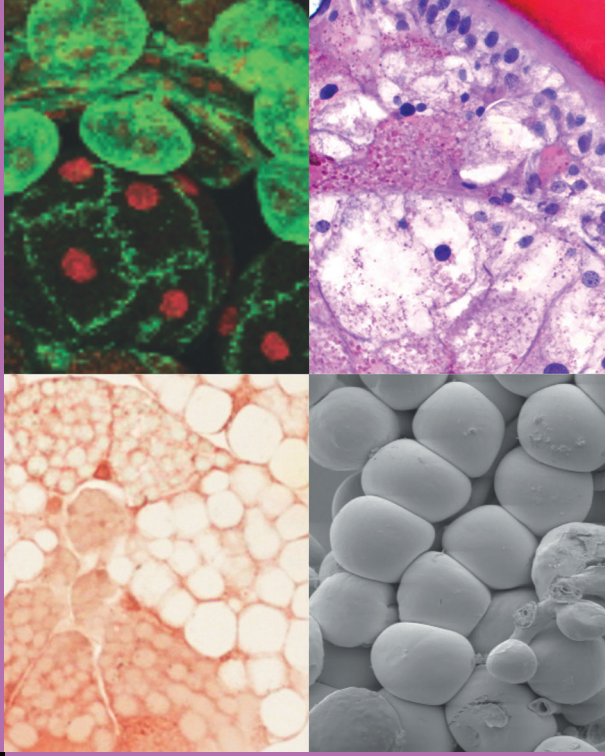

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Maria Izabel Camargo-Mathias (Ed.)

# Inside ticks

## Morphophysiology, toxicology and therapeutic perspectives

# Inside ticks: morphophysiology, toxicology and therapeutic perspectives

Maria Izabel Camargo-Mathias (ed.)

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Maria Izabel Camargo-Mathias
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## Morphophysiology, toxicology and therapeutic perspectives

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# Summary

# Preface

Ticks are blood-sucking ectoparasites, which have long been known to cause itching due their bites, and for their ability to transmit pathogens responsible for diseases that can cause physical harm to pets, livestock, wild animals, and humans, sometimes with considerable economic losses, notably in the cattle industry. The medical and veterinary importance of ticks is currently increasing in the wake of global environmental and climatic changes, which are promoting increases in some tick populations and expansion of their geographic distribution. As a consequence, contact with humans and the incidence of tick-borne diseases are on the rise.

The control of tick infestations has traditionally been carried out using chemical acaricides. However, in addition to the high costs and risks of environmental contamination associated with these chemicals, their indiscriminate use has also contributed to the selection of resistant tick populations. Therefore, there is an unquestioned need not only for the rational use of this class of acaricides, but also for the development of alternative and effective environmentally safe methods of tick control, including the use of plant extracts, biological control agents, and anti-tick vaccines.

A sound knowledge of tick biology, anatomy, and morphology are essential for an understanding of the physiology, adaptations, and evolutionary success of these organisms. Moreover, such knowledge is indispensable for the establishment of strategies for their control and management of tick-borne diseases. Tick organs represent important targets for the evaluation of a wide range of experimental conditions, including the response to pathogen infection and their distribution, the toxicological effect of substances with potential acaricidal activities and their dose-response relationship, the effects of anti-tick protein antibodies and gene silencing, the interactions between parasitoids and ticks for application in biological control, and an infinity of other issues.

Histology, although considered a basic science, has fundamental importance for understanding tissue structure and composition in relation to their specialized functions in different circumstances. Histological and histochemical techniques are rapid, powerful, and valuable tools that provide abundant visual and descriptive information, and in the past decade have contributed significantly to all fields of tick research.

In this context, the present book was conceived and written by recognized expert morphologists belonging to the Brazilian Central of Studies on Ticks Morphology (BCSTM) –Unesp, Rio Claro (SP) Brazil. The objective of this publication is to clarify tick morphohistology, through a compilation of studies conducted by these researchers, illustrating how histological techniques have been applied in tick studies.

This book was designed in an electronic format (eBook) and comprises 13 chapters that cover the following themes: histological and histochemical techniques; an overview of the biology, morphology, and taxonomy of the main tick species studied histologically; tick organ

histology, histological techniques applied to analyze acaricidal effects on ticks' organs; and new perspectives on tick salivary glands for selection of compounds with pharmacological properties. These chapters are complemented by illustrations of high quality that were carefully chosen with a view toward greatly enhancing the reader's comprehension.

Finally, we hope that this work will make a substantial contribution to the field of acarology, and that it will motivate and encourage further research in the areas described.

*Gustavo Seron Sanches*
*Maria Izabel Camargo-Mathias*

# 1
# Microscopic techniques applied in tick research

*Luis Adriano Anholeto, Patrícia Rosa de Oliveira, José Ribamar Lima de Souza, Rusleyd Maria Magalhães de Abreu, Elen Fernanda Nodari, Melissa Carolina Pereira, Izabela Bragião Calligaris, Maria Izabel Camargo-Mathias*

Histology is a useful tool that helps in the study of tissues, showing how cells and intercellular matrix interact in the same environment. The small size of the cells and components of the extracellular matrix, makes histology dependent on the use of microscopes that can be of several types. In the specific case of tissue evaluation, the use of the bright field microscope is quite common, however, in order to reach this stage, it is necessary to perform several procedures which include from the adequate fixation of the material to obtaining the histological sections.

Among the steps that involve the final obtaining of a histological slide are:

- *Collection of biological material*: which consists of removing fragments of the tissue to be analyzed

and obtained from a living organism (biopsy or surgery).

- *Fixation*: fixation is the process of placing the material in a fixative (chemical substances, heat, freezing, etc.) so that the material maintains its morphological and physiological characteristics as similar as possible to its original composition, thus avoiding the occurrence of autolysis, an effect of the action of enzymes in the cells themselves and that with the change of pH, after removal the tissue of live organism, can undergo serious modifications.
- *Dehydration*: is necessary to remove all accumulated water from the tissue so that the fixing substances can penetrate inside the material. In addition, the formulas which are used to include the tissues in the blocks to be sectioned usually carry in their composition paraffin or even plastic resins, which are hydrophobic and therefore do not homogenize with water. For the dehydration, a battery of increasing concentrations (70, 80, 90, 95 and 100%) of ethyl alcohol or acetone is used.
- *Inclusion*: includes the placement of fragments of tissues already fixed and dehydrated in substances that when polymerized will allow the section of the material to proceed. To do so, the fragments already previously infiltrated in paraffin or resins are placed in appropriate molds, filled with paraffin or resin, and transported in an incubator for polymerization (hardening).
- *Microtomy*: the next step is to obtain very thin sections (3 to 7 μm) of the material to be analyzed. The equipment used to make these sections is the microtome, to which a knife that can be made of steel, glass, tungsten, etc. is adapted.

- *Staining*: for the sectioned material to be observed under a bright field microscope, the cells and the extracellular matrix must be evidenced by specific dyes, since without the tissue exhibit a transparency which unable its visualization. Besides allowing to visualize the structures of the tissue, the dyes also show the presence of their chemical constituents (proteins, lipids and carbohydrates). Among the dyes most frequently used in histology are hematoxylin and eosin (HE), which the first staining the nucleus in violet and the second staining the cytoplams in pink. For the detection of specific tissue elements such as lipids, proteins, carbohydrates, special histochemical techniques and/or reactions are employed.

The following are summarized the histological and histochemical techniques most used in laboratory routines:

## Harris hematoxylin and aqueous eosin stain (Junqueira; Junqueira, 1983)

*Material*

Paraformaldehyde 4% (fixative)
PBS (NaCl 7.5 g/L, $Na_2HPO_4$ 2.38 g/L and $KH_2PO_4$ 2.72 g/L)
Ethanol series 70%, 80%, 90% and 95%
Historesin kit/Paraffin
Harris hematoxylin
Aqueous Eosin

*Methods*

1. The material should be fixed in paraformaldehyde 4% for 48 hours, and transferred to phosphate

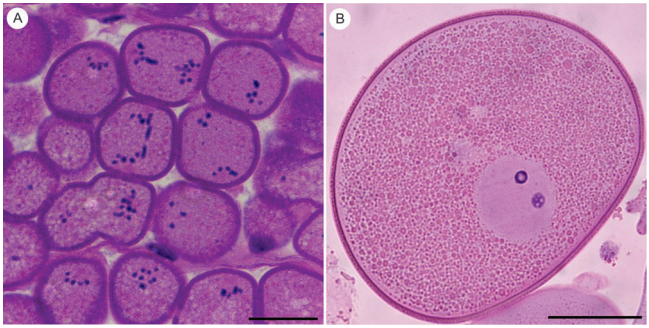
buffered saline (NaCl 7.5 g/L, $Na_2HPO_4$ 2.38 g/L and $KH_2PO_4$ 2.72 g/L), remaining for 24 hours.

2. The samples should be dehydrated in graded ethanol series 70%, 80%, 90% and 95% (30 minutes each bath).
3. Embedded in historesin containing an infiltration solution with 50 mL of (2-hydroxyethil) methacrylate and 0.5g of dibenzoylperoxide, according to the manufacturer's orientation for 24 hours.
4. Included in plastic molds containing historesin and polymerizer.
5. The blocks should be sectioned in microtome at 3 μm thickness, mounted on glass slides rehydrated in distilled water for 1 minute and stained with Harris hematoxylin for 8 minutes.
6. The material should be washed in running tap water for 3 minutes, stained with aqueous eosin for 5 minutes and washed in running tap water again. After drying at room temperature, the samples were rapidly immersed in xylol, covered with Canada balsam and a coverslip.

*Results*

At bright field microscopy, the basophilic components (nucleous for example) in general will present staining in purple and the acidophilic components (proteins for example) in general will present staining in pink (Figure 1A-B).

Figure 1 – Testes of *Amblyomma cajennense* complex stained with HE (A). Oocyte of *A. cajennense* stained with HE (B)




Bars: (A) 20 µm (B) 50 µm

## Bromophenol blue (total protein) (Pearse, 1985)

*Material*

Paraformaldehyde 4% (fixative)
Phosphate buffered saline (PBS)
Ethanol series 70%, 80%, 90% and 95%
Historesin kit/Paraffin
Bromophenol blue
Acetic acid 0.5%

*Methods*

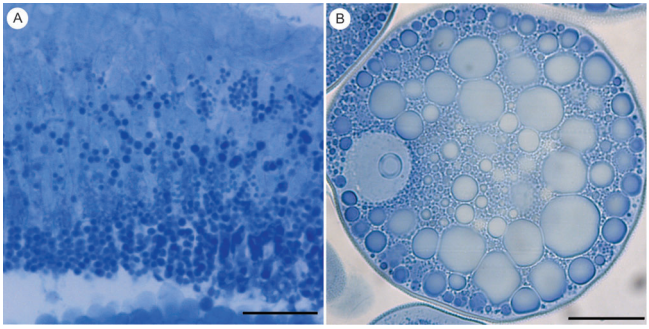
1. Fixation in paraformaldehyde 4% for 48 hours.
2. Transfer to PBS, where they stay for 24 hours.
3. The material should be dehydrated, embedded in historesin containing an infiltration solution and included in plastic molds containing historesin and polymerizer.
4. The blocks sectioned and mounted on glass slides.

5. The slides immersed in Bromophenol blue for 2 hours at room temperature.
6. The material should be washed in acetic acid 0.5% for 5 minutes and in running tap water for 15 minutes.
7. After drying at room temperature, the samples should be rapidly immersed in xylol, and covered with Canada balsam and a coverslip.

*Results*

At bright field microscopy, the protein contents will be staining in blue (Figure 2A-B).

Figure 2 – Accessory glands of testis of *A. cajennense* stained with bromophenol blue (A). Oocyte of *A. cajennense* stained with bromophenol blue (B)




Bars: (A-B) 20 µm

## PAS (Periodic Acid-Schiff) for neutral polysaccharides (Junqueira; Junqueira, 1983)

*Material*

Aqueous Bouin (fixative)

PBS (NaCl 7.5 g/L, $Na_2HPO_4$ 2.38 g/L and $KH_2PO_4$ 2.72 g/L)

Ethanol series 70%, 80%, 90% and 95%

Historesin kit/Paraffin

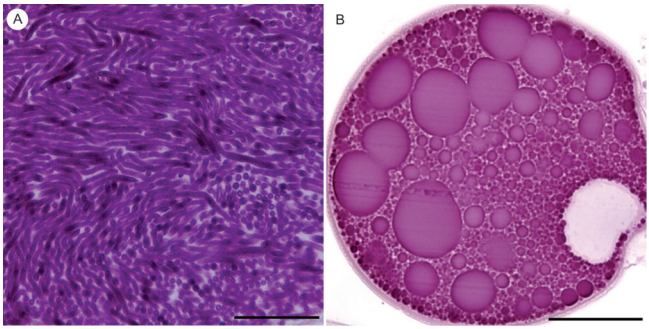
*Methods*

1. Fixation in aqueous Bouin for 5 days.
2. Transfer to PBS, where they stay for 24 hours.
3. The material should be dehydrated, embedded in historesin containing an infiltration solution and included in plastic molds containing historesin and polymerizer, the blocks sectioned and mounted on glass slides.
4. The slides should be rehydrated for 1 minute in distilled water and transferred to periodic acid solution 4% for 10 minutes.
5. Washed in distilled water for 1 minute, and immersed in Schiff reagent for 1 hour.

*Results*

PAS staining is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans). At bright field microscopy, these structures will be staining in purple-magenta color (Figure 3A-B).

Figure 3 – Seminal vesicle of *A. cajennense* stained with PAS (A) Oocyte of *A. cajennense* stained with PAS (B)




Bars: (A) 50 μm; (B) 200 μm

## Imidazole-osmium (Pereira et al., 2016)

This technique has been applied for the detection of lipids in histological preparations. The affinity of the imidazole – osmium with lipid components present in biological membranes allows a precise individualization of the granules when it reacts with the cell boundaries, through bright field light microscopy. This element is detected in the histological sections showing staining ranging from light brown to dark brown (and sometimes black) depending on the observed cell or other regions.

*Material*

Glutaraldehyde 2.5%, neutral buffered formalin 10% or formal calcium (fixative)

Phosphate buffered saline (PBS) 0.1M (pH 7.2)

Imidazole buffer 0.2M (pH 7.5) (stock solution)

Osmium tetroxide at 4% (stock solution)

*Methods*

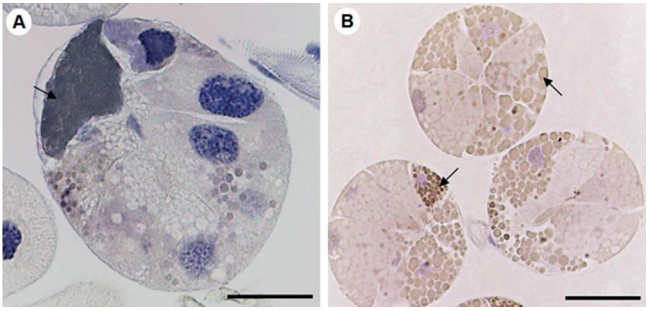
1. Fixation of the material in glutaraldehyde 2.5%, neutral buffered formalin 10% or formal calcium for 2 hours.
2. After 2 hours, wash the material twice with PBS 0.1M (pH 7.2) for 15 minutes each.
3. In a test tube, add 5 mL of osmium tetroxide at 4% and 5 mL of the imidazole buffer 0.2M (pH 7.5) to obtain the ideal concentration of imidazole-osmium solution for the technique (osmium tetroxide at 2% in imidazole buffer 0.1M).
4. In the same test tube, incubate the material in the imidazole-osmium solution for 30 minutes, in the dark, at a room temperature 25ºC.
5. After incubation, wash the material once with imidazole buffer 0.1M (pH 7.5) and twice with PBS 0.1M (pH 7.2), for 15 minutes each. The post-fixation time (including the incubation period and washing in buffer and PBS) can be longer than 15 minutes, depending on the size and thickness of the material.
6. Proceed with routine histological processing steps (dehydration, embedding, inclusion and sectioning). The histological sections obtained after sectioning can be counterstained with Harris' hematoxylin or methyl green to detect the nuclei of cells.

*Results*

After the post-fixation time (incubation in the imidazole-osmium solution), the material will present a denser and darker aspect.

At bright field microscopy, the lipid components in granules and biological membranes in general will present staining ranging from light brown to black (Figure 4 A- B).

Figure 4 – Acini type II (A) and III (B) from salivary glands of *Rhipicephalus sanguineus* sensu lato stained with imidazole-osmium technique to detect the lipid content (*arrow*) present in their cytoplasmic granules. The staining intensity can range from light brown to black depending on the chemical composition of the observed regions




Bars: (A) 50 µm; (B) 100 µm

## Acid phosphatase (Hussein et al., 1990)

This technique aims to detect the activity of acid phosphatase (Figure 5A-B), being used as a localization of lysosomes (to be part of the secretion of this organelle), detection of this free enzyme (as a constituent of secretions, for example glandular secretion) or to confirm the process of autophagic cell death.

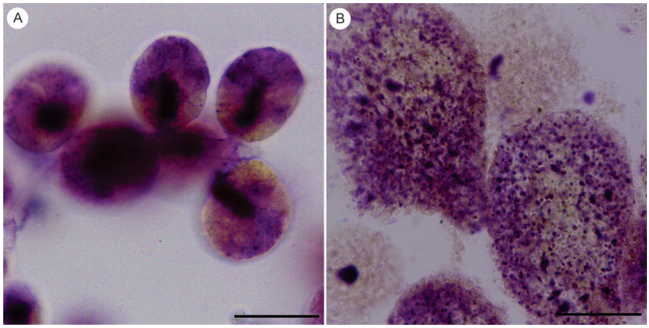
*Material*

10% buffered neutral formalin (fixative)
Sodium acetate buffer (0.05M, pH 4.8)
Naphthol AS-TR phosphate
DMSO (dimethyl sulfoxide)
10% $MnCl_2$-$4H_2O$
Violet red salt

*Methods*

1. The material should be fixed in 10% buffered neutral formalin for one hour and thirty minutes at 4ºC.
2. Subsequently, they should be washed in sodium acetate buffer (0.05M, pH 4.8) and incubated for 45 minutes at 37ºC in the following medium: naphthol AS-TR phosphate, DMSO (dimethyl sulfoxide), sodium acetate buffer (0.05M, pH 4.8), 10% $MnCl_2 \cdot 4H_2O$ and violet red salt.
3. For the preparation of the incubation medium, 3 mg of the naphthol AS-TR phosphate substrate should be dissolved in two drops of DMSO and then 10 mL of sodium acetate buffer are added.
4. Then 0.2 mL of 10% manganese chloride, 6 mg of the violet red salt should be added and, finally, the final solution will be vigorously stirred.
5. For negative control of the technique, one sample should be incubated without substrate (3 mg naphtol AS-TR phosphate).

Figure 5 – Salivary glands of *R. sanguineus* s. l. stained with acid phosphatase (A). Midgut of *R. sanguineus* s. l. stained with acid phosphatase (B)




Bars: (A) 50 µm; (B) 20 µm

## Ponceau Xylidine (Vidal; Mello, 1987)

This technique has been applied to evidence the total proteins in histological preparations.

*Material*

Paraformaldehyde at 4% or neutral buffered formalin 10% (Fixation)

Phosphate buffered saline (PBS) 0.1M (pH 7.2).

acetic acid 1%

Ponceau Xylidine

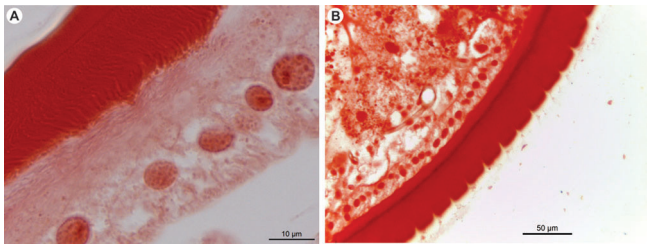
*Methods*

1. The material should be fixed in paraformaldehyde 4% or in neutral buffered formalin 10% for 72 hours.
2. Subsequently, they should be stained with Ponceau Xylidine for 30 min and washed in tap running water.
3. The material should be transferred to acetic acid 1% for 1 min, dried and mounted in Canada balsam.

*Results*

The detection of total proteins by orange staining (Figure 6A-B).

Figure 6 – Integument of *R. sanguineus* s. l. (A-B) stained with Ponceau Xylidine




Bars: (A) 10 µm; (B) 50 µm

## Toluidine blue (Mello and Vidal,1980)

This technique has been used to evidence DNA and RNA in histological preparations.

*Material*

Paraformaldehyde at 4% or neutral buffered formalin 10% (Fixation)

Phosphate buffered saline (PBS) 0.1M (pH 7.2)

Toluidine blue.

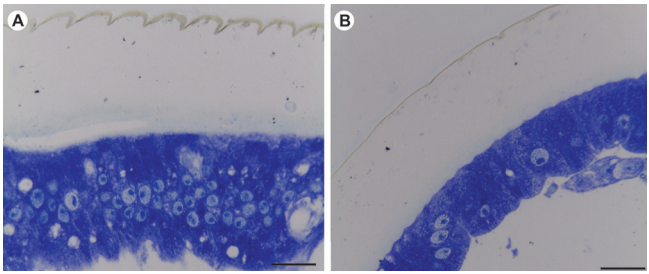
*Methods*

1. The material should be fixed in paraformaldehyde 4% or in neutral buffered formalin 10% for 72 hours.
2. Subsequently, they should be immersed in a Toluidine blue solution for 3 min at room temperature.
3. The material should be rinsed with distilled water and mounted in Canada balsam.

*Results*

The detection of DNA and RNA by blue staining (Figure 7A-B).

Figure 7 – Integument of *R. sanguineus* s. l. (A-B) stained with toluidine blue




Bars: (A-B) 50 mm

# 2
# Ticks on the magnifying glass

*Marina Rodrigues de Abreu, Raphael Vacchi Travaglini, Patrícia Rosa de Oliveira, Luis Adriano Anholeto, Renata da Silva Matos, Gustavo Seron Sanches, Maria Izabel Camargo-Mathias*

## Taxonomy and systematic

Ticks are obligatory ectoparasites which belong to the Phylum Arthropoda, Class Arachnida, Subclass Acari, Order Parasitiformes, Suborder Ixodida (Barker; Murrell, 2004). There are currently an estimated 899 species of ticks divided into the families Nutalliellidae, Argasidae and Ixodidae, the latter comprising the largest number of species, with great medical, veterinary and economic importance (Anderson; Magnarelli, 2008; Dantas-Torres, 2008; Nava et al., 2015).

The member of the family Ixodidae, constituted of 702 species (Guglielmone et al., 2014), have the mouthparts inserted in the anterior scutum of the body, as well as a chitinous scutum covering almost the entire dorsal part

in the males and almost half or one third in the females, allowing the enlargement of the body during engorgement. Ticks, therefore, present gender dimorphism in adult stage (Sonenshine; Roe, 2014). The family Argasidae comprises 193 species (Sonenshine; Roe, 2014). The main characteristic of these ticks is the absence of a dorsal scutum. Their mouthparts are located on the underside of the body and they ingest less blood in comparison with the ones belonging to the family Ixodidae (Walker, 1994).

The third family, Nutalliellidae, is constituted of a single species called *Nuttalliella namaqua,* recently reported in South Africa (Mans et al., 2012). It is considered the most basal lineage of ticks, and differs from the other families mainly for the heavily corrugated integument and the form of fenestrated plates (Roshdy et al., 1983).

Ticks in general represent a great public health concern, once they cause considerable livestock losses (*Rhipicephalus (Boophilus) microplus*), and are vectors of zoonosis that affect domestic/wild animals and the human beings as well (*Rhipicephalus sanguineus* s. l., brown dog tick, *Amblyomma sculptum,* and *Amblyomma triste)*.

## *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae)

Popularly knowed as the cattle tick, this species is endemic to tropical and subtropical areas of the world (Estrada-Peña et al., 2006). Despite having cattle as preferential hosts, they can be occasionally found parasitizing buffalo, horses, goats, sheep, dogs and wild animals (Franque et al., 2007). This species has great veterinary significance by transmitting pathogens that cause babesiosis (*Babesia bovis* and *B. bigemina*) and anaplasmosis (*Anaplasma marginale*), affecting consequently cattle

health and productivity (Giglioti et al., 2016; Mekonnen et al., 2002). Therefore, *R. (B.) microplus* is responsible for causing noteworthy economic losses in livestock industries (Grisi et al., 2014).

## *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae)

The species *R. sanguineus* sensu stricto (s. s.) was first described by Latreille in 1806. The original morphological description presented is not sufficient to allow an accurate identification of this species. Moreover, there is no type specimen available for comparisons, making its identification even more difficult (Walker et al., 2005). It is currently a group of uncertain morphological definition, once recent molecular studies demonstrated that *R. sanguineus* sensu lato (s. l.) actually consists of a complex of species (Moraes-Filho et al., 2011; Nava et al., 2015). The species remains object of discussion among taxonomists (Dantas-Torres; Otranto, 2015).

*R. sanguineus* s. l. ticks have the dog as preferential host and are vectors of pathogens, such as bacteria, protozoa and nematodes, which are responsible for transmitting several diseases to domestic animals and the human beings as well (Dantas-Torres, 2010).

## *Amblyomma cajennense* sensu lato (Acari: Ixodidae)

The *Amblyomma cajennense* complex, also called *A. cajennense* sensu lato is composed of two species in Brazil, *A. cajennense sensu stricto* (s. s.) and *A. sculptum* (Martins et al., 2016). While *A. cajennense sensu stricto* (s. s.) is

restricted to Amazonian region, *A. sculptum* can be found in coastal and central-western states (Martins et al., 2016; Nava et al., 2014). Thus, many specimens that had been classified as *A. cajennense* s. s. corresponds, in fact, to *A. sculptum*.

*Amblyomma sculptum* is popularly known as star ticks, are mainly equine parasites, and are occasionally found on other hosts including cattle, deer, domestic animals, birds, reptiles and the human being (Oliveira et al., 2003).

This species is the main transmitter of the bacteria *Rickettsia rickettsii*, the causative agent of spotted fever, a disease that seriously affects animal health in several regions of Brazil (Szabo et al., 2013; Labruna et al., 2007).

## *Amblyomma triste* (Koch, 1844) (Acari: Ixodidae)

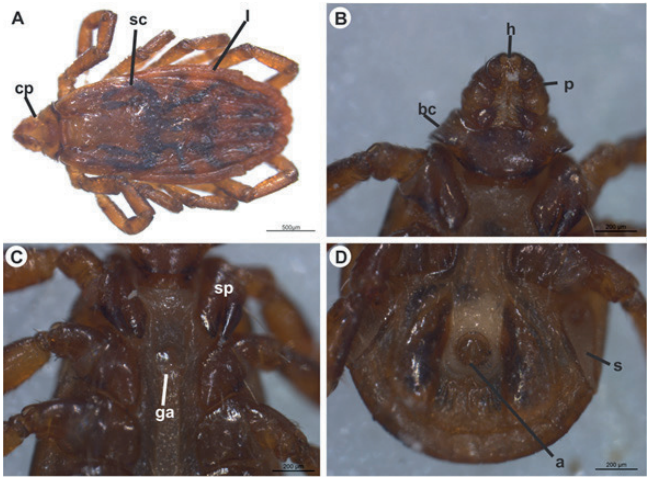
The geographic distribution of the species *Amblyomma triste* is restricted to South America. These ticks parasitize cattle, deer, capybaras (*Hydrochoerus hydrochaeris*), dogs and human beings. The species is considered the main vector of rickettsiosis, caused by the bacteria *Rickettsia conorii*. The disease is characterized by skin lesions with a black ulcerous crust (*tache noire*), high fever and severe headache (Oliveira, 2006).

## External morphology of ticks

The external structure of the ticks comprises the capitulum and the body, also called idiosoma (Figure 8A). The former contains the mouthparts, which are responsible for fixation and feeding (Figure 8B), and is constituted of: a) a pair of palps with chemiosensorial structures that

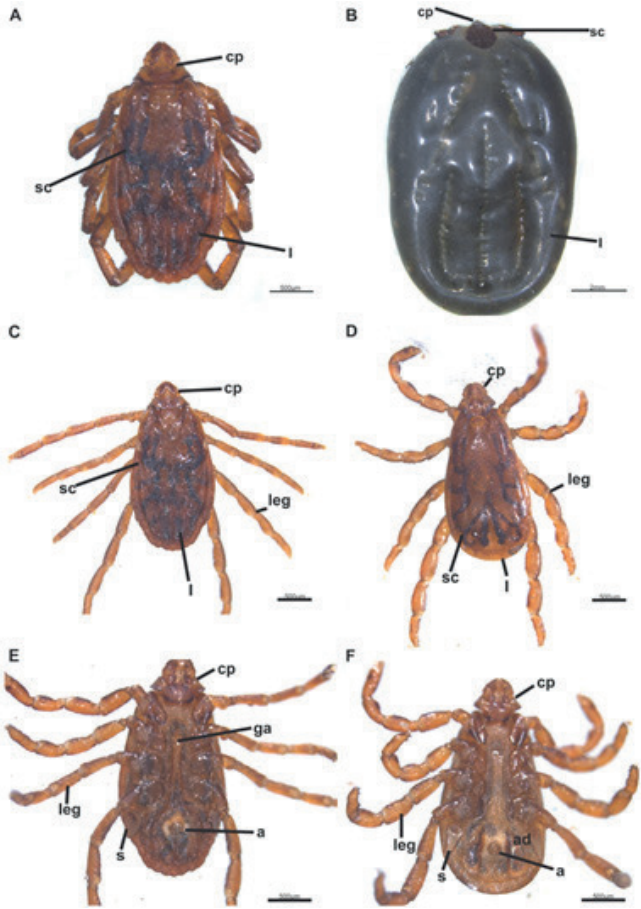
select the site on the host body where the tick will attach; b) a pair of chelicerae, with the function of perforating the host skin; and c) a hypostome, which acts as a fixation organ and as a food channel, passing out the saliva into the host and the host blood into the tick digestive tube. The hypostomal shape and dentition are important features used in the identification of species (Anderson; Magnarelli, 2008; Barker; Murrell, 2004).

Figure 8 – (A) Dorsal region of a *R. sanguineus* s. l. unfed female; (B-C) Ventral region of a *R. sanguineus* s. l. unfed female in detail; (D) Ventral region of a *R. sanguineus* s. l. unfed male in detail. a = anus; i = idiosoma; bc = basis capitulum; cp = capitulum; h = hypostome; ga = genital aperture; p = palpi; s = spiracular plate; sp = spur; sc = scutum




The legs, segmented in coxa, trochanter, femur, larva, tibia, tarsus and ambulacrum, are located in the anterior part of the body (Figure 9 C-F) (three pairs in larva, four pairs in the nymphs and adults) (Anderson; Magnarelli,

Figure 9 – (A, C) Dorsal region of a *R. sanguineus* s. l. unfed female; (B) Dorsal region of a *R. sanguineus* s. l. engorged female; (D) Dorsal region of a *R. sanguineus* s. l. unfed male; (E) Ventral region of a *R. sanguineus* s. l. unfed female; (F) Ventral region of a *R. sanguineus* s. l. unfed male; a = anus; i = idiosoma; cp = capitulum; ga = genital aperture; leg = leg; s = spiracular plate; sc = scutum; ad = adanal plate



2008; Dantas-Torres, 2008). The coxae may have spurs (Figure 8 C and Figure 9 E-F) wich are also used in tick's taxonomy. The genital pore is in the ventral anterior regions (closed in larvae and open in nymphs and adults) (Figure 8 C and Figure 9 E-F). The spiracle and anus are situated in the posterior region (Figure 8 D) (Sonenshine; Roe, 2014). Males of some tick genus can also present a group of ventral plates represented by adanal (Figure 9 F), accessory and subadanal plates (Koneman et al., 1997).

The scutum is a sclerotized plate, ornamented or no, that covers the whole dorsal region of the males, but a small anterior part in the females (Figure 9 C-D), allowing cuticle expansion during feeding and oogenesis (Figure 9 A-B) (Anderson; Magnarelli, 2008; Sonenshine; Roe, 2014).

## Tick's biology

The life cycle of ticks consists of four stages of development: egg, larvae, nymph and adult. Some species are monoxenous, as the cattle tick (*R.* (*B.*) *microplus*), i.e., they parasitize a single host during the whole life cycle. Other species are heteroxenous, feeding on different hosts, including: mammals, rodents, birds, amphibians, reptiles, other ticks, and the human beings as well (Denardi et al., 2004; Oliveira et al., 2003). An important characteristic of these ectoparasites is that, during their biological cycle, they can survive for long periods after dropping off the host, sheltered in the vegetation or soil cracks and crevices (Walker, 1994).

After fully engorged and having completed oogenesis, the females drop off the host and start oviposition, which can last approximately 18 days and produce up to 7.000 eggs, as reported for the species *R. sanguineus* s. l.

(Dantas-Torres, 2010). The female dies after laying the eggs, and the incubation period will last approximately 20 days, depending on the environmental conditions. The larvae will find and feed on a host, to eventually undergo ecdysis (Sonenshine; Roe, 2014).

Ticks need to find a host to feed on to complete their development and ensure their reproductive success. In this phase, stimuli as odor, vibration, visual appearance (some species have simple eyes located in the scutum margin) and the temperature of the host are essential parameters. The site for fixation on the host is selected by chemoreceptors located on the palps and chelicerae, used to "taste" the host. After the host dermis and epidermis are perforated by the chelicerae, the hypostome is introduced in the lesion and there remains, anchoring the tick with the aid of a protein substance named cement, secreted by the salivary glands. Through the hypostome (food channel), the tick saliva is passed out into the host, and host blood fluids sucked into the tick (Anderson; Magnarelli, 2008).

## Medical and veterinary importance

Geographically, ticks are globally spread, being found in all regions of the world (Gray et al., 2013). This wide distribution can be attributed to the diversity of hosts, including mammals, birds, reptiles, amphibians, other arachnids, other ticks (Labruna et al., 2007) and the human beings. The slow feeding process of the ectoparasites is another important factor, once ticks cover long distances transported by the hosts (Anderson; Magnarelli, 2008).

Ticks have great medical and veterinary importance, not only for the host blood spoliation, but also for being vectors of several pathogens, such as bacteria, viruses,

protozoa and helminths, affecting domestic/wild animals and the human beings as well (Dantas-Torres, 2010). These pathogens are transmitted through the saliva, which, according to Sonenshine and Roe (2014), consists of a species-specific complex mixture of molecules, varying according to the interaction parasite/host and the physiological state of the tick.

The multifunctional molecules of the saliva modulate the immunoinflammatory and hemostatic systems of the host, ensuring not only the biological success of the ectoparasites, but also their role as pathogen vectors, once its immunomodulatory action facilitates the transmission of microorganisms, minimizing the obstacles to their penetration into the host organism (Francischetti et al., 2009; Nuttall; Labuda, 2004).

# 3
# Defense and protection (integument)

*José Ribamar Lima de Souza, Rafael Neodini Remedio, Patrícia Rosa de Oliveira, André Arnosti, Rusleyd Maria Magalhães de Abreu, Maria Izabel Camargo-Mathias*

In ticks, the integument (Figure 10 A-F) is an organ of vital importance, externally covering the body and acting as a physical barrier against environmental hazards. The integument plays a key role in the biological success of the ticks, due to its versatility, physiological aspects and interaction with all the ectoparasite systems, covering and sustaining the body, protecting against mechanical impacts and regulating the hydric balance (Coons; Alberti, 1999; Sonenshine; Roe, 2014).

Histologically, the integument is constituted of a cuticle comprised of several sublayers secreted by cells that form single epithelium, which secrets all the material that will constitute the exoskeleton, responsible for the formation of epidermal appendages and dermal glands (Coons; Alberti, 1999; Hackman, 1982; Sonenshine; Roe, 2014).

The cuticle is an acellular structure comprised of two layers: a) the epicuticle, outer and thinner, with distensible and deep folds, and b) the procuticle, inner and thicker (Coons; Alberti, 1999).

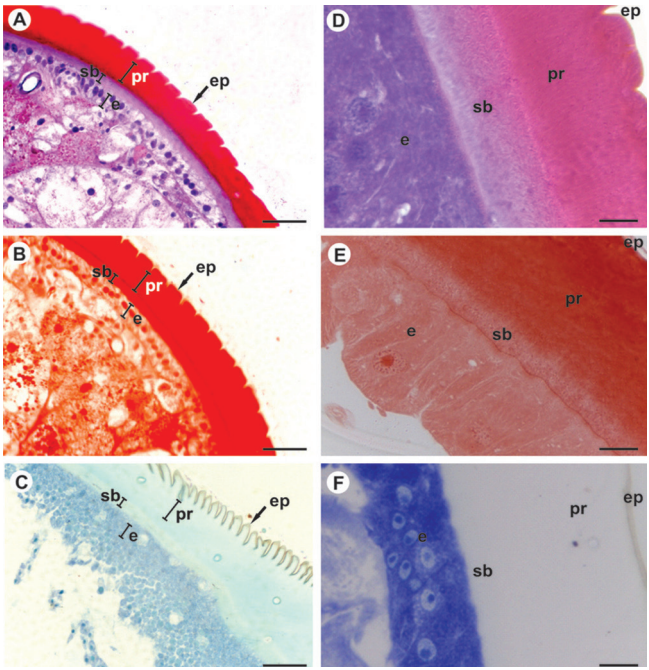
The distensibility of the integument allows the ticks (specially females in oogenesis) to increase the body volume during engorgement, accommodating the enlarged midgut, as well as the ovary in development, which contains oocytes in different stages of maturation (Bughdadi, 2008; Oliveira et al., 2009; Remedio; Nunes; Camargo-Mathias, 2014).

According to Coons and Alberti (1999), the epicuticle morphology does not present significant variations over the different developmental phases, being thinner or thicker in some species or regions of the body. Some cuticle characteristics are species-specific, e.g., argasidae ticks present a cement layer, while in Ixodidae this layer is absent. Additionally, according to Dillinger and Kesel (2002), *Ixodes ricinus* females have their body enlarged without ecdysis, once their epicuticle is highly resistant and distensible.

The procuticle is the layer between the epicuticle and the epidermis, in some cases, sclerotized. The sclerotized parts (sclerites, plates and shield) are harder and thicker than the non-sclerotized one, also called soft parts (aloscutum and membranes) (Coons; Alberti, 1999). The procuticle is subdivided into two layers: the exocuticle, in contact with the epicuticle, and the endocuticle, close to the epidermal cells (Coons; Alberti, 1999). Both sublayers contain pore channels, whose function is still nuclear; however, it has been suggested that they are involved in the exchange of gases, water and lipids between the internal compartments of the tick and the environment (Coons; Alberti, 1999). Cytoplasmic extensions can be found in the pore channels; however, these tunnels are commonly found empty. In *Rhipicephalus (Boophilus) microplus*

females, the pores are branched and less frequent (Beadle, 1972). In some cases, a subcuticular layer (deposition zone) is found between the epidermal cells and the stabilized procuticle, containing non polymerized precursors of chitin-protein complexes (Coons; Alberti, 1999).

Figure 10 – Histological sections of the integument of semi-engorged *R. sanguineus* s. l. females stained with: (A, D) Hematoxylin-eosin (HE); (B, E) stained with Ponceau Xylidine; (C, F) stained with Toluidine blue. ep = epicuticle; e = epidermis; sb = subcuticle; pr = procuticle




Bars: (A-C) 50 µm; (D-F)10 µm

According to the literature, the morphology, sublayer division and the cuticle composition may undergo alterations in the different feeding stages (unfed, semi-engorged and fully engorged) and in the different phases of the

biological cycle (larva, nymph and adult); however, such alterations differ among the species. According to Remedio, Nunes, and Camargo-Mathias, (2014), the procuticle sublayer division is not observed in unfed *Rhipicephalus sanguineus* s. l. ticks, while this division is evident in the phase of full engorgement. In *R. (Boophilus) microplus*, the procuticle presents an inner and outer endocuticle during the four days of slow engorgement.

The single epithelium is constituted of cuboidal cells, with round-shaped nuclei, small nucleoli and condensed chromatin (Amosova, 1983; Coons; Alberti, 1999). However, during the feeding process the epithelial cells can undergo morphological alterations. The epithelial cells of *R. sanguineus* s. l. are larger in the stage of full engorgement. However, the opposite occurs in *R. (Boophilus) microplus*, i.e., the epithelial cells are larger in the initial phase of the feeding process (Beadle, 1972; Remedio; Nunes; Camargo-Mathias, 2014). Recent studies have demonstrated that morphological alterations occur when the ectoparasites are exposed to acaricides, confirming that these chemicals can pass through the cuticle and reach the epithelium, causing significant alterations in the cells (Lima-de-Souza et al., 2017).

# 4

# SALIVARY GLANDS IN *RHIPICEPHALUS SANGUINEUS* S. L. TICKS: MORPHOSTRUCTURAL COMPLEXITY AND FUNCTIONAL DIVERSITY

*Karim Christina Scopinho Furquim, Luis Adriano Anholeto, Elen Fernanda Nodari, Marina Rodrigues de Abreu, Maria Izabel Camargo-Mathias*

Biological evolution has endowed ticks with characteristics that place them as ectoparasite organisms of great success. The coexistence of some factors acting in synergy allowed these organisms to reach such status in the food chain.

Among these factors, is the efficient capacity of consumption/ingestion of large amounts of blood (including skin tissue residues), the rapid digestion, the neutralization/elimination of toxic residues from the digestion and the efficient metabolization of the nutrients, which in turn allows the occurrence of physiological modifications, that, among other things, contributed for the ticks to reach sexual maturity and reproduce in large scale, fulfilling one of life's purposes, the perpetuation of the species.

Considering all the knowledge about the biology of these arthropods, a question arises: "are ticks ectoparasite organisms because they developed such biological characteristics or were these characteristics developed due to their efficient spoliation abilities, which affects mainly vertebrate animals?" It can be stated that there was a coevolution of these arthropods and their hosts, where the former acquired the capacity to produce a highly efficient salivary secretion, able to modulate and disorganize the defense systems of the latter, allowing the ectoparasite's fixation, permanence and voracious blood consumption. Furthermore, such capacity has been refined, and some tick species have specialized in the disassembly of the defense in specific hosts, stablishing the so-called parasite-host relationship for each group; i.e., species-specific in many cases.

In summary, several factors have concurred to place ticks in this position, mainly the action of their saliva, with multiple biological implications (Sonenshine, 1991). Such characteristic of the salivary secretion is a reflection of the highly complex morphostructural organization of the salivary glands from different groups of ticks, considering both males and females of the same species (Till, 1961; Binnington, 1978; Balashov, 1983; Walker et al., 1985; Fawcett et al., 1986; Gill; Walker, 1987; Sonenshine, 1991; Marzouk; Darwish, 1994; Camargo-Mathias et al., 2011; Camargo-Mathias; Furquim, 2013). The morphological complexity of the glands makes them multifunctional organs, whose physiology changes as the feeding process advances.

The salivary glands are comprised of multicellular acini, which are responsible for the production of saliva, and a system of ducts that transport and release this secretion to the buccal cavity of the tick. In the females, the glands present three types of acini (I, II, and III) (Figure 11), while

in the males the same glands comprise four types (I, II, II and IV) (Figure 12). Such morphostructural variation reflects functional differences between the respective salivary glands and is associated with the physiological and behavioral peculiarities of males and females (Table 1, Figs. 11, 13-21).

Figure 11 – Total preparation of the salivary glands of unfed female of the tick *R. sanguineus* s. l. Acid phosphatase technique. I: type I acinus; II: type II acinus; III: type III acinus; dt: principal duct; idt: intermediate duct.

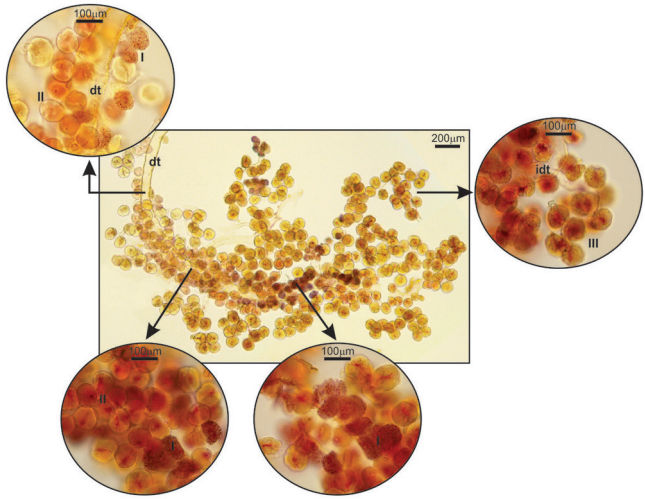


Figure 12 – Scanning electron microscopy of the salivary glands of unfed male of the tick *R. sanguineus* s. l. (C): detail of (A); (D): detail of (B). I: type I acinus; II: type II acinus; III: type III acinus; IV: type IV acinus; dt: principal duct; idt: intermediate duct.

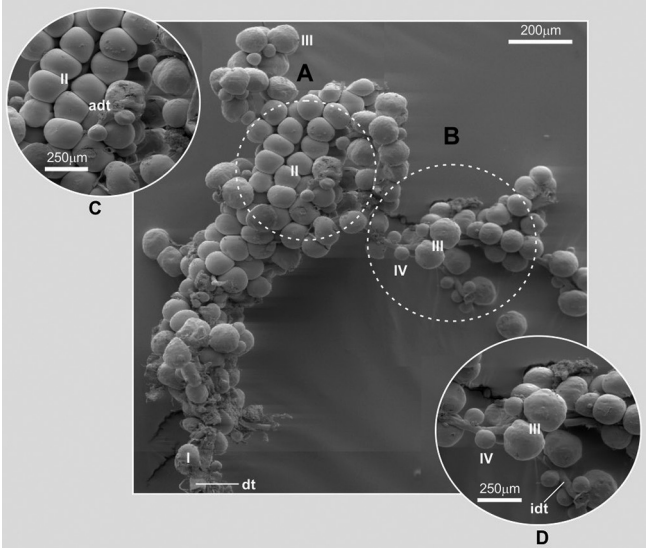


Table 1 – Morphostructural characterization and functional description of the different acini in female and male *R. sanguineus* s. l. ticks

| Type of Acinus | Cell type | Female | Male |
|---|---|---|---|
| I | central and peripheral | absorption of hemolymph salts and secretion of hygroscopic solution (hydric balance maintainance, specially in fasting periods) (1) | similarly to females |
| II | a | synthesis and secretion of cement elements (fixation on the host) | synthesis and secretion of cement elements (fixation on the host) |
| | b | synthesis and secretion of elements that modulate the host defenses | syntehsis and secretion of elements that modulate the host defenses |
| | c1 | idem | idem |
| | c2 | idem | idem |
| | c3 | idem | idem |
| | c4 | idem | idem |
| | c5 | idem | idem |
| | c6 | idem | idem |
| | c7 | absent | syntehsis and secretion of elements that modulate the host defenses |
| | c8 | idem | idem |
| III | d | synthesis and secretion of cement elements (fixation on the host) | synthesis and secretion of cement elements (fixation on the host) |
| | e | idem | idem |
| | f | synthesis and secretion of elements that modulate the host defenses and absorption of the excess of water and hemolymph ions and their release through salivation (osmoregulation) | inactive |
| IV | g | absent | synthesis and secretion of the elements related to reproduction (2) |

(1): Sauer et al. (2000) and Bowman and Sauer (2004); (2): Feldman-Muhsam et al. (1970)

Figure 13 – Schematic representation and histological sections of the salivary glands of unfed females of *R. sanguineus* s. l. ticks. Hematoxylin-eosin stainning. Characteristics of these females:

- fasting females;

- salivary glands: absence of the secretion activity; presence of the granular cells **a, c1, c3, d and e;**

**UF:** unfed females of *R. sanguineus* s. l. ticks; SGD: salivary gland description; **I:** acinus I; **II:** acinus II; **III:** acinus III; **c:** cytoplasm; **dt:** duct; **ld:** lumen of the duct; **iv:** intraacinar valve; **fc:** fibrillar cytoplasm; **cn: central** cell nucleus; **pn: peripheral** cell nucleus; **nc1: undefined** cell **1; nc2: undefined** cell **2; rc: undifferentiated** cell; **a: a** cell; **c1: c1** cell; **c3: c3** cell; **d: d** cell; **e: e** cell; **f: f** cell; **n:** nucleus.

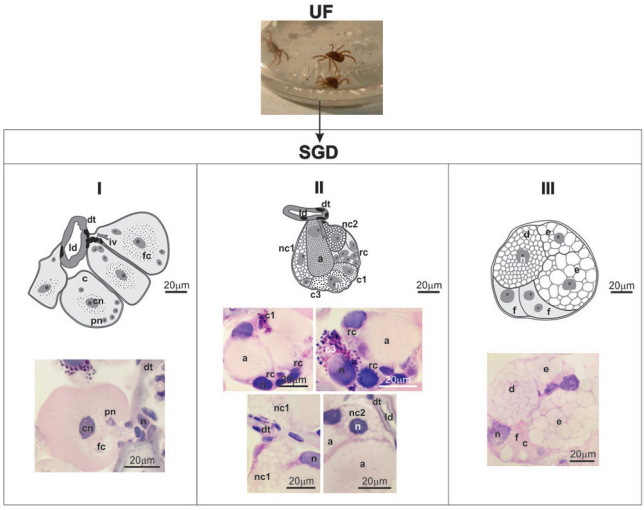


Figure 14 – Schematic representation and histological sections of the salivary glands of two-days fed females of *R. sanguineus* s. l. ticks. PAS reaction.

Characteristics of these females:

- early stage of feeding;

- salivary glands: initial glandular activity with secretion of the saliva components; presence of the granular cells **a, b, c1-c6 and d-f;**

**TDFF:** two-days fed females of *R. sanguineus* s. l. ticks; **SGD:** salivary gland description; **I:** acinus I; **II:** acinus II; **III:** acinus III; **ld:** lumen of the duct; **n:** nucleus; **dt:** duct; **cn:central** cell nucleus; **fc:** fibrillar cytoplasm; **pn:** peripheral cell nucleus; **iv:** intraacinar valve; **a: a** cell; **b: b** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **c6: c6** cell; **d: d** cell; **e: e** cell; **f: f** cell; **la:** lumen of the acinus; **c:** cytoplasm; **s:** secretion.

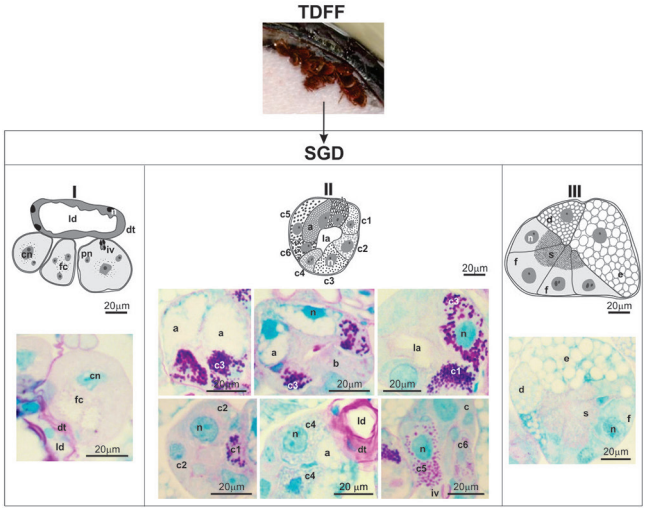


Figure 15 – Schematic representation and histological sections of the salivary glands of four-days fed females of *R. sanguineus* s. l. ticks. Hematoxylin-eosin staining.

Characteristics of these females:

- intermediate feeding stage;

- beginning of the fast engorgement phase;

- salivary glands: intense glandular activity with secretion of the saliva components and osmoregulation; presence of the granular cells **a, b, c1-c4, c6, d** end **e; FDFF:** four-days fed females of *R. sanguineus* s. l. ticks; **SGD:** salivary gland description; **I:** acinus I; **II:** acinus II; **III:** acinus III; **ld:** lumen of the duct; **dt:** duct; **iv:** intraacinar valve; **pn: peripheral** cell nucleus; **cn: central** cell nucleus; **fc:** fibrillar cytoplasm; **a: a** cell; **b: b** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c6: c6** cell; **n:** nucleus; **d: d** cell; **e: e** cell; **f: f** cell; **c:** cytoplasm; **s:** secretion; **la:** lumen of the acinus.

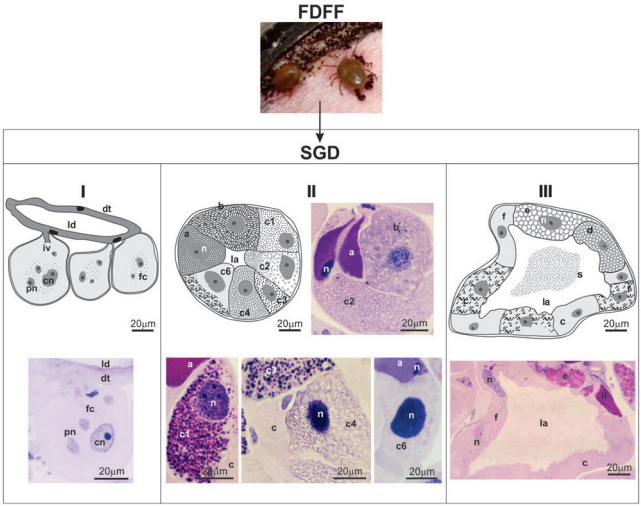


Figure 16 – Total preparation of the salivary glands of fully engorged female of the tick *R. sanguineus* s. l. Acid phosphatase techinique. **I:** type I acinus; **II:** type II acinus; **III:** type III acinus; **dt:** principal duct; **idt:** intermediate duct; **adt:** acinar duct; **la:** lumen of the acinus.

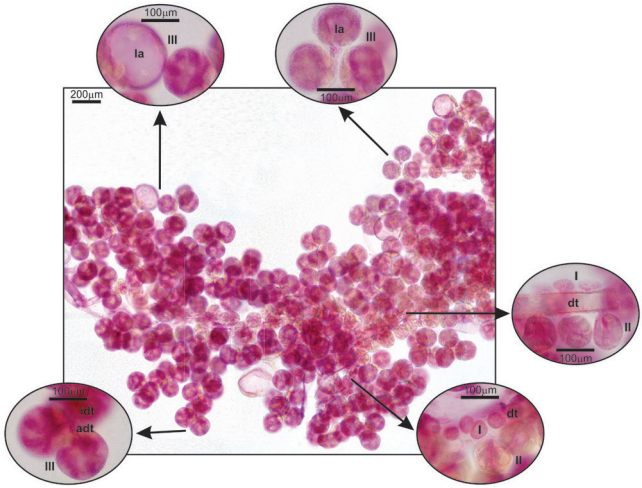


Figure 17 – Schematic representation and histological sections of the salivary glands of seven-days fed females of *R. sanguineus* s. l. ticks. Hematoxylin-eosin staining.

Characteristics of these females:

- final feeding stage;

- salivary glands: degeneration by apoptosis; presence of the granular cells in the salivary glands **a, c1, c3, d** and **e;** **SDFF:** seven-days fed females of *R. sanguineus* s. l. ticks; **SGD:** salivary gland description; **I:** acinus I; **II:** acinus II; **III:** acinus III; **ld:** lumen of the duct; **dt:** duct; **iv:** intraacinar valve; **fc:** fibrillar cytoplasm; **pn:** peripheral cell nucleus; **cn: central** cell nucleus; **c:** cytoplasm; **a: a** cell; **c1: c1** cell; **c3: c3** cell; **in: indeterminate** cell; **bb:** blebs; **arrow:** chromatin margination beginning; **chm:** chromatin margination; **fn:** fragmented nucleus; **dashed circle:** loss of cell contact; **p:** picnotic nucleus; **cm**: cytoplasmic mass; **d: d** cell; **e: e** cell; **f: f** cell; *: secretion resembling an amorphous mass; **in:** irregular nucleus.

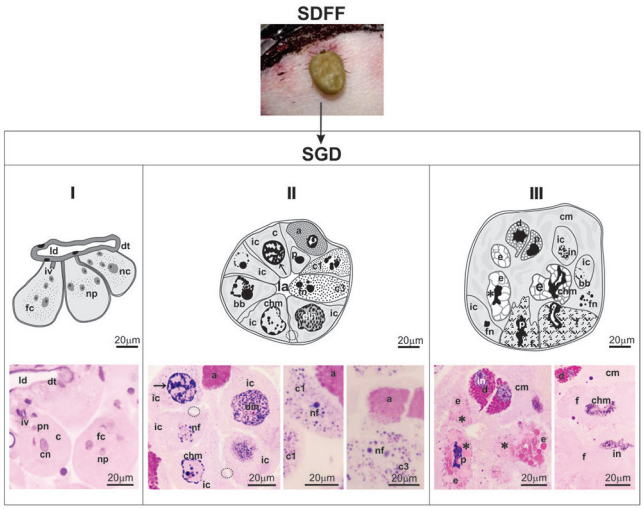


Figure 18 – Salivary glands of unfed male of *R. sanguineus* s. l. ticks. (A) Total preparation (acid phosphatase techinique); (B) Histological sections (hematoxylin-eosin staining).

**I:** type I acinus; **II:** type II acinus; **III:** type III acinus; **IV:** type IV acinus; **dt:** principal duct; **idt:** intermediate duct; **adt:** acinar duct; **np: peripheral** cell nucleus; **nc:** central cell nucleus; **c:** cytoplasm; **n:** nucleus; **a: a** cell; **c1: c1** cell; **c3: c3** cell; **nc1: undefined** cell 1; **nc2: undefined** cell 2; **rc: undifferentiated** cell; **d: d** cell; **e: e** cell; **g: g** cell.

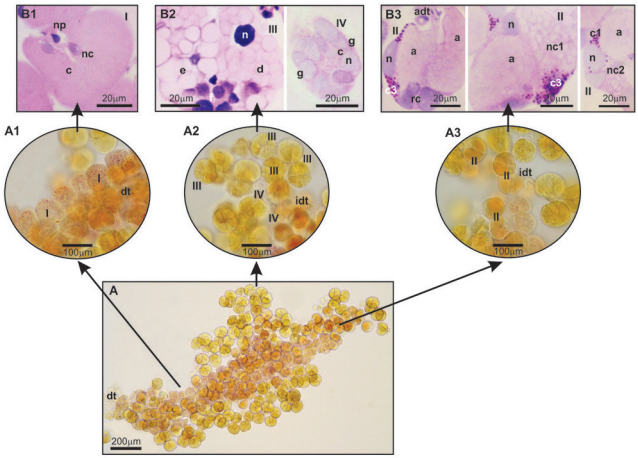
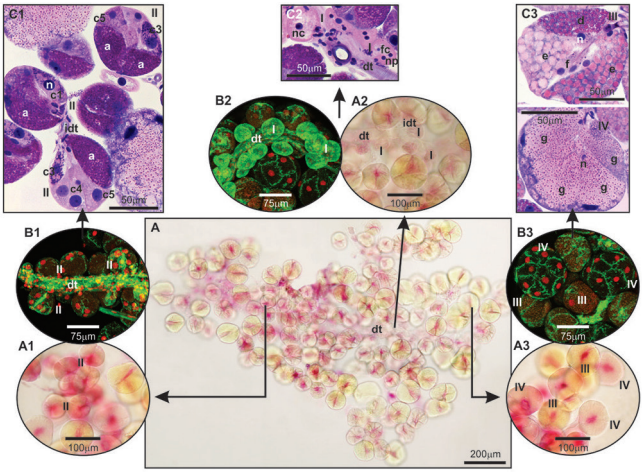


Figure 19 – Salivary glands of fed male of *R. sanguineus* s. l. ticks. (A) Total preparation (acid phosphatase techinique); (B) Confocal microscopy (cytoskeleton and nucleus staining: actin= green, tubulin= red and nucleus= red); (C) Histological sections (hematoxylin-eosin staining).
**I:** type I acinus; **II:** type II acinus; **III:** type III acinus; **IV:** type IV acinus; **dt:** principal duct; **idt:** intermediate duct; **n:** nucleus; **np: peripheral** cell nucleus; **nc:** central cell nucleus; **fc:** fibrillar cytoplasm; **a: a** cell; **c1: c1** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **g: g** cell.




Regarding the glandular secretory cycle, the synthesis and secretion capacity of the molecular complexes with different biological functions, the so-called salivary bioactives, are in the hall of the main glandular functions (Camargo-Mathias et al., 2011). However, to fulfill the purpose of signalizing/triggering cell/tissue physiological responses, the molecules produce and released

through salivation need to be bioactive to the biological system.

Cell biologists have reported that, in the biosynthetic/secretory pathways of specialized secretory cells, the products destined to oxytosis can be synthetized in an inactive precursor way and suffer a proteolytic processing to become active and efficiently perform a specific biological function. The maturation process begins in the *trans* Golgi network, when the secretion molecules are packed into structures named vesicles or secretion granules, continues in the cytoplasmic granules (immature granules) and can be extended to after oxytosis in the lumen of secretory tissue ducts (Burgoyne; Morgan, 2003).

The morphological characteristics of the secretion granule maturing process were clearly identified in the *R. sanguineus* s. l. salivary glands through histological and histochemical analyses. It is known that, as the granules mature, their contents become more concentrated (Figs. 15, 16), due to the continuous removal of membrane excess and lumen acidification. Therefore, mature secretory granules are more densely full of secretion, ready to play their biological role.

Figure 20 – Histological sections of salivary glands stained with hematoxylin-eosin. (A) Two-days fed female; (B) Fed male of *R. sanguineus* s. l. ticks. Demonstration of the maturation process (MP) of the secretion granules of the **e** and **f** cells.

**1:** maturation stage 1; **2:** maturation stage 2; **3:** maturation stage 3; **III:** type III acinus; **e1:** maturation stage 1 of the **e** cell secretion granules; **e2:** maturation stage 2 of the **e** cell secretion granules; **e3:** maturation stage 3 of the **e** cell secretion granules; **f1:** maturation stage 1 of the **f** cell secretion granules; **f2:** maturation stage 2 of the **f** cell secretion granules; **f3:** maturation stage 3 of the **f** cell secretion granules.

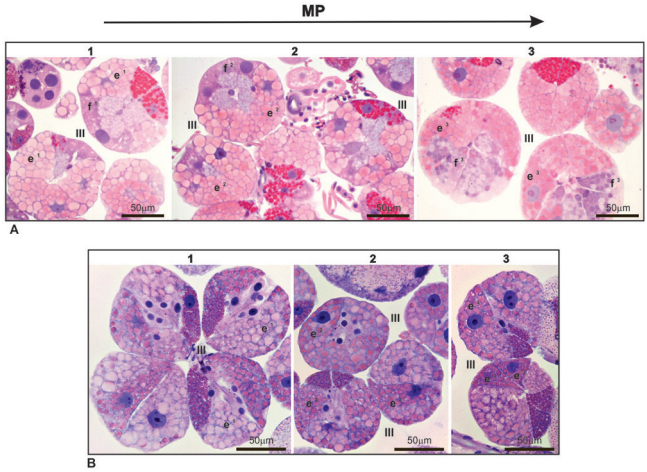
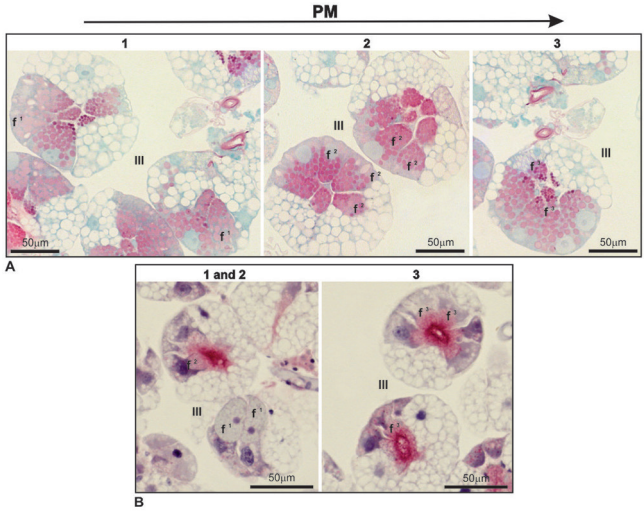


Figure 21 – Histological sections of the salivary glands of two-days fed females of *R. sanguineus* s. l. ticks. (A) PAS reaction; (B) Acid phosphatase technique. Demonstration of the maturation process (MP) of the secretion granules of the **f** cells.

**1:** maturation stage 1; **2:** maturation stage 2; **3:** maturation stage 3; **III:** type III acinus; **e1:** maturation stage 1 of the **e** cell secretion granules; **e2:** maturation stage 2 of the **e** cell secretion granules; **e3:** maturation stage 3 of the **e** cell secretion granules; **f1:** maturation stage 1 of the **f** cell secretion granules; **f2:** maturation stage 2 of the **f** cell secretion granules; **f3:** maturation stage 3 of the **f** cell secretion granules.



# 5
# Tick feeding process (digestory system)

*Elen Fernanda Nodari, Melissa Carolina Pereira, Maria Izabel Camargo-Mathias*

Ticks, obligatory hematophagous ectoparasites, are arthropods that have the digestory system involved in the digestion of a single type of food, the blood, having the hemoglobin as main source of nutrients (Caperucci; Costa; Camargo-Mathias, 2013; Sonenshine; Roe, 2014). The midgut, the main organ of the digestory system of the ticks, is of the utmost importance for their success, in addition to being the site for the development of several endoparasites and representing the major physical barrier between the tick and the immunological response of the host (Higuchi, 1987; Kocan et al., 1987).

The digestory system of the ticks is divided into three regions: a) foregut (stomodeum), constituted of the pre-oral cavity, mouth, pharynx and esophagus, with the function of transporting the food to the midgut (Caperucci;

Costa; Camargo-Mathias, 2013; Sonenshine; Roe, 2014); b) midgut (mesenteron), the largest organ of the body and responsible for the entire digestive process and nutrient absorption (Sonenshine; Roe, 2014), comprised of a central ventriculus (stomach), numerous diverticula (ceca) branching towards all the regions of the body, and a small posterior tube that conducts waste residues to the posterior region; and c) hindgut (proctodeum), divided into anal atrium and anus, with the function of eliminating the residues from the body.

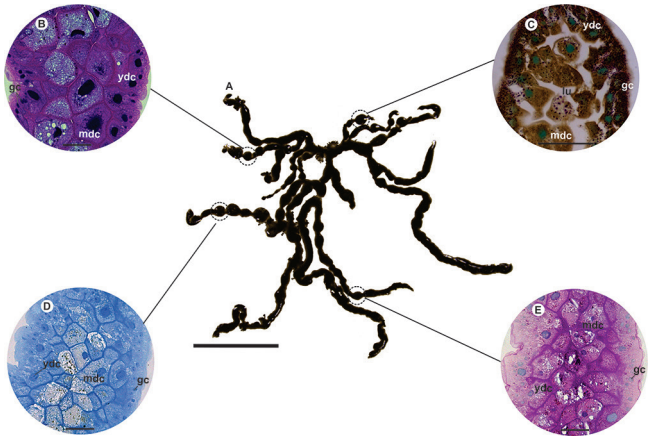
The digestion in ticks is a controversial theme. Some authors point that it would be strictly intracellular (Anderson; Sonenshine; Valenzuela, 2008; Lara; Bechara; Oliveira, 2005; Sonenshine; Roe, 2014), while others report that it would start in the midgut lumen, through a holocrine secretion, which is synthesized by the digestive/secretory cells and would perform the lysis of the red blood cells. Additionally, the digestion of the blood proteins and other molecules would occur in the interior of the epithelial cells, being therefore considered extra- and intracellular digestion (Agyei; Herbert; Runham, 1991; Agyei; Runham; Blackstock, 1992; Nodari et al., 2016).

During the digestive process, the different midgut cells undergo alterations according to how long the feeding period lasts and the stages of the ectoparasite biologic cycle, i.e., larva, nymph and adult (Agbede; Kemp, 1985; Agyei; Runham, 1995; Caperucci; Costa; Camargo-Mathias, 2013; Caperucci; Camargo-Mathias; Bechara, 2009; Coons; Alberti, 1999; Coons; Rosell-Davis; Tarnowski, 1986; Remedio et al., 2013; Sonenshine; Roe, 2014; Walker; Fletcher, 1987).

In general, the midgut is internally covered by a simple or pseudostratified epithelium supported by a basal membrane, underlain by an outer layer of muscle fibers (Sonenshine; Roe, 2014). The epithelial cells are classified

into three types: a) generative; b) digestive and c) secretory (Figure 22) (Agbede; Kemp, 1985; Agyei; Runham, 1995; Balashov, 1972a; Caperucci; Camargo-Mathias; Bechara, 2009; Sonenshine; Roe, 2014; Walker; Fletcher, 1987).

Figure 22 – Photomicrography (A) and histological sections (B-E) of the midgut of *R. sanguineus* s. l. females fed for 4 days stained with different techniques to observe the young digestive (ydc), the mature digestive (mdc) and the generative cells (gc) in the lumen (lu). (A) Photomicrography of the midgut. (B) Hematoxylin-eosin technique. (C) Acid phosphatase detection. (D) Bromophenol blue staining. (E) PAS detection




Bars: (A) 2mm; (B, D-E) 50 µm; (C) 100 µm

In addition to these types, basophilic cells have been reported in the literature (Agbede; Kemp,1985), as well as endocrine and vitellogenic cells (Coons; Alberti, 1999). Agyei et al. (1991, 1992) suggested that the secretory cells described by other authors would actually be digestive cells.

The generative cells – undifferentiated cells – are spindle-shaped and present small areas with microvillosities

towards the lumen, forming a simple cuboidal epithelium, or pseudostratified (Figure 22 B-E) (Agbede; Kemp, 1985; Caperucci; Bechara; Camargo-Mathias, 2010; Walker; Fletcher, 1987). These cells originate the digestive and secretory ones (Agbede; Kemp, 1985; Walker; Fletcher, 1987) and, according to the literature, are not involved in the digestive process itself; however, they accumulate lipids (reserves) in the cytoplasm. The generative cells are found in the initial phase of each instar, as well as in the stage known as 'slow feeding ', and are less frequent in the post-engorgement stages (Agbede; Kemp, 1985; Walker; Fletcher, 1987).

The digestive cells are regarded the main cells of the midgut epithelium (Figure 22 B-E), once they play a key role in the synthesis and secretion of digestive enzymes and are responsible for the absorption of water and other substances digested by the tick (Coons; Alberti, 1999). This type of cell, according to some authors, can undergo morphological and physiological modifications as the digestive process develops, characterizing the occurrence of specific physiological and morphological moments (Agyei; Runham, 1995).

The secretory cells are the most polemic, once they occur less frequently and have been reported for some species, in addition to presenting a morphology little distinct from the digestive ones, suggesting that digestive and secretory cells would actually be the same type. However, these cells similarities are observed in different physiological states, implying that digestive cells undergo different stages, with morphological and physiological alterations, and can be regarded multifunctional cells (Agyei; Runham; Blackstock, 1992; Nodari et al., 2016).

Ultrastructurally, the midgut epithelial cells present a relative structural complexity, having different organelles and several cytoplasmic inclusions, confirming the

hypothesis that they are multifunctional (Caperucci; Bechara; Camargo-Mathias, 2010; Caperucci; Costa; Camargo-Mathias, 2013; Nodari et al., 2016).

In summary, in addition to the digestive function, the tick midgut cells can act as nutrient reservoir, which is demonstrated by the presence of intracellular inclusions. Moreover, these cells can store proteins, carbohydrates and lipids (Balashov, 1972a; Caperucci; Costa; Camargo-Mathias, 2013), indispensable reserves for the production of eggs, which require nutrients to develop vitellogenesis and, consequently, ensure the preservation of the offspring.

# 6
# Protecting the eggs (gené's organ)

*Renata da Silva Matos, Bruno Rodrigues Sampieri,*
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On ticks' life cycle, the oviposition phase has extreme importance because it is a considerable moment of vulnerability for females, when their future descendants are placed on the ground and will need an appropriate environment for its development until the larvae hatch. Therefore, that phase of females' greater vulnerability should be considered and it becomes, consequently, very useful to apply control strategies, once the malfunction of organs involved in this process may result in the offspring non-viability (Booth; Beadle; Hart, 1984; Booth et al., 1989; Oliveira et al. 2005).

Ticks are the only arthropods that have Gené's organ, which is a specialized exocrine glands group that syntheses and releases substances that waterproof the eggs. These substances have lipid origin (waxes) and promote

protection against desiccation, predation and contamination by pathogens, also may even inhibit the absorption of substances that may be present in the environment, such as pesticides. The wax produced by the Gené's organ also assists in the eggs aggregation process, typical behavior observed in ticks, which it allows the eggs mass to remain united (Less; Beament, 1948; Cherry, 1976; Nelson; Sukkestad, 1970; Howard; Blomquist, 1982).

Some compounds synthesized by the gland cells of the Gené's organ can act as pheromones and kiromoniums, also known as semi chemicals, substances that can contribute guiding ticks' behavior, orienting activities such as aggregation in the environment, search for food, copulation and other vital activities (Howard; Blomquist, 1982; Sonenshine, 1991).

For better understand the composition of the waxes released on ticks' eggs, some studies have analyzed those waxes through different methods, such as: bright field microscopy, transmission electron microscopy (Booth, 1989) and chromatography (Booth, 1992), however, there is still a need for more complex studies that may provide further understanding about where those substances are synthetized and in which cell types these processes occur (Booth, 1992).

According to Arrieta, Leskiw and Kaufma (2006), the wax produced by the *Amblyomma africano hebraeum Koch* Gené's organ would inhibit *Escherichia coli* and *Serratia marcescens* (Gram-negative bacteria) growth, what reinforced the wax potential to protect the eggs against microorganisms' infections that could possibly be present in the microenvironment. Lima-Netto et al. (2012), in studies evaluating the *Amblyomma cajennense* s. l. Gené's organ antiviral potential against influenza and picornavirus, also found out that the secretion would inhibit both microorganisms' growth.

The morphology of the Gené's organ of several species of ticks, specifically, has been previously established by different authors who had used scanning electron microscopy (SEM) techniques. Among the species studied were: *R. (B) microplus* (Booth; Beadle; Hart, 1984), *Dermacentor reticulatus* (Schöl et al., 2001), *Hyalomma (Hyalomma) dromedarii* (El Shoura, 1987) and *Haemaphysalis longicornis* (Kakuda; Mōris; Shiraishi, 1992), However, *Rhipicephalus sanguineus* s. l. studies are more recent and have been developed.

The ultramorphological analysis, using SEM, showed that the Gené's organ of *R. sanguineus* s. l. tick females, with six feeding days, would be composed of a set of tubular glands (often called tentacles), which would have their free extremities bifurcated and ending up blind (Figure 23 A). These glands outer surface would have a smooth and homogeneous appearance, which corroborate the data already found for the other species studied by Lees; Beament (1948), Booth; Beadle; Hart, 1984, El Shoura (1987; 1988).

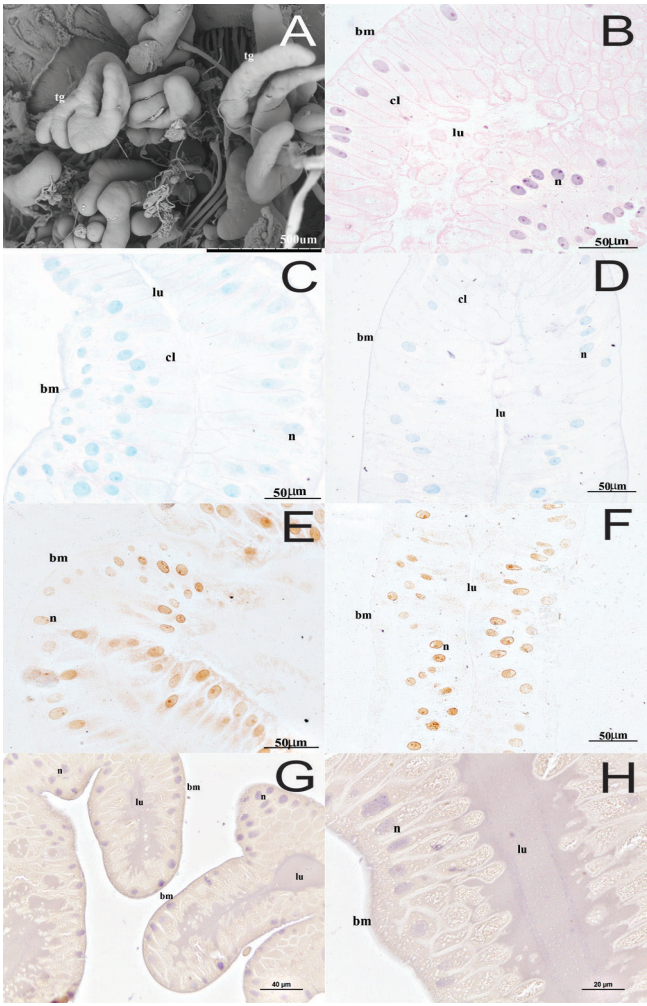
The histology of the Gené's organ of *R. sanguineus* s. l. females (Figure 23 B) in vitellogenesis stage (when they are in oviposition phase), showed that the tubular glands are composed of a secretory epithelium with cylindrical cells (typical secretory cell morphology), making a simple cylindrical epithelium, supported on a clear basement membrane and forming a tubular lumen in the gland center. The glands' secretory cells show cytoplasm with granules resulting from the release of the synthetized product, stored in the cytoplasm until the moment of its use. The studies also revealed that the secretion nature is lipid or polysaccharide.

For the secretion release into the glands' lumen, the apical portion of each cells undergoes a constriction and separates the apical portion of the cell basal portion,

released first into the glandular lumen, classifying the secretion release in these glands as merocrine type. The histological data found for *R. sanguineus* s. l. species, corroborate those found by Till (1961) for *R. appendiculatus*, by Kakuda Mōris and Shiraishi (1992) for *Haemaphysalis longicornis* and *R.* (*B.*) *microplus* by Booth (1992). The latter author also made it clear that the tubular glands of the Gené's organ would be present in larger quantities and more developed in the eggs laying phase.

The histochemical composition of the secretion synthesized and released by the *R. sanguineus* s. l. Gené's organ cells, besides the strong lipid positivity (Figure 23 G-H), would react with the weak positivity to polysaccharide elements (Figure 23 C-D), as well as calcium (Figure 23 E-F), but these elements exact function still needs to be clarified.

Figure 23 – Scanning electron microscopy (A) and histological (B) sections of the Gené's organ of *R. sanguineus* s. l. females. Reaction by PAS (Periodic Acid-Schiff) (McManus, 1946) and counter-staining with methyl green (C-D). Von Kossa staining (E-F). Imidazole osmium technique (G-H). tg = tubular glands; cl = cell limit; bm = basal membrane; lu = lumen; n = núcleo.




Bars: (A) 500 mm (B-F) 50 mm (G-H) 40 mm.

# 7
# Generating new individuals I (female reproductive system)

*Patrícia Rosa de Oliveira, Luis Adriano Anholeto, Natalia Rubio Claret Pereira, Allan Roberto Fernandes Ferreira, Maria Izabel Camargo-Mathias*

The reproductive system of female ticks consists of a single U-shaped tubular ovary located in the posterior third of the body and extending towards the posterolateral region, paired oviducts, a single uterus, and a pair of accessory glands opening at the junction of two vaginal regions (Sonenshine, 1991; Said, 1992; Sonenshine; Roe, 2014). The vagina is subdivided into a posterior muscular and a vestibular vagina (Sonenshine; Roe, 2014).

Until 2003, studies on the reproductive system of ticks have not addressed the morphology of the ovaries (Till, 1961; Balashov, 1983; Sonenshine, 1991). In 2004, Denardi et al. developed morphological studies and classified the ovaries of these ectoparasites as panoistic, i.e., all the germ cells correspond to oogonia (future oocytes);

therefore, the nurse cells – and, specifically on the ticks, the follicle ones – are not present.

The ovary of ticks, in general, consists of a lumen delimited by a wall of small epithelial cells with rounded nuclei. A large number of oocytes remains attached to this wall through the pedicel cells (ovary epithelial cells that proliferate to fix the oocyte) until they are released into the lumen (Denardi et al., 2004; Oliveira et al., 2005; Saito et al., 2005; Oliveira et al., 2006; Oliveira et al., 2007a, b; Ricardo et al., 2007; Sonenshine; Roe, 2014). Recent studies have demonstrated that, in addition to fixing the oocytes to the ovary, the pedicel synthesizes part of the vitellogenic elements that are transported to the interior of the oocytes via active transportation, which occurs mainly in the region next to the oocyte/pedicel complex (Oliveira et al., 2007a).

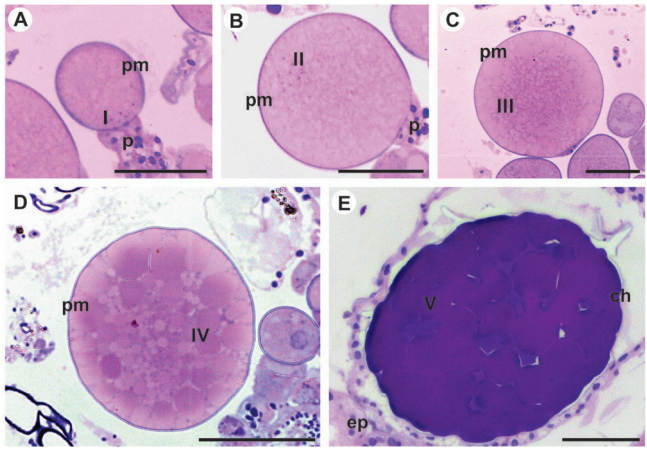
In ticks, the oocytes (Figure 24) are distributed in the ovaries on a preferential basis; i.e., the ones in early developmental stages (I and II), small and with light staining, are found in the distal region, while the more developed ones (developmental stages III, IV and V), larger and darker, are located in the proximal region. The development of oocytes is simultaneous, but asynchronous; i.e., although all the oocytes develop at the same time, the vitellogenesis occurs in different moments, enabling these cells to be fecundated also in different moments (Denardi et al., 2004; Oliveira et al., 2005).

Earlier studies on the reproductive system of ticks have suggested a classification for the oocyte developmental stages (Till, 1961; Sonenshine, 1991); however, more recent ones have improved this classification, renaming it and adding other criteria to facilitate the comprehension of the reproductive processes of these ectoparasites.

Currently, the oocytes are classified in developmental stages, according to several morphological criteria, as

follows: a) cytoplasmic aspect, b) germinal vesicle location, c) presence and constitution of yolk granules and d) presence of chorion (Denardi et al., 2004). In *Amblyomma sculptum* (Denardi et al., 2004), *R.* (*Boophilus*) *microplus* (Saito et al., 2005), *R. sanguineus* s. l. (Oliveira et al., 2005) and *A. brasiliensis* (Sanches et al., 2009) the oocytes are classified into 5 developmental stages (I-V), while in *A. triste* (Oliveira et al., 2006) they are classified into 4 stages (I-IV).

Figure 24 – Histological sections of *R. sanguineus* s. l. ovary stained by HE. (A) Detail of oocyte I. (B) Detail of oocyte II. (C) Detail of oocyte III. (D) Detail of oocyte IV. (E) Detail of oocyte V. ch = chorium; ep = ovary epithelium; I = oocyte stage I; II = oocyte stage II; III = oocyte stage III; IV = oocyte stage IV; V = oocyte stage V; p = pedicel; pm = plasma membrane




Bars: (A-D) 200 µm; (E) 100 µm

The oocyte stages are classified as follows:

- *Oocytes I*: small and elliptical cells, with central germinal vesicle (nucleus), displaying an evident nucleolus occupying great part of the cytoplasm,

which is homogeneous due to the absence of vitellogenic granulations, but rich in free ribosomes and polyribosomes, and presenting some mitochondria, located mainly in the peripheral region. These cells are surrounded by a thin plasma membrane presenting the onset of specializations, when they are called microvilli. In this stage, only structural proteins are produced (endogenous); i.e., the exogenous vitellogenesis process has not started yet (Denardi et al., 2004; Oliveira et al., 2005; Saito et al., 2005; Oliveira et al., 2006; Oliveira et al., 2007b; Sonenshine; Roe, 2014).

- *Oocytes II*: larger than oocytes I and elliptical. The germinal vesicle is still found in the central region. These cells show fine and homogeneous granulation in the cytoplasm, once in this phase the endogenous synthesis and/or the incorporation of extra-ovarian (exogenous) elements have already started. The mitochondria are the most frequent organelles, occupying the peripheral region, and numerous ribosomes, free and/or adhered to the membrane of a little developed rough endoplasmic reticulum are present. The oocytes are enveloped by a plasma membrane supported by a thick basal lamina, full of elongated microvilli. The inner region of the basal lamina is thicker, in direct contact with microvilli; while the outer region is thinner and with fibrillar aspect (Denardi et al., 2004; Oliveira et al., 2005; Saito et al., 2005; Oliveira et al., 2006; Oliveira et al., 2007b; Sonenshine; Roe, 2014).
- *Oocytes III*: these oocytes have intermediate size, and their shape range from rounded to elliptical. The germinal vesicle is translocated to the oocyte pole, due to the smaller size of the yolk granules.

The remaining cytoplasm is full of granules of different sizes, the smaller occupying the central region and the larger concentrated in the periphery, indicating the fusion of the smaller granules towards the peripheral region of the oocyte. In this developmental stage presents an accumulation of mitochondria, possibly because the cells are being prepared for an important phase of vitellogenesis, in which the organelles are required for the endogenous production and exogenous incorporation of elements that will constitute the egg yolk, such as lipids, proteins and carbohydrates (Balashov, 1972b; Sonenshine; Roe, 2014). Abundant ribosomes and rough endoplasmic reticulum are found, indicating that the oocyte is still endogenously synthesizing the vitellogenic elements. Numerous microvilli are found in the plasma membrane, which remains supported by a thick basal lamina (Denardi et al., 2004; Oliveira et al., 2005a; Saito et al., 2005; Oliveira et al., 2006; Oliveira et al., 2007b; Sonenshine; Roe, 2014).

- *Oocytes IV*: larger than the ones from the previous stage and round-shaped. The germinal vesicle occupies the oocyte pole close to the pedicel. The cytoplasm contains several yolk granules of various sizes randomly scattered, in addition to small mitochondria. The process of chorion (resistant membrane that minimizes desiccation and mechanical impacts, protects from predation and allows gas exchange between the embryo and the environment) deposition continues, demonstrated by the presence of exocytosis vesicles (surrounded by a membrane and containing material to be secreted into the external environment) that transport material to be deposited and polymerized in the

region between the basal lamina and the plasma membrane. Microvilli are no longer observed in this stage, once the process of hemolymph incorporation has already been concluded (Denardi et al., 2004; Oliveira et al., 2005; Saito et al., 2005; Oliveira et al., 2006; Oliveira et al., 2007b; Sonenshine; Roe, 2014).

- *Oocytes V*: the largest germinal cells, rounded-shaped, where the visualization of the germinal vesicle is difficult due to the presence of numerous granules in the cytoplasm. The chorion is fully deposited, and is constituted of two layers: the exochorion, outer and thinner; and the endochorion, inner and thicker, in direct contact with the oocyte, indicating the end of the vitellogenic process (Denardi et al., 2004; Oliveira et al., 2005; Saito et al., 2005; Oliveira et al., 2006; Oliveira et al., 2007b; Sonenshine; Roe, 2014).

Unlike ticks in general, the ones belonging to the species *A. triste,* the oocytes IV (final developmental stage), accumulate a smaller amount of yolk (proteins, lipids and carbohydrates), and the chorion is not subdivided into endo and exochorion.

Recent studies have pointed that the main component of the yolk is the vitellin, whose precursor is the vitellogenin, protein synthesized mainly by exogenous sources, such as the fat body and midgut, and taken into the oocytes via endocytosis (Sonenshine; Roe, 2014).

Therefore, studies on the reproductive system of Ixodidae in general bring relevant information for the comprehension of their biology, helping in the search for alternative methods to control their reproduction as well as the pathologies caused by them.

# 8
# Generating new individuals II (male reproductive system)

*Luis Adriano Anholeto, Bruno Rodrigues Sampieri, Maria Izabel Camargo-Mathias*

Ticks are dioecious arthropods that reproduce by internal syngamy (Coons; Alberti, 1999; Kiszewski; Matuschka; Spielman, 2001). Parthenogenesis is rare, occurring in the Ixodidae species *A. rotundatum* and *Haemaphysalis longicornis* (Luz et al., 2013; Oliver; Stone, 1983; Sonenshine; Roe, 2014).

The reproductive systems (Figure 25) of the families Argasidae and Ixodidae are similar in morphology; however, significant differences between the groups have been reported, and such variations are important for taxonomic and phylogenetic studies (Sampieri et al., 2014).

In both families, the male reproductive system is dorsolaterally arranged in the opisthosoma and is formed by a accessory gland complex multilobulated, a pair of tubular testes (or single testis), a pair of vas deferens, a pair of

seminal vesicle and an ejaculatory duct (Anholeto, 2015; Sampieri et al., 2014; Sonenshine, Roe, 2014).

In Ixodidae, the testes are not interconnected, i.e., the right and the left testis are individual organs. Such pattern has been observed in *Dermacentor andersoni* (Douglas, 1943), *A cajennense*, *A. scultpum* and *A. aureolatum* (Anholeto et al., 2015; Sampieri et al., 2016).

Studies performed by Sampieri et al. (2016), reported the presence of a connection between the right and left testes in *A. triste*, a thin tube found in the distal portion of each testis, also observed in *Ornithodoros moubata* (Wagner-Jevsenko, 1958). In the individuals from the family Argasidae, including those of the species *O. rostratus,* the testes are fused into a single U-shaped structure (Coons; Alberti, 1999).

The testis are responsible for the production of germ cells, which are grouped and surrounded by a simple columnar epithelium, forming the spermatocytes (Sonenshine; Roe, 2014).

The development of germ cells is basically divided into two steps: a) spermatogenesis, characterized by a sequence of mitotic and meiotic division of spermatogonia and spermatocytes, originating spermatids and b) spermiogenesis, differentiation of spermatids to originate the spermatozoa (Sonenshine; Roe, 2014).

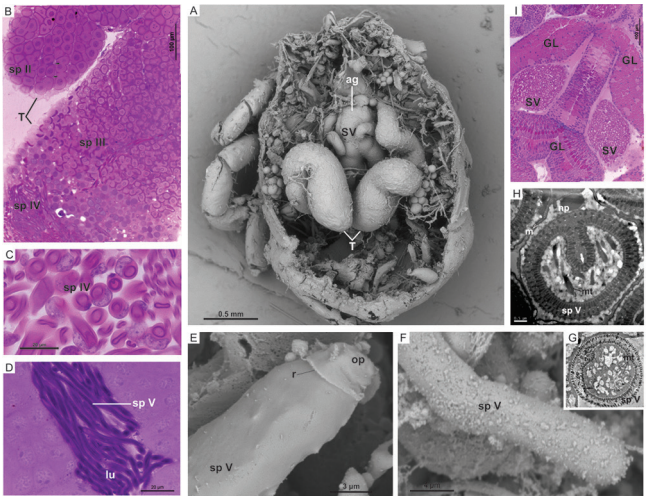
In most Ixodidae species, the spermatogenesis begins in the end of nymph stage, which was demonstrated in *R. sanguineus* s. l. nymphs (Oliveira, et al. 2012), and finishes soon after ecdysis. However, this is not a general rule, once in some species the spermatogenesis is concluded when the male reaches the adult phase and starts the feeding process (Anholeto et al., 2015; Oliver; Brinton, 1972; Sonenshine; Roe, 2014). In some Argasidae and in the extinct *Aponomma,* this process in independent of the feeding stage of the adult individual (Coons; Alberti, 1999; Dumser; Oliver, 1981).

The first staged of spermatogenesis in fed adults males occur from the distal to the proximal regions of the body (Anholeto et al., 2015; Coons; Alberti, 1999; Sampieri et al., 2014).

After differentiation, the elongated spermatids are released into the lumen of a small channel to reach the proximal region of the testes, the beginning of the deferent ducts. These ducts are in contact with the seminal vesicles, which are connected with a complex of multilobulated accessory glands, responsible for the production and secretion of spermatic fluid and for spermatophore synthesis (Anholeto et al., 2015; Coons; Alberti, 1999; Sonenshine; Roe, 2014).

The end of spermiogenesis occurs in the interior of the female genital tract, where the spermatozoa will undergo capacitation, which will enable them to fertilize the oocytes (Kiszewski et al., 2001).

Figure 25 – (A) *R. sanguineus* s. l. reproductive system overview (SEM) of a fed male, showing testis (T), accessory gland (ag) and seminal vesicles (SV) organization and anatomical position. (B-C) Histological section of *R. sanguineus* s. l. testicle distal region, with details of spermatids in different stages of development. (D) Longitudinal section of *A. triste* testicle showing mature spermatids (spV) in its lumen. (E-F) Ultramorphology (SEM) of mature spermatids of *O. rostratus* (Argasidae) and *R. sanguineus* s. l. (Ixodidae), respectively. (G) Ultrastructure of *R. sanguineus* s. l. spermatids, featured the cisternae (*arrow*) formation and mitochondria (mt) distribution. (H) Ultrastructure of *O. rostratus* spermatids. (I) Histological section of *R. sanguinues* s. l. accessory gland and seminal vesicles. gl = glandular lobe; op = operculum; sp = spermatids



# 9
# Ticks and environment (central nervous system)

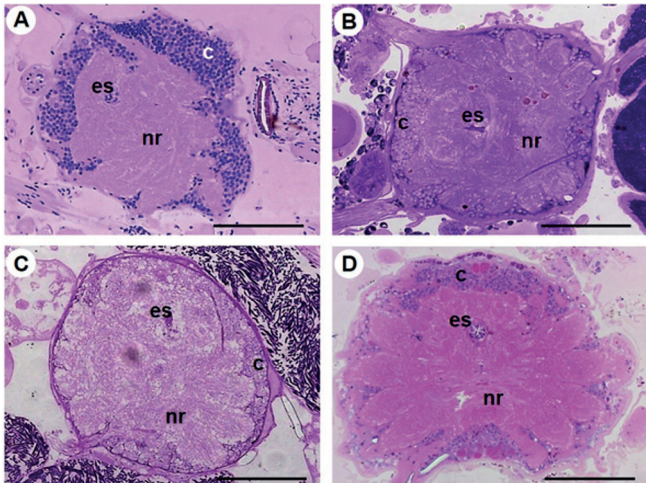
*Melissa Carolina Pereira, Patrícia Rosa de Oliveira, Renata da Silva Matos, Bruno Rodrigues Sampieri, Allan Roberto Fernandes Ferreira, Maria Izabel Camargo-Mathias*

The tick central nervous system (CNS) is a highly condensed mass of fused nerves that, according to Sonenshine and Roe (2014), derived from ancestral brain ganglia and a ventral nerve cord, forming the synganglion. In general, the CNS of tick larvae, nymphs and adults have a very similar structure, also in comparison with different species, and among the families Ixodidae and Argasidae (Figure 26 A-D) (Prullage; Pound; Meola, 1992; Sonenshine; Roe, 2014).

In adult ticks, the CNS is found between the gnathosoma and the genital pore, close to the first and second pairs of legs. The esophagus runs through the synganglion, dividing it into two regions (Figure 27 B): a) supraesophagial, the smaller portion, from which emerge the nerves responsible for controlling the systemic functions

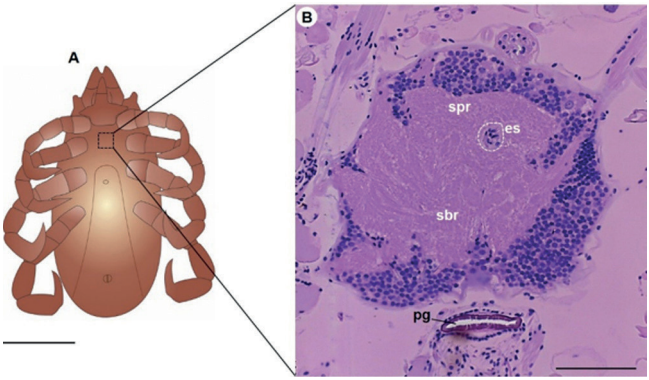
of the pharynx, esophagus, part of the salivary glands, and cheliceral movements; and b) subesophageal, the largest portion of the organ, responsible for the innervation of most internal organs, mainly the ones with reproductive and digestive functions, as well as for controlling the movement of legs and muscles in general (Roma et al., 2012; Sonenshine; Roe, 2014).

Figure 26 – Histological sections of the CNS (synganglion) of adult ticks from different species stained with Hematoxylin-eosin (HE) (A, C-D) and PAS (B) showing the esophagus (es), cortex (c) and neuropile (nr) regions. (A) *R. sanguineus* s. l. (Ixodidae). (B) *A. triste* (Ixodidae). (C) *A. sculptum* (Ixodidae). (D) *Ornithodorus rostratus* (Argasidae)




Bars: (A-D) 200 µm

Figure 27 – Graphic illustration of the ventral view (A) and histological section detail (B) of the synganglion of *R. sanguineus* s. l. female showing its location close to the genital pore (pg) and the esophagus (es) running through the organ dividing it into the supraesophagial (spr) and subesophageal (sbr) regions



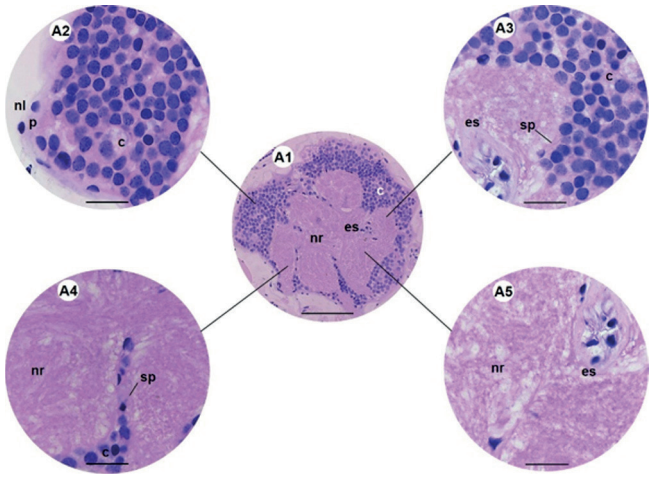

Bars: (A) 1mm; (B) 100 µm

Morphologically, the synganglion is surrounded by two peripheral membranes, which are detectable through scanning electron microscopy and difficult to be visualized under light microscopy. The first layer, outer and acellular, is the neurilemma (Figure 28 A2), constituted of homogeneous and finely granular material, 1,5-3,5 µm-thick, and mainly responsible for acting as a selective barrier for the entrance of substances into the organ, controlling the transportation of ions and nutrients from the hemolymph to the cortical region (Marzouk; Mohamed; Omar, 2001). The inner layer is the perineurium (Figure 28 A2), 2-6 µm-thick, and comprised of glial cells that support the metabolic regulation of the neural activities (Marzouk; Mohamed; Omar, 2001; Sonenshine; Roe, 2014). In Ixodidae, the perineurium is situated just below the neurilemma; however, in Argasidae it forms large spaces delimited by a membrane, probably because the

synganglion of the ticks belonging to this family has the function to store nutrients.

The cortical region (cortex) is situated just below the perineurium (Figure 28 A2-3), and is constituted of neurons, glial cells and neurosecretory centers, which synthesize important substances that will be released into the hemolymph for the regulation of the organ (Prullage; Pound; Meola, 1992; Sonenshine; Roe, 2014). According to Prullage et al. (1992), who described the nervous system of *A. americanun* (L.), the cortex also contains sites for the production of substances involved in the gametogenesis, mainly oogenesis, once they are more active in the role of producing and storing neurosecretion in the female ticks.

Figure 28 – Histological sections of the synganglion of *R. sanguineus* s. l. females stained with HE. (A1) General view. (A 2-3) Detail of the neurilemma (nl), perineurium (p), subperineurium (sp) and neuron cell bodies in the cortex (c). (A 4-5) Detail of the subperineurium, esophagus (es) and neuron fibers in the neuropile (nr)




Bars: (A1) 100 µm; (A 2-5) 20 µm

The innermost region of the synganglion is the neuropile (Figure 28 A 4-5), a mass of neural fibers, ganglions, glomeruli and a system of longitudinal (connective) and horizontal (commissures) nerves, organized into bilaterally symmetric lobules, where axons and dendrites (extensions of nerve cells) are found (Prullage; Pound; Meola, 1992; Roshdy; Marzouk, 1984; Sonenshine; Roe, 2014). The internal structural arrangement of the neuropile allows and facilitates the morphological division of the synganglion into several ganglions (Prullage; Pound; Meola, 1992). According to Sonenshine and Roe (2014), peripheral nerves emerge from this region, innervating appendixes, muscles, sensorial structures and several internal organs.

The subperineurium, a layer with varying thickness (1-7 μm), is found between the neuropile and the cortex (Figure 28, A3, A4). As the perineurium, this layer is constituted of glial cells, which play a role in the metabolic control of the neural activities. These cells have different shapes, cytoplasm with abundant mucopolysaccharides and glycogen, elongated nuclei and membrane extensions surrounding the perikarion (neuron trophic center, where the nucleus and other organelles are found) (Sonenshine; Roe, 2014).

The synganglion is an organ of the utmost importance for the metabolism of the ticks, controlling all the systemic functions of these ectoparasites; thus, it is a target organ for the action of chemical acaricides used to control these ectoparasites. Morphological studies on the biology of these arthropods are hence fundamental to provide information on their internal organs and ground further research aimed to find new efficient and less toxic methods or improve the ones currently in use.

Chemical products have had their effect on the metabolism and morphology of the central nervous system of

ticks investigated in several studies: a) synthetic chemicals, as the pyrethroids permethrin (Roma et al., 2013a, 2014) and deltamethrin (Pereira et al., 2017a); and b) natural chemicals, such as andiroba oil (Roma et al., 2013b, 2015) and neem (Remedio et al., 2014).

# 10
# Controlling ticks (methods)

*Patrícia Rosa de Oliveira,*
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Several studies have been aimed at finding efficient methods to control ticks, hematophagous ectoparasites of great economic and sanitary importance. Currently, the most effective strategy is the chemical control (via synthetic acaricides); however, this method has several disadvantages, such as the high cost of products, application and storage, and the damages caused by residues to nontarget organisms and the environment, as the chemicals impregnate living tissues (Pruett, 1999; Freitas et al., 2005; Oliveira et al., 2008, Oliveira et al., 2009).

The literature has reported that, even in minimum exposures to the different pesticides (including acaricides), the nontarget organisms can suffer dramatic consequences, such as acute poisoning. Moreover, the residues of such substances seriously affect public health through

the contamination of the food chain. The use of pesticides indirectly interferes with the entire wild life, eliminating not only the pests, but also other organisms (nontarget) which play an important role in the biological balance (Pruett, 1999).

Over the last decade, acaricides based on different chemicals (arsenic, organochlorine, organophosphate, carbamate, nitroguanidine, phenilpirazol, formamidine, pyrethroid, avermectin and benzoylphenylurea) (Häuserman et al., 1992) have been widely used. Ticks develop several survival mechanisms and strategies, decreasing the level of chemical penetration into the organism, modifying the metabolism and altering processes of storage and excretion and sites of toxic action. These mechanisms make the ectoparasites less susceptible to the products; therefore, new active ingredients are frequently launched in the market in an attempt to keep up controlling these pests (Nolan, 1985).

Crampton et al. (1999) reported that an active ingredient could lose its properties in 5 to 10 years, due to the selection of resistant strains caused by the intensive and/or incorrect use of the compounds, opposing the manufacturer recommendations (Häuserman et al., 1992). Furthermore, it is known that, once resistance to a particular acaricide is acquired, the process is irreversible, and the chemical will no longer be efficient to control the ectoparasites (Crampton et al., 1999).

On the other hand, the search for alternative control methods, less polluting, inexpensive and with lower incidence of resistant strains has been intensified over the last years (Rosado-Aguilar et al., 2010). The biological control is one of these strategies, using natural predators, such as the cattle egret (*Bubulcus ibis*) (Gonzales, 1975), or parasites, as *Escherichia colli*, *Cedecea lapagei* and *Enterobacter agglomerans* (Brum, 1988) and fungi, as

*Metarhizium anisopliae,* which parasitize the ticks (Da Costa et al., 2002).

Another alternative is the use of compounds of natural origin, i.e., products made of plant extracts, whose active ingredients have the potential to control pests (Guerra, 1985).

Acaricides based on plant extracts have been proven to be a very promising alternative, especially when the high levels of synthetic acaricide consumption and the toxic effects to the environment and nontarget organisms are considered (Martinez, 2002; Borges et al., 2011). Therefore, the development of further research on plant derivative compounds will certainly ground the formulation of safer and more suitable products to replace the synthetic ones (Panella et al., 2005; Dietrich et al., 2006).

Considering all the above information, the development of studies aimed at elucidating the action of synthetic and natural acaricides on the cells, organs and tissues of these ectoparasites is of the utmost importance, as it will ground the search for efficient and sustainable control strategies.

# 11
# Synthetic acaricides

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## Fipronil: action on the female reproductive system and salivary glands

Fipronil, manufactured by Rhône-Poulenc Agro Company in 1987 and registered in 1996, belongs to the chemical group phenylpyrazole, with acknowledged potential to control pests (Oliveira et al., 2011). It is a broad-spectrum insecticide, and in the EUA, it has been used in formulation of baits to control ants and cockroaches as well as in veterinary sprays against ticks, fleas and acari (Penaliggon, 1997; Hugnet et al., 1999; Higgins et al., 2001). The commercial formulations are: Termidor®, Frontline®, Regent®, manufactured by Bayer Cropscience (Oliveira et al., 2011).

Fipronil acts specifically on the central nervous system (CNS) of the invertebrate, preventing the flow of chloride ions to the interior of nervous cells and interfering in the GABA (Gamma-aminobutyric acid) neurotransmitters bindings to the receptors. This causes a disruption in the CNS, with an overexcitation of muscles and nerves, leading the exposed individuals to death (Rhône-Poulenc, 1995; Oliveira et al., 2008, 2009). Due to this exclusive action mechanism, the affinity to the invertebrate receptors and the fact that mammals lack glutamate chloride channels (GluCl – CNS components), fipronil has a wide margin of safety (Rhône-Poulenc, 1995). Moreover, the great efficiency in controlling resistant/tolerant pest justifies its widespread use in several countries (Oliveira et al., 2008, 2009, 2011).

## Female reproductive system

Studies developed in 2008 by Oliveira et al. brought the first information on the effects of synthetic chemical products on the female reproductive system of ticks (Figure 29, 30). Since then, the action of acaricides has been better understood, once the authors investigated the effects of different concentrations of fipronil on the germ cells and ovary tissue in semi-engorged *R. sanguineus* s. l. females. In that study, the ticks were immersed in fipronil at the concentrations of 1, 5 and 10 ppm. The chemical caused alterations in the ovaries of the individuals from all the treatment groups. The alterations were mainly regarding the number of oocytes affected, the presence, number and size of cytoplasmic vacuoles, the presence of myelin figures and cytoplasmic microtubules, and modifications in the germ cell organelles (Oliveira et al., 2008, 2009, 2011).

After the exposure to the concentration of 1 ppm of fipronil, few oocytes I displayed alterations, which occurred only in their shape, showing median constriction and few small cytoplasmic vacuoles. The individuals exposed to 5 ppm, presented a larger number of altered oocytes I with a high level of cytoplasmic vacuolation. In the females exposed to 10 ppm, oocytes I could no longer be observed, which demonstrated that, as fipronil concentrations increased, more and diverse histological alterations occurred (Oliveira et al., 2008, 2009). The vulnerability of the cell in early stages of development would be associated with the fact that the chorion is not yet deposited. The chorion is a membrane with special and typical characteristics, constitution and thickness, with the function of protecting the eggs by acting as a barrier to the toxic substance (Oliveira et al., 2009).

Oocytes II and III of the *R. sanguineus* s. l. ticks subjected to fipronil at the concentration of 1 ppm showed the onset of cytoplasmic vacuolation. The individuals subjected to the concentration of 5 ppm showed a larger number of altered oocytes II and III in comparison with the previous group, in addition to presenting extensive areas of cytoplasmic vacuolation and myelin figures, resulting from the digestion of damaged organelles. In those subjected to the concentration of 10 ppm, 75% of oocytes II and III presented an even higher level of vacuolation in most part of the cytoplasm limiting the presence of yolk granules (Oliveira et al., 2008). Such alterations indicate that the vacuoles would be autophagic, digesting cell components or cytoplasmic portions damaged by the chemical (Vendramini et al., 2012). Moreover, it was demonstrated that higher concentrations of fipronil would inhibit the development of oocytes (Oliveira et al., 2009, 2011).

Carvalho and Recco-Pimentel (2013) and Junqueira & Carneiro (2013) provided more details on the cell

alterations caused by the exposure to chemical products. The authors reported that autophagic vacuoles would be commonly observed in the cells undergoing degradation processes, and damaged portions of the cytoplasm and/or organelles would be recycled. This autophagy has the function of promoting a membrane turnover, providing elements to be reused by the cells (Roma et al., 2010).

The oocytes III from the *R. sanguineus* s. l. exposed to fipronil at the concentration of 10 ppm displayed significant alterations in the vitellogenic granules (protein), in the rough endoplasmic reticulum, in the mitochondria, and in the cytoskeleton elements. It is known that the arrangement of the cytoskeleton elements could be associated with a strategy of the cell to isolate areas of the cytoplasm (which would still be functional) from the damaged content, avoiding the spread of the damages and preserving this important cell (oocyte), responsible for the generation of a new individual (Jedrzejowska; Kubrakiewicz, 2007).

The oocytes IV from the individuals treated with fipronil at the concentrations of 1, 5 and 10 ppm showed extensive vacuolated areas, as well as a thinner chorion in comparison with the control group (Oliveira et al., 2008, 2009). The progressive increase in cytoplasmic alterations, including vacuolation, in comparison with oocytes IV from the ticks exposed to the previous concentrations, indicated the occurrence of much more intense autophagic processes, probably aiming to eliminate the damaged cytoplasmic elements more efficiently (Oliveira et al., 2009).

The oocytes V from the ticks treated with fipronil at the concentration of 1 ppm, did not show the typical characteristics of a cell capable of completing the developmental process, once the yolk granules (normally found in the cytoplasm in this stage) were replaced by extensive vacuolated areas. Additionally, the oocyte was surrounded by a thin membrane, which did not show the

same characteristics of the chorion, normally present in the oocytes in this developmental stage (Oliveira et al., 2008, 2009).

Two types of oocytes V were observed in the individuals subjected to the concentration of 5 ppm. The first type comprises those with yolk granules with varied electron-density, myelin figures, cytoplasmic vacuoles and a much thinner chorion supported by a ruptured basal lamina. The second type includes the oocytes with intact yolk granules, several vacuoles and a thicker chorion supported by an intact basal membrane (Oliveira et al., 2009). These data suggest that oocytes V react differently to the chemical agent. i.e., those with a thicker chorion would be less permeable and less susceptible to the penetration of external agents, which represents a higher level of preservation of the intracellular environment.

In the individuals subjected to the concentration of 10 ppm, the oocytes V suffered the most significant morphological alterations, confirming that this concentration of fipronil caused the greatest damage to the germ cells of *R. sanguineus* s. l. females (Oliveira et al., 2008).

Other studies on the reproductive system of ticks exposed to chemical products, such as the one developed by Friesen et al. (2003), showed that exposure to avermectin, in addition to the alterations described, caused the ovary shortening and modified the size and yolk content of the oocytes in *A. hebraeum* ticks. The authors still reported that the chemical products act directly in the inhibition of vitellogenin taken by the oocyte via hemolymph.

Thus, the high level of sensitivity of the female germ cells to the exposure to the chemical products used as acaricides was confirmed, once these substances are able to reduce fertility and cause damages to the CNS and other organs of great importance for the biological success of these ectoparasites.

Figure 29 – Schematics drawing of the histological alterations observed in oocytes (I to V) of *R. sanguineus* s. l. treated with fipronil in different concentrations (control group, 1 ppm, 5 ppm, 10 ppm). ch = chorium; gv = germ vesicle; I = oocyte stage I; II = oocyte stage II; III = oocyte stage III; IV = oocyte stage IV; V = oocyte stage V; nu = nucleolus; pm = plasma membrane; v = vacuoles; yg = yolk granules

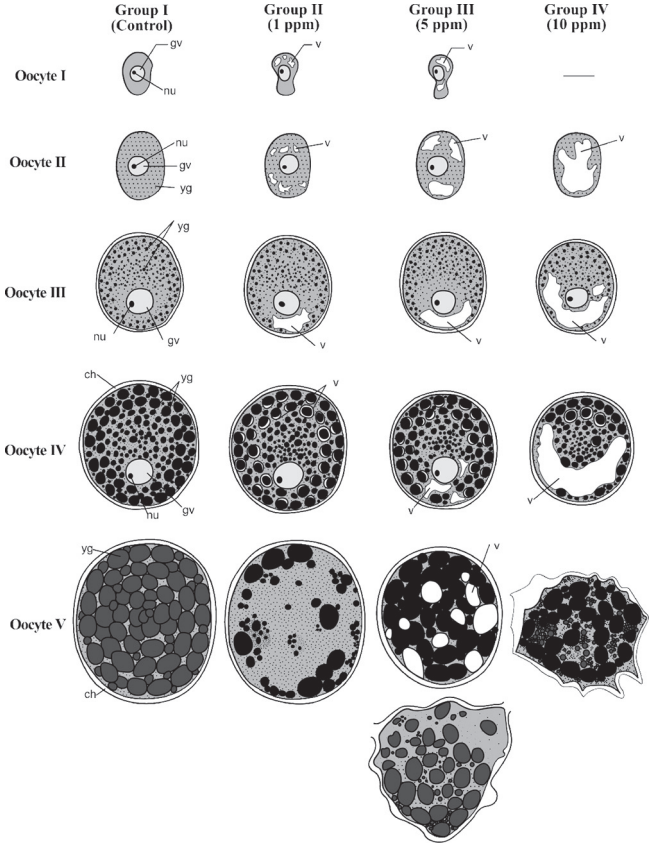


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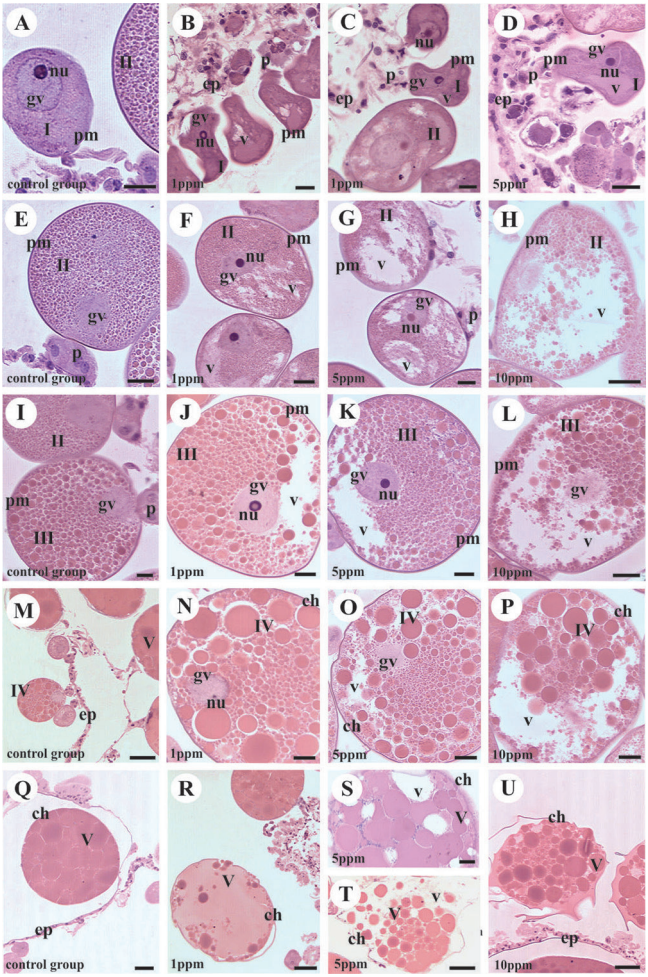
Figure 30 – Histological sections of *R. sanguineus* s. l. ovary stained by HE. (A) Detail of oocyte I of the group I (control). (B) Oocytes I of the group II (1 ppm). (C) Oocytes I and II of the group II (1 ppm). (D) Detail of oocyte I of the group III (5 ppm). (E) Detail of oocyte II of the group I (control). (F) Oocytes II of the group II (1 ppm). (G) Oocytes II of the group III (5 ppm). (H) Detail of oocyte II of the group IV (10 ppm). (I) Detail of oocytes II and III of the group I (control). (J) Detail of oocyte III of the group II (1 ppm). (K) Detail of oocyte III of the group III (5 ppm). (L) Detail of oocyte III of the group IV (10 ppm). (M) Oocytes IV and V of the group I (control). (N) Detail of oocyte IV of the group II (1 ppm). (O) Detail of oocyte IV of the group III (5 ppm). (P) Detail of oocyte IV of the group IV (10 ppm). (Q) Detail of oocyte V of the group I (control). (R) Oocyte V of the group II (1 ppm). (S) Detail of oocytes V of the group III (5 ppm). (T) Oocytes V of the group III (5 ppm). (U) Oocytes V of the group IV (10 ppm). ch = chorium; ep = ovary epithelium; gv = germ vesicle; I = oocyte stage I; II = oocyte stage II; III = oocyte stage III; IV = oocyte stage IV; V = oocyte stage V; nu = nucleolus; p = pedicel; pm = plasma membrane; v = vacuoles. Bars: (A-L, N-P, S) 0.02 mm; (M, Q-R, T) 0.1 mm; (U) 0.05 mm

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## Salivary glands

Studies on the acaricide action of fipronil on the salivary gland s of unfed and semi-engorged *R. sanguineus* s. l. females (Pereira et al., 2009) revealed the occurrence of severe alterations in these organs after exposure to different concentrations of fipronil. Additionally, it was demonstrated that this chemical does not modify the nature of the glandular secretion or the shape of the cells, but interferes in the acinar morphophysiology and accelerates the glandular degeneration.

The results revealed that fipronil at the concentration of 1 ppm modified the glandular acini type I, and the morphological alterations would be associated with the size, shape and mainly with the increase in the diameter of the lumen; however, acini II and III were not affected. It is important to note that acini type I are probably involved in the hydric balance of the tick during the fasting period (Binnington, 1978; Walker et al., 1985), or even associated with osmoregulation (excretory function) (Balashov, 1972a; Coons; Roshdy, 1973), while the acini II and III are essentially secretors.

The morphological alterations observed in acini I would represent an attempt to eliminate the fipronil (present in the circulating hemolymph) from the biological system. This confirms the role of acini I as osmoregulators, i.e., through the production of saliva, the toxic compound would be removed from the hemolymph, which was proven by the increase in the diameter of the lumen in these structures (Pereira et al., 2009)

Pereira et al. (2009, 2011), who developed studies using fipronil at the concentrations of 1, 5, and 10 ppm, demonstrated that the salivary glands of the females exposed to 1ppm showed more severe alterations than those from the control group. The alterations included loss

of turgidity and decrease in the number of acini, general tissue disorganization, irregularities in the shape of the nuclei in acinar cells, chromatin marginalization, nuclear fragmentation (Kerr et al., 1995), release of several apoptotic bodies (Kerr et al., 1995; Häcker, 2000) and intense cytoplasmic vacuolation (Bowen, 1993; Zakeri et al., 1995). Regardless the concentration, fipronil caused the salivary glands to degenerate, on a dose-dependent basis, i.e., as the concentrations increased the processes of cell death accelerated (Pereira et al., 2009, 2011).

The action of fipronil on the glandular cells was intense, once even resistant structures, as the spherocrystals found in the cytoplasm (Petit, 1970), were disorganized, with their shapes altered from round to completely irregular (Pereira et al., 2011). Another indicator of the high level of toxicity of fipronil was the disarrangement of the cytoskeleton elements, once a concentration of microfilaments was observed only in some regions of the cytoplasm, probably still intact areas, as a self-preservation strategy of the cell (Jedrzejowska, Kubrakiewicz, 2007). The disarrangement of the cytoskeleton proteins probably occurred due to the depolarization of the proteins, indicating that the cell would be in preparation for the following cell death stages (Ndozangue-Touriguine et al., 2008).

In general, studies on this thematic demonstrated that the chemical compound fipronil has the capability to accelerate glandular degeneration, interfering in the physiology of the organ and disrupting secretion synthesis and release, ultimately triggering death processes in the cells. The females are unable to complete the feeding process, which reflects on the reproductive process, impairing or preventing oviposition. Finally, the precocious glandular degeneration caused minor blood losses on the hosts and reduced the transmission of biopathogens that would normally be transported by these glands.

## Permethrin: action on the synganglion, salivary glands and female reproductive system

Permethrin, a synthetic pyrethroid, is widely used to control *R. sanguineus* s. l. ticks, with rapid action, causing paralysis to the individuals exposed via alterations in the CNS. Such alterations include the modification of the permeability of sodium channels, keeping them open in the neuron membranes (repetitive effect) and making the sensorial organs and the nerve terminations react in a particularly sensitive way, causing a state of hyperexcitation (Mencke et al., 2003; Dong, 2007). Permethrin, as well as other pyrethroids, acts on the CNS, preventing GABA neurotransmitter bindings to the receptor, blocking the flow of chloride ions to the interior of the cell and causing hyperexcitation, tremors, spasms, paralysis and, ultimately, death.

### Synganglion

The action of permethrin on the synganglion resulted in the degeneration of the organ through apoptosis involving autophagy, characterized by chromatin condensation and marginalization, formation of membrane blebs, nuclear fragmentation, alterations in the shape of nerve cells and membrane integrity, cytoplasmic retraction and decrease in the levels of acid phosphatase in the nerve tissue. These alterations were dose-dependent (Roma et al. 2013). Ultrastructurally, the neural lamella was affected, facilitating the penetration of the toxic agent into the synganglion. Significant modifications were observed in the perineurium glial cells and in the cortex neuronal cells, such as the emergence of irregular nuclei with chromatin marginalization, cytoplasm vacuolation and degeneration

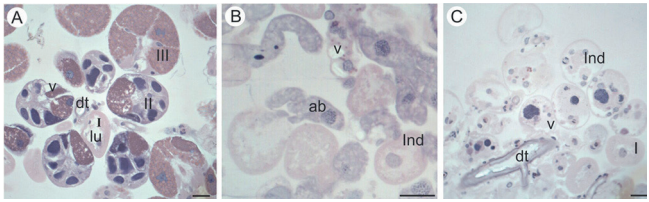
of the mitochondria. The rough endoplasmic reticulum of the neuronal cells was enlarged, indicating an intensification of the synthesis of hydrolytic enzymes used in the degradation of damaged cell structures (lysosome formation) (Roma et al. 2014).

Studies on the compound have confirmed its neurotoxic action on the nervous system metabolism in *R. sanguineus* s. l., affecting the physiology of other systems, all of them dependent on neural control. Furthermore, the chemical action is dose-dependent, once more significant alterations were observed as the concentrations increased, affecting the organism as a whole (Roma et al. 2013; 2014).

## Salivary glands

The action of permethrin on the morphophysiology of the glandular tissue (Figure 31) was confirmed, i.e., the compound caused significant alterations in the shape of the acini, intense cytoplasmic vacuolation in the acinar cells and a high level of disorganization in the glandular tissue, culminating in the progression of degeneration and consequent formation of apoptotic bodies (Nodari et al., 2011). The pyrethroids affected the salivary gland secretion mechanism, drastically reducing the synthesis of proteins, lipids and polysaccharides in the different cell types (Nodari et al., 2012a). The nucleus and the nuclear genetic material of the glandular tissue cells underwent severe alterations regarding their shape, size, chromatin marginalization and nuclear fragmentation. These cells displayed pyknotic nuclei, mainly under higher concentrations of the chemical (Nodari et al., 2012b). Cell death processes, which normally occur in the end of the female engorgement phase, were observed in the glandular cells.

Figure 31 – Histological sections of salivary glands of *R. sanguineus* s. l. semi-engorged females exposed to 206 ppm (A), 1031 ppm (B) and 2062 ppm (C) of permethrin stained with hematoxylin-eosin (HE). (A) showing I (type I acinus), II (type II acinus) and III acini (type III acinus). (B) showing I (type I acinus) and indeterminate acini (Ind). (C) showing I (type I acinus) with dilated lumen (lu), besides indeterminate acini (Ind). dt = duct; v = vacuoles; ab = apoptotic body; lu = lumen




Bars: (A-C) 25 µm

Thus, permethrin induced precocious degeneration in the glandular tissue by triggering significant morpho-physiological alterations in the acinar cells and intensified the production of enzymes associated with cell death process, affecting cell and nuclear integrity, consequently modifying the physiology.

## Female reproductive system

The action of permethrin on the ovaries of semi-engorged *R. sanguineus* s. l. females caused significant morphological alterations, changing the structure of the oocytes, as well as the emergence of large vacuolated regions in the cytoplasm, reducing the number of yolk granules and the size of the oocytes (Roma et al., 2010a). Regarding ultrastructure, the emergence of cytoplasmic vacuolation, disorganization of the plasma membrane and organelles and the presence of myelin figures can be

observed. Such alterations confirm that, even in low concentrations, the permethrin would have the potential to modify the germ cells, making the cell mechanisms unviable, including an attempt of the cell to minimize the action of the chemical by decreasing the area and surface in which the oocytes are in contact with the hemolymph. It is important to emphasize that such damages are irreversible, ultimately leading the cell to death (Roma et al., 2010b). Thus, the studies demonstrated that permethrin is a potent acaricide, able to impair reproduction in the *R. sanguineus* s. l. females exposed, even to lower concentrations of the chemical (Roma et al., 2010a, b).

## Fluazuron: action on the integument and midgut

Fluazuron belongs to a class of arthropod growth regulators (AGRs) (Taylor, 2001; Coop et al., 2002, Vieira, 2009; Vieira, 2012). The AGRs are classified into: a) juvenile hormone analogues, which prevent the activation of genetic sequences essential for the development of organs and tissues; b) benzoylphenylureas, inhibitors of the synthesis and/or deposition of chitin; and c) triazin and pyrimidine derivatives, which interfere in ecdysis (Chavasse; Yap, 1997).

Fluazuron was the first growth regulator to be registered for the control of Ixodidae ticks (Bull et al., 1996; Zenner et al., 2004), due to its systemic action via blood flow and remarkable acaricide efficacy (Catto et al., 2010; Andreotti, 2010). The commercial formulations are Acatak®, Superhion®, Tackzuron®, and Contratack®, manufactured by different companies.

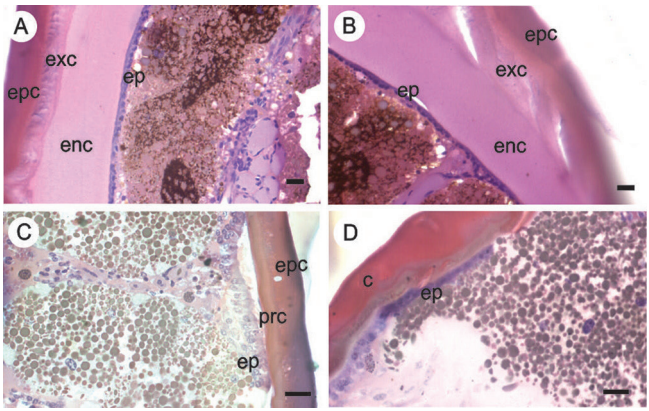
## Integument

Studies developed by Oliveira et al. (2013) provided information on the effects of synthetic chemicals on the integument of ticks, demonstrating the action of different concentrations of fluazuron (Acatak®, Novartis) (20 mg/Kg, 40 mg/Kg and 80 mg/Kg of fluazuron/body weight) applied pour-on to the hosts.

The action of fluazuron was demonstrated through the observation of alterations in the integument and midgut cells of nymphs from the different groups compared with group I (control) (Figure 32) (Oliveira et al., 2014). The alterations were mainly associated with the cuticle subdivisions, size of the digestive cells and amount of blood element taken and digestive residue accumulated, as well as with the presence of vacuoles in the cytoplasm of the digestive cells (Oliveira et al. 2013, 2014).

After the host inoculation with 20 mg/Kg, the integument of the nymphs showed thinner epicuticle and procuticle (exocuticle and endocuticle) in comparison with the control group. In the nymphs fed on the host exposed to 40 mg/Kg of fluazuron, the integument did not display the subdivisions normally found in the procuticle (exocuticle and endocuticle). In the ones fed on hosts exposed to 80 mg/Kg of fluazuron, the integument showed the most significant alterations, once all the cuticle layers (epicuticle and procuticle with exocuticle and endocuticle) seemed to be undergoing a fusion process, originating a single layer (Oliveira et al., 2013, 2014). These data could have resulted from the absence of chitin synthesis, necessary for the formation of the procuticle, which would not be synthetized and/or deposited on the integument due to the action of fluazuron, causing the formation of a single layer.

Figure 32 – Histological sections of fluazuron-treated nymphs tick *R. sanguineus* s. l. (A-D) Hematoxylin and eosin (HE) staining. (A) Control Group, (B) Group II (20mg/Kg), (C) Group III (40mg/Kg), (D) Group IV (80mg/Kg). c = cuticle; ep = epithelial cells; epc = epicuticle; enc = endocuticle; exc = exocuticle; prc = procuticle




Bars: (A-B) 10um, (C-D) 20um

The groups, except the control one, showed no evidence of ecdysis, confirming that fluazuron would inhibit the molting process in the nymphs fed on the exposed hosts, probably by preventing the synthesis and/or deposition of chitin to form a new cuticle to cover the body during periodic ecdysis (Oliveira et al. 2014).

The interference of fluazuron in the synthesis and/or deposition of chitin would cause an irregular deposition of the endocuticle, which would affect the elasticity and rigidity of the cuticle, preventing the deposition of a new cuticle and, consequently, the successful completion of ecdysis process (Oberlander; Silhacek, 1998; Palli; Rentnakaran, 1999; Oberlander; Smagghe, 2001). According to Palli and Retnakaran (1999), the interference in the synthesis and/or deposition of chitin would occur via biochemical processes, as follows: inhibition of

chitin-synthase or the flow UDP-N- acethylglycosamine through the biomembranes; interruption of chitin and cuticle protein bindings; interference in ecdysone metabolism; activation of actinases involved in chitin catabolism; inhibition of proteases that activate chitin-synthase; interruption of the conversion glucose to fructose-6-phosphate and inhibition of DNA synthesis, among others.

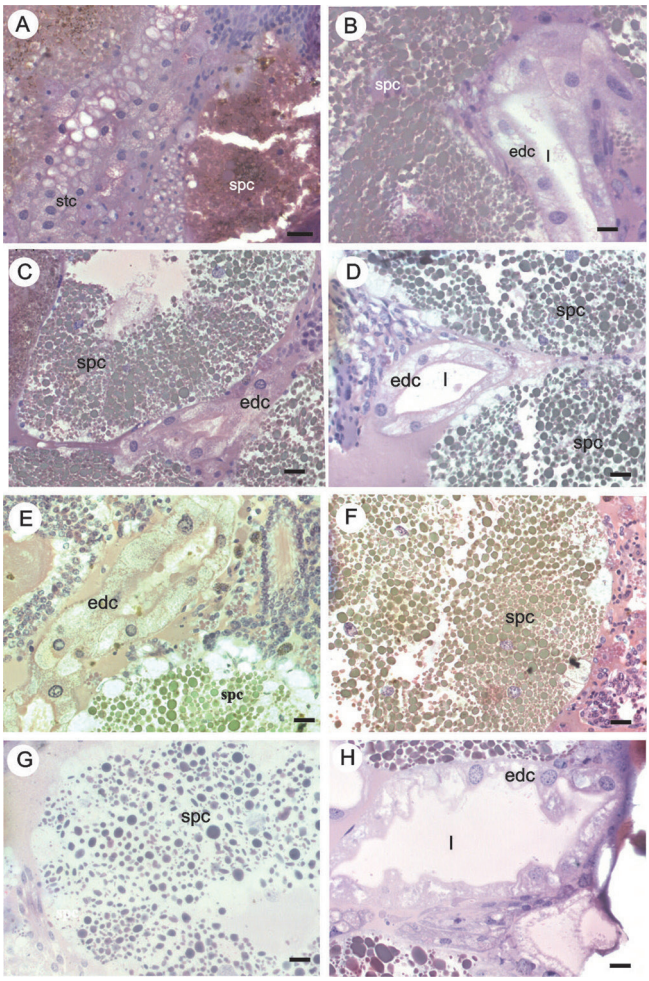
Therefore, it was demonstrated that the arthropod growth regulator fluazuron (active ingredient of Acatak®) interfered in the deposition of the integument in *R. sanguineus* s. l. nymphs fed on inoculated rabbits. The chemical impaired the process of cuticle polymeralization, affecting the development of the nymphs to adult phase. These data confirm the potential of fluazuron as a promising strategy to control *R. sanguineus* s. l. nymphs.

## Midgut

Oliveira et al. (2013, 2014) studied the effects on fluazuron on the midgut epithelium in engorged *R. sanguineus* s. l. nymphs (Figure 33). The authors demonstrated the alterations caused by the action of the compound, which included modification in the size of digestive cells, amount of blood element taken and digestive residue accumulated, as well as the presence of vacuoles in the cytoplasm of the digestive cells (Oliveira et al., 2013, 2014).

The authors used the concentrations of 20 mg/Kg, 40 mg/Kg and 80 mg/Kg of fluazuron /body weight applied pour-on to the hosts. On the first day (24 hours) after inoculation with fluazuron and distilled water (Control Group) artificial infestation was performed on all hosts, and after more 24 hours (for chamber fixation), each host was infested with *R. sanguineus* s. l. nymphs using a syringe. Soon after engorgement was completed and the engorged nymphs (metanymphs) dropped off the hosts,

Figure 33 – Histological sections of fluazuron-treated nymphs tick *R. sanguineus* s. l. (A-H) Hematoxylin and eosin (HE) staining. (A-B) Control Group I, (C-D) Group II (20 mg/Kg), (E-F) Group III (40 mg/Kg), (G-H) Group IV (80 mg/Kg). edc = Empty digest cells; l = lumen; spc = Spent cells; stc = Stem cells (generative cells)




Bars: (A) 10 mm; (B-H) 20 mm

they were placed on labelled Petri dishes. The dishes were placed in BOD incubator and monitored for 7 days. This period was established to complete 14 days after treatment with fluazuron (from the day of application of the chemical on the host): 7 days to complete the engorgement and 7 days of observation, considering that a maximum of 14 days should be enough for the chemical to cause death of the ectoparasites, (according to Acatak®, Novartis manufacturer information).

The nymphs fed on the host rabbits treated with 20 mg/Kg of fluazuron presented alterations only in the midgut cells named Spent cells, which accumulated less blood in the endosomes and digestive vacuoles, and showed fewer digestive vacuoles and hematin residual bodies (Oliveira et al., 2013). In the exposition with 40 mg/Kg of fluazuron, the individuals displayed alterations in both digestive cell stages, i.e., Spent cells and Empty cells, as well as in the midgut lumen. Spent cells are smaller, with fewer endosomes, digestive vacuoles and residual bodies, while Empty cells showed small apex undulations and the lumen is evident (Oliveira et al., 2014). The fluazuron concentration of 80 mg/Kg caused the most significant damages to the midgut. The Spent cells are much smaller and with few and small endosomes, digestive vacuoles and residual bodies throughout the cytoplasm. The Empty cells are less frequently observed and show large invaginations in the plasma membrane, as well as extensive vacuolated regions, mainly next to the lumen, which contains few digestion residues (Oliveira et al., 2013, 2014). These data suggest that fluazuron would be inhibiting the synthesis and/or deposition of chitin used to form the peritrophic membrane, normally found in the midgut of arthropods (Matsuo et al., 2003). This membrane is responsible for the selective permeability of the midgut cell plasma membrane (Terra, 2001), for

limiting the area where the digestive events occur (Shen et al., 1999; You et al., 2003) and for protecting the midgut epithelium from microorganisms and digestive enzymes (Matsuo et al., 2003). The increase in the concentrations of fluazuron intensified the damages in the midgut cells and the peritrophic membrane impairment. The epithelial cells have important functions: absorption of the blood ingested, lysis of blood cells in the digestive vacuoles, formation of digestion residues and release of nutrients after the digestion process (Agbede; Kemp, 1985; Koh et al., 1991; Agyei; Runham, 1995). The partial or complete absence of the peritrophic membrane allow the direct contact of digestive enzymes and the epithelial cells, which would be damaged and have their important functions affected (Sonenshine, 1991; Harrison, 1999). The whole metabolism of the organ would consequently be impaired, hindering the viability of the individual, once Ixodidae ticks feed once in each stage, obtaining all the nutrients they need to grow and develop from a single blood meal (Sonenshine, 1991; Harrison, 1999). Gelbic et al. (2002) reported a decrease in hemolysis (lysis of blood elements) in the midgut of *Culex quinquefasciatus* larvae subjected to methopren. Lethal mechanism were observed by Borges et al. (2004) in the midgut cells of *Aedes aegypti* subjected to diflubenzuron.

Thus, the potential of fluazuron to interfere in the digestive processes of *R. sanguineus* s. l. nymphs fed on inoculated hosts was confirmed. The chemical affected the absorption of the blood ingested, minimizing the provision of nutrients to the nymphs, which would not be able to develop and evolve to the following instar. Such evidence point this arthropod growth regulator (AGR) as a promising alternative to control the ectoparasites in this stage of development.

## Dinotefuran: action on the female reproductive system

The neonicotinoids, a chemical group with the potential of controlling several pests, have grown in the market since the commercialization of pyrethroids, and are currently considered a promising control strategy, with excellent chemical and biological properties, presenting low toxicity to the mammals (Nauen; Bretschneider, 2006).

Dinotefuran belongs to the third generation of neonicotinoids (Wakita et al., 2003; 2005; 2011), and its main commercial formulations are Vectra 3D® and Protetor Pet®.

This chemical is efficient against a wide variety of pests, including *Periplaneta americana*, *C. quinquefasciatus, Anopheles gambi, Aedes aegypti*, and is able to eliminate even strains that are resistant to other groups of insecticides, such as the pyrethroids, which demonstrates its effectiveness and excellent insecticide properties (Kiriyama; Nishimura et al., 2002; Wakita et al., 2003; Corbel et al., 2004).

Toxicological and ecotoxicological studies have demonstrated that the dinotefuran presents low toxicity for mammals, birds, aquatic animals (Kagabu, 1997; Uneme et a., 1999; Wakita et al., 2005,), and the environment as well (Wakita et al., 2005; EPA, 2009; Wakita, 2011). Its chemical, physical, biological and toxicological properties place the chemical in an important position, stimulating further research on its effects and action mechanisms.

### Female reproductive system

Studies have been aimed at analyzing the effects of different concentrations of dinotefuran on the germ cells of semi-engorged *R. sanguineus* s. l. females (Oliveira et al., 2016; 2017).

Oliveira et al. (2016) analyzed semi-engorged *R. sanguineus* s. l. exposed to the concentrations of 5000, 6250 and 8334 ppm of dinotefuran and observed that oocytes I, II and III underwent more significant alterations as the concentrations increased, including the loss of chorion, which enabled the chemical to penetrate the cell via the germ cell plasma membrane. Once the toxic is installed in the interior of the cell, it damages the organelles, which undergo lysis in the autophagic vacuoles (Oliveira et al., 2016), corroborating Oliveira et al. (2009), Roma et al. (2010) and Vendramini et al. (2012), who studied *R. sanguineus* s. l. ticks exposed to fipronil, permethrin and andiroba oil, respectively.

Moreover, the high level of vacuolation in oocytes II and III restricts the area that would be occupied by yolk granules (Oliveira et al., 2016). The decrease in the number of granules would impair, partially or totally, the development of oocytes, affecting the progression to more advanced developmental stages (IV and V) to culminate in the mature cell stage, capable of being fertilized (Oliveira et al., 2017).

Oocytes IV and V did not undergo extensive damages as the ones in initial developmental stages. The concentration of 5000 ppm did not affect these oocytes, while at the concentration of 6250 ppm rare alterations were detected, i.e., the membrane showed few pleats and rare vacuoles were observed in the cytoplasm. The individuals exposed to 8334 ppm of dinotefuran showed vacuoles among the yolk granules of oocytes IV, and oocytes V displayed pleats in the membrane, as well as less dense yolk granules, located in the central region. Ruptured granules and vacuoles were observed in the peripheral region (Oliveira et al., 2016; 2017). These data indicate that, even if the oocytes in early developmental stages managed to survive, trying to synthetize the yolk proteins on a small

scale, they could be affected by the dinotefuran in more advanced stages (IV and V) (Oliveira et al., 2008). The presence of the chorion, (Oliveira et al., 2005), responsible for preserving the structure of the future embryo and, consequently, the species (Denardi et al., 2004), would protect the eggs against mechanical impacts and temperature variations, preventing desiccation, minimizing predation and ensuring gas exchange (Hinton, 1982). As this resistant membrane represented a barrier to be overcome by the dinotefuran, it gave the oocyte more permeability, hindering the penetration of the chemical (Oliveira et al., 2017). This process was reported by Oliveira et al. (2008) and Roma et al. (2010).

Still regarding the damages caused by dinotefuran, it is important to emphasize that the ovary wall of semi-engorged *R. sanguineus* s. l. females underwent alterations after the exposure; however, only in the individuals subjected to the concentration of 8334 ppm (Oliveira et al., 2016). The epithelium, instead of small cuboid cells with rounded nuclei, showed flat cells with pyknotic nuclei (Oliveira et al., 2016). Such damages will certainly prevent the organ from performing its functions, such as forming the pedicel, structure that fixes the oocyte to the ovary wall and provides exogenous yolk elements to constitute the yolk granules, supporting the growth and development of germ cells (Oliveira et al., 2016; 2017). Therefore, the dinotefuran affected the number of mature oocytes, i.e., decreased the number of those that would complete vitellogenesis, consequently affecting the preservation of the species.

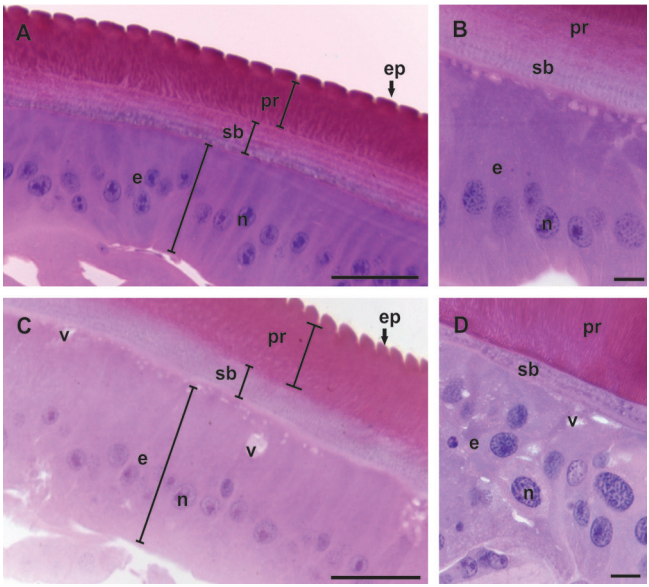
## Synergistic components: action on the integument

The extensive and indiscriminate use of acaricides contaminate the environment and the toxicity of the chemicals is affect not only the target organisms, but also other animals and the human beings as well. Moreover, the selection of resistant populations is another important consequence of this practice (Dantas-Torres, 2008).

According to Araújo (2015), several studies have demonstrated the occurrence of *R. (Boophilus) microplus* populations resistant to cypermethrin (Silva et al., 2005; Reck et al., 2014), fipronil (Castro-Janer et al., 2010; Reck et al., 2014), chlorpyrifos, amitraz, ivermectin and fluazuron (Reck et al., 2014). In *R. sanguineus* s. l., studies have reported resistance to amitraz (Miller et al., 2001; Silva et al., 2005), cypermethrin, deltamethrin and coumaphos (Miller et al., 2001; Borges et al., 2007).

Due to the great medical and veterinary importance of the ticks and the emergence of resistant populations, several studies on new bioactives have been developed, aiming to find safer and efficient products (synthetic or natural) to control these pests (Papa et al., 2011). Vectra® 3D is one of the recently launched synthetic products, a combination of permethrin (pyrethroid), dinotefuran (neonicotinoid) and pyriproxyfen (pyridine) the product has shown a persistent acaricide and repellent potential, and the combination of three active ingredients in a single acaricide results in a synergic effect able to fight infestations and cause the death of the target organisms.

Figure 34 – Histological sections of the integument of semi-engorged *R. sanguineus* s. l., stained with Hematoxylin-eosin (HE). (A-B) Group Control, (C-D) Group treatment. ep= epicuticle; e= epidermis; n= nucleus; sb= subcuticle; pr= procuticle; v= vacuoles




Bars: (A; C) 50 µm; (B; D) 10 µm

Pyriproxyfen, one of the compounds of Vectra® 3D, is manufactured by the companies *Sumitomo Chemical Co. Ltda., Syngenta and BePharm Ltda.* In the EUA, it is sold under the commercial name *Nylar,* while in Europe it is sold as *Cyclio* (Virbac) and *Exil Flea Free TwinSpot* (Emax). In Brazil, it is commercialized under the name Tiger 100 CE and has been used to protect cotton crops against flies (Ishaaya et al.,1995) and also against *Aedes aegypti*, vector of dengue and chikungunya diseases. The substance is analogue to the juvenile hormone, a growth regulator that acts on the endocrine system of the insects, interrupting the life cycle by producing infertile eggs and

blocking the development of larvae, preventing reproduction (Palma et al., 1993).

Camargo-Mathias et al. (personal communication) have developed studies to evaluate the morphological alterations caused to the integument of *R. sanguineus* s. l. females exposed to Vectra® 3D (54.45 mg/mL of dinotefuran, 396.88 mg/mL of permethrin and 4.84 mg/mL of pyriproxyfen) (Figure 34). The authors demonstrated that Vectra® 3D is efficient to control *R. sanguineus* s. l. ticks, not only for its neurotoxic action, but also for affecting the structural organization of the integument, disarranging the epithelial cells, responsible for secreting and maintaining the layers of this exoskeleton. Thus, the product interferes in the efficiency of the first protection barrier of the individuals, leaving them exposed to climatic and environmental impacts.

## Deltamethrin: action on the synganglion and female reproductive system

Deltamethrin, a semi-synthetic pyrethrin type II, is a potent calcineurin inhibitor. It was firstly described by Elliott et al. (1974) and registered by the Environmental Protection Agency – EPA (Deltamethrin Summary Document Registration Review, 2010). This chemical interferes in several cell processes and signal transduction pathways, blocking the transmission of nerve impulses and causing paralysis to the insect (Oga; Camargo; Batistuzzo, 2014; Rusnak; Mertz, 2000).

This chemical is widely used to control spiders, fleas, ticks, carpenter ants, cockroaches and bugs, causing immediate paralysis and death by altering the nervous system organs (ETN, 1995; Deltamethrin. Pesticide Information, 2004; Pereira et al., 2017). Its active ingredient is

registered as Butox P CE 25®, Butoflin®, Butoss®, Cislin®, Crackdown®, Decis®, Delsekle®, Deltaphos®, K-Othrin® and K-Otek® (Briggs; Council, 1992).

The toxic effects of deltamethrin are less harmful to mammals, once these animals have higher body temperature, are bigger and have less sensitive sodium channels (Bradberry et al., 2005). The $DL_{50}$ of deltamethrin orally inoculated in rats was 5000 mg/Kg diluted in aqueous media (Pesticide Residues In Food, 2000: Deltamethrin, 2001), for *R. sanguineus* s. l. larvae the $DL_{50}$ was 3.2 mg/Kg (Borges et al., 2007).

## Synganglion

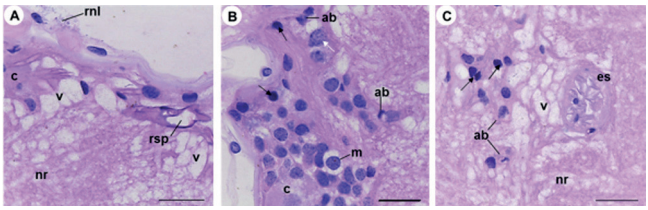
Currently, the most widely used chemical acaricides target the central nervous system (synganglion), once this organ is responsible for controlling all the systemic functions of the ticks. In this sense, Pereira et al. (2017) provided the first information on the effects of deltamethrin on the synganglion of unfed *R. sanguineus* s. l. females, using the commercial product Butox® P CE25, of which deltamethrin is the active ingredient.

In that study, the females were exposed to the concentrations of 1.5 ppm (23 times lower than the one recommended by the manufacturer), 3.12 ppm (11 times lower) and 6.25 ppm (5 times lower). The results of the morphological analyses showed dose-dependent alterations in the synganglion morphology (mainly in the regions of the cortex and neuropile), i.e., as the concentrations increased, more significant damages were observed (Pereira et al., 2017).

The concentration of 6.25 ppm caused significant alterations in all the regions of the synganglion, confirming the high toxicity of the chemical: a) damages to the peripheral membranes (neurilemma and perineurium) (Figure 35A), which act as selective barriers for the penetration of

substances in the organ and support the metabolic regulation of the neural activities through the glial cells; b) cortical region causing a high level of disorganization and degeneration in the tissue, mainly altering the cytoplasm of the neuron bodies, significant vacuolation of the cytoplasm, and cells in different stages of apoptotic cell death (Figure 35B); c) irregular folds throughout the subperineurium (membrane that separates the cortical region form the neuropile) and ruptures in some parts of the organ (Figure 35A), allowing the neuron bodies to move from the cortex region to the neuropile (Figure 35C), causing a complete structural disorganization of the organ with consequent loss of functionality; and d) disarrangement of the neural fibers in the neuropile region, causing the emergence of empty spaces in the extracellular matrix, mainly in the regions next to the esophagus opening (Figure 35C).

Figure 35 – Histological sections of the synganglion of unfed *Rhipicephalus sanguineus* l.s. females exposed to 6.25 ppm of deltamethrin and stained with HE. (A) Detail of the damages in the peripheral membranes and subperineurium. (B) Disorganization and degeneration of the cortical region (c). (C) Invasion of the neuron bodies in the neuropile region (nr) next to the esophagus (es) due to the rupture of the subperineurium (rsp). rnl = rupture of the neurilemma; v = vacuolization; ab = apoptotic body; m = chromatin marginalization; black arrow = pyknotic nuclei; white arrow = nuclear hypertrophy




Bars: (A-C) 20 µm

A similar process of gradual degeneration of the central nervous system in *R. sanguineus* s. l. was observed by Roma et al. (2013), who developed studies with another pyrethroid, the permethrin. Thus, the results found by Pereira et al. (2017), confirmed the neurotoxic potential of deltamethrin, even in lower concentrations than the ones recommended by the manufacturer, and also revealed the potential of the chemical to cause total tissue disorganization. The authors also demonstrated that deltamethrin causes alterations in systemic functions, once the synganglion is a key organ in the tick metabolic processes, and, when morphologically disorganized, is unable to control other important processes, such as feeding and reproduction (ovary development).

## Female reproductive system

The ovary of *R. sanguineus* s. l. was studied by Camargo-Mathias et al. (Camargo-Mathias et al., 2017), who analyzed semi-engorged females immersed in the concentrations of 25, 50, 100, and 200 ppm of deltamethrin (Figure 36). These concentrations were stablished according to the results of $DL_{50}$ in larvae from the same species exposed to deltamethrin (Borges et al., 2007).

This study demonstrated that the chemical was efficient to inhibit ovary growth, causing damages to the female germ cells on a dose-dependent basis, i.e., the most significant alterations were observed in the exposures using the concentrations of 100 and 200 ppm. In general, the compound affected: a) the morphology of the oocytes, which had their shape changed from round to irregular; b) the composition and organization of the yolk granules, which underwent fusion processes resulting in extremely large and mischaracterized granules; c) the cytoplasm, causing intense vacuolation, even in the

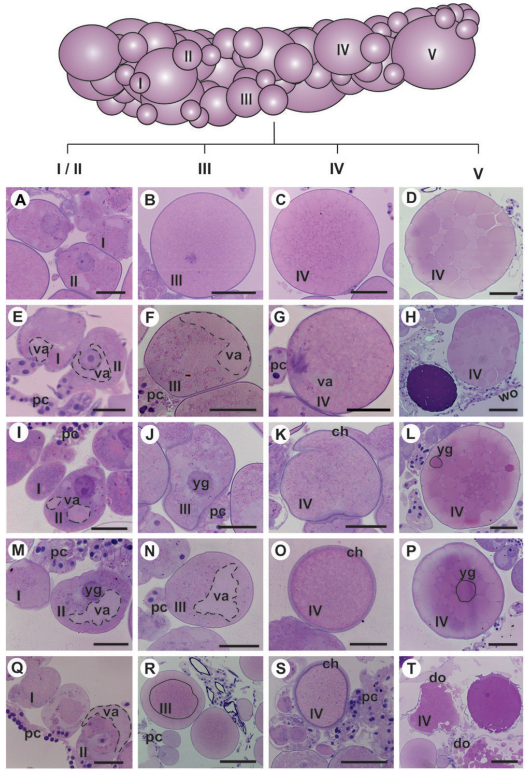
ones in early stages of development and d) the chorion, which was partially secreted, once it was thinner, probably allowing the oocyte to lose its original form (Camargo-Mathias et al., 2017).

Therefore, it was stablished that the chemical interfered directly in the vitellogenesis, causing significant cytoplasmic disorganization the oocytes, marked by intense vacuolation. Moreover, it caused serious damages to the germ vesicles (nuclei) of some oocytes, such as hypertrophy, chromatin fragmentations, and the formation of apoptotic bodies (strongly stained by hematoxylin), clearly indicating that those cells would be unviable and would not be able to advance the developmental stages. Alterations in the synthesis and secretion of the chorion (secreted in the final developmental stages) were also observed.

This study confirmed that deltamethrin acts not only on the target organ (central nervous system) of *R. sanguineus* s. l. ticks, but also on the reproductive system of the females, preventing them from originating new individuals.

Figure 36 – Vitellogenesis alterations histology of *R. sanguineus* s. l. ticks caused by the exposure to deltamethrin. Scheme showing oocytes in different stages of development (I-V) adhered to the ovary wall. (A-D) Histology of the ovary of ticks belonging to the control group where oocytes are observed at different stages of development and with preserved morphology. (E-F) Histology of the ovary of ticks belonging to the group exposed to the concentration of 25 ppm of deltamethrin in which it is evident the beginning of the loss of original rounded morphology of oocytes as well increase of cytoplasmic vacuolization (va) and with evident wall of the ovary (wo). (I-L) Histology of the ovary of ticks belonging to the group exposed to the concentration of 50 ppm of deltamethrin in wich it is evident the increase

of citoplasmatic vacuolization (va) of the oocytes in I, and the loss of yolk granules in the periphery of the oocyte in L. (M-P) Histology of the ovary of ticks belonging to the group exposed to the concentration of 100 ppm of deltamethrin in wich it is evident the increase of citoplasmatic vacuolization (va) of the oocytes in M and N. Loss of yolk granules in the periphery and evident fusion of yolk granules in P. (Q-T) Histology of the ovary of ticks belonging to the group exposed to the concentration of 200 ppm of deltamethrin in wich it is observed extensive citoplasmatic vacuolization in Q. In T it is observed a degenereted oocyte (dg) with loss of yolk granules as well as total loss of oocytes form.




Bars: (A-T) 50 mm

# 12
# Natural acaricides

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## *Azadirachta indica* (Neem)

Several methods to control the proliferation of tick species have been developed, with diverse kinds of formulations and methods for application. Some of these methods use leaves, husks and seeds of plants, such as *Azadirachta indica* (Figure 37), popularly known as neem. The neem seed oil has been proven efficient to control *Amblyomma variegatum* ticks, offering little or no harm (Ndumu et al., 1999). Azadirachtin (*A. indica* active principle) did not present significant effects when diluted in acetone and applied at high concentrations against *Hyalomma dromedarii*. However, at low concentrations, lower than 5000 mg/L, the compound caused significant reduction in the feeding activity of the larvae on camels,

with a decrease in ecdysis and consequent reduction in the body mass of engorged (Al-Rajhy et al., 2003).

The extracts obtained from neem seeds are also efficient against *H. anatolicum excavatum* ticks, altering hatchability and causing the emergence of misdeveloped larvae, (or even unable to survive), in addition to inducing death of larvae and unfed adults (Abdel-Shafy et al., 2002). The concentrations of 20% – 100% of neem oil were efficient against *R. (Boophilus) decoloratus,* causing larvae mortality (Choudhury, 2009). Neem was also proven effective against *R. (Boophilus) microplus* ticks, causing the mortality of engorged females in the first days of exposition, interfering in oviposition and causing alterations comparable with synthetic pyrethroids (Broglio-Micheletti et al., 2010; Srivastava et al., 2008).

Figure 37 – (A) *A. indica* tree (Neem). (B) Detail of leaves and fruit of neem. (C) Detail of neem flowers

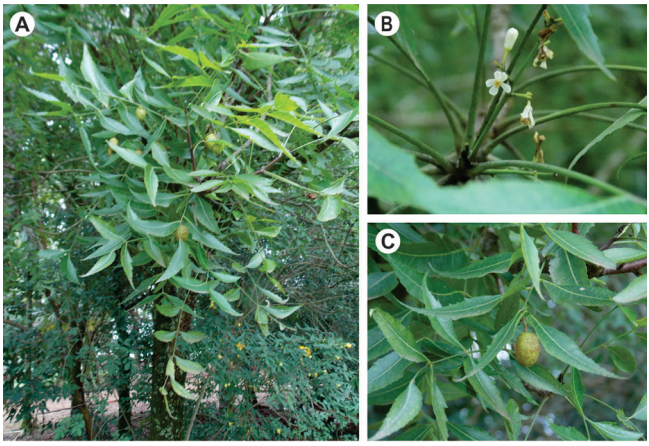



Photo by Travaglini, R.V., Piracicaba, São Paulo, Brazil

The tick feeding and reproductive processes are important physiological mechanisms, used as parameters to analyze toxicity levels of synthetic and natural

acaricides; the latter considered a very promising study line (Denardi et al., 2011).

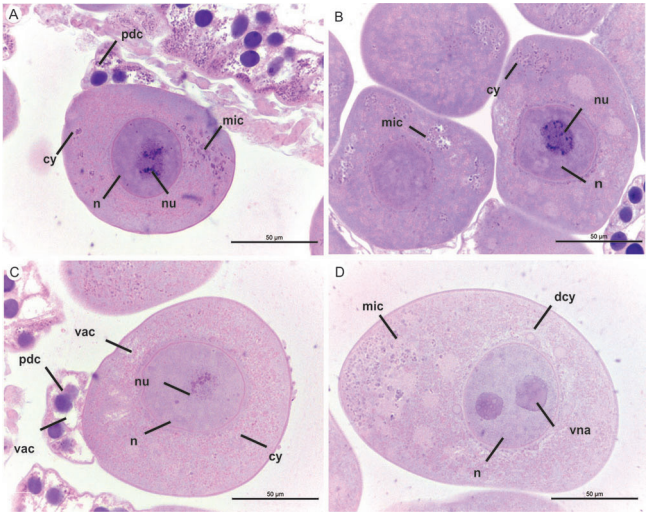
The researchers of BCSTM (Brazilian Central of Studies on Ticks Morphology, Unesp, Rio Claro, São Paulo, Brazil) exposed the ovaries of *R. sanguineus* s. l. ticks to aqueous extracts obtained from neem leaves and obtained important results regarding the use of this plant to control ticks, once it acts on reproductive processes of the ectoparasites (Denardi et al., 2010, 2011). According to the authors, neem extracts did not have the potential to cause the mortality of adult ticks; however, low concentrations were able to cause important morphological alterations in the germ cells (oocytes).

Denardi et al. (2010) exposed *R. sanguineus* s. l. to aqueous extracts of neem leaves, at the concentrations of 10 and 20%, and reported morphological alterations in the ovaries (Figure 38). The pedicel cells lost the original shape, oocytes presented cytoplasmic vacuolation, the nuclei became irregular and the nucleoli fragmented or ring-shaped, and the chorion showed folds and deformation. These results confirm the toxic potential of *A. indica* extracts. The alterations occurred on a dose-dependent basis, with evidence of cell death processes in higher concentrations. The same authors evaluated the ovary physiology using histochemical techniques and reported that the lower concentrations of neem (10%) were more efficient, with significant decrease in the level of polysaccharides in the cytoplasm of the oocytes and in the size and number of protein granules.

Denardi et al. (2012) demonstrated that the exposure to the extract affects the structure of the cells. These ultrastructural alterations included disorganization of the organelles and membranes. The morphology of young oocytes was affected: their shape changed from round to irregular; the mitochondria were damaged and some lost

the cristae, showing the accumulation of electron dense material in their interior; the rough endoplasmic reticulum (RER) cisternae were disorganized; and autophagic vacuoles and myelin bodies were found throughout the cytoplasm. The number of mature oocytes and the size of microvillosities were reduced. At the concentration of 10%, the chorion layers were no longer observed, nor the pores (commonly found in this structure, with electron

Figure 38 – Histological alterations observed in ovaries of semi-engorged *R. sanguineus* s. l. females ticks exposed to neem seed oil, stained with hematoxylin-eosin. (A) Oocytes of ticks exposed to distilled water; (B) Oocytes of ticks exposed to solution of 20% of neem oil dilluted in 10% ethanol; (C) Oocytes of ticks exposed to solution of 40% of neem oil dilluted in 10% ethanol; and (D) Oocytes of ticks exposed to solution of 60% of neem oil dilluted in 10% ethanol. cy = cytosol; dcy = disorganized cytosol; mic = microorganisms; n = nucleus; nu = nucleolus; pdc = pedicel cells; vac = vacuolization; vnu = vacuolated nucleolus.




Bars: (A-D) 50 µm

lucid aspect), which were then filled with electron dense material, suggesting that the oxygenation of eggs until larvae eclosion was interrupted. The higher concentration caused the rupture of the chorion, and the cytoplasm of the oocytes showed a large number of autophagic vacuoles and disorganized RER.

Thus, the neem extract properties of inhibiting the processes of feeding and, more importantly, oviposition in ticks, qualify the extract as a potential and sustainable strategy for the integrated control of these ectoparasites. In addition, the oil is not toxic for non-target organisms, including the human beings, once it is commonly used against infections, including those caused by ticks, due to its acknowledged fungicide and bactericide properties (Choudhury, 2009; Ndumu et al., 1999).

## *Carapa guianensis* (Andiroba oil)

The oil extracted from the seeds of *Carapa guianensis* (Figure 39), from the family Meliaceae, a large tree found in the north of South America (mainly in the Amazon basin), Central America, Antilles and tropical Africa, popularly known as andiroba, has been considered very promising to control pests, with repellent action against Arthropoda and high acaricide potential (Farias et al., 2007; Vendramini et al. 2012).

In addition to affecting fertility by inhibiting oviposition in *R. (B.) microplus*, *R. sanguineus* s. l. and *Anocentor nitens* ticks (Farias et al., 2007; 2009), the oil presents low toxicity to non-target organisms (offering less harm to the environment and public health) (Costa-Silva et al., 2006; 2007), and avoids the selection of resistant strains, which confirms its relevance as an alternative to control ectoparasites (Häuserman et al., 1992).

The first information on the effects of andiroba oil on the reproduction of female ticks was provided by Sousa et al. (2005) and Farias et al. (2007), in *in vitro* studies. The authors demonstrated 100% efficiency in the control of *Anocentor nitens* and *R. (Boophilus) microplus* ticks, respectively, through the analysis of engorged female mortality and oviposition inhibition.

Farias et al. (2009) studied *A. nitens* and *R. sanguineus* s. l. engorged females subjected to five concentrations of andiroba oil (100, 50, 30, 25 and 10%) diluted in distilled water and Tween 80 used as adjuvant, and reported that, in addition to the mortality of the engorged females, the chemical reduced oviposition, and the production of infertile eggs. The results hence confirmed the potential of andiroba oil against *R. sanguineus* s. l. and its inhibitory action on oviposition.

Vendramini et al. (2012) developed the first studies on the action of andiroba oil on the reproductive system of *R. sanguineus* s. l. females, evaluating the effects of different dilutions on the germ cells and ovary tissue of semi-engorged females. The authors exposed the semi-engorged *R. sanguineus* s. l. females to the concentrations of 5, 10 and 20%, and reported the occurrence of irreversible alterations both in the germ cells and in the epithelium. Such alterations were dose-dependent, i.e., were more severe as the concentrations increased, and were mainly regarding the shape of the oocytes, presence, size and location of cytoplasmic vacuoles, decrease in the number of yolk granules, changes in cell shape and the presence of vacuolated cells with pyknotic nuclei in the ovary epithelium.

The oocytes I from the females exposed to andiroba oil at the concentrations of 5% showed cytoplasmic vacuolation, fragmentation and the germ cells were not easily observed, which, according to Vendramini et al. (2012)

would occur due to the action of the product, causing structural damages even in lower concentrations. At the concentrations of 10 and 20%, oocytes I displayed severe alterations in the shape and in the germinal vesicle s (nuclei), which were dislocated to the periphery of the oocyte due to the accumulation of vacuoles occupying most part of the cytoplasm and causing structural damages to the cell. Similar results were found by Roma et al. (2010) for the oocytes I from the same tick species exposed to a synthetic acaricide.

The oocytes II exposed to the concentrations of 5 and 10% presented irregular shape, cytoplasmic vacuolation and a decreased number of yolk granules. These results corroborate Friesen et al. (2003), who studied the ovaries of *A. hebraeum* females exposed to avermectin and cypermethrin, and reported a drop in the production of yolk granules by the oocytes and in the synthesis of ecdysteroids as well, reducing the production of vitellogenin (main yolk protein) to the hemolymph and, consequently, the absorption by the oocyte. At the concentration of 20%, the alterations were more severe. Vendramini et al. (2012) reported cell death by autophagy at the same concentration.

Vendramini et al. (2012) observed more extensive vacuolated regions in oocytes III, an attempt of the cells to inactivate the product action by recycling and/or degrading the structures affected by the oil. The most significant alterations in all the developmental stages occurred in the oocyte pole facing the pedicel, the route through which the oil would be transferred into the oocyte.

The oocytes IV and V exposed to the concentration of 5% did not show significant morphological alterations due to the presence of the chorion (protective membrane) (Hilton, 1982). However, alteration in these cells occurred in higher concentrations, once the chorion was not able to

fully prevent the absorption of the product (Vendramini et al., 2012).

The ovary epithelium of the *R. sanguineus* s. l. females presented severe alterations, with a high level of structural disorganization, highly vacuolated cells and pyknotic nuclei, forming an amorphous mass, with the consequent impairment of the attached oocytes (Vendramini et al., 2012).

These data stimulate further studies aimed at investigating the efficiency of the product in formulations for commercial use.

Figure 39 – (A) *Carapa guianensis* tree; (B) Detail of leaves; (C) Detail of fruit

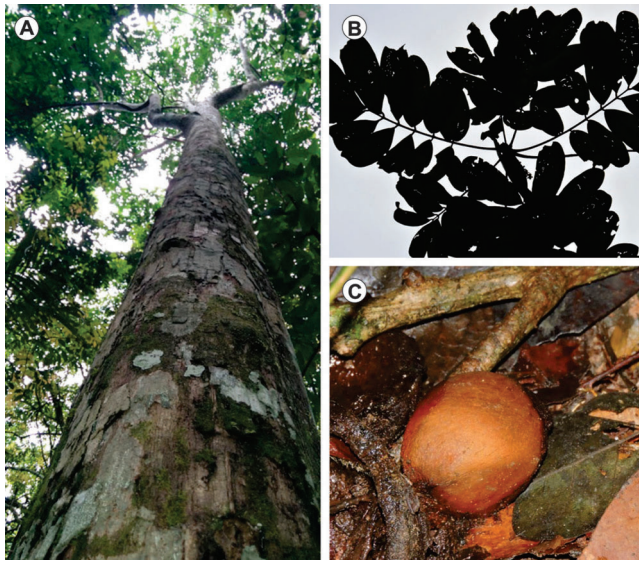



Photo by Silveira, M., Rio Branco, Acre, Brazil

## Thymol

Thymol, also known as thymic acid, isopropyl meta = cresol (= 5 methyl – 2 isopropyl – 1- phenol), is a volatile monoterpene present in the essential oils of Apiaceae, Lamiaceae and Verbenaceae plants (Figure 40) (Farmacopéia Portuguesa VIII, 2005), with acaricide action when applied to different stages and species of ticks (Novelino et al., 2007a,b; Monteiro et al., 2009;2010; Daemon et al., 2009; 2012a, b; Mendes et al., 2011; Scoralick et al., 2012; Senra et al., 2013; Matos et al., 2014a). However, further investigation is needed to clarify the acaricide action on the internal morphophysiology of ticks.

Matos et al. (2014b) analyzed the effects of thymol on the ovaries of *R. sanguineus* s. l. females and reported the presence of cytoplasmic vacuoles, membrane invaginations, damages in the chorion, and other disorders in the females fed six days and exposed to thymol at the sublethal concentrations of 1.25, 2.5 and 5.0 mg. This study also revealed that the intensity of the damages caused by the active ingredient increased in the individuals exposed to higher concentrations (2.5 and 5.0 mg/mL).

According to Hinton (1982), the chemical structure of thymol contains a hydroxyl group, which enables it to increase the affinity for water, causing damages to the cells and tissues by interfering in the permeability of the cell membrane, resulting in its rupture. These data strongly justify the damages reported by Matos et al. (2014b) after treating the females with thymol.

The chorion of the oocytes acts as a protective barrier against the environmental impacts, such as desiccation, predation and humidity/temperature variations (Hinton, 1982; Oliveira et al., 2005). Therefore, damages to the chorion facilitates the direct contact between the hemolymph (containing thymol molecules) and the oocyte

membrane, allowing the penetration of the chemical into the cell, disorganizing it. Similar results were reported by Denardi et al., apud Arnosti et al. (2011b) in studies exposing de *R. sanguineus* s. l. ovaries to the acid esters from castor oil.

Figure 40 – (A-B) *Thymus vulgaris* plant (Lamiaceae) popularly known as thyme, and which possessed thymol in its composition; (C) Thymol crystals

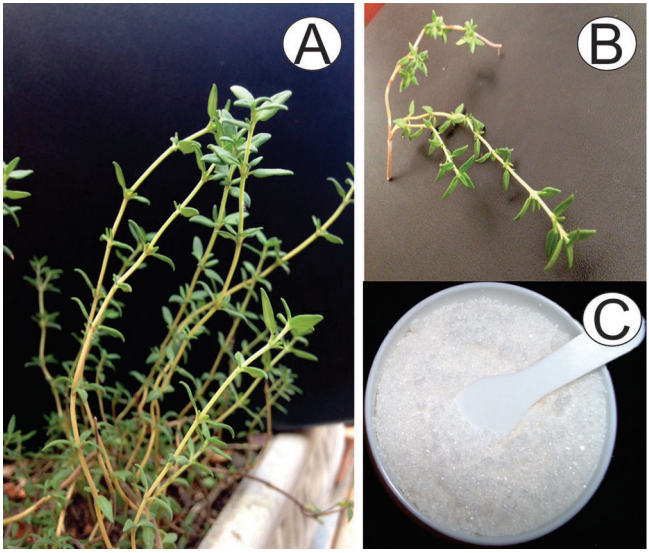



Photo by Pereira, N. R. C; Matos, R. S., Rio Claro, São Paulo, Brazil

## Ricinoleic acid esters from *Ricinus communis* (Castor oil)

Chierice et al. (personal communication) performed tests on cattle treated with ester-enriched salt and reported a significant drop in infestations, once the ticks were not able to complete their blood meal and precociously dropped off the hosts, suggesting that the esters would

interfere in the tick feeding process, providing relevant bases for further studies.

Based on these data, the researchers of BCSTM (Brazilian Central of Studies on Ticks Morphology), demonstrated that the ricinoleic acid esters from castor oil (*Ricinus communis)* (Figure 41) would represent a group with great potential to control ticks by hydrolyzing polysaccharides and its complexes (glycoproteins, proteoglycans, etc.). Such potential has been demonstrated by Messetti et al. (2010) studying *Leuconostoc mesenteroides*, and by Arnosti et al. (2011a,b), Sampieri et al. (2013a,b) and Camargo-Mathias et al. (2016) studying *Rhipicephalus sanguineus* s. l. females.

The studies used a methodology to affect the ticks indirectly, i.e., the esters would be "delivered" to the ticks through the blood of the host, whose food would be enriched with the product (stabilized in NaCl). The esters would not only interfere in the physiologic processes of the ticks, but also act as a food additive to the host. Some commercial products have used this strategy to eliminate ectoparasites, such as fleas and ticks, as is the case of Nexgard™ (Merial). However, the proposal of providing an antiparasitic product that would improve the nutritional conditions of the host makes the castor oil esters a unique product, of great medical/veterinary and economic interest (Sampieri et al., 2015).

In this sense, pioneer studies by Arnosti et al. (2011a,b), Sampieri et al. (2012; 2013a,b) and Camargo-Mathias et al. (2016), subjecting *R. sanguineus* s. l. ticks to indirect exposure to the esters (Figure 42), revealed the occurrence of significant morphological and ultrastructural alterations in the salivary glands (accelerating degenerative processes), and in the female reproductive system. Such alterations interfered in vitellogenesis, modified the yolk chemical composition and affected the chorion deposition.

Studies using *A. cajennense* s. s. have demonstrated the action of the chemical on the male reproductive system caused significant alterations to the glandular complex, which responsible for synthetizing and secreting substances for the maturation and viability of the spermatozoa, germ cells that will fertilize the eggs (Sampieri et al., 2015). All these morphological alterations implied in physiological modifications that certainly affected the processes of synthesis and release of cellular secretion, as well as the amount and composition of the yolk (in the case of females), the seminal fluid and the material that constitutes the spermatophore (in males). These events probably retarded the insemination of the male gametes in the female reproductive system and the development of embryos as well.

Thus, the ricinoleic acid esters from castor oil can be considered a group of substances with chemical properties that have the potential to interfere in the reproduction of ticks, acting directly or indirectly on the male and female systems, which makes them a promising alternative for the integrated management of these ectoparasites, in association with other methods.

Considering the importance of Ixodidae ticks and the ongoing search for efficient methods to control them, the results of the studies on this group of substances signalize that the ricinoleic acid esters of castor oil incorporated to the host feeding can support the management of these rural and urban pests.

Figure 41 – (A) *Ricinus communis* plant. (B) Detail of leaf (C) Detail of fruits

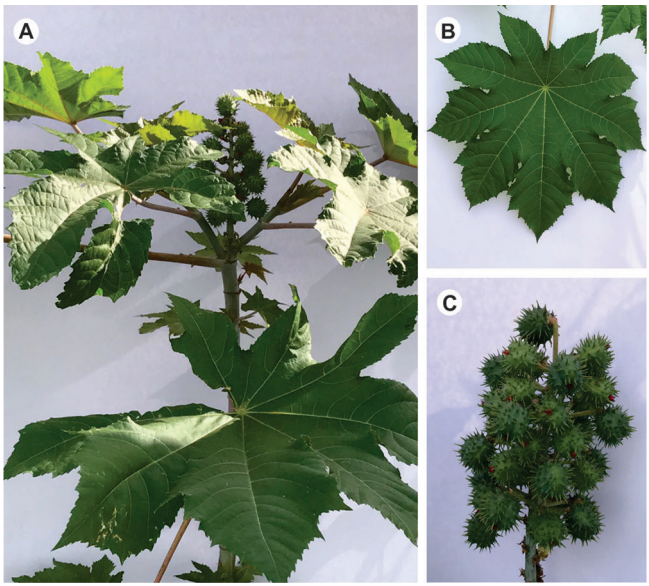
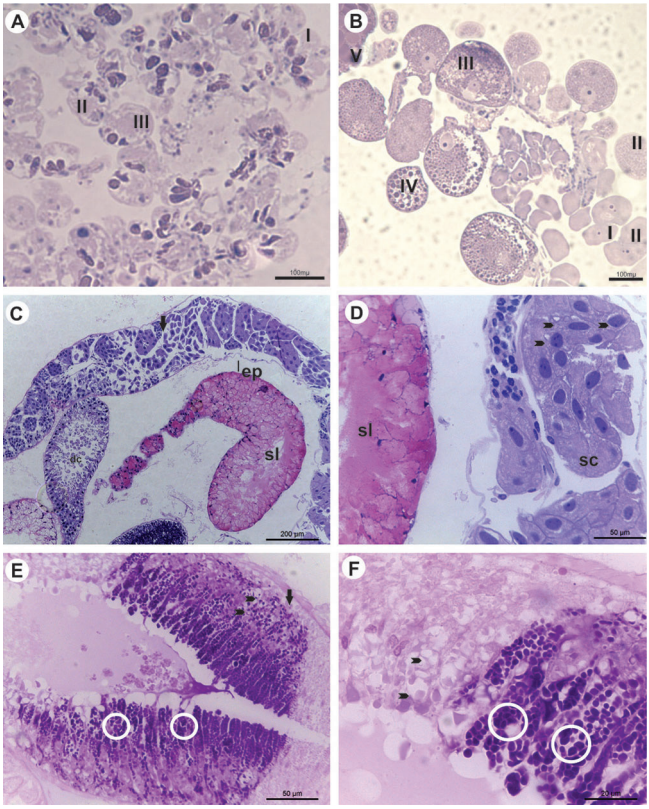



Photo by Arnosti, A., Rio Claro, São Paulo, Brazil

Figure 42 – (A) Histological sections of the salivary glands of engorged females *R. sanguineus* s. l. ticks exposed to ricinoleic castor oil, in the proportion of 5 g of ester (stabilized in NaCl/Kg) and stained with HE. I = acini I; II = acini II; III = acini III. (B): Histological sections of ovaries of *R. sanguineus* s. l. females exposed to ricinoleic castor oil, in the proportion of 5 g of ester (stabilized in NaCl/Kg) and stained with HE. (C) Overview: Testis danified by esters action, showing an epithelium detachment and a full disorganization of germ cells, including a degeneration process and cell death. (D) Spermatids inside a spermatocist, showing limit disruption and vacuolized citoplasm. (E-F) PAS reaction, showing the acessory gland secreting polysacharide and/or its conjugates, which vesicules contents apear to be in fusion process under the esters activity.

arrow = epithelium detachment; arrow head = vacuoles; circle = fused vesicules; dc = degenerative cells; ep = epithelium; sl = sponge lobe




Bars: (A-B) 100 µm; (C) 200 µm; (D-E) 50 µm; (F) 20 µm

## *Acmella oleracea* (L.) R. K. Jansen (Jambu)

*Spilanthes acmella var oleracea* or *Spilanthes oleracea* or *Acmella oleracea* (Figure 43) was introduced in Brazil and is found in the tropical regions close to the Equator in Africa, Asia and South America (Favoreto; Gilbert, 2010). In Brazil, it is popularly known as jambu, "agrião-do-pará",

"agrião-do-norte" or "agrião-bravo", and is commonly used as a food ingredient in the north region (Favoreto; Gilbert, 2010) and in traditional medicine as an efficient local anesthetic and analgesic with fungistatic, fungicidal and insecticide properties (Fabry; Okemo; Ansorg, 1996; Nigrinis; Caro; Olarte, 1986; Ramsewak, 1999; Torres; Chávez, 2001). It is an annual herbaceous perennial, up to 40-cm high, with small and yellow globe-shaped flowers (Favoreto; Gilbert, 2010). The ingestion of the aerial parts (flower, leaf and stem) cause slight tongue tingling, consequence of the anesthetic effect of secondary metabolites (Dubey et al., 2013; Favoreto; Gilbert, 2010; Ramsewak, 1999).

Figure 43 – (A) Jambu flowers, leaves and stems (*Spilanthes acmella* L. Murray), (B) Detail of Jambu leaves (*Spilanthes acmella* L. Murray). (C) Detail of Jambu flowers

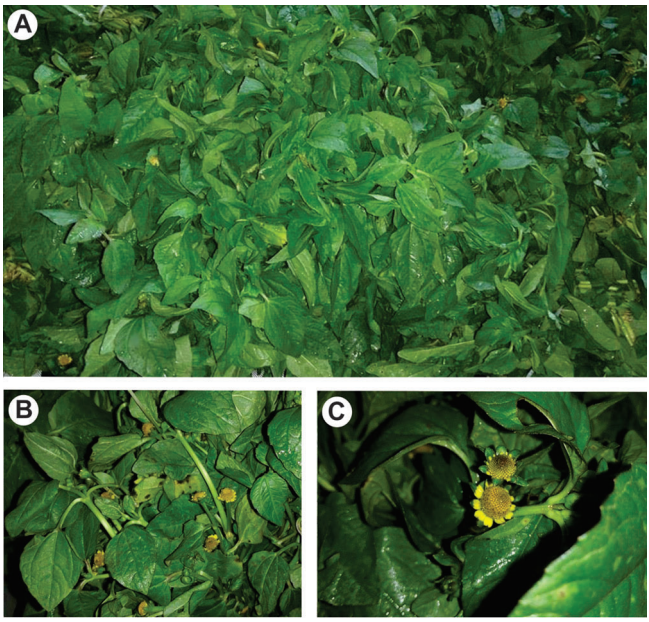



Photo by Silveira, M., Rio Branco, Acre, Brazil

The literature reports that the extract from *A. oleracea* has been efficient to control the proliferation of other arthropods, e.g., against *A. aegypti* (Dipera) larvae, *Plutella xylostella* (Lepidoptera) adults and the american cockroach (*Periplaneta americana)* (Blattodea) (Kadir et al., 1989; Ramsewak, 1999; Sharma et al., 2012).

Recent studies by Castro et al. (2014) and Oliveira et al. (2016) have demonstrated that this plant would have acaricide potential in extracts prepared with its aerial parts (flower, leaf and stem) such extracts have been used against *R. microplus*, and have showed great efficiency to control larvae and engorged females (Castro et al., 2014).

Several compounds have been identified in the extracts obtained from different parts of *A. oleracea*, such as volatile oils (beta caryophyllene, limonene and thymol), phenolic acids (vanillic and transferulic), phytosterols (stigmasterol and β-sitosterol), polysaccharides (rhamnogalacturonan), coumarin (scopoletin) and alkylamines (Barbosa et al., 2016; Cavalcanti, 2008; Prachayasittikul et al., 2013; Saraf; Dixit, 2002; Spelman et al., 2011).

The biological effects have been in general attributed to spilanthol or affinin [N-2-Methylpropyl-2,6,8-decatrienamide or N-isobutyl-2*E*,6*Z*,8*E*-decatrienamide], an aliphatic alkamide of molecular formula $C_{14}H_{23}NO$, abundant in the flowers, leaves and stems of *A. oleracea* (Ramsewak, 1999). This compound is also present in plants of the families Asteraceae, Solanaceae and Piperaceae (Prachayasittikul et al., 2013).

The toxicity of spilanthol, present in the extract of aerial parts (flower, leaf and stem) of *A. oleracea* has been investigated by Cilia-López et al. (2010), who observed that the substance acted stimulating the central nervous system of mice, increasing motor activity and irritability, effects that lasted for approximately 180 minutes, after which the animals returned to normal levels.

Nomura et al. (2013) tested the antinociceptive activity of the ethanolic extract obtained from the flowers of *A. oleracea* at the concentrations of 5, 50 and 500 mg/Kg via intraperitoneal administration in mice. The results revealed the anesthetic potential of the compound, which did not cause toxicity or mortality during the observation period (7 days).

Considering all of the above, the commercial use of spilanthol as acaricide agent has become a positive perspective. The ingredient has been already used in different formulations, such as oral hygiene, insecticides, bathing products, shampoos, soaps, creams (used to enhance the refreshing sensation), and even as therapeutic agent to treat rheumatism (Cavalcanti, 2008; Prachayasittikul et al., 2013).

Anholeto et al. (2016), who studied *A. cajennense* s.s. ticks exposed to the concentrations of 6.2; 12.5; 14.3; 16.6; 20; 25; 33; 40, 50 and 100 mg/mL reported 5 to 100% of mortality, proportionally to the increase in the concentrations. The concentration of 50 mg/mL caused 20% of mortality after 1 day of treatment, 60% after 3 days and 70% after 7 days. In the highest concentration (100 mg/mL), 100% of mortality was observed soon after 24 hours of exposure. Similar results were reported by Roma et al. (2009) and Oliveira et al. (2011), who studied *R. sanguineus* s. l. female ticks exposed to the synthetic chemicals permethrin and fipronil, widely acknowledged as efficient to control ticks, once some concentrations of these compounds caused mortality in a 100% of the individuals 24h after treatment.

The same authors demonstrated that the crude extract of *A. oleracea* at the concentrations of 25 and 50 mg/mL presented significant mortality rates after 24 of exposure. In the highest concentration (100 mg/mL) a 100% of mortality was observed 72 hours after exposure, corroborating

Castro et al. (2014), who reported an increase in mortality corresponding to 20.7, 26.6 and 59.2% in *R. (Boophilus) microplus* exposed to hexane extract of *A. oleracea* at the concentrations of 25, 50 and 100 mg/mL, respectively.

Behavioral alterations were observed in the individuals (males and semi-engorged females) of *A. cajennense* s.s. exposed to different concentrations of *A. oleracea* extract, such as decreased locomotor activity, prostration in inverted position, stretching of all legs and paralysis. Behavioral changes were also reported by Roma et al. (2009) and Oliveira et al. (2011) in *R. sanguineus* s. l. ticks exposed to permethrin and fipronil. The present study demonstrated that the *A. oleracea* extract, even in lower concentrations, presented a remarkable level of toxicity to the ticks, similarly to other natural products and extracts as azadirachtin, thymol, carvacrol, *Ageratum conyzoides* and *Artemisia absinthium* (Koc et al., 2013; Matos et al., 2014; Parveen et al., 2014; Srivastava et al., 2008).

In conclusion, the results clearly demonstrated that the use of ethanolic extract from *A. oleracea* flowers, leaves and stems is a promising strategy to control ticks, with additional advantages over the synthetic chemicals, minimizing the selection of resistant individuals and offering less harm to the environment and non-target organisms.

# 13

# A new view on the biosynthetic/secretory plurality of the salivary glands: bioactive molecules in *Rhipicephalus sanguineus* s. l. Ticks

*Karim Christina Scopinho Furquim, Luis Adriano Anholeto, Marina Rodrigues de Abreu, Maria Izabel Camargo-Mathias*

The tick saliva is as a complex mixture that comprises a diversified and efficient pharmacological arsenal, greatly responsible for the feeding success of the ectoparasites (Steen et al., 2006; Francischetti et al., 2010; Camargo-Mathias et al., 2011). The saliva of these arthropods comprises molecules of different natures, and plays a key role in the modulation of the immuno-inflammatory and hemostatic systems of the hosts (Steen et al., 2006; Hajnická et al., 2011), acting as a potential reservoir for multifunctional molecules and containing bioactive substances of great interest in several areas (Batista et al., 2010).

Among the constituting elements of the tick saliva, are the components of glycoprotein, lipoprotein and lipid origin (Camargo-Mathias et al., 2011), such as the calreticulin, acid phosphatase, esterase, aminopeptidase,

metalloproteinase, lipocalin and prostaglandin (Brossard; Wikel, 2004; Steen et al., 2006; Mulenga et al., 2007; Oliveira et al., 2011; Camargo-Mathias et al., 2011), with pharmaco- and/or immunological properties. With specific regard to females of the species *Rhipicephalus sanguineus* s. l., the saliva contains proteins, polysaccharides, lipids, acid phosphatase and calcium. The latter, according to some authors, would be probably bound to a calreticulin (Furquim et al., 2013). Studying the same species, Oliveira et al. (2011) detected the presence of non-protein molecules, prostaglandin (PGE2) and adenosin (Ado), with potent immunomodulatory properties.

According to the literature, the saliva of several tick species, including *R. sanguineus* s. l., modulates different biological steps of dendritic cells (e.g. maturation) (Oliveira et al., 2008; 2010), inhibits the proliferation of endothelial cells, have antiangiogenic action (Francischetti et al., 2010; Drewes et al., 2012), and antitumor properties as well (Kazimírová et al., 2006; Chudzinski-Tavassi et al., 2010; Simons et al., 2011; Akagi et al., 2012). Anticoagulant proteins are also found in the saliva of ticks: the tick anticoagulant peptide (TAP) extracted from the saliva of *Ornithidoros moubata* (Waxman et al., 1990), the boophilin, from *R.* (*Boophilus) microplus* (Macedo-Ribeiro et al., 2008), ixolaris and penthalaris from *Ixodes scapularis* (Francischetti et al., 2002; 2004), and Amblyomin-X, from *A. cajennense* s. l. (Batista et al., 2010). These anticoagulant proteins are currently used to treat diseases, such as thrombosis.

Considering the biochemical and functional complexity of the tick saliva, it has been object of several studies, mainly aimed at identifying and isolating the molecules with numerous biological properties. Some of these properties are: vasodilating, anti-inflammatory, anticoagulant, immunosuppressant (Oliveira et al., 2010), antitumor

(Kazimírová et al., 2006; Chudzinski-Tavassi et al., 2010; Simons et al., 2011; Akagi et al., 2012), and antigen protector (Jittapalapong et al., 2000; 2008).

## Salivary compounds with antitumor potential

There are reports in the literature about the antitumor potential of the tick saliva and Kazimírová et al. (2006) demonstrated that the molecules present in the salivary gland extracts of different species of ticks would have the capability to inhibit the growth of HeLa cells by inducing apoptosis (*R. appendiculatus* and *A. variegatum*), and its antiproliferative action (*Ixodes ricinus, R. appendiculatus*, *Dermacentor reticulatus* and *A. variegatum*). Moreover, Chudzinski-Tavassi et al. (2010) reported the presence of a protein with coagulative action in the saliva of *A. cajennense*, and this protein would have cytotoxic activity on different lineages of tumor cells. Poole et al. (2013) demonstrated the inhibiting effect of the tick saliva on the migratory and invasive capacity of breast neoplastic cells (MDA-MB-231; ATCC).

More recently, the study group Brazilian Central of Studies on Ticks Morphology (BCSTM), coordinated by professor Maria Izabel Camargo-Mathias, from the Biology Department of the Bioscience Institute, Unesp, Rio Claro *campus* (SP) Brazil, conducted a study aimed at elucidating the morphophysiological alterations caused by these components on tumor models. The results revealed that the saliva components of *R. sanguineus* s. l. females were able to inhibit the growth of tumor cells, both *in vitro* and *in vivo*.

The BCSTM group studied the action of salivary glands extracts of *R. sanguineus* s. l. females fed for two

(SGE2) and four (SGE4) days on the viability/proliferation of human tumor cells (HL-60- leukemia; SK-HEP-1- adenocarcinoma; HEP-G2- hepatocarcinoma), canine (DH82- malignant histiocytosis) (*in vitro* testing), and Walker 256. In the experiment, the tumor cells were implanted in the posterior leg muscles of female Wistar rats (*in vivo* testing).

## *In vitro* testing on cells DH82 and HL-60

The salivary glands extract of *R. sanguineus* s. l. female ticks would have the potential to cause structural alterations or affect the viability of DH82 and HL-60 tumor cells, inducing apoptosis. Such cell alterations would vary according to: a) the extract (SGE2 or SGE4), b) the concentration and c) the tumor model used.

The analysis of the effects of SGE2 revealed that the concentration of 0.2 μg/mL affected the viability of DH82 cells, which displayed clear characteristics of advanced apoptotic process (Figures 44, 45). On the other hand, the concentration of 0.5 μg/mL applied on the same tumor lineage caused structural alterations only in the cytoskeleton elements, which were disorganized, i.e., actin filaments and microtubules appeared to be depolymerized (Figures 44, 45); however, without affecting the viability of these cells. Both SGE2 concentrations (0.2 and 0.5 μg/mL) induced apoptosis in the HL-60 cells (Figures 44, 46).

Figure 44 – DH82 (A) and HL-60 (B) cells not exposed to extracts. (A, A1, B, B1). Histological sections (hematoxylin-eosin staining); (A2, B2). Confocal microscopy (cytoskeleton and nucleus staining: actin= green, tubulin= red and nucleus= blue). **c:** cytoplasm; **n:** nucleus; **v:** vacuole; **arrow head:** chromosomes.

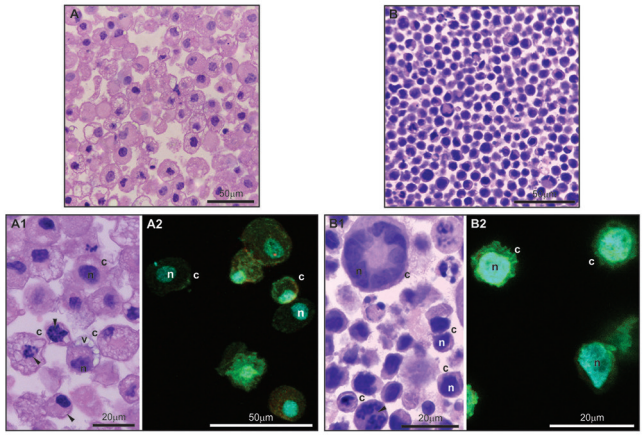


Figure 45 – Schematic representation of the procedures for **SGE2** extract obtaining and demonstration of the DH82 cells exposed to **SGE2** extract. (A, A1, A2, B, B1). Histological sections (hematoxylin-eosin staining); (A2) and (B2). Confocal microscopy (cytoskeleton and nucleus staining: actin= green, tubulin= red and nucleus= blue).

(A) Exposure to 0.2 µg/mL of **SGE2** extract.

(B) Exposure to 0.5 µg/mL of **SGE2** extract.

**TDFF:** two-days fed females of the *R. sanguineus* s. l.; **ro:** organs region; **sg:** salivary gland; **cm:** cytoplasmic mass; **ac:** apoptotic cell; **m:** chromatin marginalization; **v:** vacuole; **nf:** nuclear fragment; **bc:** broken cell; **c:** cytoplasm; **arrow:** actin filaments concentration; **circle:** microtubules concentration; **n:** nucleus.

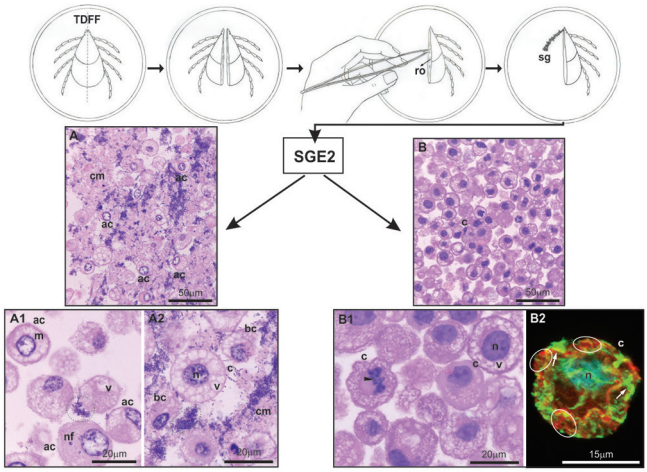
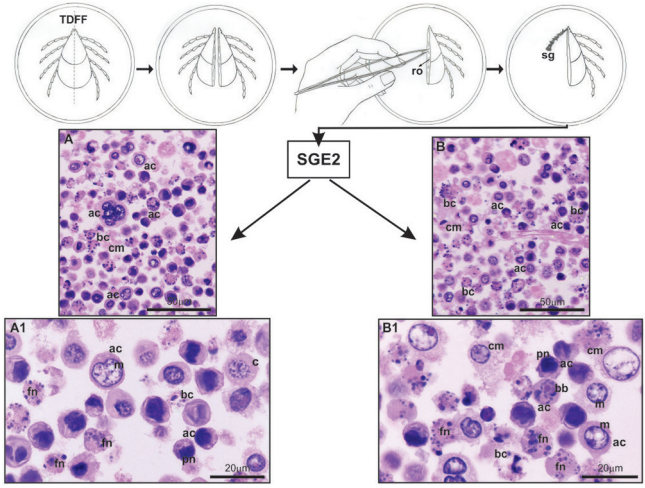


Figure 46 – Schematic representation of the procedures for **SGE2** extract obtaining and histological sections of HL-60 cells exposed to **SGE2** extract (hematoxylin-eosin staining).

(A) Exposure to 0.2 µg/mL of **EGS2** extract.

(B) Exposure to 0.5 µg/mL of **EGS2** extract.

**TDFF:** two-days fed females of the *R. sanguineus* s. l.; **ro:** organs region; **sg:** salivary gland; **ac:** apoptotic cell; **bc:** broken cell; **cm:** cytoplasmic mass; **m:** chromatin marginalization; **nf:** nuclear fragment; **c:** cytoplasm; **pn:** picnotic nucleus; **bb:** blebs;

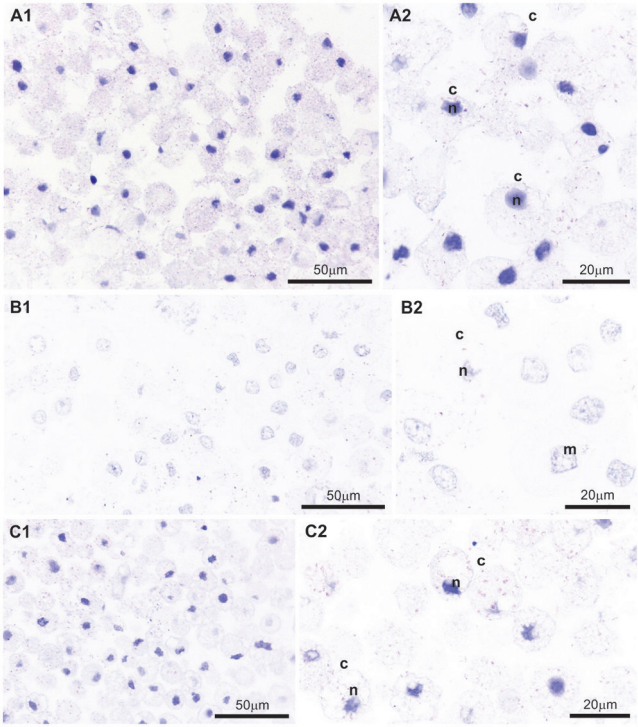



In addition, the SGE2 interfered in the physiology of DH82 cells, where the activity of acid phosphatase enzymes, which participate in the cell metabolism, was impaired (decreased or even absent in some cases) (Figure 47).

Figure 47 – Histological sections of DH82 cells not exposed (**A**) and exposed to **SGE2** extract (B, C). Acid phosphatase detection (pink scores within the cytoplasm). (B) Exposure to 0.2 µg/mL of **EGS2** extract.

(C) Exposure to 0.5 µg/mL of **EGS2** extract.

**c:** cytoplasm; **n:** nucleus; **m:** chromatin marginalization.




The viability of the tumor cells was not affected by the SGE4 extract (Figures 48, 49, 50). The extract interfered in the structural organization of HL-60 cells, causing intense depolymerization of the cytoskeleton elements, mainly in the microtubules of the ones exposed to the concentration of 0.5 µg/mL (Figure 48).

Figure 48 – Confocal microscopy of the HL-60 cells not exposed (A) and exposed to **SGE2** (B) and **SGE4** extracts (C). Cell viability test (green= living cells and red= dead cells).

(B1) Exposure to 0.2 µg/mL of **EGS2** extract.

B2) Exposure to 0.5 µg/mL of **EGS2** extract.

(C1) Exposure to 0.2 µg/mL of **EGS4** extract.

(C2) Exposure to 0.5 µg/mL of **EGS4** extract.

**n:** nucleus; **arrow:** dead cell; **double arraw:** living cell.

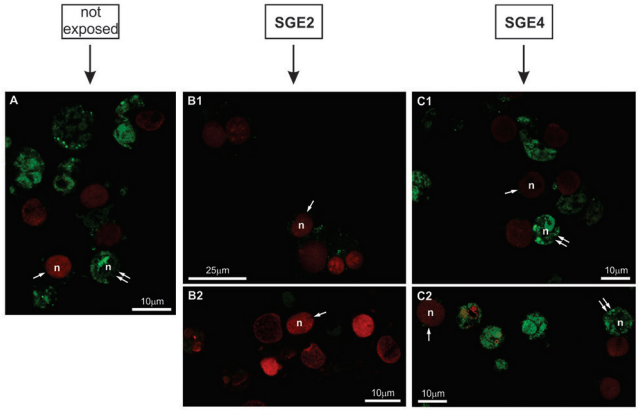


Figure 49 – Schematic representation of the procedures for **SGE4** extract obtaining and histological sections of DH82 cells exposed to **SGE4** extract (hematoxylin-eosin staining).
(A) Exposure to 0.2 µg/mL of **SGE4** extract.
(B) Exposure to 0.5 µg/mL of **SGE4** extract.
**FDFF:** four-days fed females of the *R. sanguineus* s. l.; **in:** intestine; **sg:** salivary gland; **c:** cell; **v:** vacuole; **n:** nucleus; **nu:** nucleolus; **e:** cell surface projections.

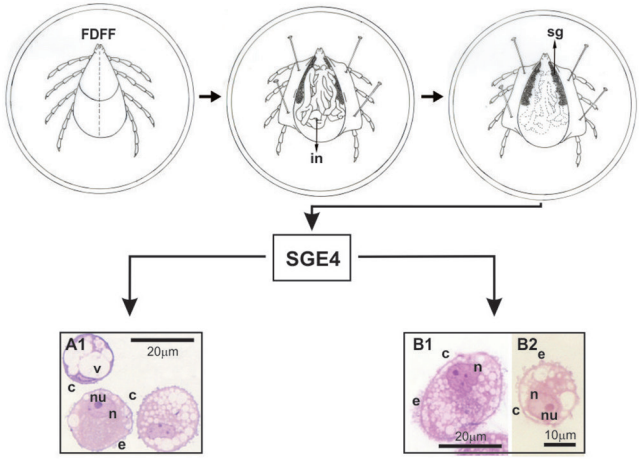
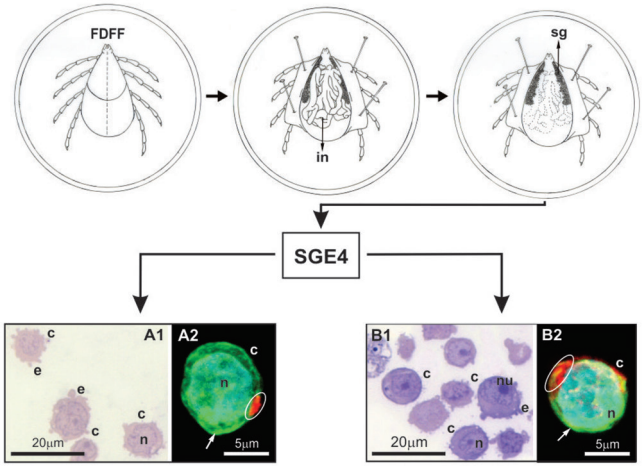


Figure 50 – Schematic representation of the procedures for **SGE4** extract obtaining and demonstration of the HL-60 cells exposed to **SGE4** extract. (A, B1). Histological sections (hematoxylin-eosin staining); (A2, B2). Confocal microscopy (cytoskeleton and nucleus staining: actin= green, tubulin= red and nucleus= blue).
(A) Exposure to 0.2 µg/mL of **SGE4** extract.
(B) Exposure to 0.5 µg/mL of **SGE4** extract.
**FDFF:** four-days fed females of the *R. sanguineus* s. l.; **in:** intestine; **sg:** salivary gland; **c:** cell; **n:** nucleus; **nu:** nucleolus; **e:** cell surface projections.




Therefore, the results showed that the extracts caused significant alterations in the morphophysiological behavior of the tumor cells, by either affecting the cell viability or inducing structural modifications (intense depolymerization of the elements that constitute the actin filaments and microtubules). This action would possibly affect the proliferative capacity of the cells, once the actin filaments and microtubules are cytoskeleton fibers weaved throughout the cytoplasm with the function of maintaining the cell

homeostasis, mainly the transportation/motility of structures and molecules/macromolecules in different metabolic pathways (microtubules specific function).

## SK-HEP-1 and HEP-G2 cells

The SGE4 had the potential to inhibit the proliferation of the tumor cell lineage SK-HEP-1 and HEP-G2, on a dose- and time-dependent basis. Additionally, the extract would act inhibiting the cell proliferations of inducing the cells to apoptosis, important processes to be regarded in the search for alternatives to control neoplasias.

## *In vivo* testing on Walker 256 tumor cells

Walker 256 tumor cells were implanted in the posterior leg muscles of female Wistar rats. The results revealed that the treatments using the SGE2 produced different results according to the concentrations used (0.2 and 0.04 μg/μL) and the number of inoculations (one or two injections).

This study showed that the animals subjected to a single injection of the extract in the concentration of 0.04 μg/μL had a decrease in the level of tumor cell invasion. In these animals, the muscle fibers were more intact, parallel, with peripheral nuclei, and showing transverse striations (similarly to a healthy muscle), with few regions of neoplasia with heavy cell degranulation, probably caused by immunoinflammatory processes.

However, the animals inoculated twice (two injections) with the extract in this same concentration presented higher tumor invasion and significant disorganization of the muscle fibers, characterized by: a) absence or rare presence of transverse striations (due to sarcomere misalignment)

and b) presence of considerable intermyofibrillar spaces, caused by the impairment of the tissue physiology.

The results for the concentration of 0.2 μg/μL (regardless the number of inoculations) revealed significant morphological alterations in the tissue: a) intense tumor invasion in the intracellular matrix (much higher in the animals inoculated twice) and b) high level of muscle fiber disorganization.

Thus, a single injection of the lower concentration of the extract (0.04 μg/μL) was more effective for the containment of the tumor invasion in addition to causing less damage to the muscle tissue. However, in all the treatments, the inhibitory action of the SGE2 on the Walker 256 tumor cells was able to reduce the tumor invasion, despite some morphological alterations in the muscles in some cases, which demonstrates that the extract inhibited tumor growth and minimized the damages to the physiology of the organism.

## Salivary compounds with immunogenic potential

According to Furquim et al. (2013), the biochemical complexity of the saliva in *R. sanguineus* s. l. female ticks is modified throughout the blood-feeding phase, once the ectoparasite needs to modulate the local hemostatic and immunoinflammatory systems of the host to ingest the blood and complete the feeding process. The authors report that the salivary secretion vary both qualitatively and quantitatively when specific periods of the glandular cycle are considered: the beginning (2-day feeding), the middle (4-day feeding) and the end (6-day feeding) of the secretion production process.

Moreover, the salivary components can have immunogenic capability, which places them as protective antigens,

providing the hosts with resistance after successive infestations or immunizations with glandular extracts, saliva or even some specific salivary molecules. It is also known that the immunogenicity level of each salivary component is variable, i.e., some are more efficient than others in developing resistance in the host subjected to reinfestations or immunizations (Jittapalapong et al., 2000).

In this sense, Furquim et al. (2011; 2013; 2014a, b) demonstrated that the secretory behavior of the salivary glands in *R. sanguineus* s. l. females would modify according to the resistance acquired by the host previously immunized with salivary bioactives. The same authors (Furquim et al., 2013; 2014a, b) reported the occurrence of glandular secretion modifications in relation to protein, polysaccharide and lipid components, as well as acid phosphatase and calcium (probably calreticulin) due to the host immunization. The salivary secretion was modified throughout the glandular cycle, in which the ticks fed on a host previously immunized with glandular extracts from females fed for two (SGE2), four (SGE4) and six (SGE6) days. According to the authors, this would be a strategy of the tick to overcome, compensate or even neutralize the resistance acquired by the host previously immunized (resistant) against the infestation by *R. sanguineus* s. l.

Furquim et al. (2013; 2014a, b) reported that the composition of the salivary secretion in relation to glyco- and lipoproteins, acid phosphatase and calcium varied according to the type of immunization (SGE2, SGE4 or SGE6), i.e., different histochemical characteristics were observed for each immunization throughout the gland secretory cycle.

Therefore, with specific regard to glycoproteins, acid phosphatase and calcium, it was verified that: a) in the salivary glands of the females fed on hosts immunized with the SGE2, the proteins displayed the most significant alterations throughout the glandular cycle, followed

by polysaccharides /acid phosphatase, and calcium b) in the females fed on hosts immunized with the SGE4, the most intense alterations were associated with the acid phosphatase, followed by proteins/polysaccharides and calcium (Figures 51-58).

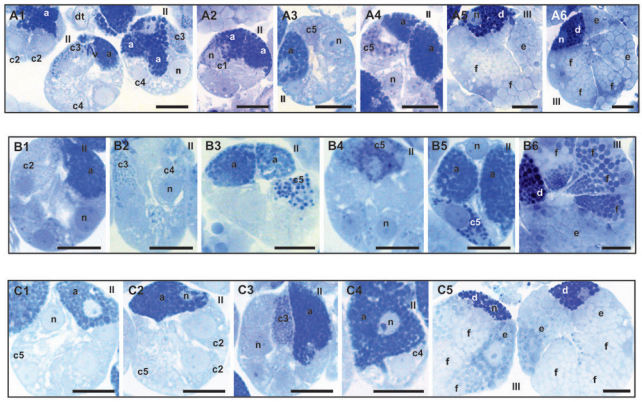
Figure 51 – Histological sections of salivary glands of two-days fed females of the tick *R. sanguineus* s. l. Bromophenol blue technique (protein stain= blue).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **dt:** duct; **v:** valve; **n:** nucleus.




Bars: (A-C) 25 µm

Reproduced from Furquim, K. C. S., Camargo-Mathias, M. I., Hebling, L. M. G. F., Roma, G. C., Bechara, G. H. (2013) Alterations in the secretory behavior of salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) feeding in resistant rabbit. Air & Water Borne Diseases, v. 2, p. 114 (doi: 10.4172/2167-7719.1000114), with permission from OMICS Group, and Furquim, K. C. S., Camargo-Mathias, M. I., Roma, G. C., Hebling, L. M. G. F., Bechara, G. H. (2014) Morphological plasticity of ticks' salivary glands and the meaning of hematophagy in hosts immunized with glandular extract of females fed for 4 days. Animal and Veterinary Sciences, New York, v. 2, n. 6, p. 194-207 (doi: 10.11648/j.avs.20140206.17), with permission from Science Publishing Group.

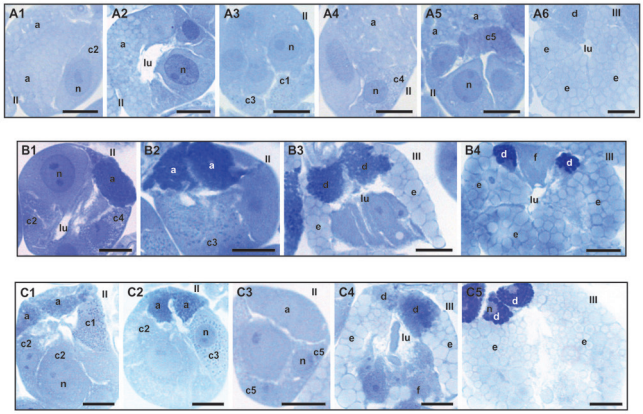
Figure 52 – Histological sections of salivary glands of four-days fed females of the tick *R. sanguineus* s. l. Bromophenol blue technique (protein stain= blue).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **dt:** duct; **lu:** lumen; **n:** nucleus.




Bars: (A-C) 25 µm

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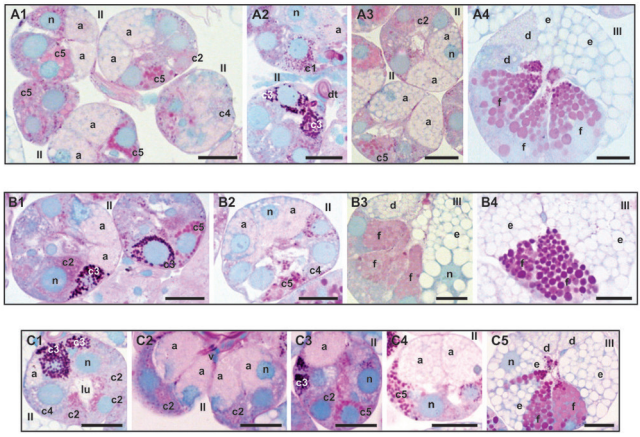
Figure 53 – Histological sections of salivary glands of two-days fed females of the tick *R. sanguineus* s. l. PAS technique (polysaccharide stain= rose/pink).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **dt:** duct; **lu:** lumen; **n:** nucleus.




Bars: (A-C) 25 µm

Reproduced from Furquim, K. C. S., Camargo-Mathias, M. I., Hebling, L. M. G. F., Roma, G. C., Bechara, G. H. (2013) Alterations in the secretory behavior of salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) feeding in resistant rabbit. Air & Water Borne Diseases, v. 2, p. 114 (doi: 10.4172/2167-7719.1000114), with permission from OMICS Group, and Furquim, K. C. S., Camargo-Mathias, M. I., Roma, G. C., Hebling, L. M. G. F., Bechara, G. H. (2014) Morphological plasticity of ticks' salivary glands and the meaning of hematophagy in hosts immunized with glandular extract of females fed for 4 days. Animal and Veterinary Sciences, New York, v. 2, n. 6, p. 194-207 (doi: 10.11648/j.avs.20140206.17), with permission from Science Publishing Group.

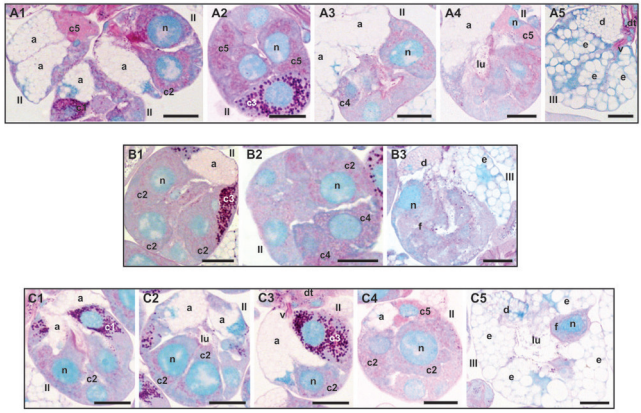
Figure 54 – Histological sections of salivary glands of four-days fed females of the tick *R. sanguineus* s. l. PAS technique (polysaccharide stain= rose/pink).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **dt:** duct; **v:** valve; **lu:** lumen; **n:** nucleus.




Bars: (A-C) 25 µm

Reproduced from Furquim, K. C. S., Camargo-Mathias, M. I., Hebling, L. M. G. F., Roma, G. C., Bechara, G. H. (2013) Alterations in the secretory behavior of salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) feeding in resistant rabbit. Air & Water Borne Diseases, v. 2, p. 114 (doi: 10.4172/2167-7719.1000114), with permission from OMICS Group, and Furquim, K. C. S., Camargo-Mathias, M. I., Roma, G. C., Hebling, L. M. G. F., Bechara, G. H. (2014) Morphological plasticity of ticks' salivary glands and the meaning of hematophagy in hosts immunized with glandular extract of females fed for 4 days. Animal and Veterinary Sciences, New York, v. 2, n. 6, p. 194-207 (doi: 10.11648/j.avs.20140206.17), with permission from Science Publishing Group.

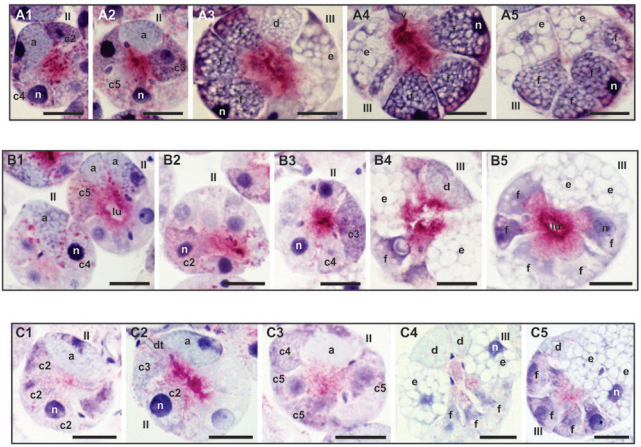
Figure 55 – Histological sections of salivary glands of two-days fed females of the tick *R. sanguineus* s. l. Acid phosphatase technique (acid phosphatase stain= carmine).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **v:** valve; **lu:** lumen; **n:** nucleus.




Bars: (A-C) 25 µm

Reproduced from Furquim, K. C. S., Camargo-Mathias, M. I., Hebling, L. M. G. F., Roma, G. C., Bechara, G. H. (2013) Alterations in the secretory behavior of salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) feeding in resistant rabbit. Air & Water Borne Diseases, v. 2, p. 114 (doi: 10.4172/2167-7719.1000114), with permission from OMICS Group, and Furquim, K. C. S., Camargo-Mathias, M. I., Roma, G. C., Hebling, L. M. G. F., Bechara, G. H. (2014) Morphological plasticity of ticks' salivary glands and the meaning of hematophagy in hosts immunized with glandular extract of females fed for 4 days. Animal and Veterinary Sciences, New York, v. 2, n. 6, p. 194-207 (doi: 10.11648/j.avs.20140206.17), with permission from Science Publishing Group.

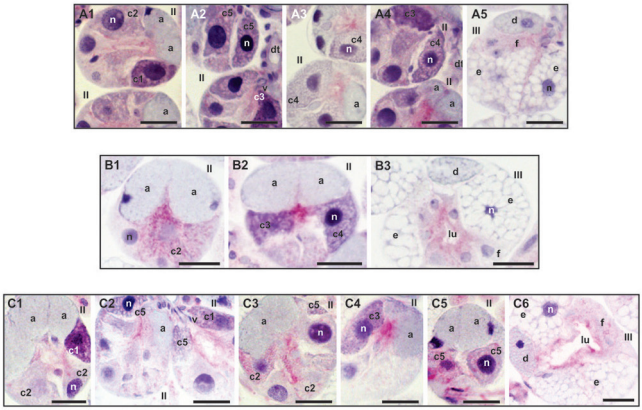
Figure 56 – Histological sections of salivary glands of four-days fed females of the tick *R. sanguineus* s. l. Acid phosphatase technique (acid phosphatase stain= carmine).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **v:** valve; **lu:** lumen; **n:** nucleus.




Bars: (A-C) 25 µm

Reproduced from Furquim, K. C. S., Camargo-Mathias, M. I., Hebling, L. M. G. F., Roma, G. C., Bechara, G. H. (2013) Alterations in the secretory behavior of salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) feeding in resistant rabbit. Air & Water Borne Diseases, v. 2, p. 114 (doi: 10.4172/2167-7719.1000114), with permission from OMICS Group, and Furquim, K. C. S., Camargo-Mathias, M. I., Roma, G. C., Hebling, L. M. G. F., Bechara, G. H. (2014) Morphological plasticity of ticks' salivary glands and the meaning of hematophagy in hosts immunized with glandular extract of females fed for 4 days. Animal and Veterinary Sciences, New York, v. 2, n. 6, p. 194-207 (doi: 10.11648/j.avs.20140206.17), with permission from Science Publishing Group.

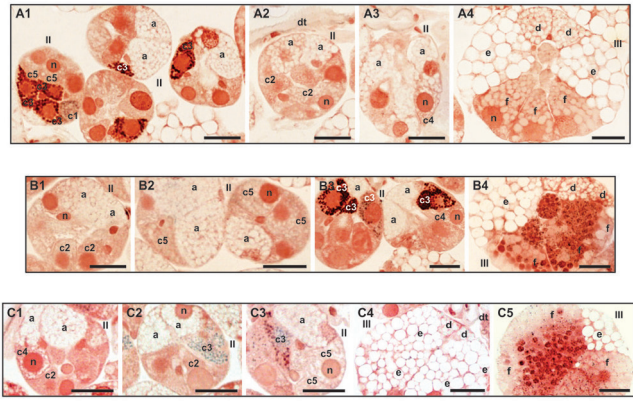
Figure 57 – Histological sections of salivary glands of two-days fed females of the tick *R. sanguineus* s. l. Calcium detection technique (calcium stain= brown).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **dt:** duct; **n:** nucleus.




Bars: (A-C) 25 µm

Reproduced from Furquim, K. C. S., Camargo-Mathias, M. I., Hebling, L. M. G. F., Roma, G. C., Bechara, G. H. (2013) Alterations in the secretory behavior of salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) feeding in resistant rabbit. Air & Water Borne Diseases, v. 2, p. 114 (doi: 10.4172/2167-7719.1000114), with permission from OMICS Group, and Furquim, K. C. S., Camargo-Mathias, M. I., Roma, G. C., Hebling, L. M. G. F., Bechara, G. H. (2014) Morphological plasticity of ticks' salivary glands and the meaning of hematophagy in hosts immunized with glandular extract of females fed for 4 days. Animal and Veterinary Sciences, New York, v. 2, n. 6, p. 194-207 (doi: 10.11648/j.avs.20140206.17), with permission from Science Publishing Group.

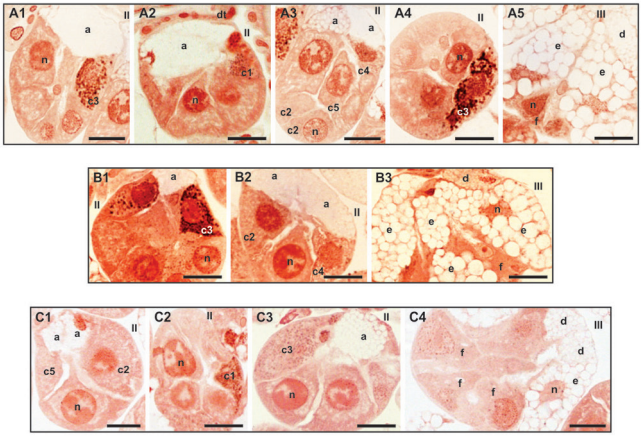
Figure 58 – Histological sections of salivary glands of four-days fed females of the tick *R. sanguineus* s. l. Calcium detection technique (calcium stain= brown).

(A) Fed females on host not immunized (Control Group)

(B) Fed females on host immunized with **SGE2** extract.

(C) Fed females on host immunized with **SGE4** extract.

**II:** type II acinus; **III:** type III acinus; **a: a** cell; **c1: c1** cell; **c2: c2** cell; **c3: c3** cell; **c4: c4** cell; **c5: c5** cell; **d: d** cell; **e: e** cell; **f: f** cell; **dt:** duct; **n:** nucleus.




Bars: (A-C) 25 µm

Reproduced from Furquim, K. C. S., Camargo-Mathias, M. I., Hebling, L. M. G. F., Roma, G. C., Bechara, G. H. (2013) Alterations in the secretory behavior of salivary glands of *Rhipicephalus sanguineus* females (Latreille, 1806) (Acari: Ixodidae) feeding in resistant rabbit. Air & Water Borne Diseases, v. 2, p. 114 (doi: 10.4172/2167-7719.1000114), with permission from OMICS Group, and Furquim, K. C. S., Camargo-Mathias, M. I., Roma, G. C., Hebling, L. M. G. F., Bechara, G. H. (2014) Morphological plasticity of ticks' salivary glands and the meaning of hematophagy in hosts immunized with glandular extract of females fed for 4 days. Animal and Veterinary Sciences, New York, v. 2, n. 6, p. 194-207 (doi: 10.11648/j.avs.20140206.17), with permission from Science Publishing Group.

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Ticks are blood-sucking ectoparasites, which have long been known to cause itching due their bites, and for their ability to transmit pathogens responsible for diseases that can cause physical harm to pets, livestock, wild animals, and humans, sometimes with considerable economic losses, notably in the cattle industry.

A sound knowledge of tick biology, anatomy, and morphology are essential for an understanding of the physiology, adaptations, and evolutionary success of these organisms. Moreover, such knowledge is indispensable for the establishment of strategies for their control and management of tick-borne diseases.

In this context, the present book was conceived and written by recognized expert morphologists belonging to the Brazilian Central of Studies on Ticks Morphology (BCSTM) – Unesp, Rio Claro (SP), Brazil. The objective of this publication is to clarify tick morphohistology, through a compilation of studies conducted by these researchers, illustrating how histological techniques have been applied in tick studies.

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