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The extensible integument of *Rhipicephalus sanguineus* female ticks in different feeding stages: a morphological approach

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Abstract

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Ticks are among the most important pathogen vectors worldwide, and the knowledge of their internal and external morphology may provide relevant information that will allow an adequate control of these animals. In this sense, the integument gains prominence, not only because of its wide capacity of expansion, but also due to the fact that this region is usually the first site of contact with natural and synthetic acaricides. Therefore, this study aimed to describe the morphology of alloscutal integument of *R. sanguineus* female ticks in three different feeding stages by means of histological and ultramorphological analysis. Significant differences were observed in all stages, with respect to the size of cells and layers, and to cuticle unfolding throughout the feeding process. Cuticle is divided into two main layers, the epicuticle and the procuticle, and is separated from a unistratified epithelium by a subcuticular layer. Procuticle, in turn, is divided into two layers, the endocuticle and exocuticle. Unfed female ticks showed cuticle with numerous folds, which decrease in quantity as the feeding expansion of the whole animal occurs. Opening of dermal glands and presence of setae also differed among groups.

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Introduction

Brown Dog The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae), is the most frequently found ixodid worldwide (Dantas-Torres 2010), being observed in almost all places inhabited by pet dogs and, consequently, by humans (Paz *et al.* 2008). This tick species is active throughout the year not only in tropical and subtropical regions, but also in some temperate areas (Dantas-Torres 2010). It is a known vector of pathogens, being considered, from the veterinary point of view, the most important species among dog disease transmitters (Dantas-Torres 2010). Although it has dogs as its main host, nymphs or even adults can infest a wide variety of rodents and small mammals, besides other animals (Blagburn and Dryden 2009; Dantas-Torres 2010). Each life stage of this species parasites the host for several days. During this period, the ticks feed mainly on blood, but also ingest lymph tissue and remnants of the dermis and/or epidermis. At the end of the parasitic period, engorged nymphs and larvae detach from the host to perform ecdysis, while the engorged females, fertilized by males, detach to lay the eggs (Labruna 2004).

In ticks, the integument presents profound and extensible folds, which distends and unfold as the feeding process occurs (Bughdadi 2008). This region consists of a single layered epidermis that secretes an acellular cuticle (Hackman 1982; Amosova 1983; Coons and Alberti 1999), exchanged during ecdysis after blood ingestion in larvae and nymphs (Coons and Alberti 1999). The cuticle forms the exoskeleton, whose primary function is the protection and support of the animal,

as well as having a role in water balance, in view of its impermeability to water (Amosova 1983). It is a heterogeneous structure, homologue to the cuticle of insects and other arthropods (Hackman 1982), and divided in two main layers: epicuticle, thinner and external, and procuticle, thicker and internal (Hackman 1982; Amosova 1983; Coons and Alberti 1999). Because it surrounds the tick, the integument is the first contact site for acaricide agents applied topically.

Thus, based on the information given, this study aimed to describe the integument morphology of *Rhipicephalus sanguineus* female ticks in different feeding stages, in order to detail this organ composition as well as to characterize the changes resulted from blood ingestion and body volume increase in these animals.

Methods

Unfed, semi-engorged and engorged female ticks of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) species were used in this experiment.

Unfed female and male ticks were obtained directly from the colony maintained in a biological oxygen demand (BOD) incubator under controlled conditions (28 ± 1 °C, 80% humidity, in a 12 h photoperiod), in the Biosciences Institute vivarium of São Paulo State University (UNESP), Rio Claro/SP, Brazil. To obtain ticks in different feeding stages, 20 couples of *R. sanguineus* were released inside special feeding chambers set on the back of naïve New Zealand white female rabbits (without prior exposure to tick infestation), following the methodology described by Bechara *et al.* (1995). Then, semi-engorged females with approximately 4 days of feeding and fully engorged females with approximately 7 days of feeding were collected and maintained under controlled conditions in a BOD incubator before dissection. For each feeding stage (unfed, semi-engorged and engorged), 10 females ticks were used, totalizing 30 specimens.

The collected ticks were dissected under stereomicroscope in Petri dishes containing phosphate-buffered saline (PBS) solution (NaCl 0.13 M, Na_2HPO_4 0.017 M, KH_2PO_4 0.02 M, pH 7.2) for withdrawal of alloscutal integument samples. The samples were obtained from the dorsal region of alloscutum, near the central portion of the tick, as outlined in Fig. 1.

All experimental procedures performed in this study were approved by the Ethics Committee in Animal Use, CEUA, UNESP, Rio Claro/SP, Brazil, protocol number 2206, decision number 021/2012.

Light microscopy

For histological analysis, the collected material was immediately fixed in 4% paraformaldehyde solution for 1 week and, then, transferred to sodium phosphate buffer solution (pH 7.2) for 24 h. The samples were subsequently subjected to dehydration in an ascending series of ethanol (70, 80, 90 and 95%, for 20 min in each solution), overnight infiltration in Leica historesin, followed by polymerization with Leica historesin plus a hardener agent.

The resin blocks containing the material were sectioned on a Leica RM2255 microtome, and the sections subjected to staining with hematoxylin and eosin technique (HE), for the observation and description of the general morphology of the tissue (Junqueira and Junqueira 1983). The microscopic slides obtained were mounted in Canada synthetic balsam, and the material was photographed in a Leica DM150 light photomicroscope, equipped with a Leica ICC50 HD camera, by means of the Leica LAS V.3.8 software.

Scanning electron microscopy

The collected samples were dehydrated in an ascending series of ethanol (70–100%, with a duration of 15 min each bath), followed by two baths in acetone, also for 15 min each. After critical point drying, the materials were stuck with double-sided tape on aluminium brackets in order to be metalized with gold sputtering. Then, they were examined and photographed in Hitachi TM3000 Scanning Electron Microscope, in the Electron Microscopy Laboratory, Department of Biology, UNESP – Rio Claro, SP, Brazil.

Results

The integument of the alloscutum of *R. sanguineus* females was outlined for each feeding stage (Fig. 1).

Externally, in unfed females, a significative amount of folds in the cuticle was observed, with numerous setae and dermal glands openings. Additionally, the cuticle arrangement was visualized in a zigzag pattern, increasing the expansion area (Fig. 2A–C). In semi-engorged individuals, cuticle expansion promoted the disappearance of most folds in the integument, and the zigzag pattern is no longer observed. The number of setae apparently decreases, and the dermal glands openings are still observed, which are possibly releasing their secretion products in the cuticle external layer, as shown by the large wax amount in the external portion (Fig. 2D–F). In the fully engorged stage, however, the integument is completely expanded, presenting a significantly lower number of folds. Setae can no longer be observed, and many dermal glands openings are present in all the cuticle extension (Fig. 2G–I).

The integument is composed, from the outer to the inner layer, of three distinct regions: cuticle, epidermis and connective tissue, which serves as a support for the epithelial cells (Figs 3A, 4A and 5A). Between the cuticle and the epidermis is found a subcuticular acellular layer. From one feeding stage to the other, individuals show variations in cuticle thickness and unfolding and in the size of epithelial cells, besides the observation of other cell types.

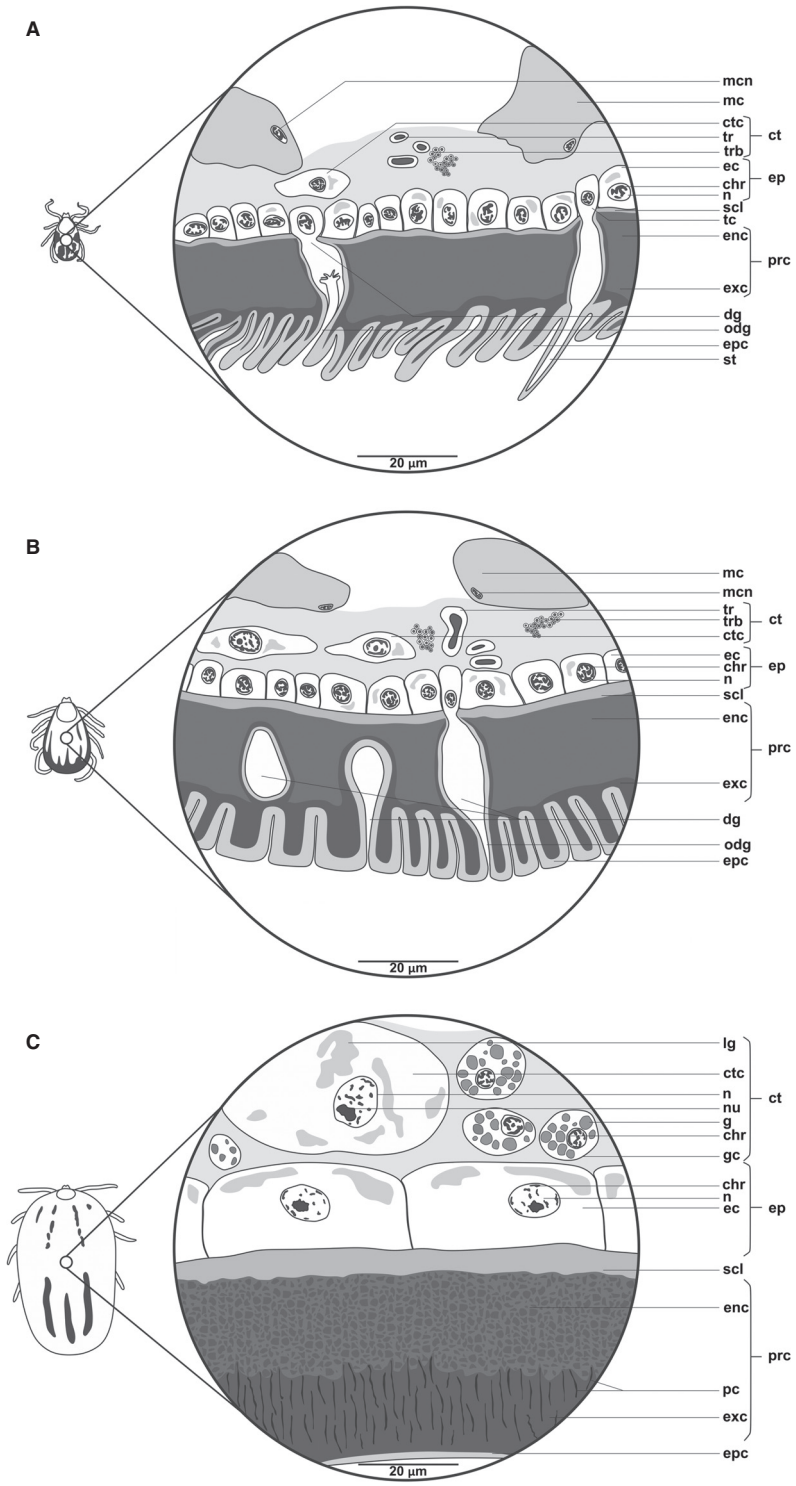

Fig. 1—Schematic drawing of the alloscutal integument of *R.* sanguineus ticks for each feeding stage: (A) unfed female; (B) semi-engorged female; e (C) engorged female. Legends: (*epc*), epicuticle procuticle (*prc*), endocuticle (*enc*), exocuticle (*exc*), subcuticular layer (*scl*), epidermis (*ep*), epithelial cell (*ec*), connecting tissue (*ct*), connecting tissue cell (*ctc*), granular cell (*gc*), cytoplasmic granules (*g*), seta (*st*), trichogen cell (*tc*), dermal gland (*dg*), opening of dermal gland (*odg*), muscle cell (*mc*), muscle cell nucleus (*mcn*), trachea (*tr*), tracheal branch (*trb*), nucleus (*n*), nucleolus (*nu*), lipid granules (*lg*), pore canals (*pc*), and chromatin (*chr*). Bars: (A–C) 20 µm.

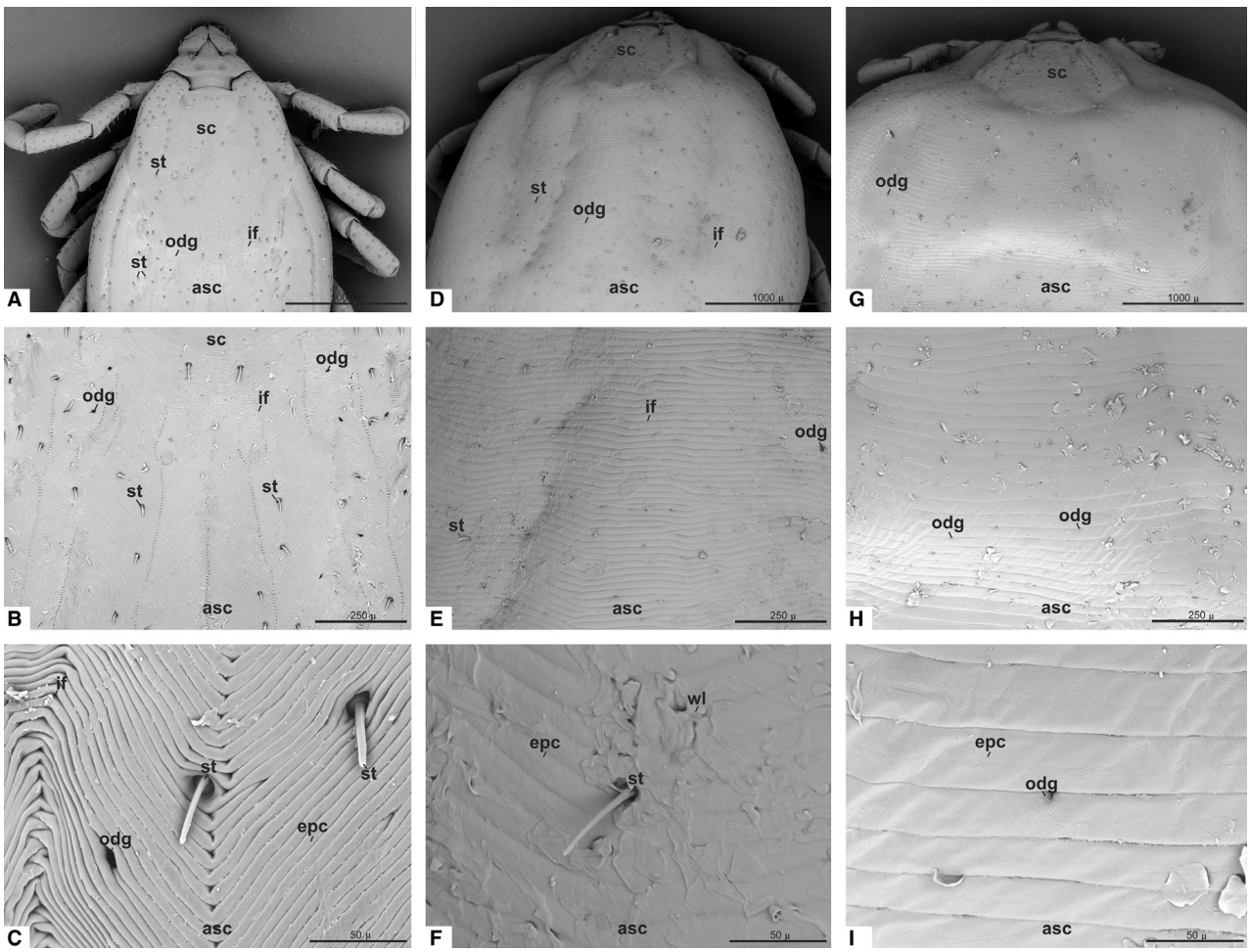

Fig. 2—Dorsal view of *R. sanguineus* female ticks in different feeding stages by scanning electron microscopy (SEM). (A–C) General view and details of the integument of unfed female ticks, showing the scutum (*sc*) and alloscutum (*asc*), with the opening of dermal glands (*odg*), seta (*st*) and integument folds (*if*). (D–F) General view and details of the integument of semi-engorged female ticks, showing the scutum (*sc*) and alloscutum (*asc*), with the opening of dermal glands (*odg*), few setae (*st*), and minor amounts of cuticle folds (*cf*). In this stage, it is possible to observe the wax layer of epicuticle (*epc*), deposited in higher quantity than in the other stages. (G–I) General view and details of the integument of fully engorged female ticks, showing the scutum (*sc*) and alloscutum (*asc*), with the opening of dermal glands (*odg*), but with virtually no setae and with the cuticle unfolded. Magnifications: (A–C) 50x; (D–F) 150x; (G–I) 800x. Bars: (A–C) 1000 µm; (D–F) 250 µm; (G–I) 50 µm.

The cuticle is the external acellular portion of the integument, covering the body of the tick. In unfed individuals, it presents numerous folds, showing less folds in semi-engorged females, and total unfolding in the fully engorged stage. It is a heterogeneous structure, in which two main distinct layers can be observed: the procuticle (internal) and the epicuticle (external). The procuticle is divided in other two distinct layers: the endocuticle, near to the epidermis, and the exocuticle, in contact with the epicuticle. The epidermis is comprised of an unistratified epithelium with cubic cells that varies in size according to the feeding stage. From the unfed to the engorged stage, the cuticle does not show great variation in its thickness, but the differentiation in the procuticle layers is more evident. The epicuticle, however, almost disappears in completely engorged females.

Unfed females

In this stage, the cuticle is composed by an evident and thickened epicuticle, when compared to other feeding stages, besides its yellow staining. The procuticle is very thick, but it is not possible the visualization of any internal structure. Staining of the two layers constituting the procuticle are subtly different. The endocuticle reacts weakly to eosin, whereas the exocuticle shows a red staining slightly stronger. Therefore, distinction between these two layers is difficult (Fig. 3D,E).

The epidermis of these individuals is constituted by a cubic cell layer, which are smaller when compared to the other stages. These cells present a round nucleus, with low condensed chromatin, reacting with medium intensity to hematoxylin. The nucleolus cannot be observed in this stage. The

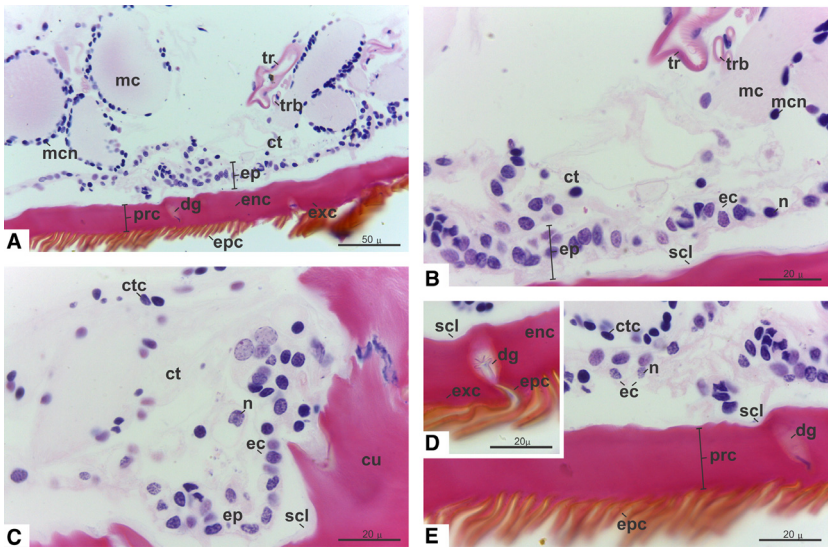

Fig. 3—(A) General view and (B–E) details of the alloscutal integument of unfed *R. sanguineus* female ticks. Legends: cuticle (*cu*), epicuticle (*epc*), procuticle (*prc*), endocuticle (*enc*), exocuticle (*exc*), subcuticular layer (*scl*), epidermis (*ep*), epithelial cell (*ec*), connecting tissue (*ct*), connecting tissue cell (*ctc*), dermal gland (*dg*), muscle cell (*mc*), muscle cell nucleus (*mcn*), trachea (*tr*), tracheal branch (*trb*), nucleus (*n*). Magnifications: (A) 400x; (B–E) 1000x. Bars: (A) 50 µm; (B–E) 20 µm.

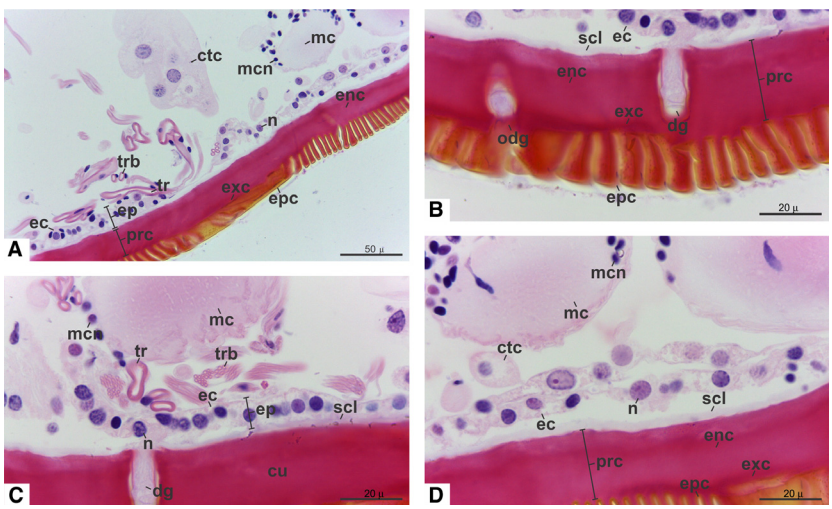

Fig. 4—(A) General view and [B-D) details of the alloscutal integument of semi-engorged *R. sanguineus* female ticks. Legends: epicuticle (*epc*), procuticle (*prc*), endocuticle (*enc*), exocuticle (*exc*), subcuticular layer (*scl*) epidermis (*ep*), epithelial cell (*ec*), connecting tissue cell (*ctc*), dermal gland (*dg*), opening of dermal gland (*odg*), muscle cell (*mc*), muscle cell nucleus (*mcn*), trachea (*tr*), tracheal branch (*trb*), nucleus (*n*). Magnifications: (A) 400x; (B–D) 1000x. Bars: (A) 50 µm; (B–D) 20 µm.

cytoplasm reacts weakly to eosin, presenting a light red staining (Fig. 3C).

Between the epidermis and the cuticle is located the subcuticular layer, with less thickness in relation to the other feeding stages, and with weak purple staining (Fig. 3B).

The connective tissue shows elongated cell, similar in size to the epithelial cells, nuclei with very condensed chromatin, which react strongly to hematoxylin, and cytoplasm with staining similar to the epithelial cells. Many trachea and its ramifications are also observed in this tissue (Fig. 3A,B).

Semi-engorged females

The cuticle of these females also presents an easily identifiable epicuticle, with yellowish staining, more intense than in unfed females, but with similar thickness. The procuticle is similar

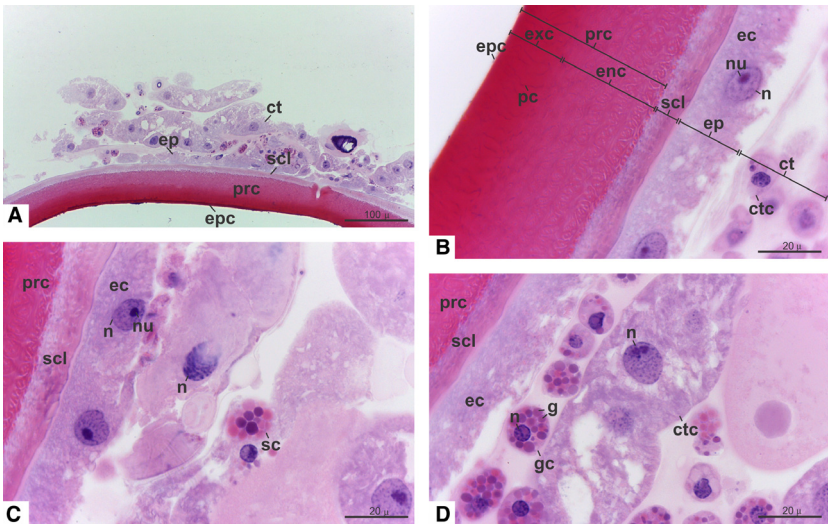

Fig. 5—(A) General view e (B–D) details of the alloscutal integument of fully engorged *R. sanguineus* female ticks. Legends: epicuticle (*epc*), procuticle (*prc*), endocuticle (*enc*), exocuticle (*exc*), subcuticular layer (*scl*) epidermis (*ep*), epithelial cell (*ec*), connecting tissue (*ct*), connecting tissue cell (*ctc*), pore canals (*pc*), granular cell (*gc*), granules (*g*), nucleus (*n*), nucleolus (*nu*). Magnifications: (A) 200x; (B–D) 1000x. Bars: (A) 100 µm; (B–D) 20 µm.

in thickness to the previous stage, not being possible the observation of structures in its interior. The endocuticle reacts more weakly to eosin and the exocuticle more intensely. Therefore, the distinction between the two layers is facilitated (Fig. 4B).

The epithelial cells are apparently bigger than in the previous stage. These cells are cubic, with a round nucleus and condensed chromatin, which reacts strongly to hematoxylin. It is not possible the distinction of the nucleolus in most epithelial cells. The cytoplasm reacts strongly to eosin, presenting a more intense staining than in unfed animals (Fig. 4C).

The subcuticular layer is slightly thicker than in individuals in the previous stage and reacts with medium intensity to hematoxylin, mainly, showing a light purple staining (Fig. 4D).

Cells from the connective tissue are elongated, very similar to the other feeding stages. They show nuclei with condensed chromatin, which react strongly to hematoxylin, besides a weakly eosin stained cytoplasm. Tracheas and its ramifications can be visualized (Fig. 4C,D).

Fully engorged females

The cuticle is composed of a thin epicuticle, of difficult observation, and a well-developed procuticle. The epicuticle is underdeveloped, almost losing the yellowish staining observed in previous stages. The procuticle, as in other stages, also reacts to eosin, although with less intensity. In the endocuticle, many structures which form the pore channels that run through the exocuticle and open in the epicuticle can be observed. These are arranged in different directions in the endocuticle and epicuticle permitting the distinction of these layers. In the endocuticle, they are anastomosing, ramified and are found in great quantity, whereas in the exocuticle, they are less numerous and less ramified. Moreover, the staining intensity of the endocuticle by the eosin is less than in the exocuticle, allowing the differentiation between these two layers (Fig. 5B).

The epidermis is composed of large cells, with round nucleus and evident nucleolus, strongly stained by hematoxylin. The chromatin, however, is partially decondensed. Spaces that are not stained by the reagents are observed. The cytoplasm reacts to hematoxylin and eosin, resulting in a purple staining (Fig. 5B,C).

The subcuticular layer reacts weakly to eosin and hematoxylin, when compared to the other layers. However, its staining is more intense than in other feeding stages, and its thickness is relatively greater (Fig. 5B).

The connective tissue shows large cells varying from elongated to oval, with a light purple stained cytoplasm, nucleus with lightly condensed chromatin, and an evident nucleolus. Cells with great quantity of granules were also observed, which were differentially stained by hematoxylin and eosin. These cells are not observed in any other feeding stage. It is also observed, in the connective tissue, a large number of tracheas and its ramifications (Fig. 5D).

Discussion

The biological success of the Phylum Arthropoda is directly associated with its integument versatility, because virtually all physiological aspects of these animals is directly affected by its locomotive, protective and support properties, besides its interaction with all body systems (Hepburn 1976). As in other

arthropods, the integument of ticks consists of a cuticle and an epidermal cell layer that secretes the cuticle, which function is to support and protect the animal, besides regulating the water balance, due to its great impermeability (Amosova 1983). The cuticle forms the exoskeleton, exchanged during ecdysis that occurs in larvae and nymphs after blood ingestion (Coons and Alberti 1999).

The capacity of the ixodid ticks to feed for long periods of time and to ingest large blood amounts is possible due to a great number of morphological and physiological adaptations (Amosova 1983). The presence of deep and extensible folds in the cuticle is common among Ixodidae, and blood ingestion causes a rapid distension and cuticular unfolding (Bughdadi 2008). This unfolding and distension of the integument allow a characteristic increase in body volume during the feeding process (Amosova 1983), occurring mainly in females and essentially in the region of the alloscutum, which accommodates the intestine distended by the large amount of food (Hackman 1982; Dillinger and Kesel 2002).

Such folds are arranged in a zigzag pattern, allowing an increase in body size, both vertically and tangentially (Coons and Alberti 1999). This pattern was observed in unfed *R. sanguineus* females and has been altered throughout blood ingestion until complete engorgement, corroborating the morphological patterns described for the group. Externally, the observation of the integument in the aloscutum of *R. sanguineus* through scanning electron microscopy (SEM) revealed striking differences for each feeding stage, once cuticular folding, as reported by Dillinger and Kesel (2002), are closely related to the animal nutritional status, differentiated in three phases, namely: fast, semi-engorgement and full engorgement. The large volume of blood ingested causes volume changes macroscopically visible not only in *R. sanguineus*, as well in many other ixodid tick species. *Ixodes ricinus* females are capable of consuming blood amounts many times its own weight, increasing its body volume without the necessity of an ecdysis, due to the high cuticular resistance of the extensible alloscutum (Dillinger and Kesel 2002). In nymphs of *R. sanguineus* ticks, however, blood accumulation before ecdysis generates changes such as body growth and increase in the thickness of the cuticle (Oliveira *et al.* 2012). In unfed *R. sanguineus* females, the great number of folds in the cuticle of the alloscutum increases the expansion area in this region. The cuticular unfolding allows a large increase in body size, observed throughout the feeding process and ended in the completely engorged phase.

The extensible region of the cuticle comprises the alloscutum of immature and female ixodid ticks, and the region between the dorsal and ventral plates in the pleural membranes of male ixodid ticks (Hackman 1982). In these stages, especially in females, it allows an increase in body volume after the ingestion of large blood volumes (Coons and Alberti 1999). In ticks, the cuticle is a complex acellular structure, comprising sublayers varying in thickness in which surface is found many setae and dermal glands openings (Hackman 1982; Coons and Alberti 1999), easily observed in *R. sanguineus* through Scanning Electron Microscopy.

The cuticle is divided in two main layers, clearly differentiated: the epicuticle externally, and the procuticle internally (Amosova 1983). This differentiation can be seen in the aloscutum of *R. sanguineus* females, corroborating the results also obtained for nymphs of *R. sanguineus* (Oliveira *et al.* 2012) and *Hyalomma (H) anatolicum anatolicum* (Bughdadi 2008), and females of *Ixodes ricinus* (Dillinger and Kesel 2002), *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus (Boophilus) decoloratus* (Beadle 1974).

The procuticle is localized between the epicuticle and the epidermis. It contains chitinous elements embedded in a protein matrix (Hackman 1982; Amosova 1983; Coons and Alberti 1999) and is found arranged in two layers: the endocuticle, closer to the epidermis, and the exocuticle, closer to the epicuticle (Hackman 1982; Coons and Alberti 1999). These layers can be easily observed and differentiated in *R. sanguineus* females during the feeding process, in agreement with what was observed in *R. sanguineus* nymphs (Oliveira *et al.* 2012), and in females of *I. ricinus* (Dillinger and Kesel 2002), *R. (Boophilus) microplus* and *R. (Boophilus) decoloratus* (Beadle 1974). However, for females of *R. sanguineus*, these layers are more easily distinguishable in more advanced feeding stages, so that all layers can be distinguished in completely engorged individuals, unlike in unfed or semi-engorged individuals. In these individuals, the layers are differentiated by subtle changes in staining by the dyes used.

According to Hackman (1982), the endocuticle is also divided in two layers, one internal and fibrous, and a dense and compact external, clearly observable in ultrastructural analysis and differentially stained in optical microscopy. However, staining by hematoxylin–eosin, in the integument of *R. sanguineus*, did not allow distinction of these two layers, being possible only the observation of one homogeneous structure in all analysed feeding stages.

Histologically, changes were observed in the thickness of each layer of the integument of *R. sanguineus*. Such modifications are probably resulting from physiological alterations during feeding, which may have caused an increase in secretion of cuticular layers by epidermal cells. According to Coons and Alberti (1999), during slow feeding, there is considerable growth in the aloscutum cuticle, nearly doubling its size in *I. ricinus* ticks. In *R. (Boophilus) microplus*, cuticle synthesis also occurs during the first 4 days of feeding, corresponding to slow engorgement (Hackman 1982). In the second feeding phase, however, the epicuticle unfolds and the procuticle is flattened, presenting almost half the thickness of that observed in a fasting tick (Hackman 1982; Coons and Alberti 1999). In *R. sanguineus* females, however, micrograph analysis showed a considerable increase in all procuticle layers, which became more evident and distinguishable as engorgement occurred, disagreeing with data from Hackman (1982) and Coons and Alberti (1999). Layers with greater thickness were observed in completely engorged individuals, that is, after the second

feeding stage. This fact differs from the observed in *R. (Boophilus) decoloratus* and *R. (Boophilus) microplus* (Beadle 1974), demonstrating the existence of differences between ticks pertaining to the same genus. In *I. ricinus* ticks, in turn, the cuticle seems to expand evidently from the first to the intermediate feeding stage, decreasing its volume to one-fourth of the initial in the second feeding stage (Dillinger and Kesel 2002), disagreeing with data observed in *R. sanguineus* females. The epicuticle, however, does not undergo morphological alterations, corroborating data observed in *I. ricinus* (Dillinger and Kesel 2002).

In the procuticle, it is possible to observe the pore channels (Coons and Alberti 1999), cytoplasmic extensions of microvilli of epidermal cells which function is still controversial (Hackman 1982). It is believed that they allow material exchange, like gases, water and lipids between the internal and external compartments (Coons and Alberti 1999), taking these substances to the cuticular surface, because they connect to the wax channels that go through the epicuticle (Hackman 1982), as observed in *H. anatolicum anatolicum* (Bughdadi 2008), *R. (Boophilus) microplus* and *R. (Boophilus) decoloratus* (Beadle 1974). In *R. sanguineus* females, these structures were clearly observed only in the completely engorged stage, in which could be noted two different layout patterns. In the endocuticle, the channels are numerous, ramified and anastomosing. In the exocuticle, they are less numerous and less ramified. This pattern is similar to the observed in *R. (Boophilus) microplus* (Beadle 1974; Hackman 1982), although in this species these observations have been characterized during the slow engorging phase. In *R. (Boophilus) microplus* and *R. (Boophilus) decoloratus*, however, irregular pore channels patterns could not be observed in completely engorged adults (Beadle 1974). To Coons and Alberti (1999), these channels would change their shape due to flattening of the endocuticle, with reduction in their density. However, in *R. sanguineus*, because the procuticle does not show any sign of flattening during the final engorging phase, these structures were easily seen. In fasting and semi-engorged phases, however, no pore channel was observed.

The epicuticle is the outermost layer of the cuticle, and it seems to present an uniform structure throughout the cuticle and may vary in thickness according to the body region or species (Hackman 1982; Coons and Alberti 1999). It is a complex region divided in many sublayers, being considered flexible, but not extensible (Amosova 1983). Generally, in ixodids, the following sublayers can be visualized from the outermost region to the innermost: wax or lipid layer, external epicuticular layer, cuticulin layer and dense homogeneous layer (Coons and Alberti 1999). As it is in direct contact with the environment, it is believed that the epicuticle is responsible for the animal protection against dehydration (Hackman 1982). In *R. sanguineus* females, the epicuticle reacted to hematoxylin–eosin differently when compared to the other cuticle layers, not showing differences throughout the feeding process. During the feeding process only the unfolding of this layer occurred, allowing the tick body expansion. However, its sublayers could not be distinguished by the technique utilized, although its lipoprotein constitution is already known in the nymph stage of *R. sanguineus*, which possibly gives these ticks protection against dehydration (Oliveira *et al.* 2012). Scanning electron microscopy, however, revealed evidences of a possible wax deposition in greater quantity in semi-engorged individuals, also observed by Beadle (1974) in *R. (Boophilus) microplus* and *R. (Boophilus) decoloratus* ticks, in which the wax structures become more common as the tick expands.

In some cases, a dense acellular subcuticular layer occurs between the epidermal cells and the stabilized procuticle, probably containing non-polymerized chitin–protein complex precursors (Hackman 1982; Coons and Alberti 1999). In *R. sanguineus*, it was possible the visualization of this layer, which showed clear differences in each feeding stage. In the final engorgement stages, it showed greater thickness and a more intense staining by eosin, indicating higher alkalinity in the tissue, and therefore variations in its composition along the blood ingestion period. These alterations are probably related to cuticular development during feeding. However, the exact function of this layer is still unclear.

Under the subcuticular layer is found the epidermis, a single layer of flattened cells, supported by a thin basal lamina (Amosova 1983; Coons and Alberti 1999). In general, the epidermal cells have a round nucleus, with a small nucleoli and condensed chromatin, with its apical portion full of microvilli and septate junctions in the lateral plasma membrane (Amosova 1983). In *H. anatolicum anatolicum*, as in other ixodid and arthropods, cuticular growth is followed by considerable changes in epidermal structure (Bughdadi 2008). The observed characteristics in fasting *R. sanguineus* epidermal cells corroborate these results.

During slow feeding, the epidermal cells increase greatly in size, demonstrating higher mitotic activity and suggesting fast cell proliferation (Amosova 1983; Coons and Alberti 1999). This increase in cell volume was demonstrated in females of *H. anatolicum anatolicum* ticks (Bughdadi 2008). In *R. (Boophilus) microplus* and *R. (Boophilus) decoloratus*, in turn, cells become distinctly columnar in this phase (Beadle 1974). The cytoplasm becomes basophilic, intercellular space is reduced and the nuclei become more active, with less heterochromatin, while the cuticle is synthesized (Amosova 1983; Coons and Alberti 1999). In *R. sanguineus*, however, the epidermal cells kept its cubic structure, becoming lightly flattened as the engorgement progressed, but always with volume increase. No increase in cell basophily was observed in this phase. In the semi-engorged phase in *R. (Boophilus) microplus* and *R. (Boophilus) decoloratus*, epidermal cells show essentially the same structure observed in young adults (Beadle 1974).

When completely engorged, *R. sanguineus* females show epidermis cells visibly larger than in previous stages, with a relevant increase in cytoplasm basophily and decrease in chromatin condensation. This fact differs from what was observed

in *R. (Boophilus) microplus* and *R. (Boophilus) decoloratus*, which showed a decrease in mitotic activity and a higher epidermal flattening, because cells from this epithelium are forced against the procuticle by the body expansion (Beadle 1974).

In the integument, there are also special epidermal cells that form dermal glands and setae (Hackman 1982; Amosova1983). Dermal glands consist of a pair of glandular cells and many other cells that form a duct which opens in the cuticular surface (Amosova 1983). They are less developed in unfed ticks, and it is believed that they are responsible for the formation of epicuticle layers, although its real function is still unknown (Hackman 1982; Amosova 1983). In *R. sanguineus* females, the number of glands apparently does not vary between stages. The number of setae, located at the top of a duct that is projected from an epidermal cell (Hackman 1982), however, apparently decrease as body expansion occurs in *R. sanguineus*. This fact may be related only to a greater distance between the setae, which gives the impression that they decreased in quantity. However, it may also indicate that the cells that form these structures are possibly not restored in the epidermis during the feeding process. These data indicate the need for further studies, by quantifying the amount of setae in the different feeding stages, associated with the measurement of the distance between them.

In general, the characteristics observed in the aloscutum integument of *R. sanguineus* females in different feeding stages is very similar to what is described in literature, differing in key aspects like the presence of larger epidermal cells with a less pronounced flattening, and an increase in cuticle thickness at the end of the second feeding stage. Between feeding stages, significant differences were found, demonstrating this organ capacity to resist the excessive increase in body volume. Thus, the data provide a solid base for future work, contributing to the better understanding of the biology of this tick species, or even with studies related to effects of certain acaricide formulations and consequent infestation control.

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Conflict of Interest

The authors have no conflict of interests to declare.

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