The Midgut Ultrastructure of the Sand Fly Vector, *Phlebotomus Langeroni* (Diptera: Psychodidae)

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**ABSTRACT**

The midgut ultrastructure of *Phlebotomus langeroni*, the vector of visceral leishmaniasis was studied by the transmission electron microscope. The midgut region warrants a special investigation since it is directly relevant to the development of *Leishmania* promastigotes. Observations performed on females at time-intervals following a bloodmeal were analyzed and compared. The epithelium of the midgut consisted of a single layer of columnar cells surrounded by the basal lamina. Under which there is a musculature consisted of circular and longitudinal fibers. The cells were characterized by large nuclei, densely packed microvilli, and intricate basal labyrinth. The cytoplasm was filled with numerous mitochondria. Morphological changes occurred in the cellular structures during the blood digestion process. The peritrophic matrix was present up to 3 days after the bloodmeal and completely disintegrated at day 4 after bloodmeal. At day 6 after bloodmeal, lipid inclusions were few within the cell cytoplasm and vacuoles were depicted. At day 8, the area of the microvilli was greatly reduced probably due to reduced functional activity along with the end of the digestive cycle. The changes depicted in the midgut of *P. langeroni* during the process of blood digestion were comparable to those described for other hematophagous diptera.

**INTRODUCTION**

*Phlebotomus langeroni* is a member of subgenus *Larroussius*. The subgenus *Larroussius* harbours important sand fly vectors or potential vectors of *Leishmania infantum* in the Mediterranean basin (northern Africa, southern Europe, and southern Iran). The principal reservoir host is the dog, while the role of cats (Asgari *et al.*, 2020) and rabbits (Díaz Sáez *et al.*, 2018) as well as various wild canids is also expected.

*Phlebotomus (Larroussius) langeroni* Nitzulescu, 1930 is the proven vector of *Leishmania infantum* in the northwestern coast of Egypt (El Sawaf *et al.*, 2012). *P. langeroni* meets the five criteria set up by Killick-Kendrick (1990) for the incrimination of a sand fly species as a vector. Essentially, it is an anthropophilic sand fly that also feeds on dogs; its spatial distribution is associated with human cases of visceral leishmaniasis; it was naturally infected by *L. infantum* and transmits the disease by bite (Kassem *et al.*, 2017). The vector species acquires its highest epidemiological relevance in El-Agamy, Alexandria, Egypt, where noticeable abundance was found.
More recently the spread of this important sand fly vector was demonstrated in southern Spain associated with wild rabbits. *Leishmania infantum* DNA was found in both *P. langeroni* and the wild rabbit *Oryctolagus cuniculus* (Diaz Sáez et al., 2018), which may increase visceral leishmaniasis incidence. Several modelled scenarios based on anticipated changes of climate variables in Europe suggest a possible spread of other *Larroussius* species into non-endemic regions (Fischer et al., 2011 and Haeberlein et al., 2013). Hence, more studies on the vector-parasite interphase will be of epidemiological significance.

In the gut of the sand fly vector, *Leishmania* parasites develop as extracellular promastigotes that are attached to the alimentary canal lining (Killick-Kendrick, 1979). Most of the developmental stages of suprapylarian *Leishmania* are confined to the midgut. In the midgut, promastigotes attach by inserting their flagella between microvilli, whereas in the foregut parasites attach to the cuticular surface by forming flagellar hemidesmosomes (Walters et al., 1989 and Warburg et al., 1989). Thus, the structure of the midgut is directly relevant to the development of *Leishmania* promastigotes within it. Some studies have been performed related to the morphology of the midgut of different female sand flies and the formation of the peritrophic matrix (Davis, 1967; Gemetchu, 1974; Rudin and Hecker, 1982; Jobling, 1987; Blackburn et al., 1988; Billingsley, 1990; Andrade-Coêlho et al., 2001 and Warburg, 2008).

In this paper, we described for the first time the ultrastructure of the midgut epithelium of *P. langeroni* after bloodmeal ingestion. The study of the midgut epithelium of *P. langeroni* is a prerequisite for future investigations on *L. infantum*/*P. langeroni* association.

**MATERIALS AND METHODS**

**Sand fly Species:**

Laboratory reared *P. langeroni* was originally collected from El-Agamy, Alexandria, Egypt. Sand fly was reared following the procedure described by Schmidt (1964).

**Study Samples:**

Females (five-day-old) were allowed to feed on anesthetized hamsters. Engorged females were taken and placed in cardboard cups provided with a piece of cotton wetted with a 30% sucrose solution and maintained at room temperature (24-26 °C ) and 60-70% RH.

**Transmission Electron Microscopy:**

Females sampled at 3, 4, 6 and 8 days after a bloodmeal were anesthetized and kept on ice and transferred individually to glass slides with a drop of saline solution and dissected. Suitable guts attached to the head were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C overnight. Then washed in the same buffer for 20 h. Post fixation was in 1% osmium tetroxide in cacodylate buffer at 4 °C for 1.5 h. Guts were rinsed in distilled water and stained with 0.5% uranyl acetate at room temperature (22 °C) for 30 min. The fixed guts were dehydrated in an ethanol/propylene oxide series, followed by embedment in fresh TAAB resin overnight at room temperature. Blocks were polymerized at 60 °C for 12-24 h. Ultrathin sections stained with 0.1% lead citrate were examined and photographed with JEOL Cx100 transmission electron microscope.

The transmission electron microscopy (TEM) was performed at the electron microscope unit. London School of Tropical Medicine and Hygiene, UK.

**RESULTS**

The ultrastructural features of the midgut epithelium of females sampled at 3, 4, 6 and 8 days after bloodmeal were described.

The epithelium of the midgut consisted of a single continuous layer of columner cells, with evident oval nuclei and densely packed apical microvilli. Underneath the columner cell layer, demarcating the epithelium was the basal lamina, made up of a complex web of invaginations, formed of
space demarcated by their membrane, and commonly known as basal labyrinths. Cells were connected together with a belt of intercellular junctions (Zonula continua). A musculature consisted of circular and longitudinal muscle fibers was present (Fig.’s 1C and D).

The sections examined 3 days after bloodmeal showed that the ingested blood was confined to the abdominal midgut and separated from the epithelium by the peritrophic matrix (PM). The PM remained mostly intact or was slightly disintegrated. The blood not completely digested. Microvilli were well defined, filled with secretions and products of blood digestion. Mitochondria were spread throughout the cytoplasm under the microvilli. Vacuoles were observed in the cytoplasm especially around the nucleus (Fig. 1A).

At day 4 after bloodmeal, the epithelial cell displayed clear vacuoles and lipid-inclusions. The PM no longer appeared and the gut-lumen was clear of any blood remnants. Mitochondria were spread throughout the cytoplasm (Fig. 1B). The epithelial cells were coated with long dense microvilli that filled almost the whole luminal space.

The sections examined at day 6 after the bloodmeal showed an undulated basal lamina and the basal labyrinths were clearly visible. The cell nuclei were large and filled with clumped chromatin granules. Mitochondria were spread throughout the cytoplasm. The microvilli were dense and arranged in parallel. Clear vacuoles and few lipid inclusions were seen in the cells (Fig.’s 1C, D, and E). At day 8 after bloodmeal, the nuclei were oval-shaped. Vacuoles were abundant within the cytoplasm. Mitochondria were few. The microvilli area was greatly reduced or even detached, suggesting the end of the digestive cycle (Fig. 1F).
Fig. 1. Electron micrographs of cross sections of the abdominal midgut of *P. langeroni* females, showing the process of blood digestion.

A (x6,000) 3 days after bloodmeal. Midgut epithelium separated from the blood by the peritrophic matrix (PM) and ectoperitrophic space (eps), small food particles penetrating among the microvilli (Mv). Basal labyrinths (BLB), basal lamina (BL), muscle (m), mitochondria (M) and intercellular junction (IJ).

B (x5,000) 4 days after bloodmeal. Clear lumen (Lu), mitochondria (M), vacuoles (V) and intense microvilli (Mv).

C (x3,300), D (x6,600) and E (x8,300) 6 days after bloodmeal. Clear vacuoles (V), few lipid inclusions (L), clear undulated basal lamina (BL) and basal labyrinths (BLB).

F (x5,000) 8 days after bloodmeal. Large nuclei (N) filled with chromatin, abundant vacuoles (V) and reduced microvilli (Mv).
DISCUSSION

The description of the midgut epithelium of *P. langeroni* based on TEM was performed for the first time. The sections examined showed that the apical end of the columnar cells contained microvilli providing a wide surface area for absorbing material from the lumen. Underneath the columnar cell layer, demarcating the epithelium, was the basal lamina, made up of invaginations called basal labyrinths, which, like the microvilli, provide a large surface across which materials can be transported. Mitochondria were found in some areas of the cytoplasm associated with the basal labyrinths. The basal lamina was granular. These structures were also described by Romoser (1996) for hematophagous insects. The Golgi apparatus was not detected in our study, probably due to the limited samples examined. However, the Golgi apparatus has already been demonstrated in the cells of other dipterans by many researchers (for e.g. Gemetchu, 1974; Rudin and Hecker, 1982 and Billingsley, 1990). We observed all the structures cited by Andrade-Coêlho *et al.* (2001) for the sand fly *Lutzomyia intermedia* except the rough endoplasmic reticulum, probably because it is not an easy structure to find or because that after the bloodmeal (as was the condition of the present study) this structure spreads around the cell cytoplasm and underwent clear morphological changes, as in *Aedes aegypti* females (Bertram and Bird, 1961). The rough endoplasmic reticulum might be responsible for all the secretory and absorptive functions ascribed to the midgut (Bertram and Bird, 1961). Gemetchu (1974) analyzed the fine structure of the midgut in *P. longipes* females and only found the whorled rough endoplasmic reticulum beginning four days after emergence.

In the present work, the PM remained intact or slightly disintegrated, in the females 3 days after a bloodmeal. The PM was present surrounding the ingested blood and performing its function of protecting the epithelium. The PM (previously known as the peritrophic membrane) is present in most insects and is classified into type I and type II. Usually, PM II occurs in the larval stage and some adult Diptera. In general, the name PM is commonly used as a reference for type I in hematophagous Diptera (Secundino *et al.*, 2005). In nematoceran Diptera including sand flies, females produce a type I PM, which is secreted by the entire midgut in direct response to the distention of the midgut caused by the blood-feeding (Jacobs-Lorena and Oo, 1996).

The blood-fed females examined at day 4 after bloodmeal indicated the absence of PM completely and showed a clear lumen. Andrade-Coêlho *et al.* (2001) found that PM was not seen 72 h after the bloodmeal in *Lu. intermedia*. We believe that the time of degradation of PM is species-specific. This agrees with the view of Gemetchu (1974) that the kinetics of PM formation and degradation is totally related to the ingestion and the time of digestion of the bloodmeal, which varies according to distinct insect species.

At day 6 after bloodmeal, which appears to represent the end of the digestive cycle. Clear vacuoles around the nucleus were evident. Lipid inclusions in the apical region of the epithelium were scarce or disappeared. These observations in *P. langeroni* are in agreement with those observed in *Lu. intermedia* (Andrade-Coêlho *et al.*, 2001) and *Lu. longipalpis* (Rudin and Hecker, 1982). Indicating that after blood digestion the cell apparatus was reduced and lipid inclusions and glycogen deposits disappeared from the abdominal region.

At day 8 after bloodmeal, it was observed that the area occupied by the microvilli was greatly reduced, and the microvilli were in a state of degeneration, probably due to reduced or no functional activity by the end of digestive cycle.

In *P. langeroni* the blood digestion process lasted over 4 days and may reach 6 days. This duration was longer than that observed for *Lu. longipalpis* (Rudin and
Hecker, 1982) and Lu. intermedia (Andrade-Coelho et al., 2001). The bloodmeal digestion process varies from one species to another depending on the type of blood and digestive enzymes activities (Dillon and Lane, 1993) and many other factors. However, the size of the bloodmeal ingested cannot be excluded. It is known that while feeding on the vertebrate, sand fly females take a bloodmeal that is directed to the abdominal midgut and surrounded by PM in the so-called peritrophic sac or endoperitrophic space (Dvorak et al., 2018). The amount of bloodmeal varies from 0.6 µl in P. argentipes or P. orientalis to 0.9 µl in P. papatasi (Pruzinova et al., 2015) and 0.85 µl in P. langeroni a closely related species to P. orientalis (Daba et al., 2004).

Finally, our study has revealed, the possible roles of the different internal structures of P. langeroni midgut. This may help in understanding the parasite infection and adaptation in this sand fly vector.

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