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# Testes of fed and unfed *Amblyomma cajennense* ticks (Acari: Ixodidae). First morphological data

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**Abstract**

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The tick species *Amblyomma cajennense* is of great medical importance, as it is the vector of the *Rickettsia rickettsii*, agent of Rocky Mountain spotted fever. The objective of this study was to perform a morphological and histological analysis of the male reproductive system of fed and unfed *A. cajennense*. The male reproductive system is formed by a pair of tubular testes dorsolaterally arranged in opisthosoma. They were divided into three regions: proximal region (next to vas deferens), median region and distal region (nearest to the blind ending of testis). Proximal regions are connected to the seminal vesicles by the deferent ducts and to accessory glands, similar to what was observed for other Ixodidae. Feeding plays a fundamental role in the development of the reproductive system, as in unfed individuals, the testes, the seminal vesicles and the accessory glands were smaller comparing with the fed individuals. In addition, the prospermia, precursors of the spermatozoa, were only observed in fed individuals. The germ cells were organized in spermatocysts, enveloped by a connective tissue. The cells in more advanced stages of spermatogenesis were localized in the distal region, in accord with studies in other ticks, but opposite to what was observed for other arthropods.

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## Introduction

Ticks are hematophage ectoparasites with great epidemiological importance for being vectors of viruses, bacteria and protozoa, which affect domestic and wild animals as well as human beings (Sonenshine 1991; Jongejan and Uilenberg 2004). Among the different species of ticks, *Amblyomma cajennense* (Fabricius, 1787), belonging to Ixodidae family, has great medical importance, mainly for being a vector of the bacterium *Rickettsia rickettsii*, the agent of the Brazilian spotted fever or Rocky Mountain spotted fever, which can lead the host to death (Labruna 2009; Szabó *et al.* 2013).

The testes of the ticks are paired tubular structures dorsolaterally arranged in the opisthosoma, and in some species, they are connected in the posterior region by a connective tissue. The first stages of spermatogenesis in the adult males occur from the distal (further away from the gland complex and nearest to the blind ending of testis) to the proximal region (in contact with the vas deferens); therefore, the germ cells of distal region are at an advanced stage of spermatogenesis process. The developing germ cells are arranged in spermatocysts covered by somatic cells (connective tissue), and in every spermatocyst, the cells present the same stage of development (Sonenshine 1991; Coons and Alberti 1999; Kiszewski *et al.* 2001; Oliveira *et al.* 2012). The spermiogenesis occurs in adult males, by the growth and differentiation of spermatids into non-motile elongated prospermia (precapacitated spermatozoa). The prospermia would be transported from the ejaculatory duct to the spermatophore and later transferred to the female during copulation. In the genital tract of the female, the capacitation, last phase of the spermiogenesis in ticks, would occur, and the prospermia would

become motile spermatozoa (Coons and Alberti 1999; Kiszewski *et al.* 2001).

The morphological description of the internal organs of the ticks is considered an important tool in parasitology, providing basis for further studies, mainly concerning chemical or natural methods of control (Camargo-Mathias and Furquim 2013). The morphology of the testes and accessory glands has been already described for some species of ticks (Sonenshine 1991; Garcia-Fernandez *et al.* 1998 ), as well as the variations of the reproductive organ according to the feeding stage, which influences the spermatogenesis (Coons and Alberti 1999; Matsuo and Mõri 2000).

In this study, the reproductive system of *A. cajennense* males in unfed and fed states is described for the first time, and the necessary feeding time for spermatogenesis to occur and the spermatids production have been established.

## Materials and Methods

### *Amblyomma cajennense* ticks

Unfed adults (males and females) of *A. cajennense* ticks were obtained from a colony maintained at the Brazilian Central of Studies on Ticks Morphology (BCSTM) at the Biosciences Institute of UNESP, Rio Claro, SP, Brazil. The ticks were kept under controlled conditions (28 ± 1 °C, 80% relative humidity and 12 h photoperiod) in a biological oxygen demand incubator and blood fed on New Zealand White rabbits.

This study was approved by the Ethics Committee in the Animal Use, CEUA, UNESP, Rio Claro, SP, Brazil, Protocol n° 942/2012.

### *Infestation*

Infestations were performed in New Zealand White rabbits, from UNESP Botucatu (SP, Brazil), according to Bechara *et al.* (1995). Thirty couples of unfed adults of *A. cajennense* were released in each chamber, and three males were collected per day for 10 consecutive days. In addition, three unfed males were sampled for comparison in each technique used.

The specimens were maintained in the refrigerator for thermal shock anaesthesia and were dissected under stereomicroscope on Petri dishes containing phosphate-buffered saline (PBS) solution (7.5 g de NaCl + 2.38 g de $Na_2HPO_4$ + 2.72 g de $KH_2PO_4$ + 1000 mL of distilled water) for the removal of testis samples processed according to different techniques.

### *Histology*

The material was fixed in 4% paraformaldehyde, dehydrated in an alcoholic series (70%, 80%, 90% and 95%) and embedded in Leica historesin for 24 h, followed by polymerization with Leica historesin. The resin blocks were sectioned on a Leica RM2255 microtome. Sections with 3 μm thickness were stained with haematoxylin and eosin (HE), for the observation and description of the general morphology of the tissue (Junqueira and Junqueira 1983). The slides were mounted in Canada synthetic balsam, and they were photographed using a LEICA DM750 photomicroscope.

### *Scanning electron microscopy (SEM)*

For the SEM analysis, testis was fixed in Karnovsky (Karnovsky 1965) for 24 h and dehydrated in a graded 70–100% acetone series. The material was processed by critical point drying, sputtered with gold and examined and photographed with a SEM Hitachi TM3000 (Tokyo, Japan).

### *Confocal laser scanning microscopy*

*Microfilaments (F-Actin)*. For the analysis of microfilaments (F-Actin), the material was fixed in 4% paraformaldehyde for 24 h at room temperature. Hence, it was permeabilized with Triton X-100 0.1% for 2 h. After rinsing it twice with phosphate-buffered saline (PBS) for five minutes each, it was incubated for 2 h in phalloidin conjugated to Alexa Fluor 488 (5 μL from the stock solution of "Alexa Fluor 488 Phalloidin" (Molecular Probes) + 200 μL of PBS + 2 μL of BSA). After rinsing with PBS twice (five minutes each), the material was mounted using ProLong® (Molecular Probes, Eugene, OR, USA) Gold reagent with DAPI for the staining of the nuclei. The nuclei were also showed with propidium iodine at 10 μg/mL for 2 h, preceded by treatment with RNAse at 10 mg/mL for 30 min. The material was analysed and photographed using confocal laser scanning microscope LEICA TCS-SP5 II, Wetzlar, Germany.

## Results

Although three males had been collected each day for ten consecutive days of feeding, here described are only the testicles of the males that showed significant differences, that is, males collected after 1, 6 and 10 days following the infestation. The testes were divided into three regions: proximal region (in contact with the vas deferens), median region and distal region (further away from the gland complex and nearest to the blind ending of testis).

### *Scanning electron microscopy*

Testes of *A. cajennense* ticks are paired tubular structures dorsolaterally disposed in the opisthosoma (Fig 1A,C). The proximal region of the testis is connected to the seminal vesicle by deferent duct (Fig 1A,C), through which elongated spermatids are delivered, giving origin to the spermatozoa in the reproductive tract of female. The male genital system of

 

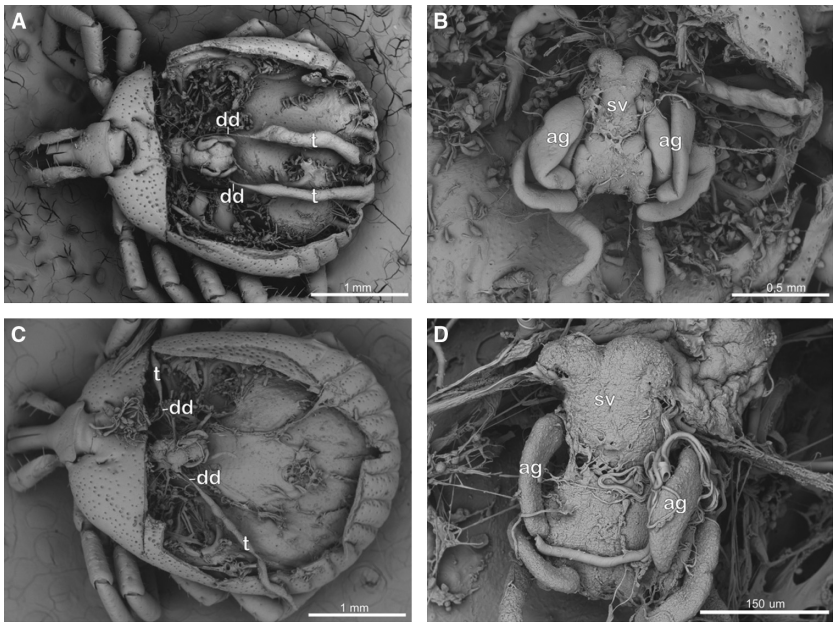


**Fig. 1**—Scanning electron microscopy (SEM) of the male reproductive system of *Amblyomma cajennense* fed —**A–B** and unfed —**C–D**. —**A**. General view of the testes and vas deferens. —**B**. General view of the male accessory genital glands and seminal vesicle. —**C**. General view of the testes and vas deferens. —**D**. General view of the male accessory genital glands and seminal vesicle. ag: accessory genital glands; dd: vas deferens; sv: seminal vesicle; t: testes.

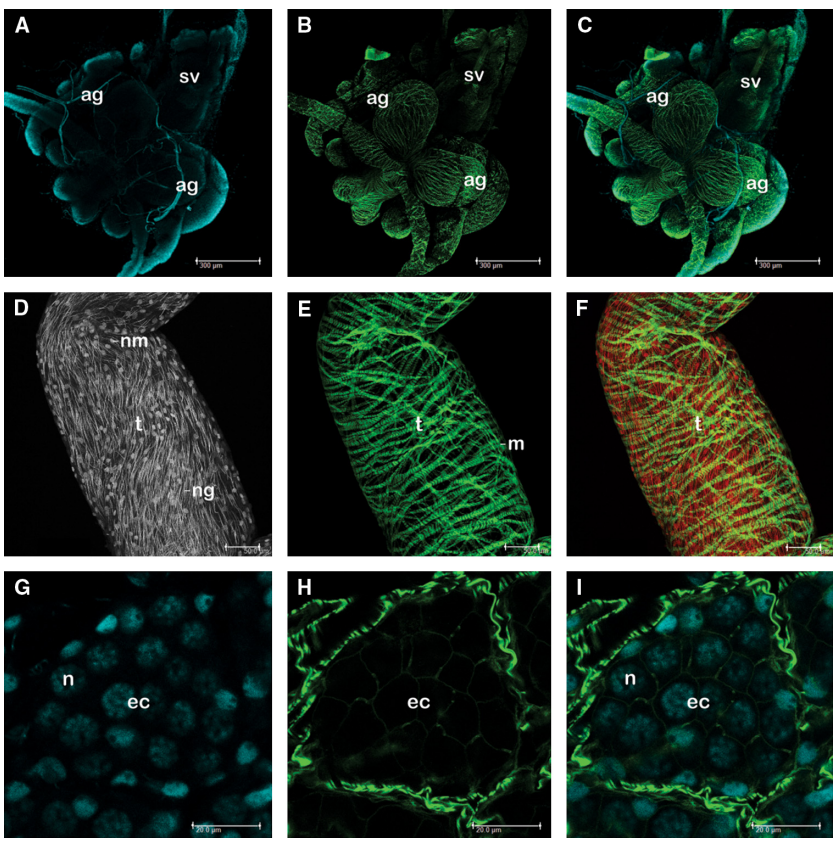


**Fig. 2**—Confocal images of the male reproductive system of *Amblyomma cajennense*. —**A–C**. General view of the male accessory genital glands and seminal vesicle. —**A**. Nuclei (DAPI). —**B**. F-Actin. —**C.** Overlap. —**D–F.** Detail of the testis. —**D**. Nuclei (Propidium Iodide). —**E**. F-Actin. —**F.** Overlap (testis). —**G–I**. Detail of the spermatocysts. —**G**. Nuclei (DAPI). —**H**. F-Actin. —**I**. Overlap. ag: accessory genital glands; ec: spermatocysts; n: nuclei; ng: nuclei of germ cells; nm: nucleus of muscular cells; sv: seminal vesicle; t: testes.

this species presents, next to the seminal vesicles, a complex of multilobulated accessory glands (Fig 1B,D). In fed males, the testes are thicker (Fig 1A,B), while in unfed males, the testes are narrow (Fig 1C,D).

*Confocal microscopy*

The results obtained through confocal laser scanning microscopy showed that the testes, the accessory glands and the

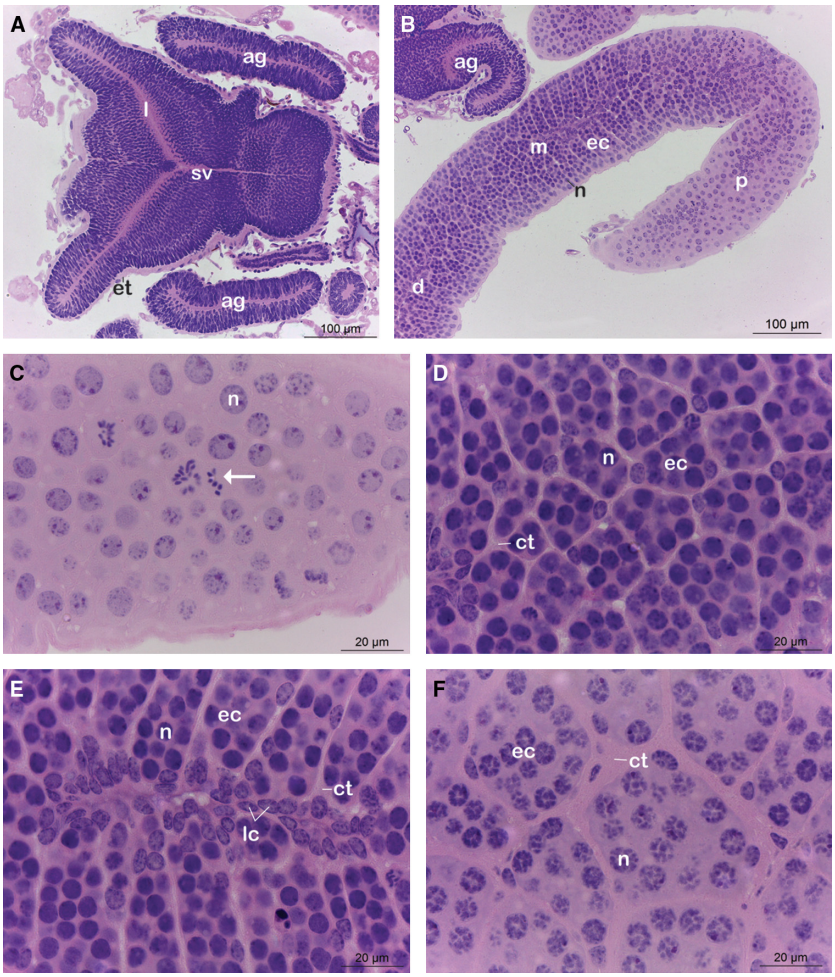


**Fig. 3**—Histological sections of the male reproductive system of unfed *Amblyomma cajennense*. —**A**. Male accessory genital glands and seminal vesicle, surrounded by epithelial tissue, with secretions within the lumen. —**B**. General view of the testis showing the proximal median and distal regions. —**C**. Detail of the proximal region showing germ cells in division. —**D–E**. Detail of the median region showing the organization of germ cells in spermatocysts. —**F**. Detail of distal region showing the organization of germ cells in spermatocysts, surrounded by connective tissue. ag: accessory genital glands; arrowhead: germ cells in division; ct: connective tissue; ec: spermatocysts; et: epithelial tissue; d: distal region of testis; l: lumen; m: median region of testes; lc: somatic cells surrounding the lumen; n: nuclei; ng: nuclei of germ cells; nm: muscular cells; p: proximal region of testes; sv: seminal vesicle.

seminal vesicle seem to be externally covered by a layer of muscle, as it was observed a great amount of actin filaments in this region (Fig 2A–F). These muscular cells apparently present round-shaped nuclei (Fig. 2D), different from the apparently elongated nuclei of the spermatids (Fig. 2D). The technique also showed the spermatocysts and the cells of a particular spermatocyst are apparently in the same stage of development (Fig. 2G–I).

### *Histology*

The histological analysis showed that the three regions (i) proximal – in contact with the deferent ducts, (ii) median (intermediate region) and (iii) distal (the posterior region, next to the ending of the testis) have different characteristics.

*Unfed males.* The glandular complex and the seminal vesicles are externally covered by pavimentous epithelium, and both present narrow lumen with little secretion in its interior (Fig. 3A).

In the unfed ticks, it is observed that the proximal region of the testis presents round-shaped germ cells and nuclei with disperse chromatin, and they are not organized in spermatocysts (Fig. 3B–C). In this region, germ cells still in process of division can be observed (Fig. 3C). In the median region, the germ cells are round shaped, arranged in spermatocysts and present nuclei with condensed chromatin (Fig. 3D–E). In the

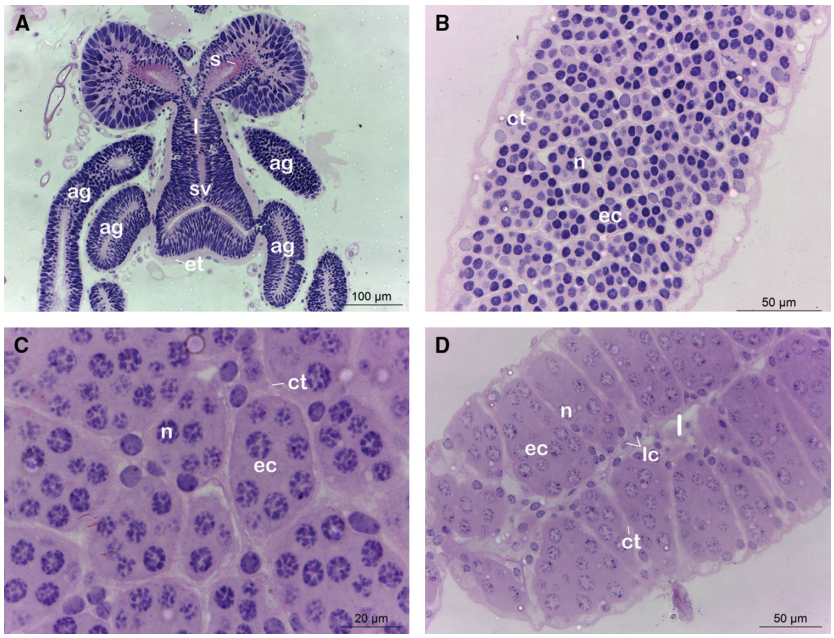


**Fig. 4**—Histological sections of the male reproductive system of *Amblyomma cajennense* after a day of feeding. —**A**. Male accessory genital glands and seminal vesicle. —**B**. Proximal region. —**C**. Detail of median region showing the organization of germ cells in spermatocysts. —**D**. Distal region**.** ag: accessory genital glands; ct: connective tissue; ec: spermatocysts; et: epithelial tissue; l: lumen; lc: somatic cells surrounding the lumen; n: nuclei; s: secretions; sv: seminal vesicle.

distal region, the germ cells have less condensed chromatin and are arranged in spermatocysts externally covered by a thin layer of connective tissue (Fig. 3F).

*Feeding males.*

*One day feeding.* In male ticks collected after 1 day feeding on the host, It was observed that both the seminal vesicles and the accessory glands present more dilated lumen in relation to those unfed (Fig. 4A), with secretion in its interior (Fig. 4A).

In the proximal region of testes, the germ cells are round shaped, grouped in small spermatocysts, and their nuclei have condensed and homogeneously distributed chromatin. These cells are covered by a connective tissue (Fig. 4B). In the median region, the germ cell nuclei are disposed in spermatocysts, presenting round-shaped nuclei with less decondensed chromatin (Fig. 4C). In the distal region, the germ cells are round shaped, arranged in spermatocysts and show nuclei with decondensed chromatin (Fig. 4D). In this region, it is also possible to observe a narrow lumen among the spermatocysts (Fig. 4D).

*Six days feeding.* In ticks collected after 6 days feeding, secretion could be found in the dilated lumen of the accessory glands and the seminal vesicle (Fig. 5A). In the proximal region of the testes, the germ cells are round shaped, arranged in spermatocysts, and their nuclei show condensed chromatin (Fig. 5B). These cells present cytoplasmic bridges among them (Fig. 5B). In the median region, it is possible to observe the round-shaped germ cells with cytoplasmic bridges (Fig. 5C–D). In the distal region, the germ cells located in the spermatocysts are elongated and called prospermia then will finally transform into the spermatozoa in the reproductive tract of the female (Fig. 5E–G). These cells present condensed and elongated nuclei (Fig. 5F–G).

*Ten days feeding.* In ticks collected after 10 days feeding, elongated spermatids in the lumen of the seminal vesicle are observed as well as a great amount of the secretion in the lumen of the accessory glands (Fig. 6A). The proximal region presents spermatocysts with voluminous and round-shaped germ cells (Fig. 6B). The presence of cytoplasmic bridges among the germ cells is also observed in this region (Fig. 6B). In the median region, the spermatocysts contain round-shaped cells, and their nuclei have little condensed chromatin (Fig. 6C). In the distal region, the prospermia formation is observed (Fig. 6D).

## Discussion

The morphological analysis of the reproductive system of *A. cajennenese* males revealed that the testes are paired tubular structures dorsolaterally arranged in the opisthosoma, and deferent ducts connect the proximal regions of testes with seminal vesicle, similarly to observed in *Dermacentor occidentalis* (Oliver and Brinton 1972), *Dermacentor andersoni* (Sonenshine 1991), *Ornithodoros moubata* (Coons and Alberti 1999), *Haemaphysalis longicornis* (Matsuo and Mõri 2000) and

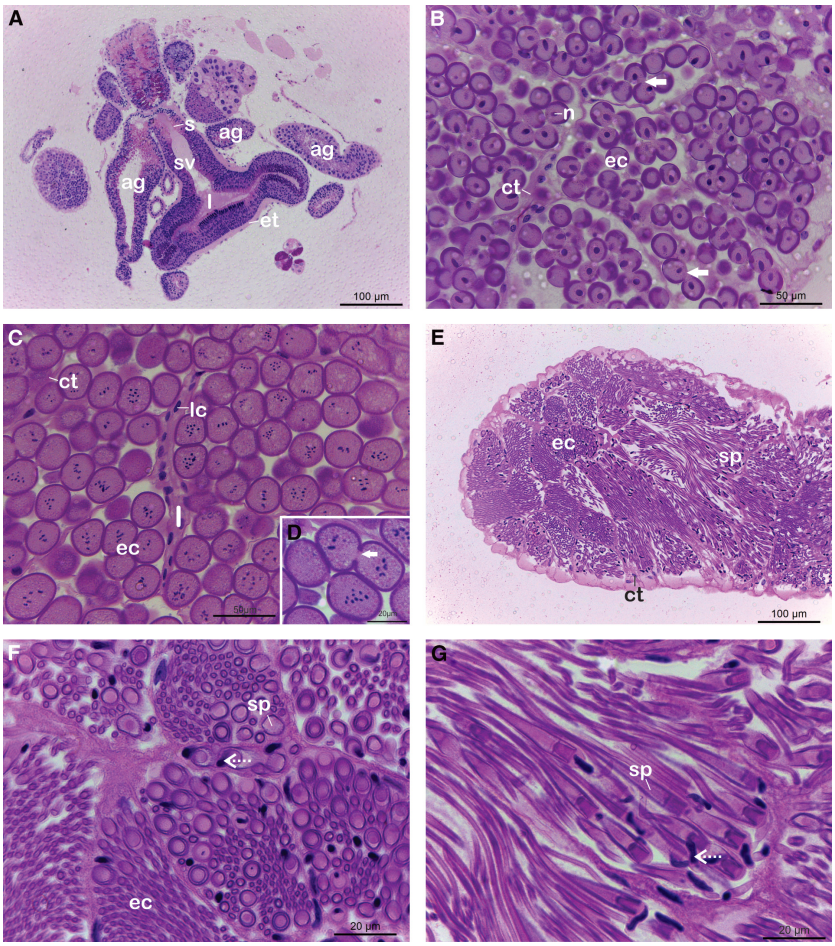


**Fig. 5**—Histological sections of the male reproductive system of *Amblyomma cajennense* after six days of feeding. —**A**. Male accessory genital glands and seminal vesicle. —**B**. Proximal region. —**C**. Median region. —**D**. Detail of the intercellular bridge between the germ cells of the median region. —**E**. Distal region showing the prospermia. —**F,G**. Detail of the prospermia showing the elongated nucleus. ag: accessory genital glands; arrowhead: intercellular bridge of germ cells; ct: connective tissue; dotted arrow: elongated nucleus of prospermia; ec: spermatocysts; et: epithelial tissue; l: lumen; lc: somatic cells surrounding the lumen; n: nuclei; s: secretions; sp: prospermia; sv: seminal vesicle.

*Rhipicephalus sanguineus* (Oliveira *et al.* 2012). The seminal vesicles, accessory glands and the testes are externally covered by a muscular cells, similarly to what was observed for *D. occidentalis* (Oliver and Brinton 1972), *D. andersoni* (Sonenshine 1991) and other arthropods as arachnids, diptera, hymenoptera and orthoptera (Ball and Vinson 1984; Valdez 2001; Ferreira *et al.* 2006).

In some species of Ixodidae ticks, the male reproductive system undergoes transformations depending on how long the ticks remain attached to the host. In unfed *A. cajennense* the testes, the seminal vesicles and the complex of accessory glands are less developed; therefore, smaller when compared with fed individuals, corroborating the data described by Matsuo *et al.* (1997a) for *H. longicornis.*

It has not been established whether feeding would be responsible only to provide the necessary nutrients for spermatogenesis or whether other stimuli would occur during feeding, which would trigger cascade events such as the release of hormones involved in spermatogenesis (Oliver and Dotson 1993).

With the exception of the proximal region of the testes of unfed *A. cajennense,* the germ cells are organized in spermatocysts, covered by connective tissue; similar configurations were described for *Rhipicephalus appendiculatus* (Till 1961), *D. variabilis* (Sonenshine 1991), *Haemaphysalis longicornis* (Matsuo *et al.* 1997a), *Boophilus microplus* (Normann 1998) and *R. sanguineus* (Oliveira *et al.* 2012). The development of the germ cells in the testes of *A. canjennense* occurs from the proximal region (next to the deferent ducts) to the distal region (next to the posterior end of the testis). Therefore, the cells of the distal region are always found in more advanced stages in the process of spermatogenesis than those of the median and proximal regions. This pattern, however, is different from what was observed for other arthropods. In insects, for example, at the distal end of each testis tubule or follicle, there is a germarium, in which proliferating germ cells pro-

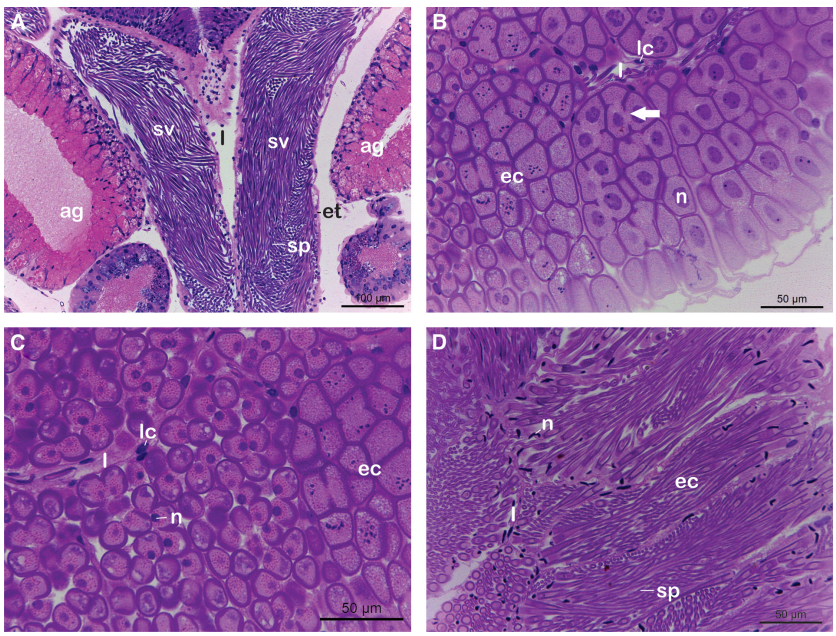


**Fig. 6**—Histological sections of the male reproductive system of *Amblyomma cajennense* after 10 days of feeding. —**A**. Male accessory genital glands and seminal vesicle. —**B**. Detail of the proximal region showing intercellular bridges between the germ cells. —**C**. Median region. —**E**. Detail of the distal region showing prospermia. ag: accessory genital glands; arrowhead: intercellular bridge of germ cells; ec: spermatocysts; et: epithelial tissue; l: lumen; lc: somatic cells surrounding the lumen; n: nuclei; sp: prospermia; sv: seminal vesicle.

duce spermatogonia, and subsequent stages of spermatogenesis are distributed towards the proximal end of the testis compartment (Sonenshine 1991; Matsuo *et al.* 1997b; Triplehorn and Johnson 2011).

The germ cells in the interior of the spermatocysts of *A. cajennense* are in the same stage of development. However, in a same region of the testis, the spermatocysts were found in different stages of its development, which has also been described for *D. occidentalis* (Oliver and Brinton 1972) and for *H. longicornis* (Matsuo *et al.* 1997b). Cytoplasmic bridges among the cells inside the spermatocysts in more advanced stages of development were also observed in *A. cajennense.* Oliver and Brinton (1972) also observed these bridges in *D. occidentalis* and reported that they would probably facilitate the synchronized differentiation of the germ cells in the spermatocysts through the distribution of regulation factors.

According to Coons and Alberti (1999) and Kiszewski *et al.* (2001), in some species of Ixodidae, the formation of prospermia would be concluded after the male has fed. In *A. cajennense,* the presence of prospermia was only observed in the ticks collected after 6 days of feeding on the host, similarly to what was observed for *D. variabilis*, where the prospermia was observed for the first time in males with 130 h of attachment on the host (Sonenshine 1991). However, in *D. occidentalis,* the spermiogenesis occurs in the male with 72 h of attachment on the host (Oliver and Brinton 1972). In the testes of *H. longicornis*, the early spermatids were observed in males fed for at least 3 days (Matsuo *et al.* 1997b) The necessary time for the complete engorgement of the females could also be related with the necessary time for the formation and transference of prospermia by the males, while the females complete their engorgement only after receiving the spermatophore; however, further studies are necessary to confirm this fact.

Thus, considering the data obtained in this study, it can be concluded that the morphology of the male reproductive system, its arrangement and the development of germ cells of *A. cajennense* ticks are similar to other Ixodidae species. The development of germ cells in *A. cajennense* begins at the proximal region advancing to the distal one, which differs from the pattern observed for other arthropods as insects but are similar to the pattern described for other ticks species. Feeding process plays a fundamental role in the development of the testes, and the spermatogenesis is completed only after the male has fed, once elongated spermatids were only observed in adult males after 6 days of feeding.

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## Conflict of interest statement

No conflict of interests to declare.

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