



Revista Brasileira de Parasitologia
Veterinária

ISSN: 0103-846X

zacariascbpv@fcav.unesp.br

Colégio Brasileiro de Parasitologia
Veterinária
Brasil

Dias de Sousa, Lorena Alessandra; Lopes Rocha, Thiago; Teixeira Sabóia-Morais,
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Revista Brasileira de Parasitologia Veterinária, vol. 22, núm. 3, julio-septiembre, 2013, pp.
339-345
Colégio Brasileiro de Parasitologia Veterinária
Jaboticabal, Brasil

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Ovary histology and quantification of hemolymph proteins of *Rhipicephalus (Boophilus) microplus* treated with *Melia azedarach*

Histologia do ovário e quantificação de proteínas na hemolinfa de *Rhipicephalus (Boophilus) microplus* tratado com *Melia azedarach*

Lorena Alessandra Dias de Sousa¹; Thiago Lopes Rocha²; Simone Maria Teixeira Sabóia-Morais²;
Lígia Miranda Ferreira Borges^{1*}

¹Centro de Parasitologia Veterinária, Instituto de Patologia Tropical e Saúde Pública, Escola de Veterinária e Zootecnia, Universidade Federal de Goiás – UFG, Goiânia, GO, Brasil

²Laboratório de Comportamento Celular, Departamento de Morfologia, Instituto de Ciências Biológicas – ICB, Universidade Federal de Goiás – UFG, Goiânia, GO, Brasil

Received January 11, 2013

Accepted July 4, 2013

Abstract

This study aimed to analyze ovary histology and quantify total protein in the hemolymph of *Rhipicephalus (Boophilus) microplus* females treated with hexane extracts from green fruits of *Melia azedarach*. Eight engorged females were immersed in the extract at 0.25% concentration, and eight in water containing 5% acetone (control). The females were dissected 72 hours after treatment, and the ovaries were weighed and subjected to standard histological techniques. The total protein concentration was measured in the hemolymph of 200 females, of which 100 were treated as described above and 100 served as a control. In the treated group, ovary weight reduction and predominance of immature oocytes were observed. In addition, there were decreases in the diameters of the cytoplasm and germ vesicle of the oocytes in the treated group, compared with the controls. The protein concentration in the hemolymph was higher in the treated group than in the controls. The morphological changes observed in the treated ovaries included: presence of vacuolization; alteration of oocyte morphology, which changed from rounded to elongated; deformation of the chorion; and disorganization of the yolk granules. These results demonstrate the action of *M. azedarach* fruit extracts on *R. (B.) microplus* oogenesis.

Keywords: *Rhipicephalus (Boophilus) microplus*, oocytes, ovary, histology, hemolymph proteins, *Melia azedarach*.

Resumo

Este estudo foi desenvolvido, visando analisar a histologia do ovário e quantificar as proteínas totais na hemolinfa de fêmeas de *Rhipicephalus (Boophilus) microplus* tratadas com extrato hexânico de frutos verdes de *Melia azedarach*. Oito fêmeas ingurgitadas foram tratadas por imersão com o extrato na concentração de 0,25%, e oito com água contendo 5% de acetona (controle). As fêmeas foram dissecadas 72 horas após o tratamento e os ovários foram pesados e submetidos a técnicas histológicas padrões. A concentração total de proteína foi mensurada na hemolinfa de 200 fêmeas, sendo 100 tratadas como descrito anteriormente e 100 como controle. Foi observada redução do peso dos ovários, predomínio de ovócitos imaturos e houve diminuição nos diâmetros do citoplasma e da vesícula germinal dos ovócitos do grupo tratado em comparação ao controle. A concentração de proteína na hemolinfa foi mais alta no grupo tratado que no controle. As alterações morfológicas observadas nos ovários tratados foram a presença de vacuolizações, alteração da morfologia dos ovócitos que mudaram de redondos para alongados, deformação do córion e desorganização dos grânulos de vitelo. Estes resultados demonstram a ação do extrato de *M. azedarach* na ovogênese de *R. (B.) microplus*.

Palavras-chave: *Rhipicephalus (Boophilus) microplus*, ovócitos, ovário, histologia, proteínas na hemolinfa, *Melia azedarach*.

*Corresponding author: Lígia Miranda Ferreira Borges
Centro de Parasitologia Veterinária, Instituto de Patologia Tropical e
Saúde Pública, Escola de Veterinária e Zootecnia, Universidade Federal de
Goiás – UFG, CP 131, CEP 74001-970, Goiânia, GO, Brasil
e-mail: borges.ligia@gmail.com

Introduction

Rhipicephalus (Boophilus) microplus is a major parasitic problem for cattle producers, because of the economic losses caused by blood spoliation, predisposition towards myiasis, damage to leather, retardation of calf development, decreased meat production, excessive expenditure on control measures and transmission of pathogens that cause diseases such as anaplasmosis and babesiosis (JONSSON, 2006).

The main method for controlling *R. (B.) microplus* is based on use of synthetic acaricides. However, frequent use of these compounds can lead to presence of toxic residues in food, biological imbalance, environmental contamination, poisoning of human beings and animals, and selection of resistant ticks (SONENSHINE, 1991).

The main motivation for searching for plant molecules as a possible tick control method arose from the perceived need to minimize the adverse effects of prolonged and intensive use of chemical acaricides (OLIVO et al., 2009). In this context, some plants such as *Melia azedarach* and *Azadirachta indica* (Meliaceae) stand out through contributing bioactive extracts for *R. (B.) microplus* control. Particularly with *M. azedarach*, Borges et al. (1994, 2003) and Sousa et al. (2008, 2011) observed high efficacy against tick reproduction, from fruit extracts. It is known that this plant has an effect on the neuroendocrine system of insects, which has been described by authors such as Schmidt et al. (1998) and Mordue and Nisbet (2000). However, there is no information on its interference with the physiology of oogenesis in ticks.

The ovary of *R. (B.) microplus* is classified as panoistic because it does not contain follicular and nourishing cells. It consists of a single tubular structure with oocytes of various sizes and at different developmental stages, attached to the epithelial wall through a pedicel. The latter structure is responsible for synthesizing and providing substances for oocyte development (SAITO et al., 2005). In ticks, vitellogenin synthesis occurs mainly outside of the ovaries: it is released into the hemolymph for subsequent ovary uptake (DIEHL; DOTSON, 1986).

Oogenesis in *R. (B.) microplus* is divided into six stages from I to VI that are defined in terms of cytoplasm appearance and presence of the germ vesicle, yolk granules and chorion (SAITO et al., 2005).

Given the above, this study aimed to analyze the histology of the ovary and quantify the total protein in the hemolymph of *R. (B.) microplus* females treated with hexane extracts from green fruits of *M. azedarach*.

Materials and Methods

Tick collection

Engorged females of *R. (B.) microplus* were obtained from naturally infested Holstein cattle that had been left without acaricide treatment for at least 30 days. After collection, the ticks were washed with water, dried and selected based on their external morphological condition. They were weighed individually in order to standardize the individuals examined at between 200 and 235 mg.

Preparation of extracts of *Melia azedarach* and immersion test

Unripe fruits of *M. azedarach* were collected in Goiânia (16° 34' 24" S and 49° 17' 32" W), because these have been found to be bioactive against the reproductive efficiency of *R. (B.) microplus* (BORGES et al., 2003; SOUSA et al., 2008). Dry and powdered ripe fruits were extracted by means of percolation in a Soxhlet apparatus using hexane as the solvent. The solvent was then evaporated using a rotary evaporator (Rotavapor®).

Eight females were weighed and immersed for 5 min in 10 mL of solution containing the extract at 0.25% concentration diluted in water plus 5% acetone. A control group with the same number of females was immersed in distilled water plus 5% acetone. They were then placed in a Petri dish and conditioned for 72 hours under controlled conditions (T = 27 °C; RH > 80%).

Histology and morphometry of the ovary

Before extraction of the ovaries, the females were anesthetized in a freezer at -5° C for 5 min. Dissection and extraction of the ovaries was performed under a stereomicroscope (Leica®), using a saline solution (NaCl 7.5 g/L, Na₂HPO₄ 2.38 g/L and KH₂PO₄ 2.72 g/L). The ovaries were weighed for subsequent calculation of the gonadosomatic index (= ovarian weight / weight of females) and were deposited individually into Eppendorf tubes with buffered paraformaldehyde solution for 24 h (pH 7.4; 4% formaldehyde). After this period, the solution was discarded and 70% alcohol was added and the material was stored at 4 °C.

Subsequently, the ovaries were dehydrated in increasing ethanol concentrations (70% to 100%); embedded in Histo-resin® (Leica, USA) in accordance with the manufacturer's specifications; and subjected to microtomy to obtain sections with 2.0 µm thickness, using an ultra-microtome (Leica Ultracut UCT, USA). The sections were stained with toluidine blue (TB) 1% (w/v) at pH 8.4 (VETEC, Brazil). Each ovary was cut at a distance of 0.5 cm from the proximal portion of the oviduct.

The histopathological analyses were performed on micrographs of the ovaries, which were obtained under a photonic microscope (Leica DMLB, USA) coupled to the image capture system (Samsung SHC 410 NAD digital color camera), using objective lenses of 10, 20 and 40X with a 100 µm scale. Oocytes I to V of each selected field were first ranked in accordance with the phases described by Saito et al. (2005) and then counted. Observations on the presence of toxic effects due to actions by the *M. azedarach* extract on the oocytes were also based on characterization of the stages of oogenesis carried out by the latter author.

The morphometric study on the stages of oogenesis was conducted using the Image Pro-Plus 6.0 software (Media Cybernetics, Silver Spring, USA). The following parameters were evaluated: largest and smallest diameters of cytoplasm in stages I to V of oogenesis; and germ vesicle corresponding to the largest and smallest diameter of oocytes I and II, since this structure is more evident in those stages of oogenesis (SAITO et al., 2005). To this end, three slides were prepared with 10 sections from each

ovary. From these, one section per slide was selected and three images of each stage of oogenesis were obtained.

Quantification of protein in tick hemolymph

One hundred engorged females were treated with the extract and one hundred with distilled water plus acetone (control group), and were conditioned for 72 h, as described earlier. The dorsal surface of each female was perforated using a glass microtube (outer diameter = 1.0 mm, internal diameter = 0.78 mm and tip diameter = 10-20 μ m), prepared in a puller (PC-10, Narishige, Japan). The tick body was subjected to gentle pressure to allow the hemolymph to extravasate. The hemolymph was then aspirated to the same microtube using a 1 mL disposable syringe connected to the tube. Hemolymph samples contaminated with blood due to leakage of intestinal contents were discarded.

The pooled hemolymph samples were placed into tubes containing 30 μ L of a protease inhibitor cocktail (Sigma-Aldrich Inhibit[®]) and 82 μ L of saline buffer (1.5 M NaCl and 50 mM EDTA) plus phenylthiourea and were kept on ice throughout sample collection. After collection, the tubes were centrifuged at 5040 \times g for 10 minutes at 4 °C. To quantify total protein in hemolymph, the modified Lowry method was followed (MARKWELL et al., 1978), using bovine serum albumin (Merck[®]) as a standard.

Statistical analysis

Statistical analyses were performed using the Statistica 7.0 software (STATSOFT, 2005), by means of the one-way ANOVA test followed by Student *t* test comparison. Results were considered significant when $p < 0.05$.

Results

The ovaries of the treated females were of lower weight than those in the control group, which resulted in a significantly lower gonadosomatic index score (0.076 ± 0.015) than in the

control group (0.148 ± 0.024). The highest total oocyte count was observed in the control group. From the individual scores, immature oocytes predominated and there was less vitellogenesis in the treated group, compared with the controls (Table 1). There was a decrease in cytoplasm diameter in all the oocytes evaluated in the treated group. The diameters of the germ vesicle were also lower in oocyte I in the treated group but there was no change in oocyte II (Table 2).

The morphological characteristics for oocytes from the control group (Figure 1) were as follows:

- Oocyte I – rounded with homogeneous cytoplasm and no yolk granules, and the germ vesicle has prominent nucleolus. At this stage, the germ vesicle occupies a large area of the cytoplasm;
- Oocyte II – higher cellular volume compared with oocyte I. At this stage, the oocyte does not have evident granules in the cytoplasm and the germ vesicle and nucleolus are still prominent;
- Oocyte III – cytoplasm is rich in coarse and evenly distributed granulation and the germ vesicle has shifted to the periphery. At this stage, it is possible to identify the chorion surrounding the oocyte, which has a sky-blue color;

Table 1. Mean \pm standard deviation of oocyte numbers in each stage (OI to OV) and total counts in the ovaries of *Rhipicephalus (Boophilus) microplus* females treated with a hexane extract of unripe fruits of *Melia azedarach* at 0.25% concentration. The control group was treated with water + acetone.

Oocytes	Control	Treated
OI	13.54 \pm 4.26 ^a	18.8 \pm 3.58 ^b
OII	12.67 \pm 3.37 ^a	22.83 \pm 3.63 ^b
OIII	15.75 \pm 3.15 ^a	20.79 \pm 3.52 ^b
OIV	31.25 \pm 7.9 ^b	15.00 \pm 2.94 ^a
OV	26.71 \pm 5.82 ^b	12.33 \pm 2.82 ^a
Total	99.92 \pm 10.81 ^b	89.54 \pm 7.94 ^a

Means followed by different lowercase letter in the lines differ significantly according to Student *t* test ($p < 0.05$).

Table 2. Mean (μ m) \pm standard deviation of largest (LCD) and smallest (SCD) cytoplasm diameters, and largest (LGVD) and smallest (SGVD) germ vesicle diameters of *Rhipicephalus (Boophilus) microplus* oocytes treated with a hexane extract of unripe fruits of *Melia azedarach* at 0.25% concentration (T) and control group (C).

		LCD	SCD	LGVD	SGVD
OI	C	58.70 \pm 10.76 ^b	37.88 \pm 13.48 ^b	28.57 \pm 7.50 ^b	15.63 \pm 7.9 ^b
	T	39.76 \pm 19.80 ^a	28.00 \pm 14.27 ^a	17.33 \pm 10.39 ^a	12.24 \pm 7.67 ^a
OII	C	74.03 \pm 9.96 ^b	45.91 \pm 12.79 ^a	14.13 \pm 16.20 ^a	6.79 \pm 5.12 ^a
	T	63.38 \pm 11.20 ^a	43.95 \pm 13.39 ^a	13.53 \pm 9.03 ^a	8.55 \pm 5.94 ^a
OIII	C	91.34 \pm 13.10 ^b	62.01 \pm 17.93 ^b	-	-
	T	79.49 \pm 11.55 ^a	57.31 \pm 11.88 ^a	-	-
OIV	C	120.84 \pm 24.74 ^b	89.86 \pm 28.21 ^a	-	-
	T	91.13 \pm 10.27 ^a	65.38 \pm 14.61 ^a	-	-
OV	C	168.66 \pm 28.81 ^b	130.95 \pm 34.63 ^a	-	-
	T	110.27 \pm 15.29 ^a	87.94 \pm 16.81 ^a	-	-

Means followed by different lowercase letters in columns differ significantly according to Student *t* test ($p < 0.05$), in comparisons between oocytes at the same stage of development (OI to OV).

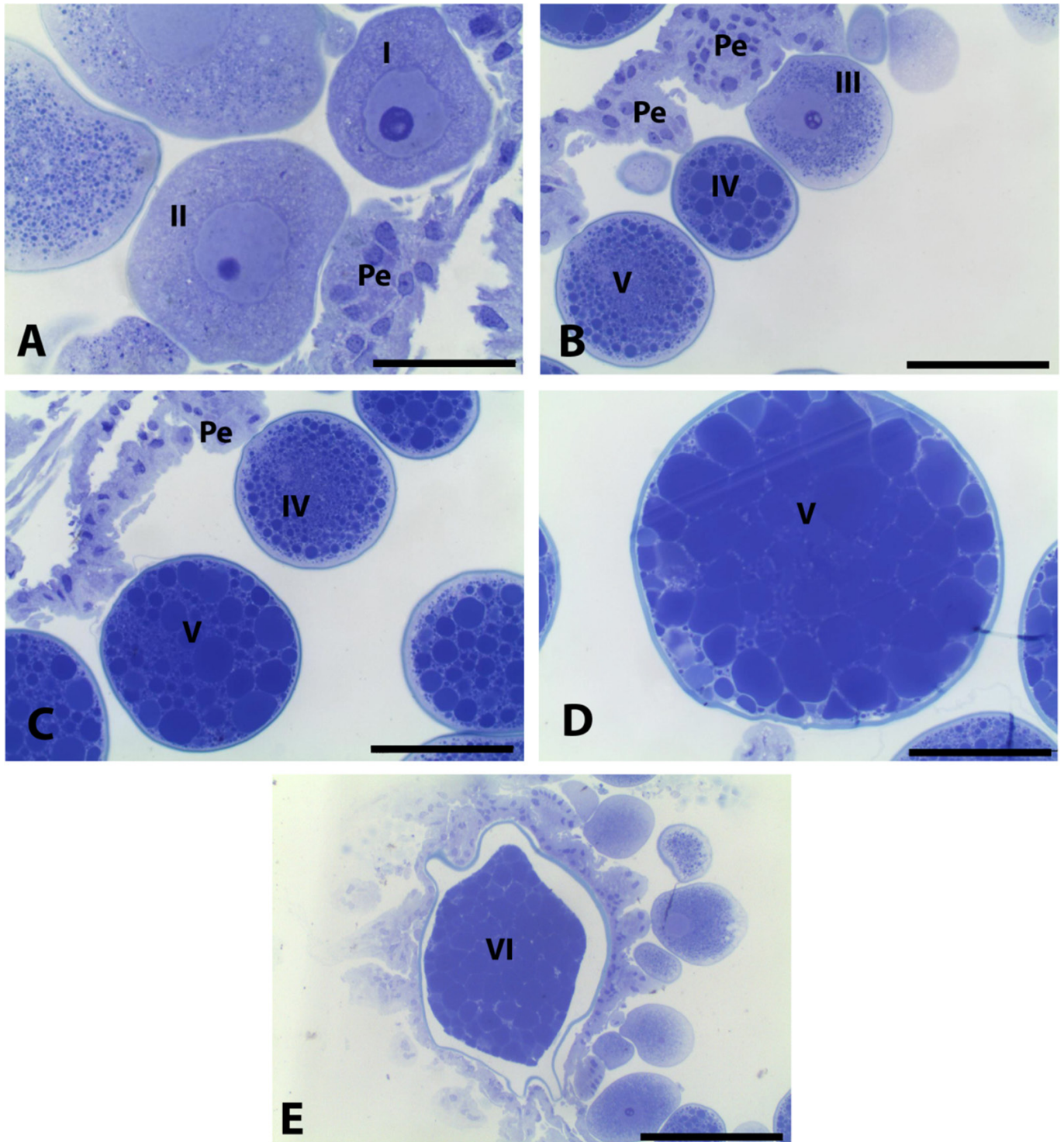


Figure 1. Histological sections through ovaries of engorged females of *Rhipicephalus (Boophilus) microplus* stained with toluidine blue, from the control group. (A) I - oocyte I, II - oocyte II, Pe - pedicel; (B) III - oocyte III, IV - oocyte IV, V - oocyte V, Pe - pedicel; (C) IV - oocyte IV, V - oocyte V, Pe - pedicel; (D) V - oocyte V; (E) VI - oocyte VI. Bar = 100 μ m.

- Oocyte IV - cytoplasm with large and small yolk granules. In this phase, the germ vesicle and nucleolus are peripheral and the chorion is evident;
- Oocyte V - cytoplasm is completely filled with large yolk granules and the chorion is thicker;

- Oocyte VI - cytoplasm is disorganized and the chorion has folds, making it irregular.

In ovaries from the group treated with *M. azedarach*, large vacuolated regions were seen to be present in pedicels, and the oocytes were seen to show alteration of morphology, which changed

from rounded to elongated, with deformation of the chorion and disorganization of yolk granules (Figure 2).

The engorged females treated with *M. azedarach* extract had higher protein concentrations in the hemolymph (130.57 $\mu\text{g}/\mu\text{L}$) than shown by females in the control group (112.7 $\mu\text{g}/\mu\text{L}$).

Discussion

The results from this study demonstrate the effect of crude hexane extracts from the fruits of *M. azedarach* on *R. (B.) microplus* oogenesis. These results corroborate investigations that have already been carried out using *M. azedarach* extracts, which described interference from the plant compounds on the reproductive parameters of females of *R. (B.) microplus*, with inhibition of egg conversion and larva hatching (BORGES et al., 2003; SOUSA et al., 2008).

The main changes observed here were decreased numbers of mature oocytes, increased numbers of immature oocytes, decreased cytoplasm and germ vesicle diameters and cellular deformities. The observed predominance of immature oocytes

at the expense of vitellogenic oocytes revealed that blockage of the development process of oogenesis in *R. (B.) microplus* had occurred. Morphological alterations such as vacuoles and cellular disorganization indicated cell intoxication caused by the extract of *M. azedarach*. Vacuolization is an attempt by the cell to isolate the toxic substances or even the cytoplasm that was damaged by the action of the compound, so that the cell can still perform its metabolic processes. In addition to toxic substances and debris, many vacuoles can also contain entire organelles that are unable to perform their metabolic functions (ARNOSTI et al., 2011).

The toxic effect observed in oocytes and nearby cell structures have also been observed in other studies using plants. Denardi et al. (2011) and Arnosti et al. (2011) evaluated the activity of neem (*A. indica*) and ricinoleic acid on *R. sanguineus*, and observed that these had inhibitory effects on oocytes, thereby preventing them from reaching advanced stages of development. In addition, there was morphological damage such as vacuolization, cellular disorganization and deformation. Likewise, these findings are similar to those found by Friesen and Kaufman (2003, 2004) in an evaluation on the inhibitory effect of cypermethrin against 20-hydroxyecdysone. This

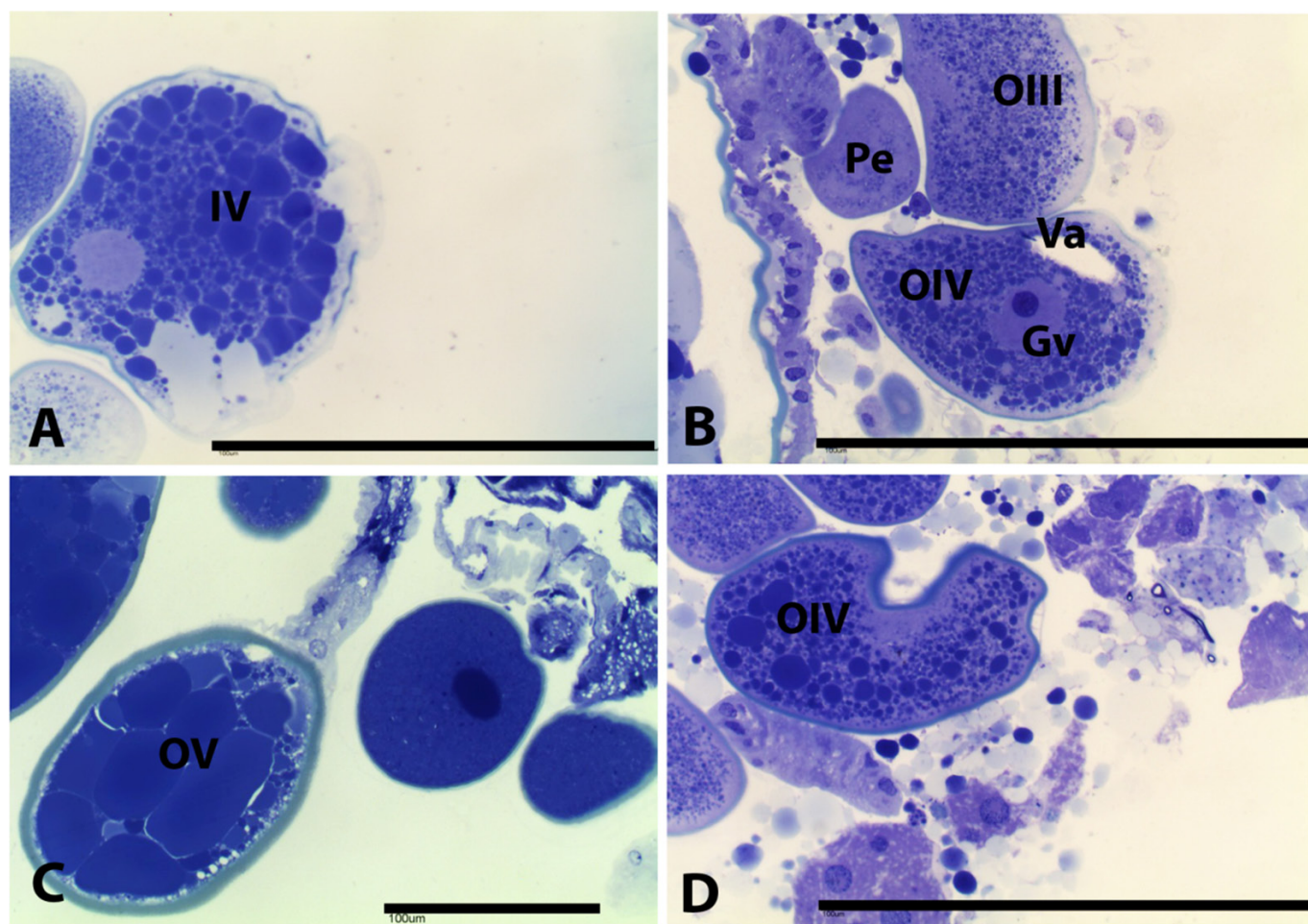


Figure 2. Histological sections of ovaries of *Rhipicephalus (Boophilus) microplus* stained with toluidine blue, from the group treated with hexane extract of green fruits of *Melia azedarach* at 0.25% concentration. (A) Chorion deformation, IV – oocyte IV; (B) oocyte vacuolization, OIII – oocyte III, OIV – oocyte IV, Gv – germ vesicle, Pe – pedicel, Va – vacuole; (C) OV – oocyte V; (D) Cellular deformation, OIV – oocyte IV. Bar = 100 μm .

acaricide caused suppression of vitellogenesis and egg development in *Amblyomma hebraeum*.

The effects attributed to *M. azedarach* are largely ascribed to the action of limonoids, especially azadirachtin, salannin, melianthol and nimbin (YAMASAKI et al., 1988). These compounds can inhibit the molting process through interfering with the neuroendocrine system, thereby altering the hemolymph ecdysteroid levels (SCHMIDT et al., 1998). In *Drosophila melanogaster*, *Aedes aegypti* and *Manduca sexta*, azadirachtin, salannin, nimbin and 6-desacetyl nimbin were found to inhibit the activity of ecdysone in a dose-dependent manner (MITCHELL et al., 1997).

In the present study, higher protein concentration was observed in the hemolymph of engorged females treated with hexane extract of *M. azedarach* than in the controls. Vitellogenin is the most abundant protein in tick eggs and represents approximately 11% of the total protein in hemolymph (SONENSHINE, 1991). Its concentration in hemolymph increases with engorgement, and is regulated by its uptake in oocytes (SEIXAS et al., 2010). Since the treated females predominantly presented immature oocytes and had fewer vitellogenic oocytes, it can be assumed that the accumulation of protein in the hemolymph occurred because vitellogenin was not taken up by oocytes.

Reproduction and embryogenesis in ticks are regulated by ecdysteroid hormones (CABRERA et al., 2009). 20-hydroxyecdysone is the major ecdysteroid hormone, accompanied by its precursor, ecdysone (DELBECQUE et al., 1978; DEES et al., 1984). However, the specific process that triggers vitellogenin uptake has not been established. The most favored hypothesis is that a "vitellogenin capture factor" may exist (FRIESEN; KAUFMAN, 2004; SEIXAS et al., 2010). Thus, it can be suggested that this accumulation occurred through a direct toxic effect from the plant on oocytes, thereby making them unable to capture this protein, probably through interference with this capture factor.

Acknowledgements

We are grateful to Fundação de Apoio à Pesquisa do Estado de Goiás (FAPEG) for the financial support.

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