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The Midgut Muscle Network of *Anopheles aquasalis* (Culicidae, Anophelinae): Microanatomy and Structural Modification After Blood Meal and *Plasmodium vivax* (Haemosporida, Plasmodiidae) Infection

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Abstract

The mosquito midgut is divided into two regions named anterior midgut (AMG) and posterior midgut (PMG). The midgut expands intensely after the blood ingestion to accommodate a large amount of ingested food. To efficiently support the bloodmeal-induced changes, the organization of the visceral muscle fibers has significant adjustments. This study describes the spatial organization of the *Anopheles aquasalis* (Culicidae, Anophelinae) midgut muscle network and morphological changes after bloodmeal ingestion and infection with *Plasmodium vivax* (Haemosporida, Plasmodiidae). The midgut muscle network is composed of two types of fibers: longitudinal and circular. The two types of muscle fibers are composed of thick and thin filaments, similar to myosin and actin, respectively. Invagination of sarcoplasm membrane forms the T-system tubules. Sarcoplasmic reticulum cisternae have been observed in association with these invaginations. At different times after the bloodmeal, the fibers in the AMG are not modified. A remarkable dilation characterizes the transitional area between the AMG and the PMG. In the PMG surface, after the completion of bloodmeal ingestion, the stretched muscle fibers became discontinued. At 72 h after bloodmeal digestion, it is possible to observe the presence of disorganized muscle fibers in the midgut regions. The *Plasmodium* oocyst development along the basal layer of the midgut does not have a significant role in the visceral musculature distribution. This study provides features of the visceral musculature at different blood feeding times of *An. aquasalis* and shows important changes in midgut topography including when the mosquitoes are infected with *P. vivax*.

Key words: midgut muscle network, bloodmeal, *Plasmodium vivax*

Hematophagous mosquitoes evolved efficiently to support bloodmeal-induced changes. The insect gut is an organ composed of a monolayer formed by epithelial cells that rests on the basal lamina and topographically divided into three distinct regions: foregut, midgut, and hindgut (Clements 1992). The midgut is divided into two regions: anterior midgut (AMG) and posterior midgut (PMG), which during the digestion process have distinct functions. The AMG is responsible for

the sugar absorption. The PMG stores the bloodmeal and is responsible for the digestion processes. Immediately after the blood ingestion, the midgut expands intensely to accommodate a significant amount of ingested food. To efficiently support the bloodmeal-induced changes, the organization of the visceral muscle fibers has fundamental importance in the digestion process. The visceral muscles are associated with the distension and the contraction of the midgut, and peristaltic

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movements control and determine the functional compartmentalization of the passage of food along the gut (Billingsley and Lehane 1996, Hecker 1977, Chapman 1998, Jones 1960, Jones and Zeve 1968, Park and Shahabuddin 2000, Okuda et al. 2007).

The organization of the visceral muscle is constituted of a gut muscle fiber network that has fundamental importance in the digestion process in addition to providing the structural maintenance of the midgut in blood-feeding insects. This midgut muscle fiber network consists of the arrangement of two striated muscle fiber types: longitudinal fiber (LF) and circular fiber (CF) along the entire organ. The two muscle fibers are orthogonally arranged and create a knitted structure that helps to maintain the integrity of the epithelium toward the gut lumen (Goldstein and Burdette 1971, Smith et al. 1966, Priester 1971, Richards 1975). Consequently, after the ingestion of the bloodmeal, the gut physiology depends mainly on the structure and rearrangement of this muscle network.

In arthropods, the ultrastructural aspects of the midgut muscle network have been described in several orders (Smith et al. 1966, Nagai and Graham 1974, Chapman 1998) including the Diptera that include species of mosquito vectors with public health importance, such as the Anophelinae which transmit malaria. The mosquito *Anopheles aquasalis* is an important malaria vector in South and Central America, which breeds in brackish coastal marsh waters (Deane 1986). This mosquito species is distributed predominantly along the Atlantic Coast, including Venezuela, where it is the primary coastal malaria vector of *Plasmodium vivax* (Berti et al. 1993, Chadee and Kitron 1999, Laubach et al. 2001). A Brazilian strain of *An. aquasalis* has been colonized for several years under laboratory conditions (da Silva et al. 2006) and has become a valuable experimental model for studies about vector-*Plasmodium* interaction (Pimenta et al. 2015).

We describe the spatial organization of the midgut muscle network of *An. aquasalis* and the resultant morphological changes after bloodmeal ingestion and induction by the infection with *P. vivax*. We provide a better understanding of the organ physiology in relation to the bloodmeal ingestion and also regarding to the entry of the *Plasmodium* parasite, which may contribute to future studies that aim to target this important vector of human malaria in coastal areas of South America.

Materials and Methods

Mosquito Rearing

Anopheles aquasalis were reared at the insectaries of the Laboratory of Medical Entomology at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), Manaus, Amazonas State, Brazil, and the Instituto de Pesquisa René Rachou (Fiocruz-MG), Belo Horizonte, Minas Gerais State, Brazil. Colonies were kept at a 24–26°C and a relative humidity of 70–80% on a 12:12 light–dark cycle. Larvae were hatched at room temperature, in water containing salt at a final concentration of 2 g/liter, and ground TetraMin fish food was provided daily. Larvae were allowed to pupate and become adults in an enclosed mesh-covered cage with water provided and were fed ad libitum with a 10% sucrose solution until 2 d before being given the infective blood meals (Pimenta et al. 2015).

Membrane Blood Feeding Assay and *P. vivax* Infection

Three- to five-day-old adult female mosquitoes were sugar-starved overnight prior to infection via membrane feeding assays. Briefly, normal blood-fed (NBF) or *P. vivax*-infected blood-fed (IBF) samples were offered to two groups of 50 *An. aquasalis* females for 30 min through membrane feeders covered with Parafilm at 37°C, as described previously (Ríos-Velasquez et al. 2013, Pimenta et al.

2015). The two sets of experimental blood feeding (NBF and IBF) were performed and compared with unfed mosquitoes (UF) to study the effect of blood intake and the consequence of the *P. vivax* ingestion in the *An. aquasalis* midgut muscle network. Three replicate independent assays were developed for each experimental group.

Blood Collection and Ethics Statement

Adult volunteers (aged >18 yr) residing in the region of Manaus, who presented themselves at the FMT-HVD with microscopically confirmed *P. vivax* malaria, were invited to participate in this study. About 3 ml of blood was collected by venipuncture and placed into a sterile heparinized vacutainer tube. All patients signed a written informed consent form. After blood collection, patients were treated according to Brazilian Health Ministry guidelines (Ministério da Saúde, 2010). This study was approved by the Committee of Ethics of the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (033595/2014) and the Brazilian National Ethics Committee Board (CONEP 686297).

Dissection, Fixation, and Morphometric Study of *An. aquasalis* Midgut

The three groups of mosquitoes, UF, NBF, and IBF, were dissected at distinct time intervals. All mosquito midguts were dissected over glass slides in a drop of phosphate-buffered saline (PBS) pH 7.4 under a stereoscope where they were measured or fixed for further microscopic studies. The mosquitoes from the NBF group were carefully dissected at 5, 15, 30 min, 1, 3, 6, 12, 24, 48, 72, and 96 h after bloodmeal ingestion.

The measurements of the width changes of the dissected midguts were analyzed and compared using five samples of each experimental group under a stereomicroscope using the Axiovision software 4.8. The Shapiro-Wilk test was conducted to test the null hypothesis that data were sampled from a Gaussian distribution. The Mann-Whitney test was performed since the data have nonparametric distribution. Data were analyzed using GraphPad Prism 5.0 software (Graph Prism Inc., San Diego, CA) and $P < 0.005$ was considered significant.

The mosquitoes from the IBF group were dissected at 5, 7, and 9 d after the infection with *P. vivax*. The mosquitoes from the UF group were examined at the same times so that the two other groups could be used as a comparative control. Immediately after dissection, the midguts were fixed at room temperature for 24 h with a 2.5% glutaraldehyde solution in 0.1M cacodylate buffer (Pimenta and De Souza 1983).

Transmission Electron Microscopy and Scanning Electron Microscopy

The glutaraldehyde-fixed midguts were postfixed with a 1% osmium tetroxide solution in 0.8% potassium ferricyanide. They were dehydrated in an ascendant concentration of acetone and routinely embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and processed for observation at transmission electron microscopy (TEM; Zeiss EM-900) as previously described in details on Pimenta and De Souza (1983). Also, another set of fixed samples was dried using a critical point apparatus and after covered with a layer of 10 nm of gold particles in a sputter to be observed in the scanning electron microscopy (SEM; Jeol X-100 SEM) as described in details in Secundino et al. (2005a,b)

Results

Size Changes of the *An. aquasalis* Midgut Induced by the Bloodmeal

To recognize the midgut changes induced by the bloodmeal ingestion the widths of the *An. aquasalis* midguts were measured before (UF

group) and at different time intervals after the intake of the normal bloodmeal (NBF group), until the digestion completion at 96 h. The maximum increase in the midgut size occurred after 30 min after the bloodmeal intake. After the bloodmeal digestion and excretion, the NBF midguts showed similar size to the UF midguts. Statistical differences were detected among the width measurements of the midguts before (UF) and after the bloodmeal (NBF). The comparison of the UF with the NBF midgut sizes showed significant differences in the midgut widths of each experimental group ($P \leq 0.005$) with the exception of the 72 h and 96-h NBF midguts (Table 1).

General Ultrastructure of the *An. aquasalis* Midgut Muscle Network Revealed by TEM

The midgut muscle network is located outside the midgut in direct contact with the hemocoel and just below the basal lamina that sustains the epithelial cells situated toward the organ lumen (Fig. 1A). The midgut muscle network is composed of LF and CF. The LFs are superimposed over the CFs (Fig. 1A and B). Occasionally, tracheas are seen in direct contact with the muscle fibers (Fig. 1C). The muscle fibers are maintained attached to the midgut via a narrow layer of connective tissue (Fig. 1E). Some muscle fibers are connected to each other by structures similar to tight junctions or desmosomes (Fig. 1B and C). Each muscle fiber is surrounded externally by a basal lamina (Fig. 1C–F). The basal lamina of the muscle fibers is very thick and is composed by several filamentous and parallel layers (Fig. 1G). Also, sarcoplasmic reticulum cisterna has been observed in association with sarcoplasm membrane invaginations (Fig. 1D and E) forming a structure similar to T-system tubules described in other muscle systems (Schaefer et al. 1967). In cross sections of longitudinal muscle, some tubules of T-system appear to fold back on themselves and enclose electron-dense substance (Fig. 1D). Also, this structure can pass from one side of a muscle fiber to another (Fig. 1E).

The two muscle fibers, the LFs and the CFs, are composed of thick and thin filaments, structurally similar to the contractile filaments, myosin and actin (Fig. 1F and G). Each myosin-like thick filament is surrounded by thin actin-like thin filaments (Fig. 1G). In high magnification of the transversal section of the muscle fiber, it is

Table 1. Midgut size changes of *An. aquasalis* considering width measurements at sequential periods of time after bloodmeal ingestion

Time	Width mean of PMG (Min–Max) ^a	Proportion of the size increase ^b
Unfed ^c	102 (99–105)	0
5 min	324.7 (224–326)	3.18
15 min	423.3 (415–438)	4.15
30 min	540.7 (516–600)	5.30
1 h	491.5 (449–520)	4.82
3 h	489.7 (423–555)	4.80
6 h	485.8 (472–500)	4.76
12 h	474.5 (465–491)	4.65
24 h	425.0 (419–440)	4.17
48 h	291.8 (270–300)	2.86
72 h	181.2 (150–220)	1.78
96 h	123.8 (100–200)	1.21

^aThe width mean was obtained with the measured of 5 midguts under a stereomicroscope.

^bThe proportion of the size increase is the value compared with the unfed midgut.

^cThe unfed midguts are from dissected mosquitoes that never ingested any bloodmeal.

possible to observe the Z lines and the A zones of the sarcolemma units (Fig. 1F).

Microanatomy of the *An. aquasalis* Midgut Muscle Network Revealed by SEM

Muscle Network of the UF *An. aquasalis* Midgut

The midgut muscle fibers of the UF mosquitoes are structured as well-organized muscle network over the midgut surface. They have similar morphological aspects in the AMG and PMG. The LFs are overlapping on the CFs and are arranged orthogonally, with the LFs parallel to each other (Figs. 2A, 3A, and 4A). The CFs are arranged as several rings regularly spaced throughout the midgut surface with the variable width depending on the midgut region. Apparently, a single circular fiber appears to surround the entire midgut circumference. Fifty-seven CF units were observed along the mosquito midgut (data not shown).

Muscle Network of the Blood-Fed *An. aquasalis* Midgut

The Anterior Midgut

Observation of the midgut of all experimental groups, until the time of 72 h after the blood ingestion, showed none morphological changes in the AMG muscle network among the NBF midguts compared with the UF midgut. Protruding epithelial cells appear among the LFs and CFs (Fig. 2A–F). The LFs have sinuous relaxed surfaces (Fig. 2D–F), whereas the CFs are difficult to visualize because they are surrounded by protruding epithelial cells that appear to be delimited by the muscle fibers (Fig. 2A–F). Some relaxed LFs are linked by small fibers (Fig. 2D). Also, several branched tracheas can be seen in this region and sometimes appear to be emerging between the epithelial cells (Fig. 2B–F).

The Transitional Area of the Midgut

The transitional area between the AMG and PMG of the UF midgut showed a slight enlargement (Fig. 3A) compared with the NBF midguts (Fig. 3B and C), where it is seen as a prominent contrast between these two regions mainly observed in the 1-h NBF and 6-h NBF midguts (Fig. 3B and C). The PMG is an expanded midgut region and the muscle fibers are compacted, juxtaposed, and continuous with the fibers of the AMG, which are fully distended (Figs. 3C and 4F) as seen observed in the 6-h NBF through the 24-h NBF. These uninterrupted LFs acquire a new arrangement and become more distant from each other and more juxtaposed over the PMG surface as can be observed at the sequential time after the blood ingestion (Fig. 3B, C, and E). Also, the CFs are more evident in the PMG when compared with the AMG (Fig. 3B and C). The two muscle fibers have sinuous surfaces and some bleb formations (Fig. 3D). Protruding epithelial cells are seen among the muscle fibers (Fig. 3B and F). The muscle network of the transitional area in the 48-h NBF is more relaxed showing regular orthogonal arrays of the LFs and CFs (Fig. 3F).

The Posterior Midgut

The PMG muscle network is morphologically altered after the blood feeding in all NBF midguts (Fig. 4B–H) and remains modified during all the digestive process compared with the UF midgut (Fig. 4A). The muscle fibers are well individualized and evident in PMG surface. The enlargement of the NBF midguts, at different times of the blood digestion, permits us to observe the general arrangement of the muscle network. Immediately after the bloodmeal ingestion, the CFs and LFs are extremely stretched and flat, mainly in the 10-min

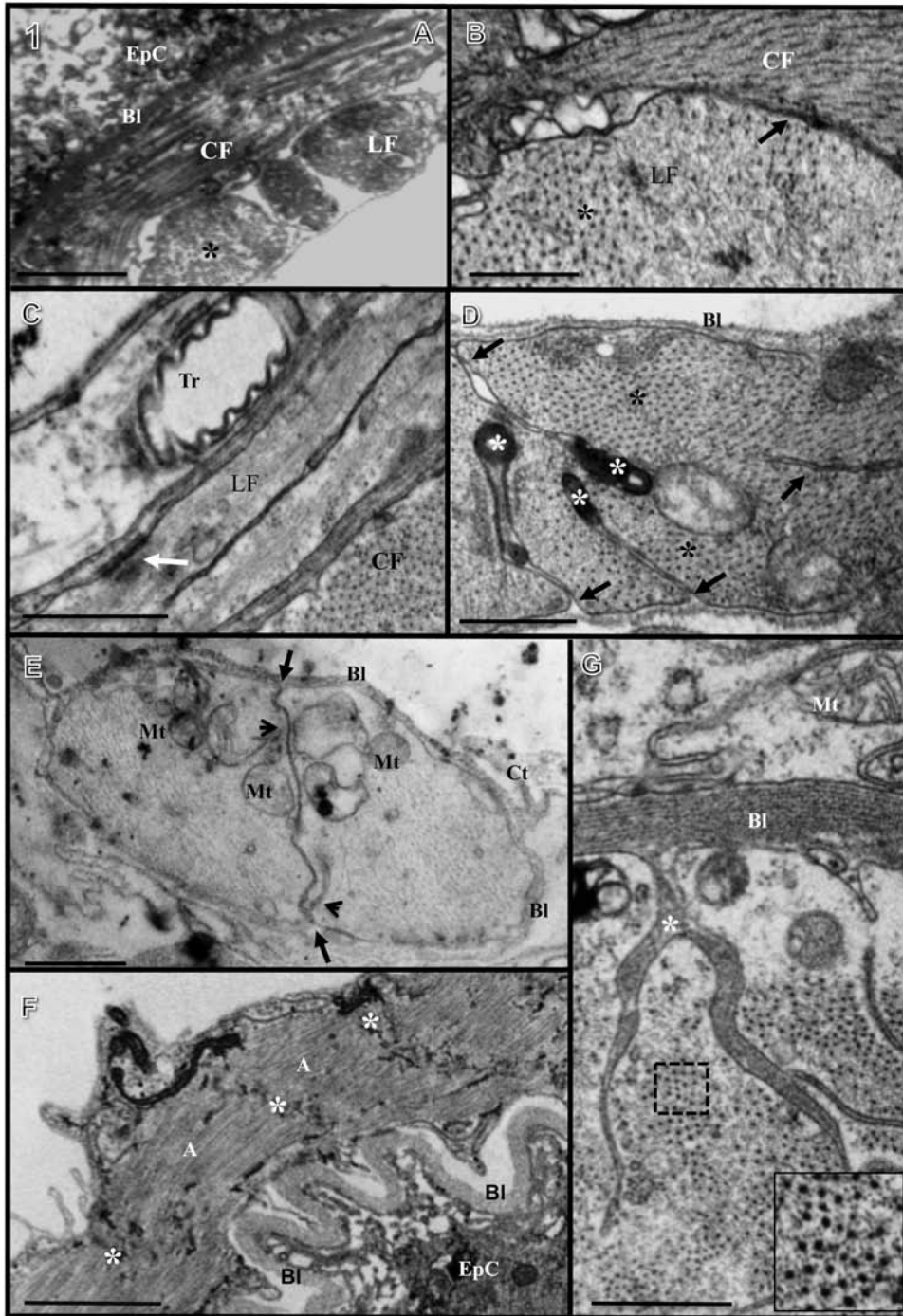


Fig. 1. Ultrastructure of midgut muscle network of *An. aquasalis*. (A–C) The connection between CF and LF. (A) General view of the contact region between LF and CF composed of myofilaments. The CF is in direct contact with the basal laminae (BL) located below the midgut epithelial cells (EpC). (B and C) Large magnification of the connections between a LF with a CF and LF with a trachea (Tr). In (B), it is seen the arranged actin and myosin filaments (asterisks) and a tight junction like-structure (black arrow) between the two muscle fibers (LF and CF). In (C) observe the existence of a desmosome (white arrow) between the two muscle fibers (LF and CF) and the LF with a Tr. Bars: 2 μ m (A), 0.5 μ m (B), and 1 μ m (C). (D and E) The T-system tubules. (D) A cross-section of an LF showing T-tubule system composed by a double membrane, the sarcolemma foldings (arrows). Observe electron-dense substances (white asterisks) inside the T-tubules and cross-sectioned microfilaments (black asterisks) distributed all over the cytoplasm. (E) Details of a T-tubule crossing an entire muscle fiber (black arrows) forming branches of invaginations (arrowheads). Note the presence of mitochondria (Mt) close to the T-tubule. BI, basal lamina; Ct, connective tissue. Bars: 0.5 μ m (D) and 2 μ m (E). (F and G). Ultrastructure of the myofilaments. (F) Details of a longitudinal section of a muscle fiber showing typical regions. The dark Z lines (white asterisks) are separating sarcomeres and A bands (A) are located between them. (G) Large magnification is showing a thick basal laminae with several filamentous layers (BI) and branched invaginations of the sarcolemma (asterisk). Insert is a higher contrasted image delimiting the disposition of thick filaments surrounded by thin filaments, respectively, similar to myosin and actin. Mt, mitochondria. Bars: 5 μ m (F and G).

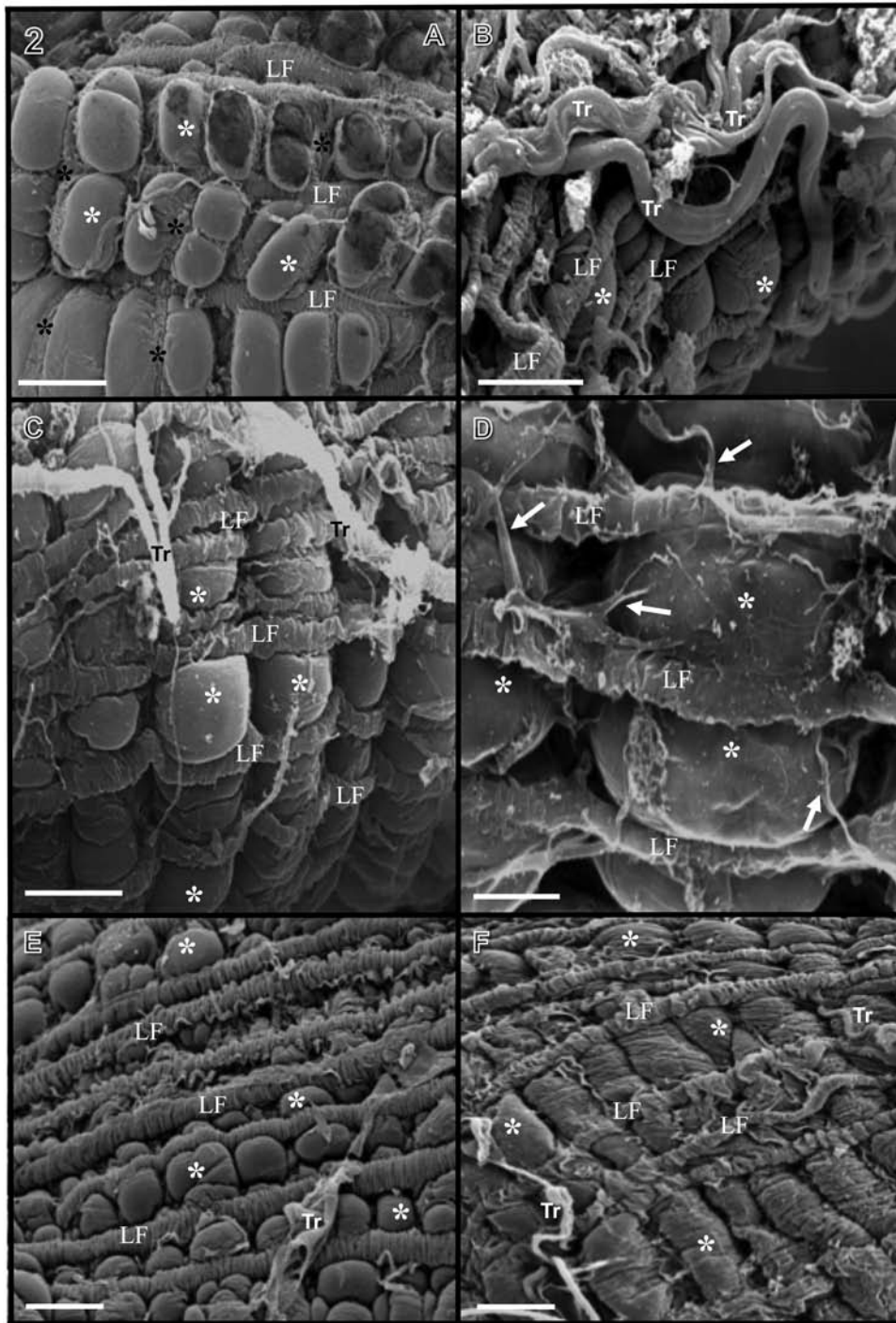


Fig. 2. Microanatomy of AMG muscle network of the unfed and blood-fed *An. aquasalis*. SEM pictures are showing details of the AMG muscle networks of UF (A) and at sequential times after the normal blood ingestion: 1-h NBF (B), 6-h NBF (C), 12-h NBF (D), 48-h NBF (E), and 72-h NBF (F). In the UF midgut (A), the basis of the epithelial cells (black asterisks) is protruding between the LFs and the almost hidden CFs (white asterisks). Few changes were observed in the muscle networks of the AMG of the NBF mosquitoes. In the 1-h NBF (B) and 6-h NBF (C), several branching Tr are seen lying on the PMG and several relaxed LFs are delimiting the stressed epithelial midgut cells (white asterisks). The large magnification view of the PMG muscle network in the 12-h NBF (D) is showing details of the relaxed LFs linked by several small fibers (arrows) and the protruding epithelial midgut cells (asterisks). In the 48-h NBF (E), observe the LF closed to each other and it not possible the see the CFs hidden by the protruding epithelial cells (asterisks), whereas in the 72-h-NBF (F), the epithelial cells (asterisks) are less protuberant and with the unstressed surface. Bars: 20 μm (A), 10 μm (B, D, E, and F), and 5 μm (C).

NBF until the 24-h NBF midguts (Fig. 4B–F). Actually, the extremely stretched 10-min NBF midgut shows juxtaposed and branched LFs in some areas of the muscle network (Fig. 4B). In truth, the muscle network of 30-min NBF and 1-h NBF are still very stretched on the surface of the 30-min NBF midguts (Fig. 4C and D). In some areas

of the muscle network of the NBF midguts, the LFs and CFs present finely attached bifurcated filaments (Fig. 4E–G). In the 12-h NBF midgut, some LFs join to form pleats (Fig. 4F). This aspect was also similar in the muscle network of the 24-h NBF midgut (Fig. 4G). Nonetheless, the protruding epithelial cells are easily observed

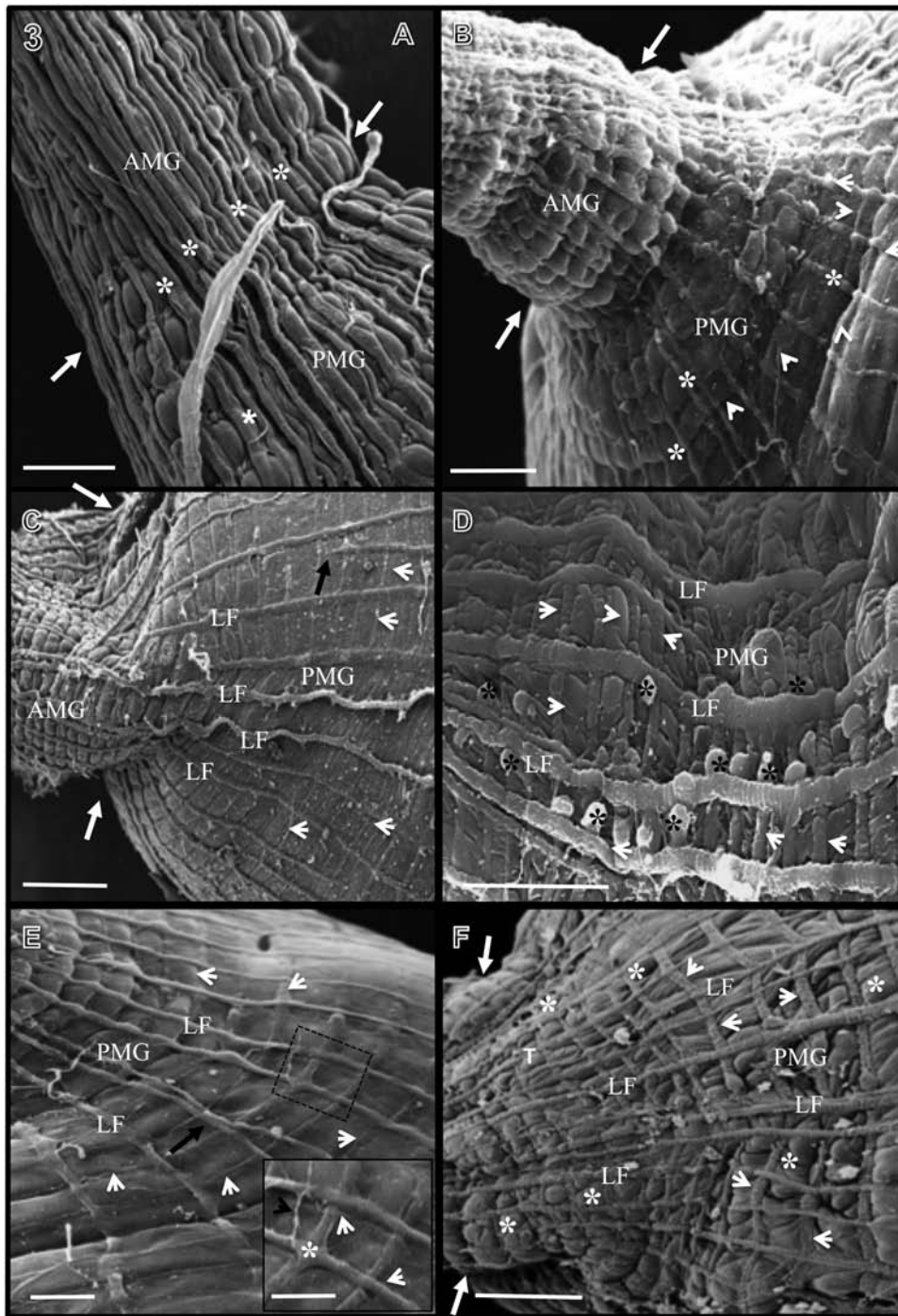


Fig. 3. Microanatomy of the transitional area between the AMG and PMG of UF and blood-fed *An. aquasalis*. In the UF midgut (A), there is a slight change in the general aspects of the aspect of the side-by-side LFs. They are compacted fibers (white asterisks) and uninterrupted between the two midgut regions (white arrows). In 1-h NBF (B) and 6-h NBF (C), the transitional area of the PMG side is hugely dilated compared with the AMG. The white arrows are showing abrupt transition between the two midgut regions. The muscle networks are presenting individualized LFs (white asterisks) and CFs (arrowheads), which become more evident in the 6-h NBF midgut (C) with some broken fibers (black arrow). In D (6-h NBF), the muscle network of the transitional area in the side the PMG is showing relaxed LFs linked by the parallel CFs (arrowheads). Note the presence of several lateral blebs (black asterisks) in some longitudinal fibers. In 24-h NBF (E), the muscle network of the transitional area in the PMG is showing the perfect morphological features of the LFs and CFs (arrowheads). The LFs are seen linked to each other (black arrow). The inset is showing details of a magnified area of LFs positioned over the CFs (arrowheads), linked by small fibers (black arrow) or showing fusion areas (asterisk) between the two types of fibers. In 48-h NBF (F), the muscle network of the transitional area (white arrows) is showing relaxed LFs and CFs (arrowheads) and protruded epithelial cells of the midgut (asterisks). Bars: 50 μ m.

among the muscle fibers in the 48-h NBF midgut (Fig. 4H). The muscle network of the 72-h NBF midgut after the blood digestion is completed, is not stretched, and maintains a sinuous aspect on the surface midgut with straight LFs and partial aspects of the CFs that are covered by projections of the epithelial cells (Fig. 4H).

Muscle Network of the *P. vivax*-Infected *An. aquasalis*

The *P. vivax* oocysts start to be apparent in the muscle network of the *An. aquasalis* midgut around the fifth day after infection and only in the PMG region when the blood digestion is completed. Therefore, we observed the distribution and development of oocysts

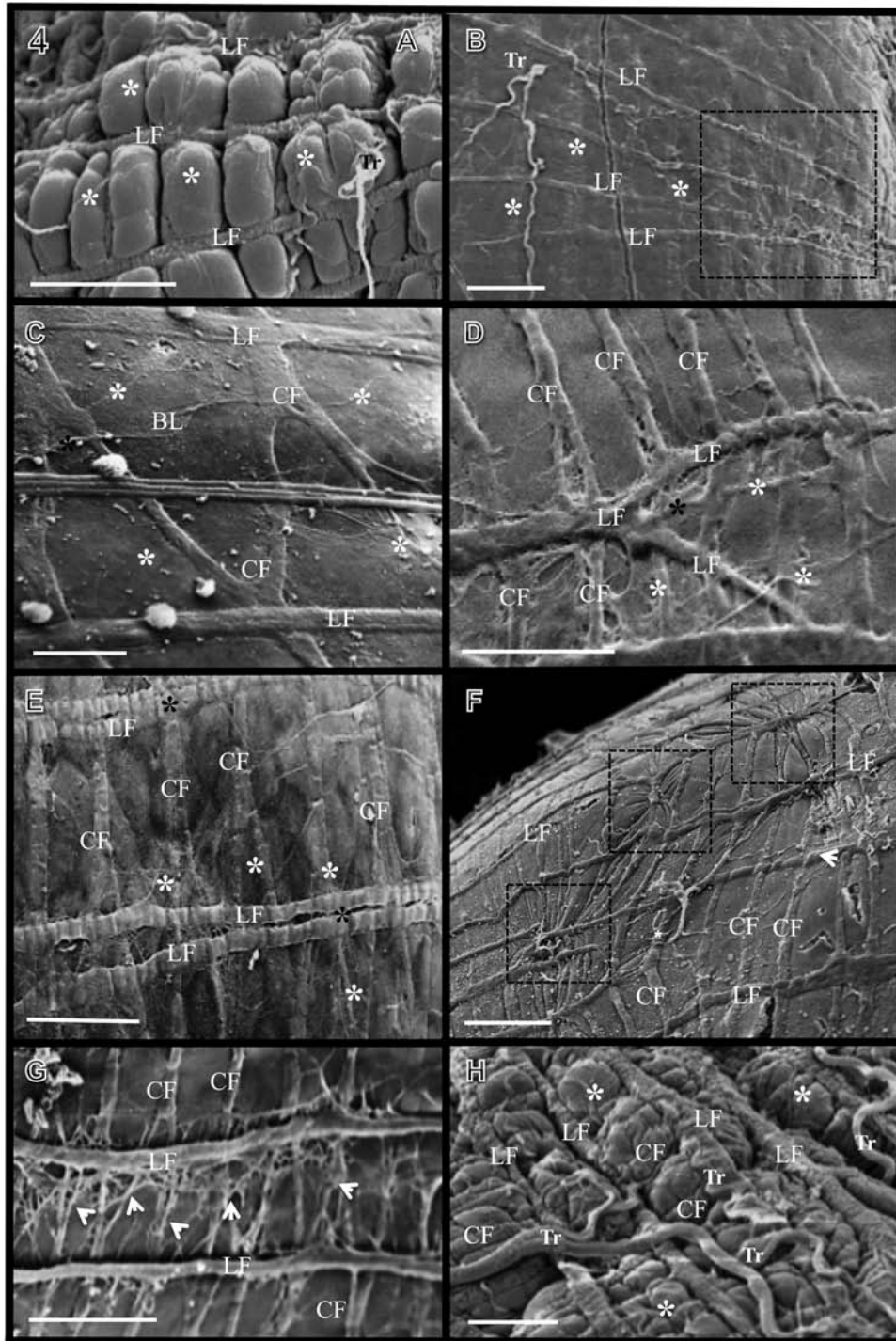


Fig. 4. Microanatomy of PMG muscle network of the UF and blood-fed *An. aquasalis*. SEM pictures are showing details of the PMG muscle networks of UF (A) and at sequential times after the normal blood ingestion: 10-min NBF (B), 30-min NBF (C), 1-h NBF (D), 12-h NBF (E), 24-h NBF (F), 48-h NBF (G), and 72-h NBF (H) midguts. The muscle network of unfed midgut (A) is showing individualized LFs but is not possible to see the CFs hidden by the protruding epithelial cells (asterisks). Evident changes occur in the muscle networks of the PMG of the NBF mosquitoes. In the (B), 30-min NBF (C) and 1-h NBF (D), the muscle network is extremely stretched on the surface of the PMG. As seen in 10-min NBF (B), in some areas, the LFs are juxtaposed and branched (white square) and CFs are branched very extended and almost nonvisible (asterisks). In 1-h NBF (C), the PMG is even more stretched and the basal lamina (BL) is presenting several stressed areas (asterisks). Note the branched CFs. On the surface of the 1-h NBF (D) PMG, there are specific connections between the branched LFs and CFs that are forming an intricate arrangement involving small filaments (asterisks). Also, note the LFs are linking together (black asterisk). In 12-h NBF (E), the muscle network is very stretched. The LFs are linked each other (black asterisks) and positioned above the CFs that are forming branches (white asterisks). The muscle network is more relaxed on the PMG of the 24-h NBF (F) and is composed of concentrated arrays (squares) of twisted LFs and CFs. The 48-h NBF (G) midgut also presents a well-convoluted muscle network with parallel LFs linked by CFs but with a large amount of small filaments (arrowheads) between them. In the 72-h NBF (H), the muscle network is disorganized presenting evident LFs and partial aspects of the CFs that are covered by projections of the epithelial cells (asterisks). Several tracheas are also seen. Bars: 20 μ m (A–C), 100 μ m (D), 50 μ m (E–H).

in the midgut muscle network in the PMG of the IBF mosquitoes at different intervals of time after infection (Fig. 5A–E). In the 5-d IBF midguts, small rounded oocysts are adhering to the basal membrane,

which are located in the center of two muscle fibers or sometimes laterally positioned adjacent to a muscle fiber (Fig. 5A). The oocysts have smooth surfaces and as the period of the infection increases, their

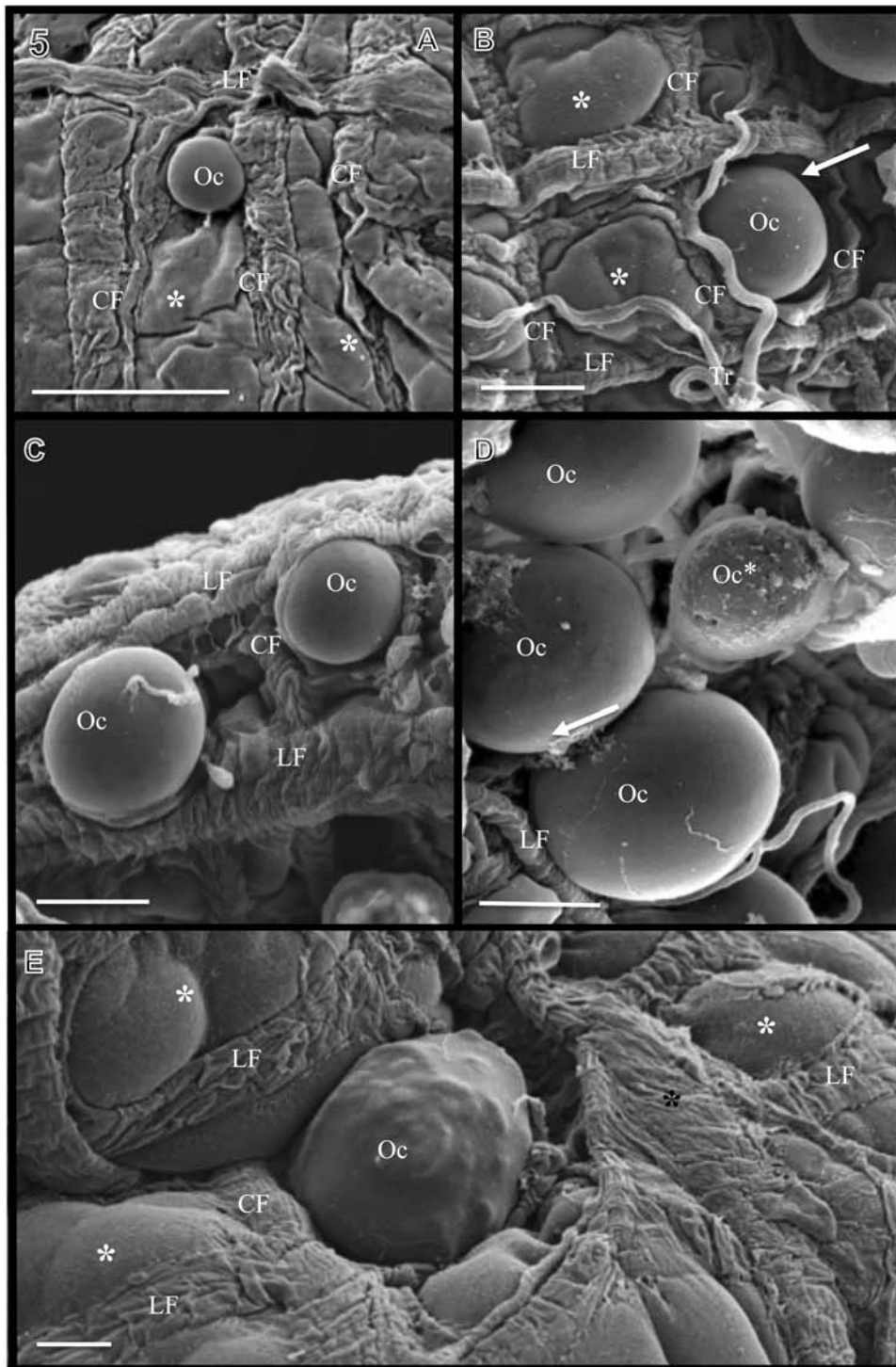


Fig. 5. SEM of the BFI *An. aquasalis* PMG at distinct time of the *P. vivax* infection. In the infected PMG, the oocysts (Oc) are delicate rounded structures with smooth surfaces rising in the muscle network as are seen at different times after parasite ingestion: 5-d IBF (A), 7-d BFI (B and C), and 10-d BFI (D and E). In 5-d IBF (A), small isolated and rounded Oc are seen rising among well-structured LFs and CFs. In 7-d IBF midguts (B and C), single (B) or paired Oc (C) are rising among the LFs and CFs. The 10-d IBF midguts (D and E) are presenting distinct aspects of the Oc. The D is showing three well-developed Oc inserted in the midgut wall (arrow) and small one undeveloped and dead Oc* with a holed surface. The rounded oocysts increased in size and occupied almost all of the space bounded by the muscular fibers. The (E) is showing a mature Oc with the characteristic corrugated surface. Note that the Oc is deepen-inserted in the PMG surface surrounded by a complex arrangement of LFs and CFs, which are amalgamated in some areas (black asterisk). Tr, trachea, black asterisks, epithelial cell protrusions. Bars: 50 μ m.

sizes increase, but not equally as can be observed (Fig. 5C and D). Also, in the 7-d IBF and 10-d IBF midguts, the oocysts are completely surrounded by the muscular fibers (Fig. 5C and E).

Discussion

Information about the microanatomy and ultrastructure of midgut muscle network of the hematophagous mosquito vector would help to increase our understanding of their functions. Furthermore, it is essential to know how the midgut is structurally organized, which, in turn, may lead to a better understanding of blood ingestion, digestion, and development of vector-borne pathogen and consequently, the transmission to vertebrate hosts.

The integrity of the entire gut depends mostly on the structure, arrangement, and maintenance of the muscle network that holds the tissue together during the bloodmeal and the whole time of the digestion. The muscle network confers the peristaltic actions of the gut, allowing the movement of ingested bloodmeal and the secretion of the digested residues. This study analyzed the spatial organization of the muscle network that comprises the *An. aquasalis* midgut by focusing on structural aspects of UF and blood-fed mosquitoes following modifications until the completion of the blood digestion. Moreover, the midgut muscle network in *P. vivax*-infected mosquitoes was analyzed when the parasite infection is established in the vector midgut. We characterized morphological aspects of the arrangement of these muscle fibers in different blood-feeding intervals; from the beginning when the midgut expands to store a large quantity of bloodmeal until the digestion is finished and the midgut has to reduce its volume. Therefore, we emphasize here the importance of the muscle network in the morphological maintenance of the midgut in regards to the organ changes during the process of blood feeding and digestion, furthermore, when *P. vivax* infects the mosquito vector and when they develop oocysts in the midgut surface.

The midgut of the *An. aquasalis*, as with other mosquitoes, undergoes a marked anatomical change after bloodmeal ingestion, which is easily seen through the PMG dilation. The maximum volume is seen 30 min after the blood ingestion and starts to decrease until recovering its initial size ~72 h after blood feeding when the digestion is finished. During this period, the midgut muscle network needs to be expanded during the blood ingestion and the organ volume decreases during the digestion until the time when the midgut is empty. Usually, in Diptera, the bloodmeal digestion is finished between 48 and 72 h (Clements 1963) and depends on several factors including the type of ingested bloodmeal and the temperature of the environment (O'Gower 1956). During this entire process, the midgut muscle network is responsible for maintaining the organ integrity.

The microanatomy visualized by the SEM revealed the general topography of the *An. aquasalis* external midgut surface. The midgut muscle network is composed of a well-arranged complex of two types of fibers according to their positions in the midgut surface, the CFs and LFs. These muscle fibers form an intricate arrangement that holds the monolayer of epithelial cells beneath the basal lamina. The CFs are interconnected and form rings around the midgut surface. These rings become gradually larger in diameter toward the widest portion of the midgut and surround the entire circumference of the organ, although in some regions it is possible to observe their coalition or bifurcation and sometimes the CFs bind forming double rings. The LFs are in parallel displays to each other, thicker and evident, though not all of them are continuous from the beginning to the end of the midgut, some end in the middle portion of the midgut,

whereas some originate there. In some places, new bundles of LFs originate by the branching of single existing fiber, or they start randomly at the midgut surface. Similar aspects, of individual branching of midgut muscle fibers, were seen in *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) midgut (Park and Shahabuddin 2000, Bernick et al. 2007). Actually, in general aspects, the *An. aquasalis* midgut has similar aspects of the midgut muscle network of other Diptera including hematophagous insects like the sand fly (Secundino et al. 2005a) and the mosquitoes, *Ae. aegypti*, *Anopheles gambiae* (Park and Shahabuddin 2000), and *Anopheles quadrimaculatus* (Schaefer et al. 1967).

We observed the structural differences in the organization of the midgut muscle network in the AMG and PMG. The muscle network organization of the AMG is not modified or distended after the bloodmeal ingestion. This midgut region maintains its general structural aspect during and after the bloodmeal ingestion and digestion. Actually, the CFs and LFs are closer to each other forming a tight-condensed muscle network helping to support the tubular shape of the AMG. In most of the hematophagous insects studied, little importance has been attributed to the PMG region, since it is only recognized as a tube for the passage of the bloodmeal in the direction of the PMG, and actually, not actively involved in the storage and digestion of blood (Rudin and Hecker 1976, Hecker 1977, Billingsley 1990). Additionally, the transitional area between the AMG and PMG is easily recognized and characterized by a remarkable dilation of the organ that induces the straining of the muscle network. The LFs are observed in parallel arrays with some ramifications and with the presence of several tiny fibers connecting them or connecting them to the stretched CFs.

Immediately after the blood ingestion by the *An. aquasalis*, there is a complete distinction between the aspects of muscle network of the PMG when it is compared with the AMG, similar to what has been identified in other anatomical studies of other vectors (Schaefer et al. 1967, Park and Shahabuddin 2000, Secundino et al. 2005a). In the 24-h BF midguts, the muscle fibers are more densely distributed and the muscle fibers are less thick. After the 24 h-NBF, the muscle fibers are more relaxed and organized. The longitudinal fibers become more spaced out from each other and form an expanded network, and more branches of muscle fibers can be observed. As the blood digestion progresses, the fibers become juxtaposed and their surface has sinuosity. In the PMG surface, after the completion of bloodmeal ingestion, the stretched muscle fibers became discontinued in some areas of the organ, probably due to the midgut stress. These CFs and LFs even presented some splits and bifurcations not evident in the UF midguts. These aspects were also observed in other mosquito vectors such as *An. gambiae* and *Ae. aegypti* (Park and Shahabuddin 2000). After the bloodmeal, in the 72-h NBF midguts, it is possible to observe the disorganization of the muscle fibers. The LFs seem to lose their original parallel arrangements, different from the CFs that seem to maintain their arrangement. Interestingly, we observed that after the bloodmeal digestion of the *An. aquasalis*, the midgut muscle network continues to be disorganized similar to what has been described in the sand fly midgut (Secundino et al. 2005a). It was suggested that the disturbance in the special arrangement of the muscle network could help the recognition of individual females that have already had a bloodmeal and digested it. Mosquitoes, as with other hematophagous insects, develop their eggs after the bloodmeal. As suggested for sandflies (Secundino et al. 2005a), the observation of a disorganized midgut muscle network in the *An. aquasalis* may be used as a marker to identify individual mosquito females that already ingested a bloodmeal and, consequently, developed their gonadotrophic cycle, i.e., have already produced and laid their eggs. Since

mosquitoes can take more than one bloodmeal during their life cycle, this morphological aspect can contribute to a better understanding of the state of an individual mosquito vector in a determined epidemic area adding to the knowledge of the vectors' blood feeding habit.

Ultrastructural description of the midgut musculature of mosquitoes through TEM is scarce in the literature. Schaefer et al. (1967) is the only study to describe ultrastructural aspects of the general organization of the midgut muscle network in the *An. quadrimaculatus* by using TEM. This muscle network is composed of LF and CF with myosin filaments surrounded by actin, similar to the arrangement observed by us in the *An. aquasalis*. Actually, the arrangement of the muscle fibers in the UF *An. aquasalis* midgut resembles the *An. quadrimaculatus* midgut, including the presence of cisternae of the sarcoplasmic reticulum and the double-membraned T-system vesicles. Also, the arrangement of the filaments in the muscle fibers follows a pattern of actin fibers surrounding a filament of myosin observed in *An. quadrimaculatus* and *Ephesia kühniella* (Smith et al. 1966, Schaefer et al. 1967). In the *An. aquasalis*, the sarcomeres were more clearly observed in the muscle fibers of the AMG. It was possible to identify the Z lines and the A bands in the transversal section of the muscle fibers. Distinct aspects of these bands were also seen in sarcomeres of the muscle fibers of the *An. quadrimaculatus* midgut (Schaefer et al. 1967).

Anopheles aquasalis is an important mosquito vector in the coastal of the South American continent including in the Amazon region where is an established vector of malarial parasites (Ríos-Velasquez et al. 2013, Pimenta et al. 2015, Orfanó et al. 2016a,b). Anophelines ingest the *Plasmodium* gametocytes along the bloodmeal in individuals with malaria and about 15 min after ingestion, the gametocytes take the rounded form, leave the red blood cells, and differentiate into gametes. Fertilization gives rise to diploid zygotes that subsequently differentiate into mobile forms, called ookinetes. Approximately 12–24 h after the bloodmeal, the ookinete must cross the peritrophic matrix and subsequent midgut invades. After the invasion of the epithelium, the ookinete differentiates in oocysts and adheres to the basement membrane of the epithelium, and become in direct contact with the hemocoel. Between 5 and 10 d after ingestion of the parasite, the oocysts reach maturity and within 10–14 d after the onset of infection escape the oocysts. Once in the hemocoel, the sporozoites that survive the immune activation of the vector, follow the salivary glands, invade the distal portion of the lateral lobes and meet in the salivary duct. In the salivary duct, the sporozoites can be transmitted to vertebrate hosts through a bite (Sinden 2002, Pimenta et al. 1994, Lobo and Kumar 1998, Ghosh et al. 2000, Dinglasan et al. 2009, Smith et al. 2014). An earlier study from our group has described the final development of distinct *Plasmodium* species in their main mosquito vectors showing the oocysts disposition in the mosquito vector midgut, as well as how the sporozoites escape from the oocysts (Orfanó et al. 2016a). Here, we observed that the appearance of the *P. vivax* oocyst, as well as their growth in the *An. aquasalis* midgut wall along the outside of the organ, does not interfere with the general aspects of the organization of the midgut muscle network. It seems that the emergence of the oocysts in the midgut wall is a well-adapted process and not a fundamental disturbing phenomenon. The fact that the midgut muscle network remains intact guarantees the continuity of the *Plasmodium* life cycle.

Conclusion

In conclusion, the general microanatomy and ultrastructure of the midgut muscle network of *An. aquasalis*, an American vector of human *Plasmodium*, are similar to other Diptera as have been described by Old World vectors, *An. gambiae*, *Ae. aegypti*, and *An.*

quadrimaculatus (Schaefer et al. 1967, Park and Shahabuddin 2000). However, as not shown before in other mosquito midgut investigations, this detailed study adds several particular aspects of the midgut muscle network of the *An. aquasalis* at different times after the blood feeding. It was essential to differentiate between the midgut muscle network of the AMG and PMG and relate it to the physiological state of the organ. Moreover, it was also shown that the development of the *P. vivax* in the infected *An. aquasalis* does not disturb the midgut muscle network allowing the complete maturation of the oocysts in the organ surface. Thus, this study provides a better understanding of blood digestion and highlights aspects of a New World vector of malaria that may also help studies related to the transmission of human pathogens by vectors.

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