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Effect of ricinoleic acid esters from castor oil (*Ricinus communis*) on the oocyte yolk components of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae)

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ABSTRACT

Rhipicephalus sanguineus are bloodsucking ectoparasites, whose main host is the domestic dog, thus being present in urban areas and closely located to people. Eventually, this tick species parasitize humans and can become a potential vector of infectious diseases. Methods to control this type of pest have been the focus of many research groups worldwide. The use of natural products is increasingly considered nowadays, due to the low toxicity levels to the host and low waste generation to the environment. This study tested the effect of ricinoleic acid esters from castor oil (as an potential acaricide) on the reproductive system of *R. sanguineus* females, more specifically on the vitellogenesis process. For this, two groups were established: the control group (CG) and the treatment group (TG) with five rabbits in each (New Zealand White), used as hosts. NaCl and ester were added to rabbits' food and offered to the hosts. After full engorgement, the females were collected and had their ovaries extracted. The ticks ovaries were submitted to histochemical techniques so the effects of esters could be observed over polysaccharides, proteins and lipids yolk. Changes in the deposition of yolk components were observed. This caused modifications on elements of polysaccharide origin and on glycoprotein compounds, interfering in the final yolk synthesis and compromising the development of the future embryo.

1. Introduction

Ticks of the Ixodidae family are bloodsucking ectoparasite arthropods of wide geographical distribution with high potential for disease transmission to animals and humans (Sonenshine, 1991; Walker, 1994). In addition to being one of the main problems faced by kennel owners, *Rhipicephalus sanguineus* can be common in the domestic and peridomestic environment of people living with the main urban host of this ectoparasite: the domestic dog (Paz et al., 2008).

Currently, synthetic acaricides are the main way to control Ixodidae. However, the emergence of individuals resistant to these products has been reported. The induced selection of *R. sanguineus* individuals resistant to arsenic, organophosphate, carbamate and organochlorine acaricides has been reported in several countries since the 1970s (Nolan, 1985). In Brazil, the first studies on the chemical control of *R. sanguineus*, as well as the first report on the selection of ticks resistant to acaricides and insecticides, emerged only in the mid-1990s (Fernandes et al., 2000).

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In this sense, biological control using entomopathogenic fungi (Garcia et al., 2005) and natural compounds (Denardi et al., 2010; Arnosti et al., 2011a, 2011b) has been intensified in order to minimize the selection of resistant individuals. Furthermore, these alternatives aim to control the tick with a low impact on the environment and non-target organisms.

Leonardo et al. (2001) and Mandelbaum et al. (2003) published the first studies on ricinoleic acid esters from castor oil and their antimicrobial activity. Later, Messetti et al. (2010) investigated the practical applications of these esters as biocides in the control of *Leuconostoc mesenteroides*, a bacteria of great importance in the sugar and alcohol industries.

The results of morphological studies developed by researchers at the BCSTM (Brazilian Central of Studies on Ticks Morphology) have recently revealed the use of ricinoleic acid esters from castor oil as a promising alternative to control *R. sanguineus*. These compounds act by modifying the morphophysiology of ovaries and salivary glands of ticks, preventing two important processes feeding and reproduction (Arnosti et al., 2011a, 2011b).

Thus, the aim of this study was to characterize the way of action of ricinoleic acid esters from castor oil (*Ricinus communis*) on the vitellogenesis of *R. sanguineus* ectoparasites. Histochemical techniques were applied to show alterations caused in the deposition of vitellogenic elements (lipids, proteins and carbohydrates) in this ticks oocytes.

2. Materials and methods

2.1. Bioassays

To carry out this study, two groups were established: the control group (CG) and the treatment group (TG). Five rabbits (New Zealand White), never having been infested with ticks, were used as hosts in each group. NaCl and ester concentrations used were based on the rabbits' live weight and were not intended to kill the ticks, thus allowing the observation of histochemical results.

Control group (CG): The animals in this group were fed with a commercial diet for rabbits with added NaCl at a concentration of 5 g NaCl/kg of food. Each host was infested with 25 pairs (adults) of *R. sanguineus*.
Treatment group (TG): The animals in this group were fed with a commercial diet for rabbits with added esters from castor oil stabilized in NaCl at a concentration of 5 g of esters/kg of food. Each host was infested with 25 pairs of *R. sanguineus*.

The food was offered to hosts on the day of infestation, which was performed according to methodology proposed by Bechara et al. (1995).

Procedures performed in this study were approved by the ethics committee from UNESP *Comitê de Ética no Uso de Animal* (*CEUA*) Protocol 006/2009.

2.2. Histochemistry

After complete engorgement of *R. sanguineus* females and their voluntary detachment from the host, they were anesthetized by thermal shock in refrigerator, dissected and had their ovaries removed. The organs were fixed according to the techniques applied, and then dehydrated in increasing ethanol concentrations (70, 80, 90 and 95%) for 15 min each. After dehydration, the material was soaked in historesin (Leica) for 24 h, embedded and 3 μm sections were made, which were collected on glass slides for subsequent staining.

2.3. Detection of total lipids by the Baker's staining technique

The ovaries were fixed in calcium–formaldehyde for 2 h. After collection on glass slides, they remained for 18 h in calcium dichromate, being subsequently washed in distilled water. The slides remained for 5 h in a hematein solution. Shortly afterwards, a second and last wash in distilled water was carried out. Once dried, the slides were mounted with glycerin and coated with a coverslip. The photo-documentation was performed with a Motic BA 300 photomicroscope on the day the technique was completed to prevent discoloration (Baker, 1946).

2.4. Detection of proteins by the bromophenol blue staining (according to Pearse, 1985)

According to Pearse (1985), the material was fixed in 4% paraformaldehyde for 2 h and the glass slides containing the histological sections were stained with bromophenol blue for 2 h, at room temperature. After this period, they were washed with 0.5% acetic acid for 5 min and with running water for 15 min. Then, they were quickly immersed in tertiary butyl alcohol and left to dry at room temperature for subsequent mounting in Canada balsam. Afterwards, the histological sections were photographed with a Motic BA 300 photomicroscope.

2.5. Simultaneous staining of acid and neutral polysaccharides by the Alcian blue/PAS (periodic acid Schiff) reaction

Initially, the material was fixed in aqueous Bouin's solution for 12 h and the histological sections were rehydrated for 1 min in distilled water. The material was then stained with 1% Alcian blue in 3% acetic acid for 30 min and washed in distilled water. The sections were transferred to 1% periodic acid solution for 5 min and washed for 10 min in distilled water. After 15 min in Schiff reagent, the material was washed again for 7 min in running water. After drying, the slides were mounted in Canada balsam to be later observed and photographed with a Motic BA 300 photomicroscope (McManus, 1946; Junqueira and Junqueira, 1983).

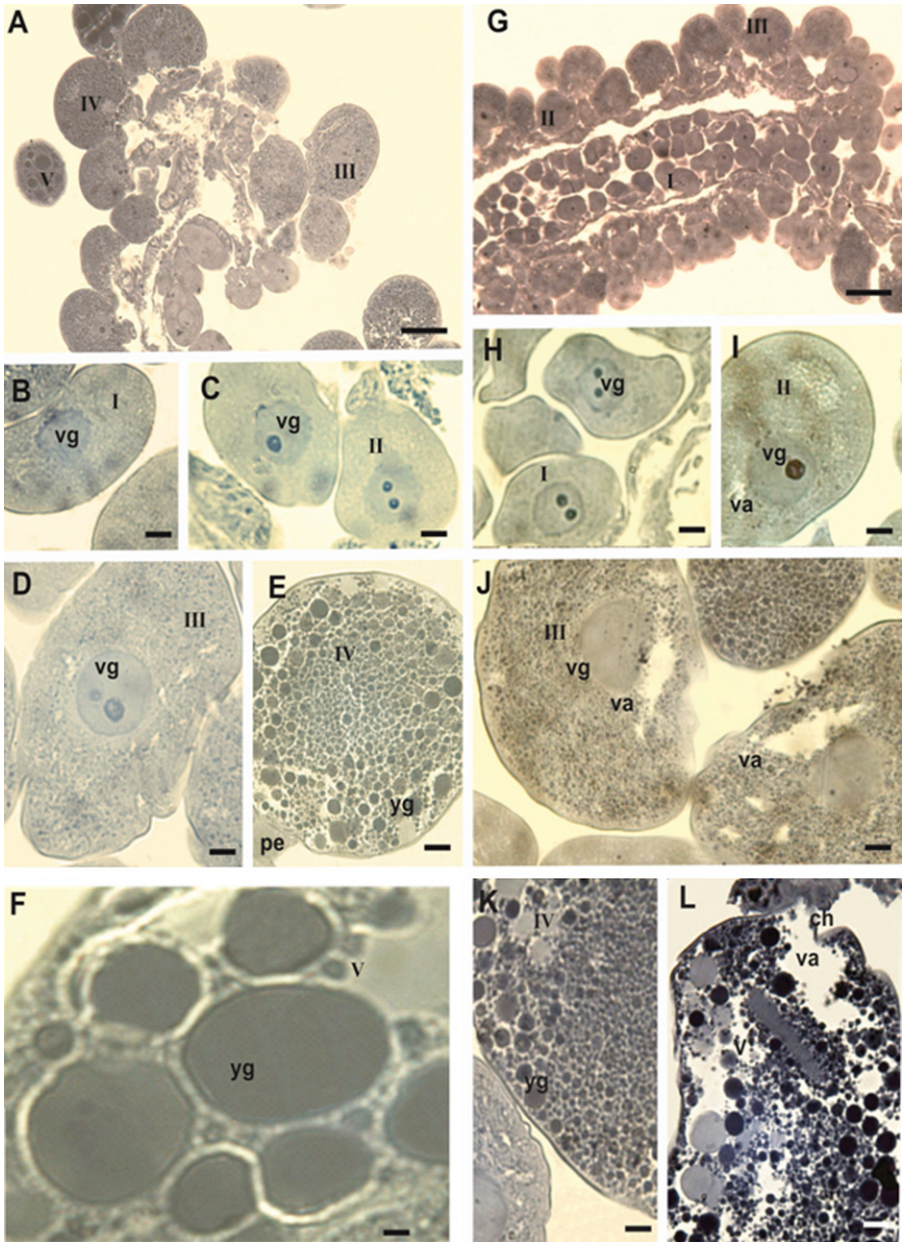

Fig. 1. Photomicrograph of ovaries sections of engorged *R. sanguineus* females from **CG** (**A–F**) and **TG** (**G–L**) individuals stained with PAS/Alcian blue to identify total polysaccharides. (**A** and **B**) Overview of the ovary with oocytes at different development stages and detail of oocyte II of **CG** individuals, (**C–F**). Detail of oocytes at stages III, IV and V of development of **CG** individuals; (**G–I**) Overview of the ovary of **TG** individuals with many immature and mature oocytes already showing cytoplasm with vacuoles, with detail of oocyte II weakly stained by the technique used, (**J–L**) Detail of oocytes III, IV and V of **TG** individuals showing changed morphology and compromised vitellogenesis. **Ch** = chorion; **Pc** = pedicel cell; **Va** = vacuole, **Gv** = germinal vesicle; **Yg** = yolk granules.

3. Results

3.1. Detection of lipids

The histological sections of the ovaries show a larger amount of oocytes in more advanced development stages in CG individuals (Fig. 1A) when compared to TG individuals (Fig. 1G). However, a stronger positive staining for lipids in oocytes I from the TG (Fig. 1H) than those from the CG is observed (Fig. 1B).

In oocytes II from CG individuals (Fig. 1C), the cytoplasm shows little positive lipid staining, while TG individuals show moderately positive cytoplasmic staining. The beginning of negative cytoplasm vacuolation in oocytes II from TG individuals can be observed (Fig. 1I).

In oocytes III from TG individuals, positive staining for lipids is intense (Fig. 1D). In the CG, the oocytes are negative to this test. The cytoplasm from TG oocytes has large areas of cytoplasmic vacuolation negative to this test (Fig. 1J).

Oocytes IV from both groups exhibit granules stained for lipids. In CG individuals, positive lipid granules are homogeneously distributed throughout the cytoplasm (Fig. 1E) and in TG individuals, the central regions of the cytoplasm are the prevalent location (Fig. 1K).

In oocytes V from CG individuals, the lipid yolk is homogeneously distributed (Fig. 1F) and strongly positive to the technique applied (Fig. 1L). Large vacuoles negative to the test and chorion disruption are shown in oocytes V from TG individuals (Fig. 1L).

3.2. Detection of proteins

In histological sections showing ovaries from CG individuals, there is a prevalence of oocytes in more advanced development stages, richer in protein granules when compared to the TG (Fig. 2A and G).

Oocytes I from CG individuals have cytoplasm and germinal vesicle negative or weakly positive to the test applied, while oocytes from TG individuals have weakly positive fine granules, as well as small vacuoles negative to the test, irregularly distributed throughout the cytoplasm (Fig. 2B and H).

In oocytes II from CG individuals, the protein granules are small and some are strongly marked and homogeneously distributed throughout the cytoplasm (Fig. 2C). In the TG, the small granules are weakly positive and are concentrated in the central region of the oocyte (Fig. 2I).

In oocytes III, from both the CG (Fig. 2D) and the TG (Fig. 2J), there are small granules, strongly positive and homogeneously distributed throughout the cytoplasm; however, in the TG, there are vacuolated regions in the cytoplasm, which have no protein content. In the case of CG individuals (Fig. 2D and E), protein granules have a greater size than those observed in TG individuals (Fig. 2J). The germinal vesicle stains more strongly in the TG (Fig. 2J), where the nucleolus is more compact.

Oocytes IV exhibit strongly positive granules in both groups, whereas in the CG, the largest granules occur preferentially at the periphery of oocytes (Fig. 2E) and in the TG, the cytoplasm of oocytes shows smaller granules (Fig. 2K). In the TG, the cytoplasm of oocytes IV are permeated by large vacuolation and the germinal vesicle can still be observed despite being weakly positive to the test (Fig. 2K).

Oocytes V from CG and TG individuals have large vitellin protein granules strongly positive and homogeneously distributed throughout the cytoplasm (Fig. 2F and L). However, TG individuals clearly show the presence of extensive vacuolation between protein granules (Fig. 2L).

3.3. Detection of polysaccharides

The histochemical test for the detection of polysaccharides in the ovaries of *R. sanguineus* exposed to ricinoleic acid esters from castor oil showed significant differences when compared to results obtained from the CG. In addition to inhibiting development of oocytes attached along the ovary wall, there is a reduced staining for polysaccharides in the treated ovaries shown in Fig. 3F (TG). This does not occur in the CG (Fig. 3A), where even oocytes in the early development stages show strong PAS staining.

When oocytes II are observed in detail, it is clear that TG individuals show strong and intense positive PAS staining, which is observed in the CG and not observed in the TG (Fig. 3B and G).

In oocytes III and IV from CG individuals, there is a progression of positive PAS staining from stages III to IV, with positive granules of various sizes taking almost the entire cytoplasm in stage IV (Fig. 3C and D). In addition to oocyte deformation, smaller-size positive granules sparsely distributed throughout the cytoplasm are observed in oocytes III of treated individuals (Fig. 3H). Unlike the CG and according to the same oocytes III pattern, oocytes IV from this group have smaller size and are more scattered, showing the presence of many cytoplasmic vacuoles in the middle of the vitelline granulation (Fig. 3I).

Oocytes V from CG individuals have strong PAS positive staining throughout the cytoplasm (as well as pedicel cells) and yolk granules have large dimensions (Fig. 3E).

In treated oocytes, the grain size decreases and they are permeated by large areas of vacuolated cytoplasm. Unlike what was observed in CG individuals, pedicel cells are negative to the PAS test (Fig. 3J).

The summary of histological results is shown in Table 1.

4. Discussion

The present study provides further information on the action of ricinoleic acid esters from castor oil on oocyte and vitellogenesis of *R. sanguineus* ticks, showing the effects on the synthesis and deposition of lipid, protein and polysaccharide elements. In many animal species, the accumulation of these elements in the oocyte during the vitellogenesis occurs for further use during embryonic development (Camargo-Mathias and Fontanetti, 1998). In arthropods in general, these elements are deposited in the oocyte in the form of yolk granules, in a deposition sequence where lipids are the first, followed by proteins and polysaccharides (Ramamurty, 1968).

Specifically in ticks, previous works have reported that the oocyte yolk was formed only by lipids and proteins (Balashov, 1983). However, more recent studies demonstrated the presence of other elements, such as polysaccharides (Ricardo et al., 2007), which is also confirmed in this study.

The search for acaricides having lower environmental impact and less damage to non-target organisms has been intensified in the last decade. Thus, the use of ricinoleic acid esters from castor oil has proven to be a potentially interesting. Arnosti et al. (2011a, 2011b) have already demonstrated the action of these substances on the reproductive process of *R. sanguineus*. The esters acted on oocytes in the early development stages (I and II), which showed smaller size due to the impaired synthesis and incorporation of vitelline elements, making these cells unviable due to the action of the toxic product.

The quality of the oocyte growth in arthropods is measured by the amount of proteins, lipids and carbohydrates

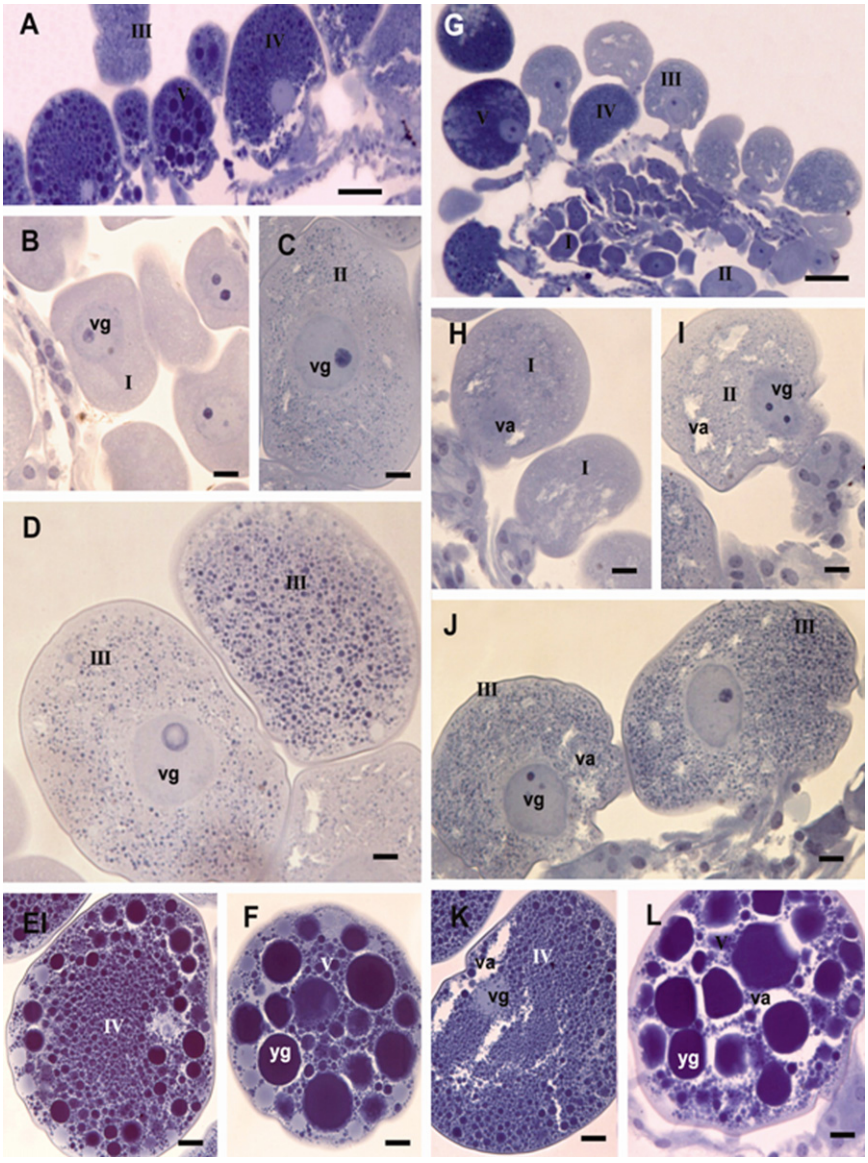

Fig. 2. Photomicrograph of ovaries sections of engorged *R. sanguineus* females from **CG** (**A–F**) and **TG** (**G–L**) individuals stained with bromophenol blue to identify proteins. (**A–C**) Overview of the ovary with oocytes I and II of **CG** individuals, (**D–F**) Details of oocytes III, IV and V of **CG** individuals; (**G–I**) Overview of the ovary of **TG** individuals showing vacuolation at more mature stages, with oocytes I and II weakly stained and with the presence of vacuoles, (**J–L**) Details of oocytes III, IV and V moderately and strongly stained, but with lower grain size and presence of large vacuoles of **TG**. **Pc** = pedicel cell; **Va** = vacuole, **Gv** = germinal vesicle; **Yg** = yolk granules.

Table 1
Summary of results from the application of histochemical tests for the detection of lipids, proteins and polysaccharides in oocytes of *Rhipicephalus sanguineus* in the control group (CG) and treatment group (TG) with esters from castor oil.

Histochemical tests	Oocytes I		Oocytes II		Oocytes III		Oocytes IV		Oocytes V	
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
Backer (lipids)	+	++	–	++	–	++	+++	+++	+++	+++
Bromophenol Blue (proteins)	–	+	+	+	++	++	+++	+++	+++	+++
PAS/Alcian Blue (polysaccharides)	+	–	++	–	++	+	+++	++	+++	+++

(–) negative; (+) weakly positive; (++) medium positive; (+++) strongly positive.

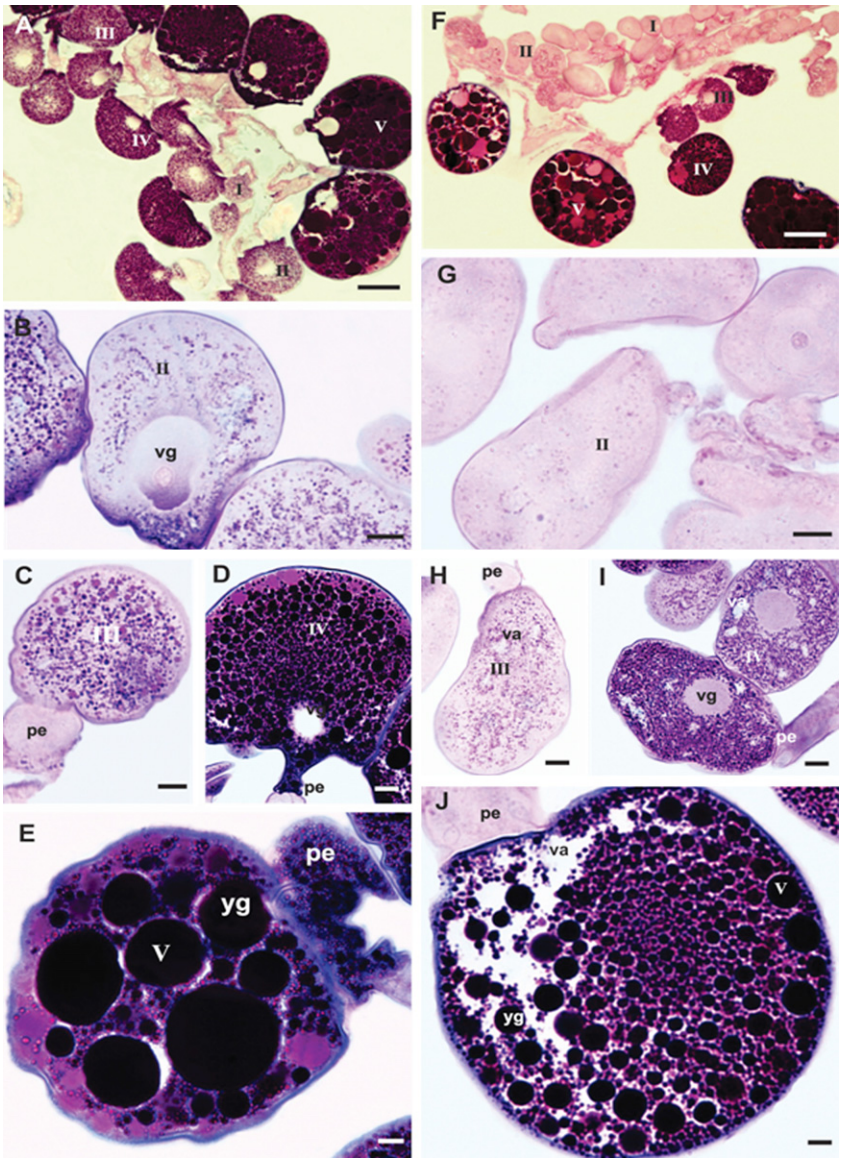

Fig. 3. Photomicrograph of ovaries sections of engorged *R. sanguineus* females from **CG** (**A–E**) and **TG** (**F–J**) individuals stained with the Baker technique (1946) to identify total lipids. (**A–C**) Overview of the ovary with oocytes at different development stages and detail of oocytes I and II of **CG** individuals; (**D–E**) Detail of oocytes III, IV and V of **CG** individuals; (**F–H**) Overview of the ovary of **TG** individuals with several immature and mature oocytes, already showing cytoplasm with vacuoles, with the detail of oocytes I and II weakly stained by the technique used; (**I–J**) Detail of oocytes III, IV and V of **TG** individuals showing changed morphology and compromised vitellogenesis, moderately and strongly stained, but with smaller vitelline granules and cytoplasm with vacuoles. **Ch** = chorion; **Pc** = pedicel cell; **Va** = vacuole, **Gv** = germinal vesicle; **Yg** = yolk granules.

incorporated during the formation of yolk granules. Despite controversies about the way of acquisition (endogenous or exogenous) of lipid components deposited inside the cytoplasm, their presence is related to important functions, such as a nutritional reserve for the future embryo and the structuring of the oocyte chorion (Camargo-Mathias and Fontanetti, 1998).

In the present study, lipid components were more evident in oocytes from all stages in TG individuals when compared to CG individuals it seems that, as with the protein components, there is an indirect effect of the action of esters on the synthesis of lipids. Ticks could be using lipids of oocytes as the main source of energy to compensate for the reduction or absence of carbohydrates that had their synthesis affected by the ester. This explains the increased presence of lipids in the cytoplasm of TG oocytes demonstrated by the strong staining through the technique applied.

In addition to the oocyte participation in the synthesis of yolk components (endogenous), there is also the participation of other cells and structures in the vitellogenesis of ticks. According to Oliveira et al. (2007), pedicel cells also play an important role in the vitellogenesis of ticks, synthesizing and transferring different substances into the

oocyte. The present study found the occurrence of extensive vacuolated areas often located in the oocyte region that makes direct contact with the pedicel cell, suggesting that the toxic agent circulating in the hemolymph could reach the oocyte via pedicel cells.

Similar results were obtained by Roma et al. (2011) for ticks exposed to permethrin and by Denardi et al. (2010) when studying the effect of aqueous extract of neem leaves on the vitellogenesis of ticks. Thus, the part of the oocyte in direct contact with the pedicel cells would be the first region to receive the toxic agent and the first to suffer from its action.

According to Oliveira et al. (2006), the protein components of the yolk are only deposited in the form of granules in *R. sanguineus* oocytes in the most advanced development stages (VI and V). However, it could be observed that in oocytes I, II III, there is positive staining for proteins ranging from weakly to moderately positive, with the exception of oocytes I from CG individuals, which are negative to the technique used. It is known that the main protein present in the yolk of oocytes is vitellin, which originates from the conversion of vitellogenin into these cells, being initially synthesized in the intestine and later in the fat body of these animals.

This conversion of vitellogenin into vitellin probably occurs due to enzyme action, as well as other processes that require more energy. Therefore, similarly to the proteins that compose the yolk, these enzymes would also be positively stained for the technique used.

Positive staining for proteins in oocytes I from TG individuals suggests a more intense participation of these compounds in the physiology of oocytes, unlike what was observed in CG individuals, in an attempt to neutralize the toxic component arising from esters and preserve the cell that originates a future individual.

According to Oliveira et al. (2007), the collection and synthesis of protein components during vitellogenesis are carried out by endogenous and exogenous processes. Oocytes IV from TG individuals show smaller protein granules irregularly distributed in the periphery of the oocyte, while in CG individuals, granules are larger and more spherical, suggesting the interference of esters in the mechanism of absorption and deposition of protein yolk components. Oocytes V from TG individuals showed vacuolated areas that permeate large protein granules. Oocytes V from CG individuals had smaller protein granules and lipid droplets, which shows an attempt to isolate the toxic compound from the yolk granules already deposited.

Vitellin, the main yolk protein, is a glycolipoprotein molecule. Individuals treated with esters from castor oil showed smaller protein granules in oocytes V when compared to the CG, demonstrating the action of esters on biomolecules probably hydrolyzing and causing glycoproteins fragmentation (vitellin).

Data reported by Arnosti et al. (2011b) is corroborated by this study, which demonstrated that *R. sanguineus* females treated with esters would have yolk synthesis and/or incorporation inhibited. In the case of polysaccharide components in oocytes at stage II from TG individuals, this inhibition was clear. According to Ricardo et al. (2007), the absorption or production of carbohydrates started in oocytes II, having pedicel cells and hemolymph as exogenous sources. In the present study, the results observed for oocytes II from TG individuals indicated that ricinoleic acid esters from castor oil acted on the hydrolysis of polysaccharides, which led to a delay in the synthesis and/or incorporation of carbohydrates observed in TG individuals.

In contrast, for oocytes at stage IV of development, it was observed that TG individuals showed higher positive carbohydrate staining than CG individuals. In ticks, oocytes at stages IV and V of development were at the end of vitellogenesis, which is when the deposition of carbohydrates is performed on a larger scale (Ricardo et al., 2007). The data suggest that the greater positivity for carbohydrates in oocytes IV of individuals exposed to esters is due to an increase in the production of these elements, since the presence of esters leads to a greater lysis of polysaccharide granules, which must be compensated with a higher production.

Studies conducted with esters from castor oil, Messetti et al. (2010) demonstrated the biocide action of these compounds on *Leuconostoc mesenteroides*, acting in the hydrolysis of polysaccharides present on the cell wall. In this sense, it was possible to confirm the action of esters on carbohydrates incorporated into oocytes during the vitellogenesis of ticks, either those intended for the yolk granules for the use of embryo, or polysaccharides for the formation of the chorion or sugars that compose the complex glycoprotein molecules present in the oocytes of *R. sanguineus*.

5. Conclusion

Esters acted on the vitellogenesis of *R. sanguineus*, with increased vacuolation in the oocyte, including those at the final stage (V), when the oocyte seems to be in total cytoplasmic disarrangement, ending up with the chorium disruption.

These new data open up a new range of possibilities for further studies on the embryonic development of eggs from individuals treated with esters, since the changes observed in vitellogenesis can act on the development of the new individual. Thus, ricinoleic acid esters from castor oil become a product with high potential for environmental control of *R. sanguineus*.

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