticks were distributed in six groups of 10 individuals, among treated and control groups. The control group I (C1) was exposed to placebo (distilled water) and the control group II (C2) was exposed to 3% DMSO solution. The treated groups contained increasing concentrations of acetylcarvacrol diluted in 3% DMSO solution: group T1 (3.0µL/mL), T2 (3.5µL/mL), T3 (4.0 µL/mL), T4 (4.5µL/mL). For each group, the ticks were immersed for 5 minutes in beakers containing the above concentrations of acetylcarvacrol as well as the control solutions. Afterwards, ticks were dried on soft absorbent paper and placed in Petri dishes at room temperature for 7 days, as suggested by Oliveira et al. (2008). Subsequently, five ticks of each group were randomly selected for histological analysis.

2.4. Histology

All the ticks were maintained in refrigerator for thermal shock anesthesia, then dissected on Petri dishes containing phosphate buffered saline solution (NaCl 0.13 M, Na2HPO4 0.017 M, KH2PO4 0.02 M, pH 7.2), under a stereomicroscope for collection of ovaries. The samples were then fixed in 4% paraformaldehyde solution for 72h, dehydrated in

an alcoholic series (70, 80, 90, and 95 %) for 20-minute intervals, embedded in Leica historesin for 24 hours at 4oC, and transferred to plastic molds. After resin polymerization, 16 histological sections were obtained from each block at a thickness of 3.0 µm using a Lupetec MRP09 microtome and distributed on two glass slides. Sections were then stained with hematoxilin and eosin. The slides were dried and covered with Entellan® and a coverslip. Afterwards, sections of the ovary were examined and photographed using a capture system and image analysis, consisting of trinocular Olympus CX31 microscope (Olympus Optical Ltd. Brazil, São Paulo, SP, Brazil) and camera (SC30 Color CMOS Camera for Light Microscopy, Olympus Optical Ltd. Brazil, São Paulo, SP, Brazil).

2.5. Semi-quantitative and morphometric analysis

Semi-quantitative analysis of tick ovaries was based on work published by Marinho et al. (2014). From information available in literature, the main alterations described in the ovary of ticks subjected to acaricidal treatment were displayed in Table 1. Each alteration received an importance factor (w), ranging from 1 to 3, according to their relevance for development and surviving of ovarian cells: (1) minimal importance, when easily reversible after interruption of exposure to the compound; (2) moderate importance, when mostly reversible; and (3) high importance, when usually irreversible. After histological analysis, and based on information present in Table 1, the morphological changes found in this work were classified in scores (α), varying from 0 to 5. Zero represents features similar to control groups, and 5 means that the morphological changes are present in all cells evaluated. The individual index (Indexind) was calculated by the sum of each alteration index (w x α), as follows: Indexind = Σ(w x α). The individual indexes were compared statistically by means of the Kruskal-Wallis test, followed by Dunn's multiple comparison post-hoc test ($\alpha<0.01$), using GraphPad Prism software (version 7.00).

Morphometric analysis of oocytes was performed on Image J software (NIH). The oocytes were classified in accordance with the developing stages described for *R. microplus* (I to VI) (SAITO et al., 2005). Then, measurements of cytoplasmic area of 10 oocytes for each stage for each animal were taken. Analysis of oocytes I, II and III was performed only in cells in which germinal vesicle was evident. Because the germinal vesicle is rarely observed in stages IV and V, these cells were randomly selected for the measurements (BARBOSA et al., 2016). Type VI oocytes were not found. The cytoplasmic area of the oocytes was compared

statistically by means of the one-way ANOVA test, followed by Tukey's post-hoc analysis ($\alpha<0.05$), using GraphPad Prism software (version 7.00).

Table 1. Histological and ultrastructural changes observed in ovaries of ixodid ticks and their respective importance factors (w) for semiquantitative analysis.

Morphological alterations	Importance Factor (w)	References
Irregular chorion	1	(ARNOSTI et al., 2011; BARBOSA et al., 2016; DENARDI et al., 2010, 2011; MATOS et al., 2014; OLIVEIRA et al., 2016; ROMA et al., 2010a, 2010b, 2011;)
Non existent chorion	3	(OLIVEIRA et al., 2009; ROMA et al., 2010a)
Fragmented chorion	3	(CAMARGO-MATHIAS et al., 2017; DENARDI et al., 2011; OLIVEIRA et al., 2009, 2016; SREELEKHA et al., 2017)
Thicker chorion	1	(SAMPIERI et al., 2012)
Changes in the size of oocytes	1	(BARBOSA et al., 2016; MATOS et al., 2014; OLIVEIRA et al., 2017; ROMA et al., 2010b)
Irregular oocyte shape	2	(CAMARGO-MATHIAS et al., 2017; DENARDI et al., 2010, 2011; MATOS et al., 2014; OLIVEIRA et al., 2017; SAMPIERI et al., 2012; VENDRAMINI et al., 2012)
Oocyte disappearance	3	(OLIVEIRA et al., 2016)
Irregular basal lamina	2	(OLIVEIRA et al., 2009, 2016, 2017; ROMA et al., 2010a)
Reduction in the amount of microvilli	1	(OLIVEIRA et al., 2009, 2017)
Absence/alteration of microvilli	1	(OLIVEIRA et al., 2009; SAMPIERI et al., 2012)
Cytoplasmic vacuolization	2	(ARNOSTI et al., 2011; BARBOSA et al., 2016; CAMARGO-MATHIAS et al., 2017; DENARDI et al., 2010, 2011; MATOS et al., 2014; OLIVEIRA et al., 2009, 2016, 2017; REMEDIO et al., 2014; ROMA et al., 2010a, 2010b, 2011; VENDRAMINI et al., 2012)
Cytoplasmic disorganization	2	(OLIVEIRA et al., 2016; REMEDIO et al., 2014; SREELEKHA et al., 2017)
Presence of acidophilus areas in the cytoplasm	1	(REMEDIO et al., 2014)

Continue…

Morphological alterations	Importance Factor (w)	References
Increase of yolk granules size	1	(ARNOSTI et al., 2011; BARBOSA et al., 2016; CAMARGO-MATHIAS et al., 2017; DENARDI et al., 2010; MATOS et al., 2014; OLIVEIRA et al., 2016)
Decrease in size/amount of yolk granules	1	(ARNOSTI et al. 2011; OLIVEIRA et al., 2017; VENDRAMINI et al., 2012)
Breaching/fusion of yolk granules	2	(MATOS et al., 2014; SREELEKHA et al., 2017)
Presence of myelinic figures	2	(DENARDI et al., 2012; OLIVEIRA et al., 2017; ROMA et al., 2010a)
Decreasing in the amount of lipid droplets	1	(OLIVEIRA et al., 2009, 2017)
Degenerating/Irregular/Fragmented Protein Granules	1	(DENARDI et al., 2011; OLIVEIRA et al., 2009; ROMA et al., 2010a)
Irregular mitochondria	1	(DENARDI et al., 2012; OLIVEIRA et al., 2009)
Absence/alteration of mitochondrial inner membrane	2	(DENARDI et al., 2012; OLIVEIRA et al., 2009; REMEDIO et al., 2014; ROMA et al., 2010a; SAMPIERI et al., 2012;SREELEKHA et al., 2017)
Strongly electrondense mitochondrial matrix	1	(REMEDIO et al., 2014)
Decrease in the amount of mitochondria	1	(OLIVEIRA et al., 2017; SAMPIERI et al., 2012)
Disorganized/dilated/vesiculated RER	2	(DENARDI et al., 2011, 2012; OLIVEIRA et al., 2009; REMEDIO et al., 2014)
Disorganized cytoskeletal elements	2	(DENARDI et al., 2012)
Irregular nuclear envelope	1	(ARNOSTI et al., 2011; DENARDI et al., 2010, 2012; OLIVEIRA et al., 2009; REMEDIO et al., 2014; ROMA et al., 2011; SAMPIERI et al., 2012; SREELEKHA et al., 2017)
Picnotic/degenerated nucleus	3	(ARNOSTI et al., 2011; OLIVEIRA et al., 2016; VENDRAMINI et al., 2012)

Continue…

Morphological alterations	Importance Factor (w)	References
Disorganized/vacuolized/fragmented nucleolus	3	(ARNOSTI et al., 2011; DENARDI et al., 2010, 2011; OLIVEIRA et al., 2009; REMEDIO et al., 2014; ROMA et al., 2010a; VENDRAMINI et al., 2012)
Disappearance of the nucleus	3	(DENARDI et al., 2012; OLIVEIRA et al., 2016)

3. RESULTS

3.1. Histopathological analysis

Control Group I (Distilled water)

The results obtained in the analysis of the ovaries of ticks exposed to distilled water corroborate with the description of the female reproductive system of *R. microplus* (SAITO et al. 2005). Below, a summary of the morphological characteristics of the oocytes in this group (Fig.2A-B):

Oocytes I represent the smallest cells of the line, increasing progressively throughout the vitellogenesis. These cells presented a rounded shape, slightly irregular, with homogeneously basophilic cytoplasm. The germinal vesicle was more basophilic than the cytoplasm, presented a round shape and was located in the central region of the cell. The nucleolus was poorly evident, but stained more intensely in purple than the nucleus.

Oocytes II presented an elliptical shape, with fine and weakly basophilic homogeneous granulations. The germinal vesicle exhibited a slightly irregular shape and evident nucleolus. Externally, involving the oocyte, a basal membrane stained strongly in purple was observed. Oocytes with intermediate characteristics between stages II and III presented an elongated region in contact with the pedicel, without reactivity to the dyes.

Oocytes III exhibited rounded shape and small uniform acidophilic granulations in the cytoplasm. The germinal vesicle displayed characteristics similar to those observed in the previous stage, although it was eccentric and close to the pedicel. It was observed the beginning of the chorion deposition by the appearance of a thin acidophilus layer externally to the basal membrane.

Oocytes IV presented a rounded shape, with a slightly irregular surface and a thicker chorion than the previous stage. The nucleus, rarely observed, was located at the basal pole of the cell. In the cytoplasm, yolk granules with rounded or irregular shape were visualized, the smaller ones being located in the central region. In addition, distinct reactivity of the yolk granules was observed, some more basophilic and others more acidophilc, with the presence of small spaces between them without reactivity to the dyes.

Oocytes V, the largest cells of the vitellogenesis, exhibited a fully deposited chorion and thicker than in the previous stage. The yolk granules were larger, with uniform size and round shape. The same distinct staining pattern of the yolk granules presented in oocyte IV was observed in this stage.

Control Group II (3% DMSO)

This group (Fig.2C-D) showed no differences in comparison to the control group I. However, one specimen had cytoplasm regions slightly more vacuolated than the others, especially in oocytes III and IV.

Treatment Group I (3.0 µL/mL)

In this group (Fig.3A-B), oocytes I and II showed small vacuolated regions in the cytoplasm and the germinal vesicle was weakly stained. The presence of vacuolated nucleolus was observed in two specimens. In stages III and IV, the cells were slightly vacuolated. In stage V, irregular cell shape and chorion detachment were observed. In general, the changes were observed in small extensions of the tissue.

Treatment Group II (3.5 µL/mL)

In this group (Fig.3C-D), oocytes I and II exhibited characteristics similar to the previous treatment, although more vacuolated. In the transition stage between stages II and III the vacuolization was more evident than in the controls, occupying, in some cases, about half of the cell. Some oocytes in these stages presented a ring-shaped nucleolus. In the subsequent stages, the chorion was thicker in two specimens, evidenced by the presence of a thick basophilic region around the cell. Chorion detachment and irregular oocyte shape were also observed in this treatment.

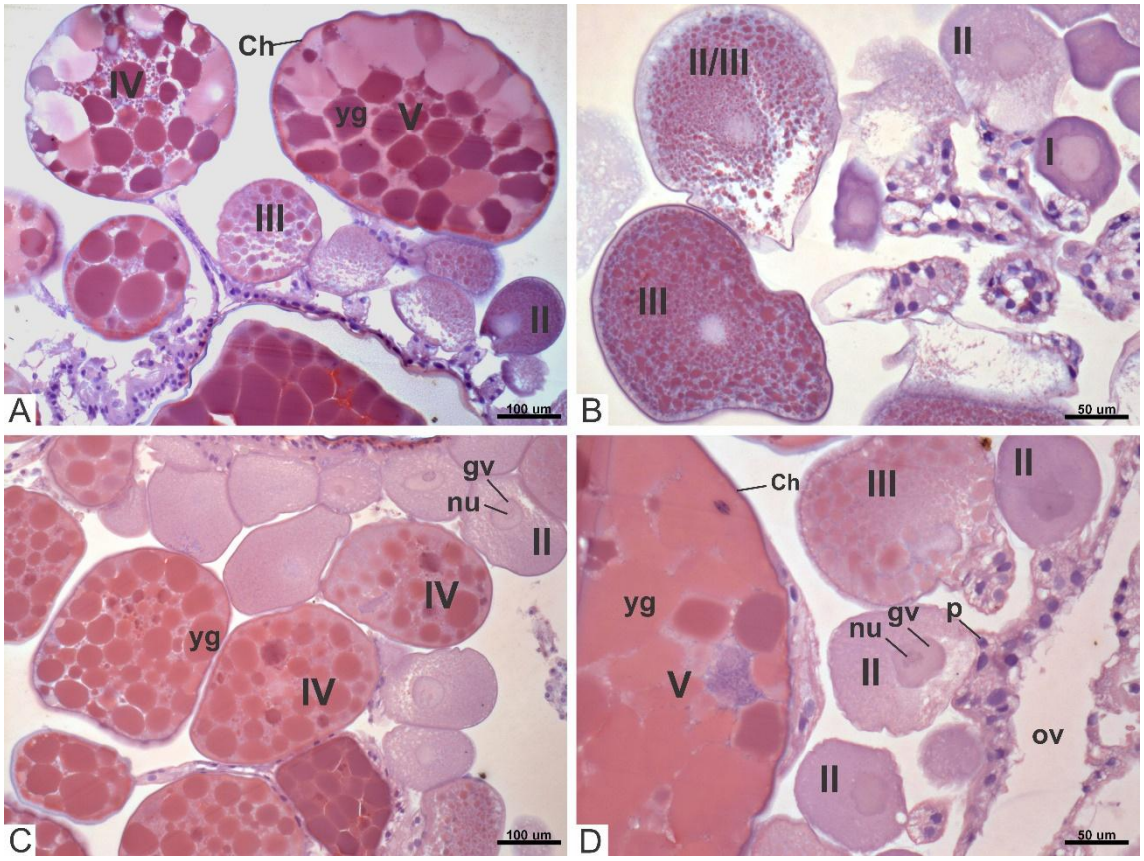

Fig.2. Histological sections of ovaries of *Rhipicephalus microplus* engorged ticks (control groups). (A–B) Control Group I (distilled water); (C–D) Control Group II (3% DMSO solution). Legends: (I-V) stages of oocyte development, (Ch) chorion, (gv) germinal vesicle, (nu) nucleolus, (ov) oviduct, (p) pedicel, (yg) yolk granules. Bars: (A, C) 100µm; (B,D) 50 µm.

Treatment Group III (4.0 µL/mL)

Ovaries of the individuals exposed to this treatment presented similar alterations to the previous treatment (Fig.3E-F). Vacuolization was observed at all stages of the vitellogenesis. Oocytes V exhibited irregular and detached chorion as well as apparent decrease in cell size, which was confirmed by morphometric analysis.

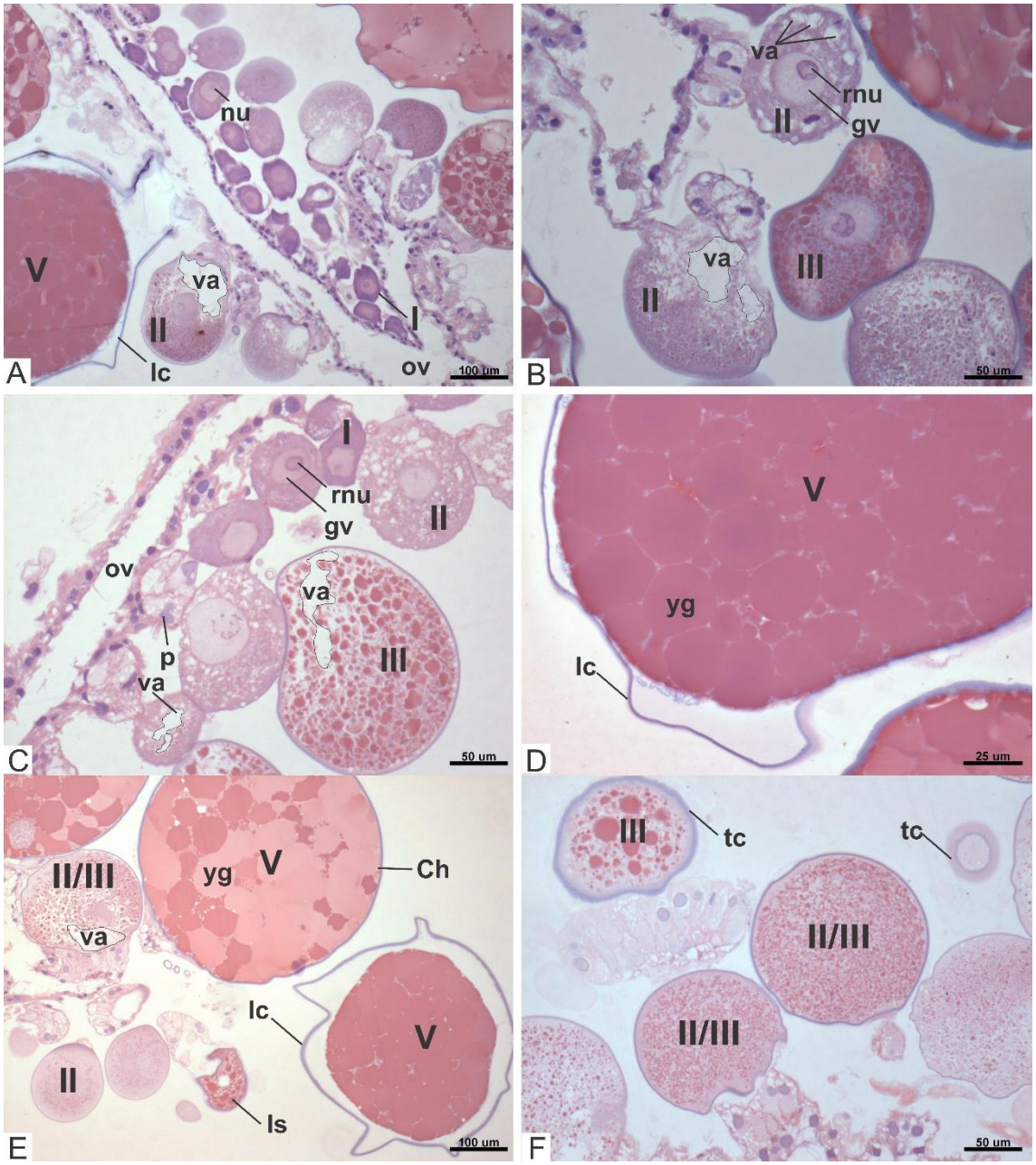

Fig.3. Histological sections of ovaries of *Rhipicephalus microplus* engorged ticks exposed to acetylcarvacrol. (A–B) Treatment Group I (3.0 µL/mL); (C–D) Treatment Group II (3.5 µL/mL); (E–F) Treatment Group III (4.0 µL/mL). Legends: (I-V) stages of oocyte development, (Ch) chorion, (gv) germinal vesicle, (Ic) irregular chorion, (Is) irregular oocyte shape, (nu) nucleolus, (ov) oviduct, (p) pedicel, (rnu) ring-shaped nucleolus, (tc) thicker chorion, (va) cytoplasmic vacuolization, (yg) yolk granules. Bars: (A, E) 100 µm; (B,C, F) 50 µm; (D) 25 µm.

Treatment Group IV (4.5 µL/mL)

Ovaries of the individuals in this group showed the most significant alterations when compared to the other groups (Fig.4A-F). Oocytes I and II presented irregular shape and less intense staining of the germinal vesicle in comparison to controls. Most of the cells exhibited a ring-shaped nucleolus. In addition, early deposition of the chorion was observed in this treatment. In stages IV and V, the cells presented great vacuolization, with irregular shape and chorion thicker than the controls. Also, it was observed the fusion of yolk granules (Fig. 3D) and vacuoles around them (Fig. 3E) in oocytes V. An inverse relationship between chorion thickening and vacuolization of oocytes was observed in this treatment. In other words, the more vacuolated cells showed a similar chorion to the controls and the ones that presented thicker chorion were less vacuolated. In addition, oocytes in all development stages were visibly smaller when compared to controls.

3.2. Morphometric analysis

Measurements of cytoplasm area of oocytes (I-V) showed decreased values in all treatment groups in comparison with the control groups (Table 2). Control groups I and II did not show statistical differences. The oocytes I were equally affected by carvacrol acetate, showing no difference between treatments. For stages II to V, it was observed that treatment group IV had the most significant diminution of cytoplasm area of oocytes. This group, which was exposed to the highest concentration of carvacrol acetate, had the cytoplasm area heavily reduced when compared to the other groups ($p<0.05$).

3.3. Semi-quantitative analysis

The morphological changes found in ovaries of *R. microplus* ticks with their respective alteration indexes are shown in Table 3. Control group I did not exhibit any alterations while one specimen of control group II showed slight changes in the size and shape of oocytes. The sum of the alteration indexes for each animal in each group was used to calculate the individual index, which is shown in Table 4. Control groups I and II did not show statistical differences in the values. Treatment groups I to IV are statistically equal. However, treatment group IV presented a higher (15.20 ± 4.21) and statistically different individual index when compared to the control groups ($p<0.01$).

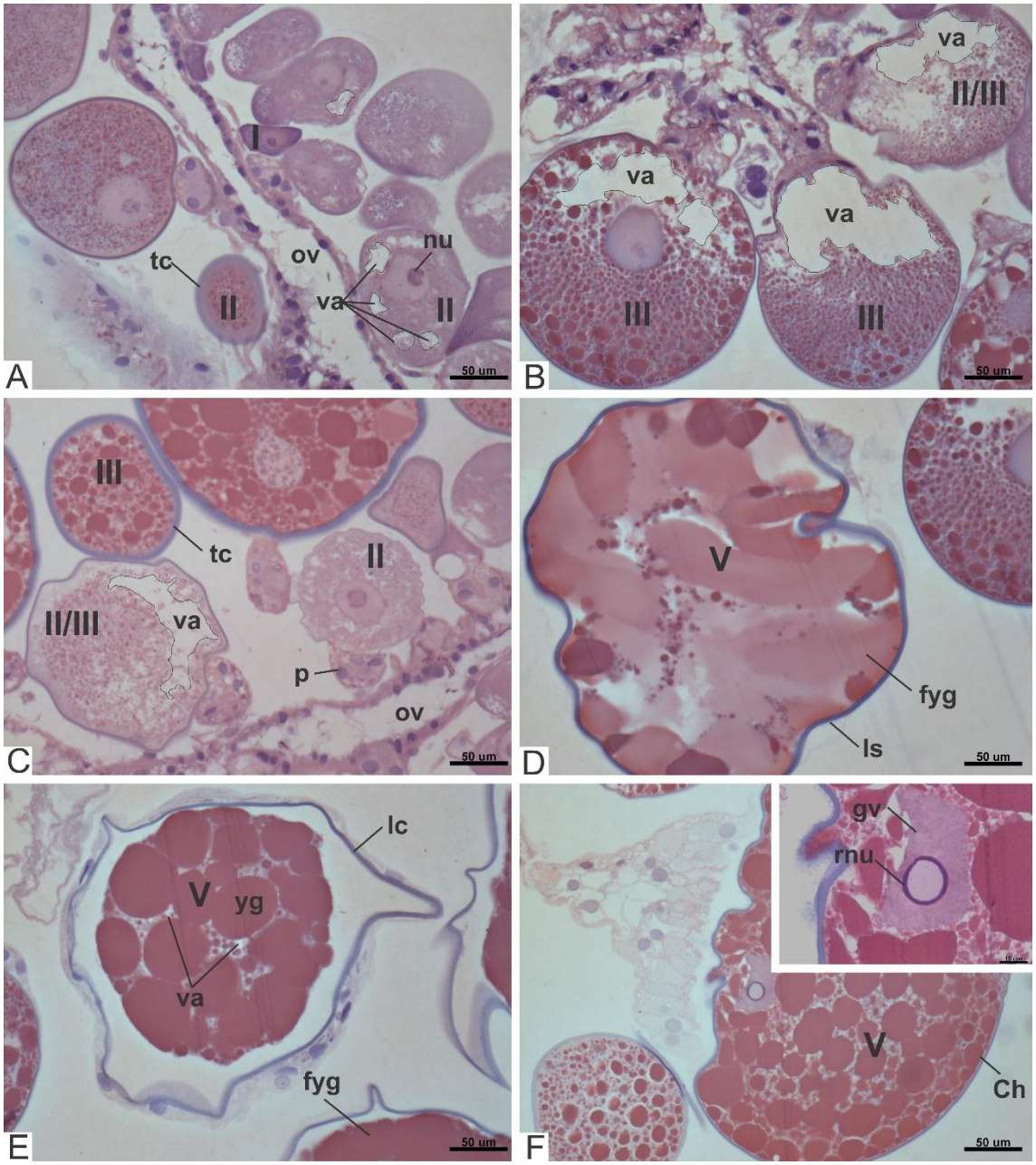

Fig.4. (A-F) Histological sections of ovaries of *Rhipicephalus microplus* engorged ticks exposed to the highest concentration of acetylcarvacrol (4.5 µL/mL). Legends: (I-V) stages of oocyte development, (Ch) chorion, (fyg) fused yolk granules, (gv) germinal vesicle, (Ic) irregular chorion, (Is) irregular oocyte shape, (nu) nucleolus, (ov) oviduct, (p) pedicel, (rnu) ring-shaped nucleolus, (tc) thicker chorion, (va) cytoplasmic vacuolization, (yg) yolk granules. Bars: (A-F) 50 µm.

Table 2. Mean ± SD (µm²) of the oocyte cytoplasm area of the cattle-tick *Rhipicephalus microplus* exposed to different concentrations of acetylcarvacrol.

Oocyte stages	H_2O	DMSO	T1	T2	T3	T4
I	1,304.89 ± 149.57[a]	1,236.64 ± 117.22[a]	1,016.37 ± 164.35[b]	900.87 ± 150.56[b]	940.49 ± 83.66[b]	884.81 ± 162.52[b]
II	4,985.78 ± 792.10[a]	5,041.06 ± 524.84[a]	4,132.32 ± 120.97[b]	3,696.93 ± 350.54[b, c]	4,088.76 ± 140.58[b]	3,552.11 ± 746.77[c]
III	8,932.36 ± 820.38[a]	9,378.39 ± 547.56[a, b]	8,981.27 ± 777.48[a, b, c]	8,439.11 ± 856.64[a, c, d]	7,796.12 ± 433.80[d,e]	7,236.52 ± 731.40[e]
IV	21,397.01 ± 2,245.67[a]	20,526.02 ± 2,172.63[a, c]	18,045.09 ± 2,306.30[b]	18,343.87 ± 1,785.75[b, c]	18,390.64 ± 703.43[b, c]	15,478.15 ± 3,324.35[c]
V	36,059.08 ± 3,786.75[a]	34,773.20 ± 3,130.71[a]	32,344.72 ± 3,350.21[a, b]	29,947.19 ± 1,668.20[b]	29,206.97 ± 1,825.82[b]	25,111.81 ± 6,550.86[c]

Means followed by different lowercase letters in lines differ significantly (One way ANOVA; $P < 0.05$).

Table 3. Mean ± SD of the alteration indexes observed in oocytes of *Rhipicephalus microplus* exposed to different concentrations of acetylcarvacrol.

		Alteration index (w x α)					
Morphologica lchanges	Importancefactor (w)	CI	CII	TI	TII	TIII	TIV
Irregular chorion	1	0 ± 0	0 ± 0	0.80 ± 0.45	0.80 ± 0.45	1.40 ± 0.55	1.20 ± 0.84
Thicker chorion	1	0 ± 0	0 ± 0	0 ± 0	0.60 ± 0.55	0.60 ± 0.55	1+00 ± 0.71
Changes in the size of oocytes	1	0 ± 0	0.20 ± 0.45	0.60 ± 0.55	1.60 ± 0.55	1.40 ± 0.55	2.00 ± 0.71
Irregular oocyte shape	2	0 ± 0	0.40 ± 0.89	1.20 ± 1.10	1.60 ± 0.89	2.00 ± 0.00	2.80 ± 1.10
Cytoplasmatic vacuolization	2	0 ± 0	0 ± 0	1.60 ± 0.89	3.20 ± 1.10	2.40 ± 0.89	2.80 ± 2.28
Disorganized / vacuolized / fragmented nucleolus	3	0 ± 0	0 ± 0	1.20 ± 1.64	1.20 ± 1.64	0.60 ± 1.34	5.40 ± 2.51

Table 4. Mean ± SD of the individual indexes calculated for each cattle-tick exposed to sublethal concentrations of acetylcarvacrol.

	Individual index Σ(w x α)					
Individual	CI	CII	TI	TII	TIII	TIV
I	0	3	5	9	10	14
II	0	0	4	10	8	20
III	0	0	5	10	8	19
IV	0	0	6	9	8	10
V	0	0	7	7	8	13
Mean ± SD	0 ± 0^{a}	0.60 ± 1.34^{a}	$5.40 \pm 1.14^{a, b}$	$9.0 \pm 1.23^{a, b}$	$8.40 \pm 0.89^{a, b}$	15.20 ± 4.21^{b}

Means followed by different lowercase letters in lines differ significantly (Kruskal-Wallis; $P < 0.01$).

4. DISCUSSION

Many plant species synthesize secondary metabolites to protect them against pathogens and pests (BENELLI, 2015; ERB; ROBERT, 2016; SHITAN, 2016). These self-defense mechanisms can have fungicidal (TAYEL et al., 2016), bactericidal (TIAN et al., 2018) and/or antioxidative properties (SHAH et al., 2014). Certain plants used within the medical and food industries are known to be acaricidal, or toxic against ticks (BENELLI et al., 2016). Among the various acaricidal compounds that have been isolated, carvacrol, which is used as a food preservative, stands out as a promising research candidate in the search for more efficacious strategies to manage tick infestations (MAHIAN; SANI, 2016; NOVATO et al., 2015). The promise of carvacrol, as in many other research chemicals, arises from the ability to chemically alter its functional groups to create semi-synthetic analogs that may be more biologically active than the parent molecule itself (KIM et al., 2015). Several semi-synthetic carvacrol derivatives have already been shown to exhibit antihelminthic (MORAES et al., 2013), insecticidal (BAGUL et al., 2018) and acaricidal properties (NOVATO et al. 2018; RAMÍREZ et al., 2016). Additionally, acetylcarvacrol presented a greater mortality rate on *R. microplus* larvae when compared to carvacrol (RAMÍREZ et al., 2016). Besides mortality itself, sublethal concentrations of many chemicals are able to affect fundamental biological processes associated with the survival of ticks (CAMARGO-MATHIAS et al., 2017).

The reproductive system of ticks has been the target of several studies aiming at inhibiting the ovary development by using sub lethal concentrations of various drugs (CAMARGO-MATHIAS et al., 2017; MATOS et al., 2014; OLIVEIRA et al., 2016; REMEDIO et al., 2014). These chemicals frequently act slowly on the tick's physiology, and that is the reason of an observation period of seven days established in this study (ROMA et al., 2010a). Thus, this research brings relevant data on the impact of acetylcarvacrol on the female germ cells of *R. microplus*, crucially important in a scenario in which reports of tick resistance to acaricides are increasing (KLAFKE et al., 2017).

The morphological alterations found in *R. microplus* ovary exposed to acetylcarvacrol were dose-dependent, as evidenced in treatment group IV, which was exposed to the highest concentration of the drug (4,5 µL/mL). Morphological alterations found in the female germ cells were irregular and thicker chorion, decreasing in size and irregular shape of oocytes,

cytoplasmatic vacuolization as well as ring-shaped nucleolus. Similar results were found in germ cells of semi-engorged *Rhipicephalus sanguineus* sensu lato female ticks using 50 μL/mL carvacrol (SOUZA et al., 2019). It is worth pointing out that this concentration of carvacrol tested by Souza et al. (2019) is over 10 times greater than the concentration tested in treatment group IV in this study. This indicates that acetylation of carvacrol enhances enormously its capacity to impair oogenesis.

After mating, ovary asynchronously engages in oogenesis to produce eggs, being possible to find oocytes in different development stages (SAITO et al., 2005). During the vitellogenesis, a process by which protein precursors and other molecules are produced by ovarian and extraovarian tissues and accumulated inside the germ cells, the size of the oocytes increases considerably (ESTRELA et al., 2010; XAVIER et al., 2018). Our results showed that oocytes I presented smaller cytoplasm area when compared to the control groups, but they were equally affected by different concentrations of carvacrol acetate. Interestingly, for stages II to V, it was observed that treatment group IV had the most significant diminution of cytoplasm area of oocytes. Therefore, acetylcarvacrol seems to impact not only the oogenesis itself by damaging the oocytes morphology, but also the vitellogenesis by impairing the synthesis of macromolecules. As the intake of the precursors that are responsible for increasing in size of oocytes is more significant after stage I (XAVIER et al., 2018), the dose-dependent diminution of cytoplasm area of the germ cells in stages II to V indicates that it is probably caused by the drug. Thus, acetylcarvacrol may affect the endocytosis of macromolecules or their synthesis or both. Consequently, the reduction in size of oocytes may impair *R. microplus* reproduction by generating eggs with reduced amount of nutrients.

According to Saito et al. (2005), the chorion of *R. microplus* is composed of an electron-dense material produced by the oocyte. As the chorion is produced, it fuses to the plasmatic membrane of the oocytes, starting at stage III of oogenesis (COONS; ALBERTI, 1999). This material serves as a protective barrier against adverse situations, such as desiccation, mechanical shocks, predation, and changes in humidity and temperature. In addition, the chorion has micropores that allows the oxygenation of the embryo (HINTON, 1981; SAMPIERI et al., 2012). We observed that the chorion of treatment groups II, III and IV was considerably thicker when compared to control groups. Also, it exhibited irregular format in all of the treatment groups. These findings corroborate reported data by various authors (ARNOSTI et al., 2011; BARBOSA

et al., 2016; MATOS et al., 2014; SAMPIERI et al., 2012). We believe that chorion thickening is probably due to a protective response against carvacrol acetate. However, as the chorion allows the input of oxygen inside the egg, a thicker layer would prejudice the survival of the embryo by interfering in gas exchange.

Among all morphological alterations described in the ovary of ticks subjected to acaricidal treatment, cytoplasmic vacuolization is the most frequent one. Cytoplasmic vacuoles were present in oocytes of all treatment groups. Several authors describe this vacuolization process as an attempt of the cell to isolate toxic compounds or even as an autophagic process of degradation or recycling of damaged cytoplasmic components (ARNOSTI et al., 2011; ROMA et al., 2011). Interestingly, the inverse relationship between chorion thickening and vacuolization of oocytes observed in treatment group IV suggests that acetylcarvacrol entered more considerably in cells that were vacuolated. The uptake of the drug possibly occurred through the pedicel, which is one of the main entrance routes of chemicals (OLIVEIRA et al., 2009; SAMPIERI et al., 2012). A fact that supports this idea is that more vacuolated regions appeared closer to the pedicel. Furthermore, the presence of small vacuoles in the cytoplasm of oocytes with intermediate characteristics between stages II and III of control groups demonstrates that permeability of these cells is probably increased, as the input of macromolecules starts to rise considerably in this stage of oogenesis (SAITO et al., 2005). Matos et al. (2014) also observed the presence of vacuoles in the cytoplasm of type II oocytes of *Rhipicephalus sanguineus* exposed to 30% ethanol as well as in treatment groups exposed to different thymol concentrations. In fact, it is well known that besides the increase of permeability of the germ cells in earlier stages, which could lead to a greater intake of carvacrol acetate, oocytes in the beginning of development are more vulnerable to chemicals due to the absence of the chorion (REMEDIO et al., 2014). We believe that the lipophilic chain of acetylcarvacrol could destabilize the membrane of pedicel cells in contact with oocytes and increase even more the permeability of these cells. Then, once the drug is inside the germ cells, it causes several damages as shown in our results. In addition, as acetylcarvacrol possibly affects permeability of oocytes, larger vacuoles generated with greater concentrations of the drug could be due to the loss or degradation of cytoplasm components. This could lead to a decreasing of cytoplasm contents, which explains the size diminution of oocytes in treatment group IV. The increase of permeability could also

lead to a greater intake of liquids. However, the cytoplasm area might still decrease due to a reduction of yolk granules, since all cell metabolism may have been impaired.

Vacuolated regions in the nucleolus of oocytes of ticks exposed to acetylcarvacrol were also a remarkable morphological alteration. These nucleoli exhibited a compact ring-shaped mass with a central vacuole, which was also reported by numerous authors (DENARDI et al., 2010, 2011; REMEDIO et al., 2014; VENDRAMINI et al., 2012). These authors suggest the occurrence of genetic material degeneration leading to the death of the oocytes.

The semi-quantitative analysis was proposed for the first time in this study, to the best of our knowledge, to evaluate morphological alterations in ovaries of ixodid ticks. It demonstrated to be a straightforward and more reliable method to determinate the efficiency of any chemical based on extension and impact factor of morphological alterations. Our results showed that treatment group IV exhibited the individual index statistically different from control groups. This demonstrates the impact of acetylcarvacrol in the reproductive system of *R. microplus* by causing several damages in the oocytes.

Control methods using sublethal concentrations of natural based chemical compounds are usually less harmful to the environment and also safer for hosts. The morphological alterations observed in the oocytes of ticks exposed to sublethal concentrations of acetylcarvacrol may affect the viability of the embryos and probably contribute to mitigate tick infestation. Additionally, further evaluation of the reproductive performance of these ticks, through estimation of the egg production indexes and hatching rates, may provide complementary information that will allow the understanding of the effects of acetylcarvacrol in *R. microplus* offspring. Finally, we would like to propose the use of the semi-quantitative analysis performed in this research in further studies in order to facilitate the comparison of the effects of new acaricidal compounds in the reproductive system of ticks.

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PARTE III – Artigo II

Low concentrations of acetylcarvacrol induce drastic morphological damages in ovaries of surviving *Rhipicephalus sanguineus* sensu lato ticks (ACARI: IXODIDAE)

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Low concentrations of acetylcarvacrol induce drastic morphological damages in ovaries of surviving *Rhipicephalus sanguineus* sensu lato ticks (ACARI: IXODIDAE)

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ABSTRACT

Rhipicephalus sanguineus sensu lato (s. l.) ticks are targets of acaricidal treatments due to their great veterinary and medical importance. Acetylcarvacrol stands out as a promising acaricidal substance for its increased biocidal activity and stability. Additionally, its toxicity to mammals is reduced when compared to the parent molecule, carvacrol. The present study aimed to evaluate the effect of acetylcarvacrol on the morphology of ovaries of engorged *R. sanguineus* s. l. ticks. The animals were subjected to the Adult Immersion Test to calculate the lethal concentrations (LC_{50} and LC_{90}) of acetylcarvacrol. Subsequently, the surviving ticks were dissected for collection of the ovaries. The samples were processed through routine histological techniques. The histological sections were stained with hematoxylin and eosin, bromophenol blue (protein detection) and PAS (polysaccharide detection). The alterations found in the ovaries were evaluated by morphometric and semiquantitative analysis. The LC_{50} and LC_{90} were 17.805 and 26.164 µL/mL, respectively. The most severe morphological alterations were disappearance of the female germ cells (oocytes), nucleolus vacuolization, thicker and irregular chorion, and decrease in size of the oocytes. Also, the content of proteins and carbohydrates in the oocytes were heavily affected by the chemical, as evidenced by a non-homogeneous staining pattern. The group exposed to the highest concentration of acetylcarvacrol (20 µL/mL) exhibited a statistically greater score in the semiquantitative analysis when compared to the other groups. The morphological changes in the ovaries may reduce the ticks' offspring production or generate descendants that will struggle to carry out essential biochemical processes during their lives. Thus, acetylcarvacrol may be a promising alternative to control tick infestation by impairing the reproduction of this parasite.

Keywords: Brown dog tick. Tick control. Ovary. Toxicity. LC_{50}.

1. INTRODUCTION

Rhipicephalus sanguineus sensu lato (s. l.), commonly referred to as the brown dog tick, has a vast global distribution (DANTAS-TORRES, 2008). It is a blood-sucking ectoparasite that requires three hosts to complete its life cycle (DANTAS-TORRES, 2010). *R. sanguineus* s. l. feeds primarily on dogs, but may occasionally infest humans and other animals (RODRÍGUEZ-VIVAS et al., 2016). This tick is also the vector of more than a dozen pathogens for both humans and dogs, and its bite causes pain and discomfort to the hosts (DANTAS-TORRES; OTRANTO, 2015). For this reason, *R. sanguineus* s. l., as many other tick species, is a target of control methods based on acaricidal treatment (REY-VALEIRÓN et al., 2018; BURGIO et al., 2016).

The use of synthetic chemical compounds is the most common method to control tick infestation (VUDRIKO et al., 2018). Selection of resistant tick strains is a natural process that requires continuous search for cost-effective chemical substances with acaricidal activity (ROBBERTSE et al., 2016). Additionally, the indiscriminate use of acaricides available on the market may accelerate this process (KLAFKE et al., 2017). Moreover, synthetic acaricides are usually expensive and toxic to hosts and environment, requiring specialized handling (TSABOULA et al., 2016; ABBAS et al., 2014). Thus, the use of plant extracts and essential oils has emerged as promising alternatives to mitigate tick infestation (OLIVEIRA et al., 2016; REMEDIO et al., 2014). This is mainly due to the enormous abundance of plant secondary metabolites with biocidal activity, their low cost and relatively lower toxicity to the hosts (BORGES et al., 2012).

Carvacrol, one of the major constituents present in essential oils of *Origanum* sp. and *Thymus* sp., has recognized acaricidal activity (PEREIRA-JUNIOR et al., 2019; NOVATO et al., 2018; ARAÚJO et al., 2016). It stands out among others compounds for having a hydroxyl group that can be easily modified, aiming at increased stability and biological activity, while decreasing host toxicity (ANDRE et al., 2016; CACCIATORE et al., 2015). Among a multitude of possible chemical modifications, acetylation has been shown to increase acaricidal activity of carvacrol against unfed larvae of *R. microplus* (RAMÍREZ et al., 2016). Additionally, oral administration of acetylcarvacrol caused reduced acute toxicity to mice (*Mus musculus*) when compared to carvacrol (ANDRE et al., 2016). Finally, a number of other biological activities has already been

reported to acetylcarvacrol, such as bactericidal (MARINELLI et al., 2018), anti-inflammatory (DAMASCENO et al., 2014) and anti-helminthic (MORAES et al., 2013).

Besides acaricidal activity itself, sublethal concentrations of natural compounds can affect the morphophysiology of organs particularly important for tick survival (CAMARGO-MATHIAS, 2018), such as salivary glands (REMEDIO et al., 2016), ovaries (KONIG et al., 2019), synganglion (ROMA et al., 2013), and integument (REMEDIO et al., 2014). Through histological and histochemical techniques, it is possible to evaluate the toxic effects of such products in these organs (FONTANETTI et al., 2010). Chemical compounds that affect ovaries are of great interest due to the capability of a single engorged female to lay thousands of eggs (TROUGHTON; LEVIN, 2007). Therefore, control methods based on the reproduction of this parasite stand out as notable alternatives to mitigate tick infestation (CAMARGO-MATHIAS et al., 2017; OLIVEIRA et al., 2016; SAMPIERI et al., 2012).

Thus, this study aimed at evaluating the acaricidal action of acetylcarvacrol and the effects of sublethal concentrations of this compound in the oocyte development of *R. sanguineus* s. l. engorged females. Morphological changes and alterations in composition of macronutrients in the oocytes were analyzed through histological and histochemical techniques and compared by semiquantitative analysis.

2. MATERIAL AND METHODS

2.1. *Rhipicephalus sanguineus* s. l. ticks

All experimental procedures were conducted as instructed and approved by the Ethics Committee on Animal Use (CEUA, UFLA - Lavras/MG, Brazil), protocol nº. 043/185 of 05/24/2018. Ticks were provided by the *R. sanguineus* s. l. colony maintained in a Biological Oxygen Demand (BOD) incubator under controlled conditions (27°C, 85% humidity, and 12-h photoperiod) in the Laboratory of Parasitic Diseases in the Federal University of Lavras (UFLA), Brazil. A total of 180 *R. sanguineus* s. l. couples were fed on New Zealand White male rabbits without prior contact with ticks or acaricides, as described by Bechara et al. (1995). The rabbits

were maintained in cages and received water and food *ad libitum*. After engorgement, which lasted for approximately nine days, female ticks were weighed and subjected to the bioassays.

2.2. Acetylcarvacrol

Carvacrol (5-isopropyl-2-methylphenol) was purchased from Sigma-Aldrich Co. (St Louis Mo, USA) with 99% purity and acetylated to obtain acetylcarvacrol. In the synthesis, 10 mL of carvacrol was added to a volumetric flask containing 50 mL of the catalyst, 10% sodium hydroxide solution, at room temperature. Then, 11 mL of acetic anhydride was added to the flask under cooling. The solution was kept under stirring for 15 minutes. Finally, acetylcarvacrol was separated from the reaction mixture as an oil and characterized according to its melting point and by infrared (IR) spectroscopy (MORAES et al., 2013).

2.3. Adult Immersion Test

The Adult Immersion Test was performed according to Drummond et. al. (1973). In the experiment, 90 females of *R. sanguineus* s. l. were washed in a sieve with tap water and dried on soft absorbent paper. Subsequently, the ticks were randomly divided in nine groups of 10 ticks with homogeneous weights ($p > 0.05$). In the control groups, the ticks were exposed to distilled water (CI) and 3% DMSO solution (CII). In the treated groups, the ticks were exposed to seven different concentrations of acetylcarvacrol, diluted in 3% DMSO solution: 10.0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 µL/mL. The ticks were immersed for five minutes in beakers containing the different solutions for control and treated groups. Afterwards, they were dried on soft absorbent paper and kept in Petri dishes at room temperature. A duplicate was performed with 90 other engorged female ticks following the same experimental procedures. Mortality was recorded every 24 hours during seven days (OLIVEIRA et al., 2008). The ticks that were considered dead presented no movement after stimuli to carbon dioxide and also exhibited a rigid and blackened integument. The mortality data obtained was subjected to Probit analysis using the software Statistica v. 7.0 to calculate the lethal concentration for 50% and 90% of ticks (LC_{50} and LC_{90}) and their 95% confidence intervals.

2.4. Histology

Ticks that remained alive for seven days after the Adult Immersion Test were randomly selected for histological analysis. Five ticks from the control groups I and II and treatment groups I (10 µL/mL), II (15 µL/mL) and III (20 µL/mL) were randomly chosen for dissection, totalizing 25 ticks.

All the ticks 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), under a stereomicroscope for collection of ovaries. The collected samples were immediately fixed in 4% glutaraldehyde solution for 72h, weighed, dehydrated in an alcoholic series (70, 80, 90, and 95%) for 20 minutes' intervals each, embedded in *Leica* historesin for 24 hours at 4^{o}C, and transferred to plastic molds. After resin polymerization, a minimum of 16 histological sections were obtained from each ovary at a thickness of 3.0 µm using a Lupetec MRP09 microtome and distributed on two glass slides. Sections were then stained with hematoxilin and eosin. The slides were dried and covered with Entellan® and a coverslip. Subsequently, sections of the ovaries were examined and photographed using a capture system and image analysis, consisting of trinocular Olympus CX31 microscope (Olympus OpticalLtd. Brazil, São Paulo, SP) and camera (SC30 Color CMOS Camera for Light Microscopy, Olympus Optical Ltd. Brazil, São Paulo, SP).

2.5. Histochemistry

A glass slide containing six histological sections for each animal was used for each histochemical analysis. These techniques were applied to evaluate changes in composition of proteins and polysaccharides in the oocytes.

2.5.1. Periodic acid–Schiff technique – polysaccharide detection

The histological sections were immersed for 30 min in 1% periodic acid. Then, the slides were washed with distilled water for 20 min, and immersed in Schiff's reagent for 30 min in the absence of light. Afterwards, the slides were washed with tap water for 30 min. After drying, slides were clarified for 5 min with xylol and mounted in Entellan® (JUNQUEIRA; JUNQUEIRA, 1983).

2.5.2. Bromophenol blue staining– protein detection

All slides were stained with bromophenol blue for 2 h at room temperature. Subsequently, they were washed with 0.5% acetic acid for 5 min and rinsed in tap water for 15 min. Then, the histological sections were quickly immersed in tertiary butyl alcohol, allowed to dry at room temperature, clarified for 5 min with xylol, and mounted in Entellan® (PEARSE, 1985).

2.6. Morphometric and semiquantitative analysis

The gonadosomatic index was used to quantify the interference of acetylcarvacrol in the ovary development, as proposed by Barbosa et al. (2016). It was calculated by dividing the body weight of the ticks by their respective ovary weight for both control and treated groups. The data was subjected to the Shapiro-Wilk normality test. Statistical comparison was made by means of the Kruskal-Wallis test, followed by Dunn's *post-hoc* test ($\alpha<0.05$), using GraphPad Prism software (version 7.00).

Measurements of cytoplasmic area of oocytes were performed on ImageJ software (NIH) for the morphometric analysis. Firstly, oocytes were classified in accordance with the developing stages described for *R. sanguineus* s. l. (I to V) (OLIVEIRA et al., 2005). Then, the cytoplasmic area of 10 oocytes of each stage for each animal was measured. Analysis of oocytes I, II and III was performed only in cells in which germinal vesicle was evident. In the stages IV and V, the cells were randomly selected for the measurements because the germinal vesicle is rarely observed in these stages (BARBOSA et al., 2016). The cytoplasmic area of the oocytes was compared statistically by means of the one-way ANOVA test, followed by Tukey's *post-hoc* analysis ($\alpha<0.05$), using GraphPad Prism software (version 7.00).

Morphological and histochemical alterations found in the ovary of the female ticks subjected to treatment with acetylcarvacrol were evaluated through a semiquantitative analysis proposed by Konig et al. (2019). Each alteration received an importance factor (w), ranging from 1 to 3, according to their relevance for development and survival of ovarian cells: (1) minimal importance, when easily reversible after interruption of exposure to the compound; (2) moderate importance, when mostly reversible; and (3) high importance, when usually irreversible. Changes

not reported by Konig et al. (2019) were inserted and received an importance factor assigned by the authors of the present study. Additionally, a score (α), varying from 0 to 5, was given for each alteration according to the extension of the morphological/histochemical change. Zero represents features similar to control groups, and five means that the morphological changes are present in more than 80% of the cells evaluated. The individual index ($Index_{ind}$) was calculated by the sum of each alteration index (w x α), as follows: $Index_{ind} = \Sigma(w \times \alpha)$. The individual indexes were compared statistically by means of the Kruskal-Wallis test, followed by Dunn's multiple comparison *post-hoc* test ($\alpha<0.05$), using GraphPad Prism software (version 7.00).

3. RESULTS

3.1. Lethal concentration (LC)

The mean weight ± standard deviation (SD) of the *R. sanguineus* s. l. female ticks was 296,9 ± 49,2 mg and no statistical differences were observed between groups ($p > 0.05$). The percentage of dead ticks during each day after the Adult Immersion Test (AIT) is shown in Table 1. The percentage of knockdown ticks, which are characterized for expressive reduction in movement, is also shown after seven days of the treatment. Most of the ticks were dead after the first day of exposition to acetylcarvacrol. In the group exposed to the highest concentration of acetylcarvacrol (25 µL/mL), for example, more than 80% of the total mortality happened 24h after the experiment. The mortality data recorded in the seventh day after AIT was used to estimate the lethal concentrations for acetylcarvacrol in engorged *R. sanguineus* s. l. female ticks. The LC_{50} and LC_{90} values and their confidence intervals (CI) are shown in Table 2.

3.2. Morphological analysis

3.2.1. Control groups I (distilled water) and II (3% DMSO)

Ovaries of the ticks from Control groups I and II presented a morphology consistent with that described in the literature for this species (OLIVEIRA et al. 2005). No differences were observed between groups. Herein, a summary of the main morphological characteristics.

Oocyte I

Rounded or elliptical in shape, with homogeneously basophilic cytoplasm, and a slightly evident germinal vesicle (nucleus) that occupies a great part of the central cytoplasm and has an evident nucleolus. These cells are attached to the oviduct through the pedicel, composed of a single layer of epithelial cells with an evident nucleus. They were weakly intense for carbohydrates. However, it is possible to observe small granules that reacted with PAS. Pedicel and oviduct did not show reactivity with this technique (Fig. 2A). The cytoplasm and nucleolus of type I oocytes were strongly and homogeneously stained by Bromophenol blue. Nucleus, pedicel and oviduct were weakly stained (Fig. 3A).

Oocyte II

The cells are elliptical in shape and larger than stage I. Oocytes II have thin and homogeneous cytoplasmic granulations. The germinal vesicle is rounded in shape, poorly basophilic, located mostly at the basal pole of the cell, and with a very evident nucleolus (Fig. 1A). Fine magenta granulations in PAS were observed homogeneously distributed throughout the cytoplasm (Fig. 2A). The same granulation pattern observed in the PAS technique was seen in Bromophenol blue staining. The germinal vesicle is less stained than in stage I, but with an evident nucleolus (Fig. 3A).

Oocyte III

Type III oocytes are rounded and larger than in the previous stage. The nucleus, poorly stained and located in the basal pole of the cell, has a strongly basophilic nucleolus. Around the cell, it is possible to observe the beginning of the deposition of the chorion as a membrane weakly stained by hematoxylin. The yolk granules are acidophilic, mostly small and homogeneously distributed (Fig. 1B). In PAS technique, these granules were evident and intensely magenta. They varied in size, with the smaller ones located in the center of the oocyte (Fig. 2B). The same homogenous pattern of granulations was observed in Bromophenol blue staining, but with more strongly stained yolk granules. The chorion was very poorly stained (Fig. 3B).

Table 1: Percentage of dead and knockdown engorged *R. sanguineus* s. l. female ticks exposed to different concentrations of acetylcarvacrol.

Concentration of acetylcarvacrol (µL/mL)	Percentage of dead ticks/days of treatment (%)							Knockdown ticks (%)
	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	7 th
Control I (distilled water)	0	0	0	0	0	0	0	0
Control II (3% DMSO)	0	0	0	0	0	0	0	0
10.0	5	5	5	5	5	5	5	5
12.5	25	35	35	35	35	35	35	0
15.0	35	35	40	40	40	40	40	5
17.5	30	40	50	50	50	50	50	5
20.0	20	35	35	40	45	45	55	25
22.5	40	60	60	60	60	65	65	5
25.0	75	90	90	90	90	90	90	10

Table 2: Probit analysis results based on the mortality of engorged *R. sanguineus* s. l. female ticks exposed to acetylcarvacrol.

LC_{50} (95% confidence interval)	17.805 limits = 17.645 - 17.965
LC_{90} (95% confidence interval)	26.164 limits = 25.844 - 26.485
Regression equation	y = -0.3498 + 0.0477x
Coefficient of determination (R^2)	0.99741418

Oocyte IV

These cells are rounded in shape and bigger than in stage III. The nucleus, which is rarely observed at this stage, is poorly basophilic. The chorion is more evident than in stage III and attached to the oocyte. Yolk granules are well delimitated, strongly acidophilic, and vary in size (Fig. 1C). These granules were strongly and homogeneously stained by Bromophenol blue, and reacted with the PAS reagent intensely (Fig. 2C, 3C).

Oocyte V

Oocytes V are the biggest cells of the germ line. The chorion is totally deposited, weakly basophilic and adhered to the cell. Yolk granules are homogenous in size, strongly acidophilic and larger than stage IV (Fig. 1D). They were strongly magenta, as evidenced in the PAS technique. Close to the chorion is possible to observe smaller yolk granules, well delimitated, and strongly stained (Fig. 2D). These granules were also intensely stained by Bromophenol blue (Fig. 3D).

3.2.2. Treatment group I (10 μL/mL)

The oviduct of one specimen presented an abnormal morphology (Fig. 1I). In this region, a cluster of cells was observed in an unordered array of multiple layers. In addition, the pedicel exhibited an increased number of epithelial cells. In stages III to V, the oocytes of two animals presented slightly thickened chorion (Fig. 1J-K). In stage V, the cells had a greater spacing between the yolk granules. Also, some V oocytes showed fragmented yolk granules and chorion detached from the oocyte (Fig. 1L). In stages II to V, the oocytes exhibited a non-homogeneous PAS staining pattern (Fig. 2J). Additionally, in stage V, regions close to the chorion showed greater spacing, poorly magenta (Fig. 2L). In Bromophenol blue staining, oocytes I and II of 60% of the ticks showed lower protein content when compared to controls (Fig. 3I). In addition, a non-uniform protein staining pattern was observed in about 40% of the oocytes of all animals evaluated (Fig. 3J-L). In addition, oocytes V had weakly stained regions at the edge of the cell (Fig. 3L).

3.2.3. Treatment group II (15 μL/mL)

Epithelial cells of the oviduct were disposed in several layers with an unorganized arrangement. An apparently reduced number of oocytes I and II was observed in 40% of the ticks (Fig. 1M). Some type I oocytes presented cytoplasmic vacuolations around the nucleus. Oocytes III showed thickened chorion (Fig. 1N). In stage V, the chorion was irregular in some cells and detached from the cell edge. In addition, the yolk granules showed fragmented appearance in some oocytes V and large spacing between them. The cell limit showed irregular shape, weak staining and an apparent destruction of the yolk granules (Fig. 1P). The non-homogeneous staining pattern in PAS observed in the previous treatment was intensified in this group. Stages II and III presented non-stained regions close to the cell edge (Fig. 2M-P). Oocytes I and II were also less stained by Bromophenol blue when compared to the control groups. In the following stages, the same non-uniform staining pattern was found in the oocytes (Fig.3 N-P).

3.2.4. Treatment group III (20 μL/mL)

This group presented the most severe morphological alterations when compared to the others. Regions of the oviduct with abnormal morphology were present in 60% of the evaluated animals. In these regions, it was not possible to observe oocytes in stages I and II (Fig. 1Q). In addition, oocytes I to III exhibited ring-shaped nucleolus and cytoplasmic vacuolization (Fig. 1R). Irregular shape, thickening and irregularities of the chorionic membrane were also frequent changes in this treatment (Fig. 1S). About half of the oocytes V showed fragmentation and destruction of the yolk granules as well as loss of the cell limits, as evidenced by poorly stained regions in the cell edge (Fig. 1T). Oocytes in stages I, II and III presented less intense reaction with PAS than in the controls (Fig. 2Q). Non-stained regions were observed in the oocytes of all ticks, especially in the edge of the cells. In addition, the non-homogeneous staining pattern of yolk granules became more evident in this treatment, especially in stage V (Fig. 2R-T). The same non-uniform staining pattern was also observed in the oocytes stained by Bromophenol blue, especially in stage V (Fig. 3Q-T).

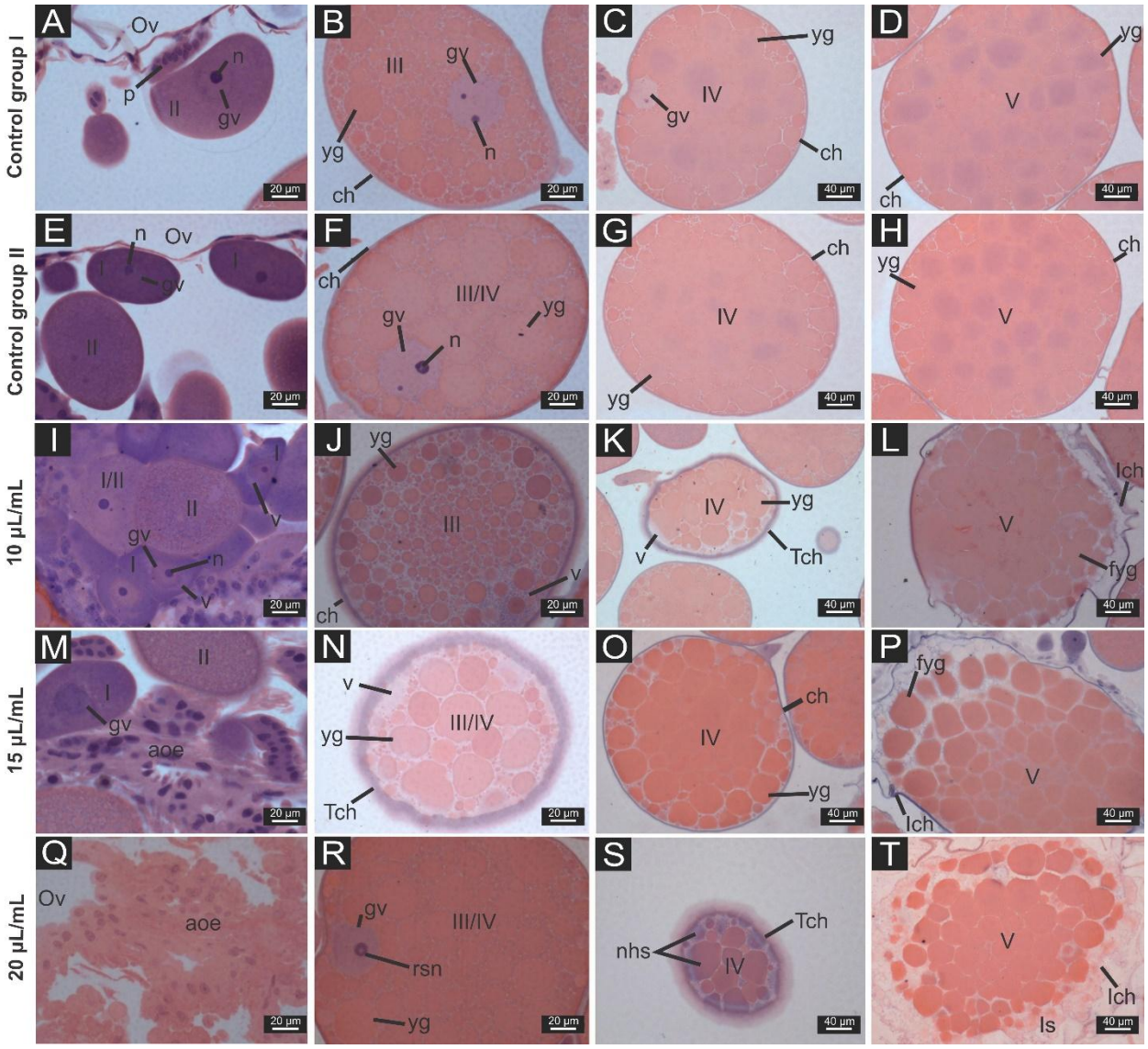

Fig. 1 - Histological sections of ovaries of engorged *R. sanguineus* s. l. stained with Hematoxylin and Eosin. (A, B, C, and D) Control Group I; (E, F, G, and H) Control Group II; (I, J, K, and L) Group TI (10 µL/mL); (M, N, O, and P) Group TII (15 µL/mL); (Q, R, S, and T) Group TIII (20 µL/mL). **Legends:** (I) Oocyte I; (II) Oocyte II; (III) Oocyte III; (IV) Oocyte IV; (V) Oocyte V; (ch) Chorion; (Ich) Irregular chorion; (Tch) Thicker chorion; (Is) Irregular oocyte shape; (Ov) Oviduct; (aoe) Abnormal ovary epithelium; (gv) Germ vesicle; (nu) Nucleolus; (rsn) Ring-shaped nucleolus; (p) Pedicel; (yg) Yolk granules; (fyg) Fragmented yolk granules; (v) Vacuolization; (nhs) Non-homogeneous staining. **Bars:** A-B, E-F, I-J, M-N, Q-R = 20 µm. C-D, G-H, K-L, O-P, S-T = 40 µm.

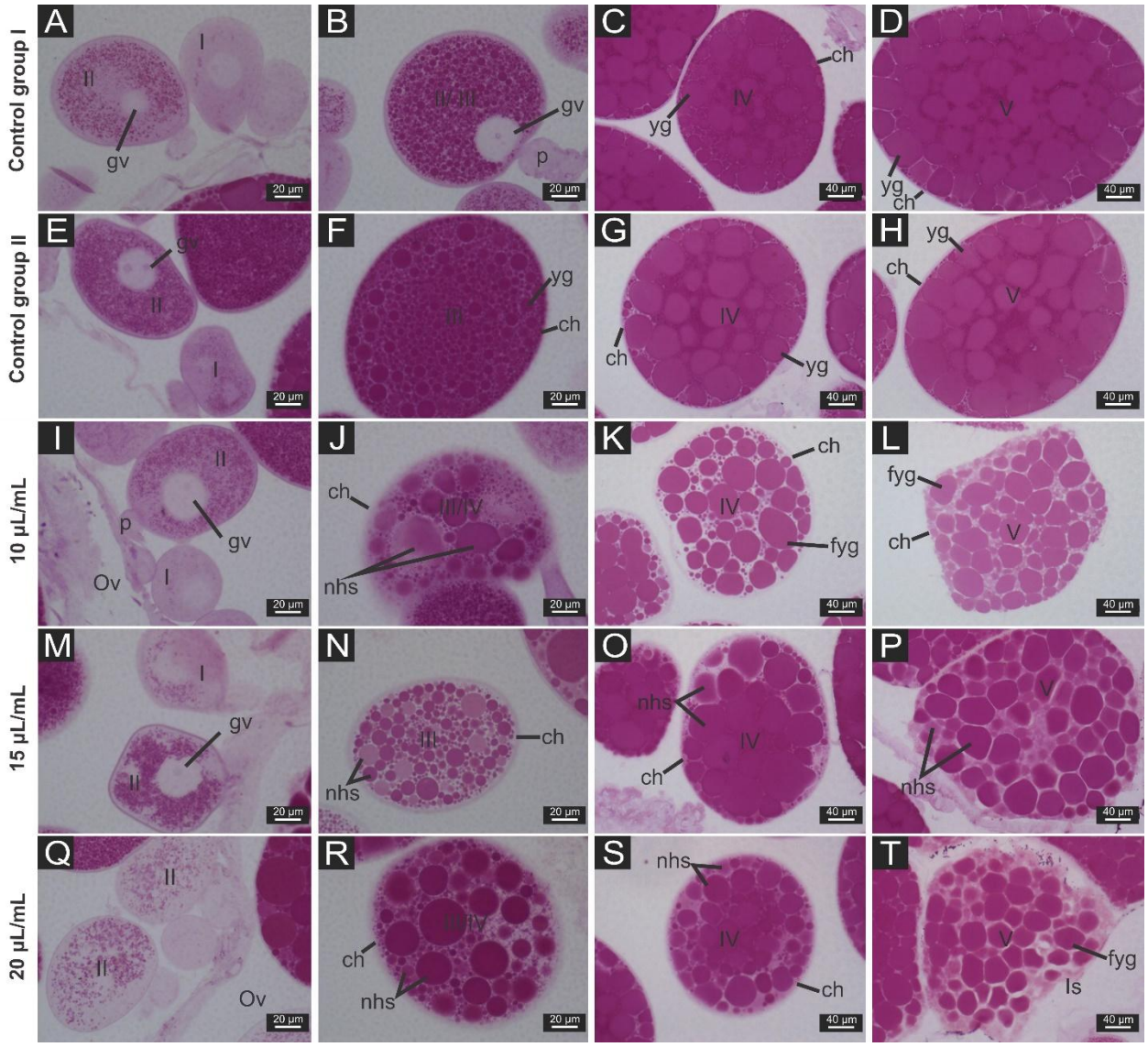

Fig. 2 - PAS technique of histological sections of ovaries of engorged *R. sanguineus* s. l.. (A, B, C, and D) Control Group I; (E, F, G, and H) Control Group II; (I, J, K, and L) Group TI (10 µL/mL); (M, N, O, and P) Group TII (15 µL/mL) (Q, R, S, and T) Group TIII (20 µL/mL). **Legends:** (I) Oocyte I; (II) Oocyte II; (III) Oocyte III; (IV) Oocyte IV; (V) Oocyte V; (ch) Chorion; (Ich) Irregular chorion; (Is) Irregular oocyte shape; (gv) Germ vesicle; (p) Pedicel; (Ov) Oviduct; (yg) Yolk granules; (fyg) Fragmented yolk granules; (nhs) Non-homogeneous staining. **Bars:** A-B, E-F, I-J, M-N, Q-R = 20 µm. C-D, G-H, K-L, O-P, S-T = 40 µm.

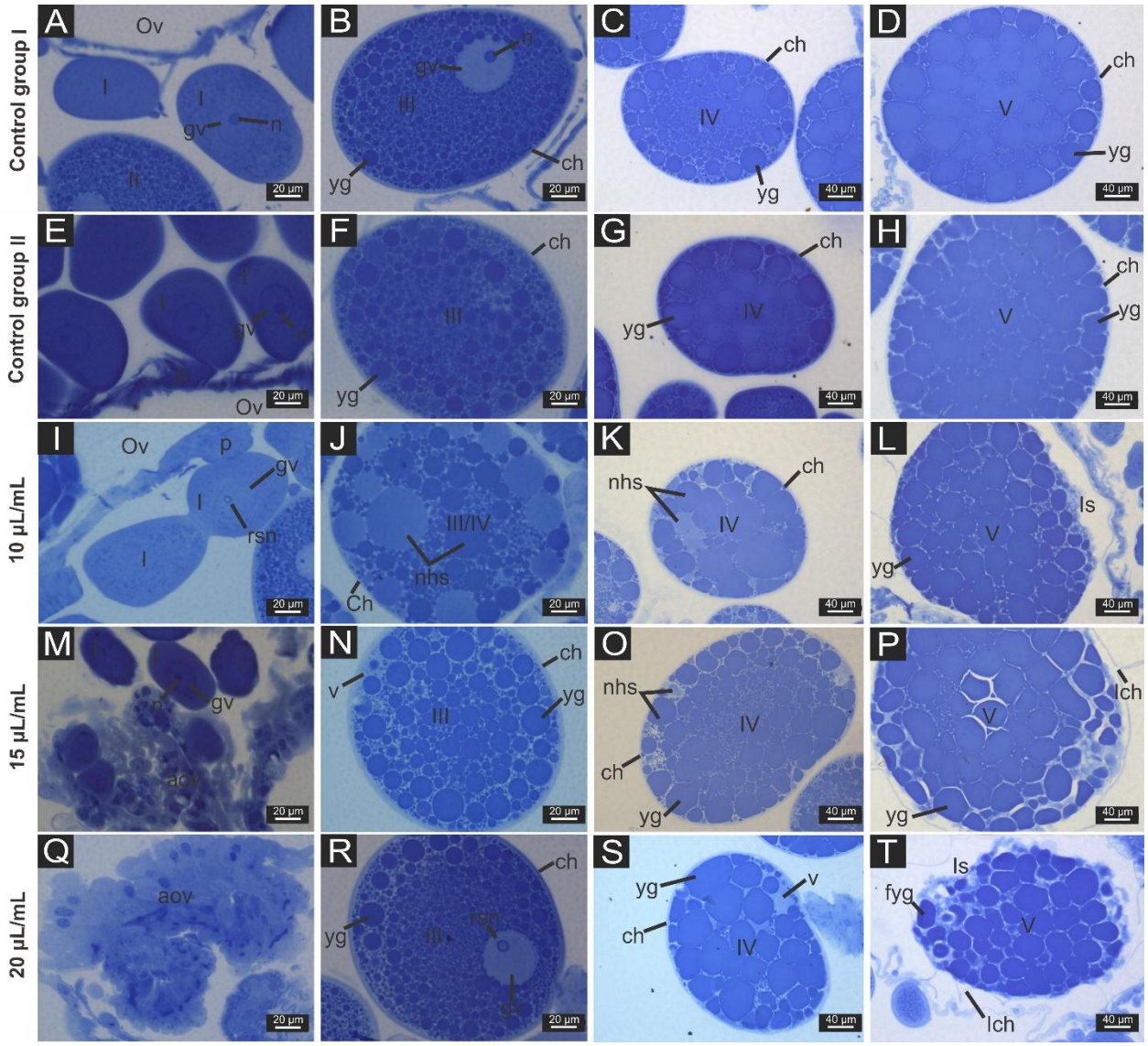

Fig. 3 - Histological sections of ovaries of engorged *R. sanguineus* s. l. stained with Bromophenol blue. (A, B, C, and D) Control Group I; (E, F, G, and H) Control Group II; (I, J, K, and L) Group TI (10 µL/mL); (M, N, O, and P) Group TII (15 µL/mL); (Q, R, S, and T) Group TIII (20 µL/mL). **Legends:** (I) Oocyte I; (II) Oocyte II; (III) Oocyte III; (IV) Oocyte IV; (V) Oocyte V; (ch) Chorion; (Ich) Irregular chorion; (Is) Irregular oocyte shape; (Ov) Oviduct; (aoe) Abnormal ovary epithelium; (gv) Germ vesicle; (nu) Nucleolus; (rsn) Ring-shaped nucleolus; (p) Pedicel; (yg) Yolk granules; (fyg) Fragmented yolk granules; (v) Vacuolization; (nhs) Non-homogeneous staining. **Bars:** A-B, E-F, I-J, M-N, Q-R = 20 µm. C-D, G-H, K-L, O-P, S-T = 40 µm.

3.3. Morphometric analysis

The gonadosomatic index is shown in Figure 4. As seen in the control groups, approximately 20% of the total mass of the ticks is composed of ovaries. No significant difference in the gonadosomatic index was observed between control and treated groups. However, a diminution in cytoplasm area of oocytes was noted in the treated groups when compared to control groups (Table 3). No significant difference was observed between control groups I and II. In the group exposed to the highest concentration of acetylcarvacrol (20 µL/mL), except for stage IV, all of the oocytes had a decrease in the cytoplasm area when compared to the control groups ($p < 0.05$).

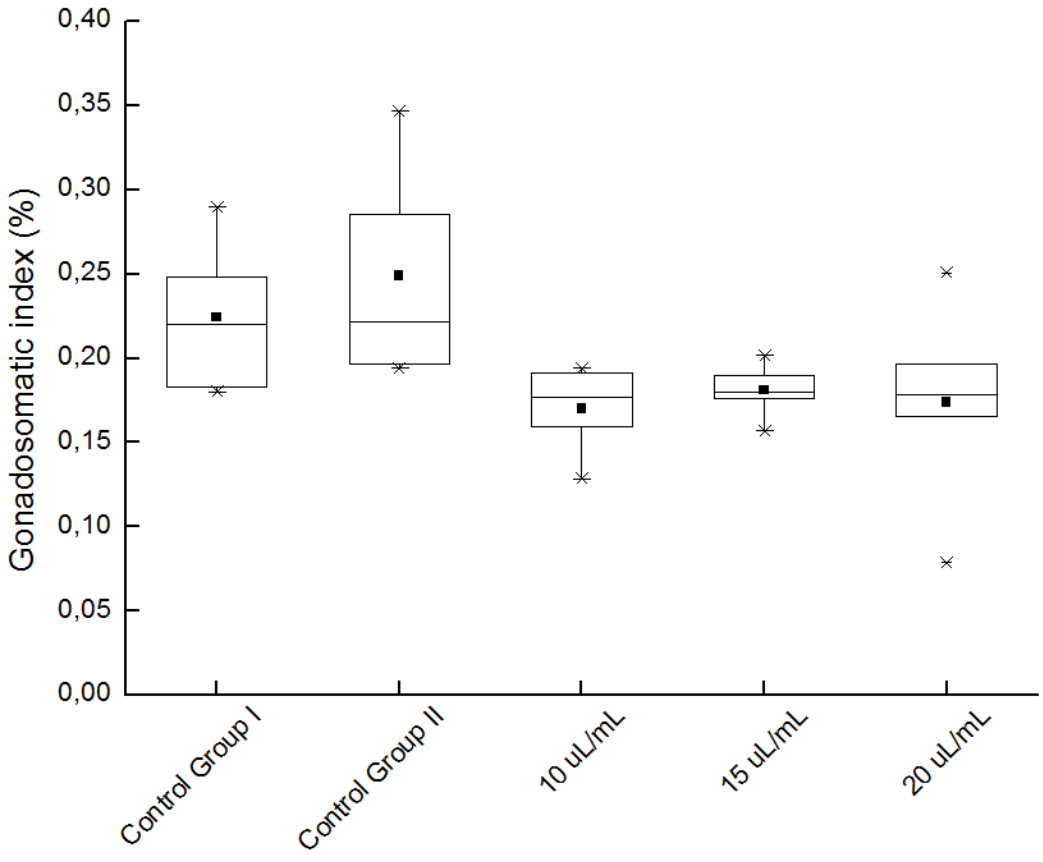

Fig. 4. Gonadosomatic index of *R. sanguineus* s. l. ticks exposed to acetylcarvacrol. Means did not differ significantly (Kruskal-Wallis; $p > 0.05$).

Table 3: Mean ± SD (µm²) of the oocyte cytoplasm area of *R. sanguineus* s. l. ticks exposed to different concentrations of acetylcarvacrol.

Oocyte stages	H_2O	DMSO	T1 (10 µL/mL)	T2 (15 µL/mL)	T3 (20 µL/mL)
I	1,668.50 ± 176.75[a]	1,647.88 ± 223.25[a]	1,371.09 ± 162.46[b]	1,301.13 ± 157.60[b]	1,229.95 ± 84,34[b]
II	5,014.84 ± 331.98[a]	5,008.16 ± 651.02[a]	4,200.72 ± 154.44[b]	3,914.45 ± 335.17[b]	3,779.00 ± 384.70[b]
III	8,669.08 ± 295.22[a]	8,364.90 ± 725.94[a,b]	7,827.47 ± 770.76[a,b]	8,276.16 ± 445.52[a,b]	7,574.76 ± 398.36[b]
IV	18,427.20 ± 1,420.38[a]	18,120.92 ± 1,378.85[a]	18,419.62 ± 1,049.44[a]	18,025.25 ± 1,292.83[a]	17,800.05 ± 610.29[a]
V	29,496.38 ± 1,381.23[a]	28,129.08 ± 1,267.68[a]	25,649.71 ± 914.57[b]	25,451.66 ± 1,009.88[b]	25,629.42 ± 708.08[b]

Means followed by different lowercase letters in rows differ significantly (One way ANOVA; $p < 0.05$).

3.4. Semiquantitative analysis

The alteration index for each morphological change found in the ovaries of the engorged *R. sanguineus* s. l. female ticks are shown in Table 4. Control groups I and II did not exhibit any morphological alterations. The individual indexes were calculated by the sum of the alteration indexes for each animal, as shown in Table 5. The individual index calculated for Treatment group III (23.4 ± 4.15) was statistically greater than the control groups ($p < 0.05$).

Table 4: Mean ± SD of the alteration indexes observed in ovaries of *R. sanguineus* s. l. exposed to different concentrations of acetylcarvacrol.

		Alteration index (w x α)				
Morphological changes	Importance factor (w)	CI	CII	TI (10 µL/mL)	TII (15 µL/mL)	TIII (20 µL/mL)
Irregular chorion	1	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.4	1.0 ± 0.0	1.4 ± 0.5
Thicker chorion	1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.5	0.4 ± 0.5	0.6 ± 0.5
Changes in the size of oocytes	1	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.7	1.4 ± 0.5	1.8 ± 0.7
Changes in protein content*	1	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.5	1.8 ± 0.4	2.4 ± 0.5
Changes in polysaccharide content*	1	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 0.8	2.2 ± 0.4	2.6 ± 0.5
Breaching/fusion of yolk granules	2	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 1.4	2.8 ± 1.1	4.0 ± 0.0
Irregular oocyte shape	2	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 1.1	3.6 ± 0.9	3.6 ± 0.9
Cytoplasmic vacuolization	2	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 1.1	1.2 ± 1.1	1.6 ± 0.9
Abnormal morphology of oviduct*	3	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 1.3	2.4 ± 2.5	3.0 ± 3.0
Ring-shaped nucleolus	3	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 1.3	0.6 ± 1.3	1.2 ± 1.6

* Alteration not described by Konig et al. (2019). Importance factors were given by the authors of the present study according to the degree of reversibility of the alterations.

Table 5: Mean ± SD of the individual indexes calculated for each engorged *R. sanguineus* s. l. female tick in the experimental groups.

	Individual index Σ(w x α)				
Individual	CI	CII	TI (10 µL/mL)	TII (15 µL/mL)	TIII (20 µL/mL)
I	0	0	10	19	25
II	0	0	12	16	25
III	0	0	20	18	26
IV	0	0	11	20	16
V	0	0	9	19	25
Mean ± SD	0 ± 0^{a}	0 ± 0^{a}	$12.4 \pm 4.4^{a, b}$	$18.4 \pm 1.5^{a, b}$	23.4 ± 4.1^{b}

Means followed by different lowercase letters in rows differ significantly (Kruskal-Wallis; $p < 0.05$).

4. DISCUSSION

The present study demonstrated that the LC_{50} of acetylcarvacrol in *R. sanguineus* s. l. engorged females is almost four times lower (17.80 µL/mL) than the LC_{50} calculated for carvacrol in semi-engorged ticks of the same species (64.19 µL/mL) (LIMA-SOUZA et al., 2019a). It is important to mention that semi-engorged females have thinner integument when compared to engorged ones (REMEDIO et al., 2015). As the integument represents the first barrier to toxic compounds (LIMA-SOUZA et al., 2017), a thinner layer would facilitate the penetration of these substances. In this sense, acetylation of carvacrol considerably raised its acaricidal activity even when testing this compound in a more resistant stage of development.

The estimated LC_{50} of carvacrol in *R. microplus* engorged females was 20.58 µL/mL (PEREIRA-JUNIOR et al., 2019). Although this value is similar to our results, different tick species usually present distinct susceptibility to acaricidal treatment (DAEMON et al., 2012). In fact, it is speculated that *R. sanguineus* s. l. may be less susceptible to acaricidal treatment due to the lower cuticle permeability of this tick (DAEMON et al., 2009). The decrease in cuticle permeability is probably an adaptation to xeric conditions, as this species is originated from the African continent (DANTAS-TORRES, 2008). Indeed, the LC_{50} of carvacrol for unfed larvae of *R. sanguineus* s. l. (3.29 mg/mL) was almost two times greater than the LC_{50} for *R. microplus* (1.76 mg/mL) under the same conditions (ARAÚJO et al., 2016). Finally, the increased biocidal activity of acetylcarvacrol is in accordance with the results of Ramírez et al. (2016). These authors found that the mortality rate of acetylcarvacrol (1% w/v) on *R. microplus* larvae was considerably greater (67.83 ± 2.07) than carvacrol (35.85 ± 3.18).

Rhipicephalus sanguineus ticks invest in reproduction great amount of the energy acquired during engorgement. A single engorged female of this species is able to lay over 2500 eggs under laboratory conditions (DANTAS-TORRES et al., 2011). In our work, the gonadosomatic indexes of the control groups I and II were greater than 20%. Although no statistical difference was observed in the gonadosomatic index between control and treated groups, various morphological changes were found in the ovaries of the treated groups. These alterations include irregular chorion, changes in the size and shape of oocytes, ring-shaped

nucleolus, cytoplasmic vacuolization, abnormal morphology of oviduct and modifications in yolk granules' composition and structure. Thus, this demonstrates the potential of acetylcarvacrol to impair the reproduction of this tick.

Vitellogenin is a high molecular weight protein that is synthesized in the gut, fat body and ovaries of ixodid ticks (ROSELL; COONS, 1992). The synthesis of this protein starts after mating and increases in the ovipositing period (THOMPSON et al., 2007). As vitellogenin is synthesized, it is released into the haemolymph and processed into smaller subunits to be taken up by the developing oocytes as vitellin (RAIKHEL et al., 2002). Additionally, a proteomic study of the *R. microplus* vitellogenesis revealed that besides vitellin, the ovary of engorged female ticks has over 3000 proteins particularly important for the development of the oocytes (XAVIER et al., 2018). In our work, the presence of a vacuolated nucleolus, also called ring-shaped nucleolus, is thought to be responsible for affecting protein content in this tick. The nucleolus, which is the site of ribosome biogenesis, plays a pivotal role in the synthesis of proteins (SCHEER; HOCK, 1999). A number of authors suggest that the presence of vacuolated nucleolus in the oocytes of ticks can lead to the cell death, although the mechanisms by which the death occurs are not discussed (REMEDIO et al., 2014; DENARDI et al., 2011, 2010). We believe that nucleolus alterations promote an abnormal synthesis of proteins notably important for the oocyte development. Thus, if the cell death does not occur, this alteration may generate descendants that will struggle to carry out fundamental biochemical processes during their lives.

Variations in proteins and carbohydrates content were observed in all treatments of this study. Oocytes I and II in the treated groups showed reduced amount of proteins, as evidenced by the decrease in affinity to the histochemical stain. Also, after stage III, compositions of proteins and carbohydrates showed similar alterations, mainly a non-homogeneous staining pattern of the yolk granules. The intake of these molecules starts to raise considerably in stage III. The deposition of proteins begins first, followed by carbohydrates (OLIVEIRA et al., 2005). Thus, it is suggested that acetylcarvacrol not only prejudices the synthesis of proteins in stages I and II, but also the intake of exogenous sources of these nutrients in the following stages of development. The non-uniform staining pattern found in both histochemical techniques in our study confirms that the composition of yolk granules may be impaired. Oliveira et al. (2019) also found similar histochemical changes in the oocytes of semi-engorged *R. sanguineus* s. l. ticks

exposed to *Acmella oleracea* extracts. The authors argue that the extract affected the synthesis and storage of proteins and polysaccharides in the germinative cells of the treated *R. sanguineus* females. The changes in macronutrients content may be the cause of deformation and fragility of the germ cells, preventing the emergence of new individuals (OLIVEIRA et al., 2019).

Decrease in size of oocytes is an alteration commonly found in ticks subjected to acaricidal treatment (KONIG et al., 2019; BARBOSA et al., 2016; MATOS et al., 2014; ROMA et al., 2011). The cytoplasm area diminution found in our results also demonstrates the influence of acetylcarvacrol in the synthesis of endogenous and in the synthesis/absorption of exogenous macromolecules. This was evidenced by the decrease in size of oocytes I and II, in which the endogenous synthesis predominates, as well as in oocytes V, in which the exogenous intake is the main source of nutrients (OLIVEIRA et al., 2005).

Drastic alterations in the pedicel and oviduct morphology were caused by acetylcarvacrol in the present study. In the treatment II (15 µL/mL), ovary wall and pedicel cells presented hyperplasia, and the number of oocytes I and II were apparently reduced. Moreover, in the treatment III (20 µL/mL), the oocytes I and II became a mass of cells with complete loss of the cell morphology. Oliveira et al. (2016) also observed an heterogeneous mass in the oviduct of engorged *R. microplus* female ticks exposed to 50 mg/mL of *Acmella oleraceae* extract. In this group, occytes I were no longer observed and oocytes II, rarely observed, showed an irregular morphology with the absence of germinal vesicle and various vacuoles. Moreover, a study with semi-engorged *R. sanguineus* s. l. female ticks exposed to 100 µL/mL of carvacrol found severe damages in oocytes I and II with irregular morphology and unpreserved boundaries (LIMA-SOUZA et al., 2019b). It worth pointing out that the concentration of carvacrol employed by Lima-Souza et al. (2019b) is five times greater than the one used in our study. These alterations found in oocytes I and II are due to the absence of the chorion, which makes undeveloped oocytes more susceptible to the absorption of external products from hemolymph (LIMA-SOUZA et al., 2019b; REMEDIO et al. 2014). By damaging oocytes in initial stages, acetylcarvacrol can certainly reduce the amount of viable eggs deposited by females in the environment.

The chorion is a barrier that protects the egg against mechanical shocks, changes in humidity and temperature, and desiccation (SAMPIERI et al. 2012; HINTON, 1981). Oocytes of

all treatment groups of this study exhibited thicker or irregular chorion when compared to the control groups. Thicker chorion was reported by Sampieri et al. (2012) in ovaries of engorged *R. sanguineus* female ticks treated with esters of ricinoleic acid from *Ricinus communis*. Konig et al. (2019) also observed thicker chorion in oocytes of engorged *R. microplus* ticks exposed to acetylcarvacrol. These authors stated that chorion thickening is a protective response of the cell against acetylcarvacrol. However, as the chorion allows the gas exchange of the embryo, a thicker layer would impair the input of oxygen inside the egg. Thus, the survival of the embryo would be prejudiced (KONIG et al., 2019). Additionally, irregularities in the chorionic membrane were observed in *R. sanguineus* exposed to different thymol concentrations (MATOS et al., 2014). Denardi et al. (2011) also found irregular chorion with folds and deformations in all the surface of oocytes of engorged *R. sanguineus* ticks exposed to aqueous extract of *Azadiractina indica* A. JUSS. These authors state that these deformations could facilitate the entrance of chemicals in the cell, which could interfere in its maturation and ability to generate a new individual. It is important to note that, since ticks were observed for seven days after the application of acetylcarvacrol in our work, the changes observed in the chorion may have been a result of both the impaired synthesis of its components by young oocytes and direct damage caused by the products in maturing oocytes.

Fragmentation of yolk granules and loss of the cell limits were also remarkable morphological alterations found in our results. These alterations were mostly observed in oocytes in which the chorion exhibit several irregularities and was detached from the oocytes. We believe that acetylcarvacrol was able to pass through the chorion, causing alterations in the yolk granules as well as destroying the cell limits. A fact that supports this idea is that the oocytes V, in the present study, showed changes in the yolk granules surrounding the edge of the cells. This indicates the presence of acetylcarvacrol in the haemolymph, which is in contact with the oocytes, and is another entrance route for chemicals besides the pedicel (OLIVEIRA et al., 2019). A study that evaluated the effects of fipronil on ovaries of semi-engorged *R. sanguineus* found similar results (OLIVEIRA et al. 2008). The authors emphasize that even though the chorion is a membrane considered sufficiently resistant, the more developed oocytes (IV and V) can also be heavily affected by chemicals.

The morphological and histochemical changes observed in oocytes of all treatment groups were dose-dependent, as shown by the semiquantitative analysis in our results. Furthermore, the significant increase in the individual index (23.4 ± 4.1) of the animals in the treatment group III (20 µL/mL) demonstrated that their ovaries were heavily affected by acetylcarvacrol. Konig et al. (2019) also found greater individual index (15.20 ± 4.21) in engorged *R. microplus* in the group exposed to the highest concentration of acetylcarvacrol (4.5µL/mL). The morphological alterations reported by Konig et al. (2019), although somehow similar, are not the same observed in the present study. The ovaries of *R. microplus* exposed to acetylcarvacrol exhibited vacuoles occupying about 40% of the cell, suggesting loss of cell permeability. In the present study, on the other hand, acetylcarvacrol altered the morphology of oocytes I and II and the pedicel cells, but keeping the cytoplasm contents in these cells. Thus, we believe that acetylcarvacrol may act differently in both species. Other alterations such as ring-shaped nucleolus, irregular or thicker chorion and changes in size and shape of oocytes were found in both species.

In conclusion, our results demonstrate the potential of a natural based chemical compound to cause drastic morphological and histochemical changes in ovaries of engorged *R. sanguineus* s. l. ticks. This will probably lead to a diminution in the ticks' offspring production or generate descendants that will struggle to realize essential biochemical processes during their lives, thus contributing to a long-term control of tick infestation. Future studies to evaluate life cycle characteristics and parasitism efficiency of these descendants are needed to prove this a long-term control hypothesis.

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PARTE IV

1. DISCUSSÃO GERAL

Fêmeas ingurgitadas de carrapatos ixodídeos são capazes de realizar a postura de milhares de ovos (DANTAS-TORRES, 2010). De fato, nosso estudo demonstrou que o índice gonadossomático de *R. sanguineus* sensu lato (s. l.) em condições normais é superior a 0,2; ou seja, mais de 20% da massa total do carrapato é composta por ovário. Após a postura, a eclosão das larvas ocorre em cerca de 32 dias sob condições laboratoriais. Inicia-se, então, um novo ciclo com a busca de milhares de larvas por um hospedeiro (TROUGHTON; LEVIN; 2007). Desse modo, é evidente que métodos de controle baseados na reprodução de fêmeas ingurgitadas atuam em uma etapa estratégica para diminuir a infestação por carrapatos (CAMARGO-MATHIAS, 2018).

O primeiro artigo desse trabalho demonstrou o efeito de concentrações subletais de acetilcarvacrol na morfologia dos ovócitos de fêmeas ingurgitadas de *R. microplus*. Além disso, um método de análise semiquantitativa baseado no grau de reversibilidade e extensão das alterações morfológicas foi proposto para padronizar e facilitar a comparação de diferentes drogas que afetam a morfologia dos ovários de carrapatos ixodídeos. Já no segundo artigo, a atividade acaricida de acetilcarvacrol foi descrita para *R. sanguineus* s. l.. Os efeitos desse composto no ovário de fêmeas ingurgitadas de *R. sanguineus* s. l. também foram avaliados por meio de técnicas histológicas e histoquímicas através da análise semiquantitativa proposta no primeiro artigo.

Sabe-se que diferentes espécies de carrapatos apresentam susceptibilidade distinta a substâncias acaricidas (ARAÚJO et al., 2016). Desse modo, faz-se necessária a investigação do potencial acaricida de determinado composto em diferentes espécies. De fato, nosso estudo evidenciou que o padrão de alterações morfológicas encontradas em ambas as espécies, embora semelhantes, não são idênticos. A vacuolização citoplasmática, por exemplo, mostrou-se bastante evidente em *R. microplus* expostos ao acetilcarvacrol, mas esteve menos intensa nos ovócitos de *R. sanguineus* s. l.. Além disso, uma concentração maior de acetilcarvacrol foi necessária para causar mortalidade em *R. sanguineus* s. l.. Não obstante, os carrapatos que sobreviveram a essas concentrações elevadas tiveram seus ovários severamente danificados. As alterações morfológicas no oviduto, devido a exposição ao acetilcarvacrol, provocaram a diminuição do número de ovócitos I e II no grupo TII (15 µL/mL) e culminaram na ausência dessas células no grupo TIII (20 µL/mL) foram certamente

as mais drásticas desse estudo. Esses dados permitem concluir que, possivelmente, acetilcarvacrol atua de forma distinta nas duas espécies. Mesmo assim, independentemente da variabilidade observada nas alterações morfológicas nos ovários, sugere-se que prejuízos à sobrevivência dos descendentes e um provável controle reprodutivo das espécies tenham sido resultados alcançados em ambos os casos.

A comparação dos efeitos de diferentes substâncias químicas capazes de prejudicar a reprodução de carrapatos ixodídeos foi facilitada com a tabela de análise semiquantitativa proposta no primeiro artigo. Todavia, é provável que aprimoramentos sejam feitos no futuro para tornar a comparação mais fidedigna. Dado a susceptibilidade distinta de diferentes espécies, a CL_{50} pode ser um parâmetro confiável para ser relacionado com os indexes obtidos na avaliação morfológica.

Por fim, cabe ressaltar o papel da química semissintética no aumento da estabilidade e atividade biológica de compostos químicos. A acetilação do carvacrol é uma reação simples, rápida e de relativo baixo custo (MORAES et al. 2013). Através dessa reação a atividade do carvacrol foi aumentada consideravelmente. Isso se deve, possivelmente, ao aumento da lipofilicidade do composto (SOLOMONS et al., 2016). Substâncias mais hidrofóbicas supostamente tendem a interagir mais fortemente com o tegumento dos carrapatos, dado a composição química desse órgão (REMEDIO et al., 2015). Desse modo, fica evidente o aumento da atividade biológica e estabilidade do acetilcarvacrol.

2. CONCLUSÃO GERAL

Concentrações subletais de acetilcarvacrol foram capazes de alterar a morfologia dos ovócitos dos carrapatos *R. sanguineus* s. l. e *R. microplus*. As alterações morfológicas comuns nas duas espécies foram vacuolização citoplasmática e nucleolar, diminuição de tamanho dos ovócitos, alteração do formato da célula e do córion, além de espessamento do córion. Essas alterações foram avaliadas e comparadas de acordo com a tabela de análise semiquantitativa proposta em nosso estudo. Alterações morfológicas nos ovários dos carrapatos podem reduzir a produção ovos ou gerar descendentes com dificuldades para realizarem processos bioquímicos essenciais durante suas vidas. Dessa forma, acetilcarvacrol pode ser considerado uma alternativa para a controle a logo prazo desses parasitos.

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