

# Peritrophic matrix of *Phlebotomus duboscqi* and its kinetics during *Leishmania major* development

Jovana Sádlová · Petr Volf

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**Abstract** Light microscopy of native preparations, histology, and electron microscopy have revealed that *Phlebotomus duboscqi* belongs to a class of sand fly species with prompt development of the peritrophic matrix (PM). Secretion of electron-lucent fibrils, presumably chitin, starts immediately after the ingestion of a blood meal and, about 6 h later, is followed by secretion of amorphous electron-dense components, presumably proteins and glycoproteins. The PM matures in less than 12 h and consists of a thin laminar outer layer and a thick amorphous inner layer. No differences have been found in the timing of the disintegration of the PM in females infected with *Leishmania major*. In both groups of females (infected and uninfected), the disintegration of the PM is initiated at the posterior end. Although parasites are present at high densities in the anterior part of the blood meal bolus, they escape from the PM at the posterior end only. These results suggest that *L. major* chitinase does not have an important role in parasite escape from the PM. Promastigotes remain in the intraperitrophic space until the PM is broken down by sand-fly-derived chitinases and only then migrate anteriorly. Disintegration of the PM occurs simultaneously with the morphological transformation of parasites from procyclic forms to long nectomonads. A novel role is ascribed to the anterior plug, a component of the PM secreted by the thoracic midgut; this plug functions as a temporary barrier to stop the forward migration of nectomonads to the thoracic midgut.

**Keywords** Chitinase · Midgut · Digestion · Peritrophic matrix · *Phlebotomus duboscqi* (Insecta) · *Leishmania major*

## Introduction

The peritrophic matrix (PM, previously referred to as the peritrophic membrane) is an acellular chitin-containing envelope, which, in most insects separates the gut lumen from the midgut epithelium; it is composed of chitin, proteins, and proteoglycans (Peters 1992; Lehane 1997). In nematoceran Diptera, including sand flies, females produce a type 1 PM, which is secreted by the entire midgut in direct response to the distension of the midgut caused by blood feeding (Jacobs-Lorena and Oo 1996).

The role of the PM is associated with the protection of the midgut epithelium against pathogens and abrasion by food particles and with the compartmentalization of digestion between the ecto- and endoperitrophic spaces (Peters 1992; Lehane 1997). In mosquitoes and other hematophagous insects, the PM also performs a central role in heme detoxification (Pascoa et al. 2002). Although electron-microscopy studies on the PM of sand flies have been published (Gemetchu 1974; Blackburn et al. 1988; Walters et al. 1993, 1995; Andrade-Coelho et al. 2001; Secundino et al. 2005), the data presented by the different authors are often fragmentary. Moreover, descriptions of PM formation vary, even in studies when two groups have worked on the same sand fly species (Reznik and Kuznetsova 1983; Blackburn et al. 1988).

Detailed knowledge of the formation and morphology of the sand fly PM is important as sand flies are vectors of *Leishmania* parasites, causative agents of serious human diseases. In the early phase of infection, when *Leishmania* are vulnerable to proteolytic damage, the PM is supposed to

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protect the parasites against the rapid diffusion of digestive enzymes (Pimenta et al. 1997). On the other hand, following digestion of the blood meal, the PM can adversely influence the development of *Leishmania* promastigotes by creating a potential physical barrier that prevents their escape from the endoperitrophic space (for a review, see Bates and Rogers 2004). In unnatural parasite-vector combinations, Feng (1951) and Walters et al. (1992) have reported that the PM does not break down during blood digestion, and that parasites are excreted.

*Leishmania* promastigotes produce chitinase (Shakarian and Dwyer 1998, 2000), and experiments with chitinase-overexpressing mutants of *L. mexicana* have confirmed the damaging effects of the enzyme on the stomodeal valve of the sand fly (Rogers et al. 2008). The role of chitinase in parasite interactions with the sand fly PM is still not clear. Schlein et al. (1991) have reported that *L. major* promastigotes cause the disintegration of the anterior end of the PM of *P. papatasi* by means of their own chitinase. However, in most vector-parasite combinations, parasite escape from the PM seems to coincide with the breakdown of the PM by sand-fly-derived chitinases (Ramalho-Ortigao and Traub-Cseko 2003; Ramalho-Ortigao et al. 2005).

In this study, we describe the PM in the natural vector-parasite pair of *Phlebotomus duboscqi* Neveu-Lemaire infected with *Leishmania major* Yakimoff et Schokhor. We have studied the morphology, ultrastructure, and timing of assembly and degradation of the PM. In contrast to previous descriptions, we have found that *Leishmania* parasites do not cause the disintegration of the PM at the anterior end but migrate through posterior openings that exist in the PM even in non-infected females. In addition, we suggest a novel role for the anterior plug (AP), a PM projection separating the abdominal (AMG) and thoracic (TMG) regions of the midgut. This structure seems to temporarily limit the anterior migration of *Leishmania* into the TMG.

## Materials and methods

### Sand flies

The colony of *P. duboscqi* was maintained on 50% sucrose at 26°C under a 14-h light/10-h dark photoperiod (for more details, see Benková and Volf 2007).

### Parasites and sand fly infections

The *Leishmania major* strain LV561 (LRC-L137; MHOM/IL/1967/Jericho-II), which is highly virulent in BALB/c mice (Sádlová et al. 1999), and the green-fluorescent protein (GFP) line from the same strain were kindly

provided by Dr. J. Votýpka and Dr. D. Folková (Charles University, Prague). BALB/c mice were inoculated with  $10^7$  promastigotes in the rump to generate amastigotes for sand fly infections. The lesions from infected animals were dissected and homogenized in Schneider's *Drosophila* medium with gentamicin (40 mg/100 ml). Sand fly females were fed through a chick-skin membrane on a suspension of amastigotes mixed 1:1 with rabbit erythrocytes. The final concentration of parasites was  $10^6$  (wild strain, GFP line) or  $10^7$  (GFP line) amastigotes/ml. Engorged sand flies were maintained under the same conditions as those described above.

### Light microscopy

A total of 186 blood-fed females were dissected at various time intervals after feeding on anesthetized mice; 6 within 10 min (T0) post-blood-meal (PBM) and 20 for each of the following times PBM: 1, 3, 6, 12, 24, 48, 72, 96, 120 h (T1–T120). In addition, 257 females fed through a chick-skin membrane were dissected for *Leishmania* infections at T48, T72, and T96. Dissections were carried out in isotonic saline solution, and then the gut from each fly was briefly washed in distilled water for better isolation of the PM (Gemetchu 1974). Slides were observed under an Olympus BX51 microscope with Nomarski contrast and photographed with an Olympus D70 camera and software (DP Controller). Parasite loads were graded according to Myskova et al. (2008) as light (<100 parasites/gut), moderate (>100 and <1000 parasites/gut), or heavy (>1000 parasites/gut).

### Morphometry of parasites

Gut smears of females infected with *L. major* were dissected at T72 and T96, fixed with methanol, stained with Giemsa, and examined under the light microscope with an oil-immersion objective. Three smears were made for each of four categories of PM (intact, slightly disintegrated, opened/heavily disintegrated, and defecated), and 40 randomly selected promastigotes were measured in each smear. The body length, flagellar length, and body width were measured, and the position of the kinetoplast in relation to the nucleus was noted. Five morphological forms were distinguished based on Čiháková and Volf (1997) and Rogers et al. (2002): (1) procyclic promastigotes (body length <14 μm and flagellar length ≤ body length), (2) elongated nectomonads (body length ≥14 μm), (3) short nectomonads (leptomonads; body length <14 μm and flagellar length > body length but ≤ twice body length), (4) metacyclic promastigotes (body length <14 μm and flagellar length > twice body length), (5) paramastigotes (kinetoplast lateral to the nucleus).

## Histology

Unfed females and females at T0–T96 time intervals PBM were fixed at 4°C in AFA solution (formaldehyde:ethanol:acetic acid:distilled water, 1.5:12.5:1:10). After being washed in phosphate-buffered saline (pH 7.6) and 70% ethanol, the samples were embedded in JB-4 following the manufacturer's protocol (Polysciences). Histological sections (4–6 µm thick) were stained with Ehrlich's acid hematoxylin and 0.2% eosin, mounted on glass slides with Plastic UV Mount (Polysciences), and observed under an Olympus BX51 microscope. Three to five females were used for each of the time intervals.

## Electron microscopy

Unfed females or gut samples and blood-fed females or gut samples were dissected at T0–T72, fixed in modified Karnovsky fixative (Karnovsky 1965), and post-fixed with a 2% osmium tetroxide solution (both at 4°C). The samples were dehydrated in ascending concentrations of ethanol and embedded in Poly/Bed 812/Araldite 502 (Polysciences). Semithin sections (400 nm) and ultrathin sections (70 nm) were obtained by using a Reichert-Jung Ultracut E ultramicrotome. Semithin sections were stained with toluidine blue for light microscopy. Ultrathin sections stained with uranyl acetate and lead citrate (Reynolds 1963) were observed and photographed with a JEOL 1011 transmission electron microscope. Three to seven females were used for each of the time intervals.

## Statistical analysis

PM development in the groups of infected and uninfected females was compared by using the Chi-squared test. Measurements of parasites and the representation of morphological forms were compared by using an analysis of variance and the Chi-squared test, respectively. All statistical evaluations were performed with statistical software (SPSS version 12).

## Results

### Development of the PM: light microscopy

Immediately after blood feeding (T0), the blood meal was spread throughout the TMG and AMG with no apparent agglutination of erythrocytes (data not shown). Within 1 h, the TMG was free of blood, and the whole blood meal was concentrated in the AMG. Erythrocytes were agglutinated to a compact dark central bolus surrounded by a light periphery formed from extruded blood plasma. In some of

the flies, a sharp line separating the blood bolus from both the plasma in the periphery and the midgut epithelium indicated the first trace of the future PM (Fig. 1a).

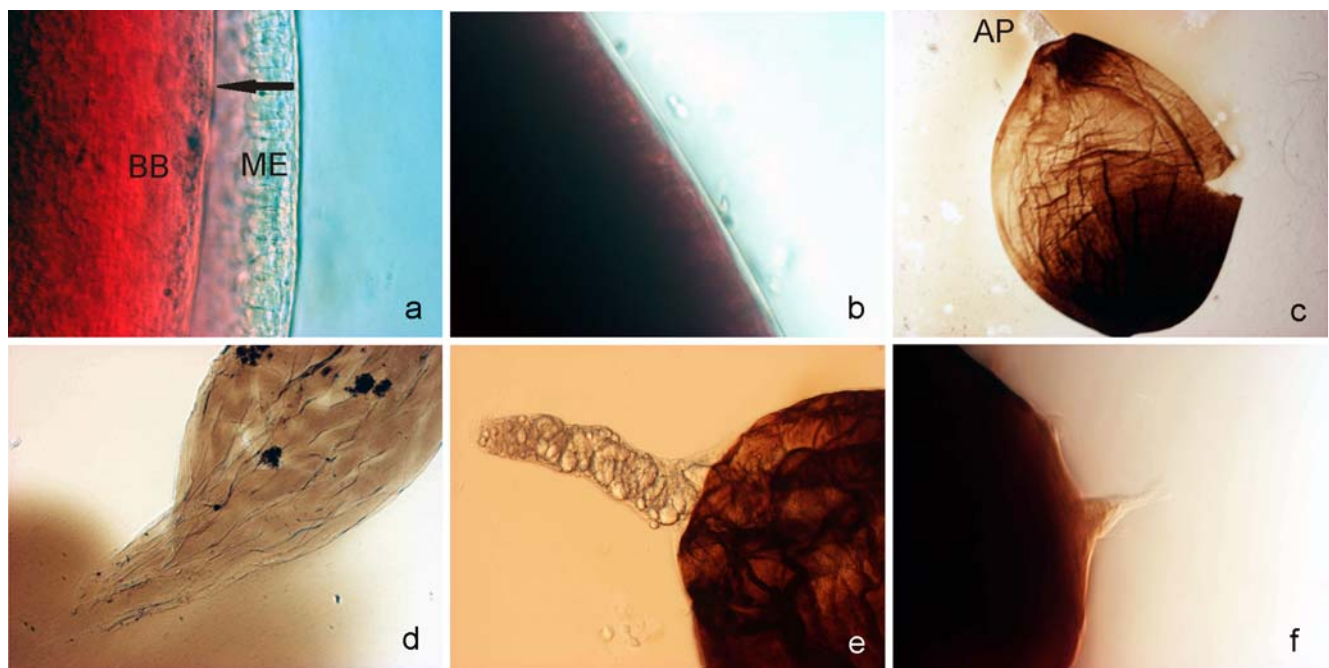
At T3, a thin transparent PM had formed in half of the dissected females (Figs. 1b, 2). In some cases, it did not cover the whole surface of the blood bolus but only the anterior part. At T6, the PM was strong enough to enable dissection of the entire peritrophic sac in all studied females (Fig. 2). By T24, the PM darkened as it became encrusted with heme, a product of erythrocyte degradation (data not shown). This process affected both the anterior and posterior ends of the PM in most of the flies (85% of females) and less frequently affected only the anterior (5%) or posterior ends (10%). At T48, further encrustation with heme led to a denser PM with a paler median region (Fig. 1c).

By T72, the erythrocytes had been disintegrated, and the PM showed a reduced volume and a folded surface and began losing its dark color in some flies. In most females, the PM was only slightly disintegrated or intact, but in some cases, the posterior end had widely opened (Fig. 2). At 4 days PBM (T96), most females had defecated (Fig. 2); their midgut was free of the blood meal and often contained air bubbles. Rarely, slightly disintegrated PM, posteriorly opened PM (Fig. 1d), or heavily disintegrated PM was found. By day 5 PBM (T120), all but one female had defecated.

The mature PM of *P. dubosqi* took the form of a closed sac in all females. The anterior and posterior regions of the PM protruded into structures termed here the AP and posterior tail (PT), respectively. The AP represented the part of matrix secreted by the TMG and, because of peristaltic contractions of the midgut, regressed to the anterior end of the peritrophic sac (Richardson and Romoser 1972). Both the AP and PT were visible by T6 (in 50% and 5% of females, respectively) and, at T24, were well formed in all females studied. These structures were not in a direct contact with blood and therefore remained transparent, without a heme encrustation (Fig. 1e, f)

### Development of the PM: histological sections

During the first hour PBM, the irregular marginal line of the blood meal mass changed to a clear line separating the red blood cells from the extruded plasma and the midgut epithelium. This line, the future PM, was often found in specific regions of the midgut from T1 to T3 (data not shown). Heme deposits were visible in histological sections from earlier time points when compared with native observations; both the anterior and posterior ends became colored with heme at T6 (Fig. 3a). At T24, the PM was intensely brown, and several distinct layers were observed (Fig. 3b). By T48, the heme encrustations became even more pronounced (Fig. 3c) and affected all parts of the PM, except for the AP and PT. The AP region, with heme absent



**Fig. 1** Development of the peritrophic matrix (PM) in *P. dubosqi* females observed by light microscopy. **a** The sharp line (*arrow*) separating the blood bolus from the midgut epithelium is the first sign of the future PM at T1 (*BB* blood bolus, *ME* midgut epithelium).  $\times 400$ . **b** Detail of the peritrophic sac dissected from the gut at T3; a thin PM

surrounds the blood bolus.  $\times 400$ . **c** Heme incrustation at T48 reaches both the anterior and posterior ends of the PM; the anterior plug (*AP*) remains transparent.  $\times 40$ . **d** The PM opens widely at the posterior end at T96.  $\times 200$ . **e** Anterior plug in a female dissected at T72.  $\times 200$ . **f** Posterior tail in a female dissected at T24.  $\times 400$

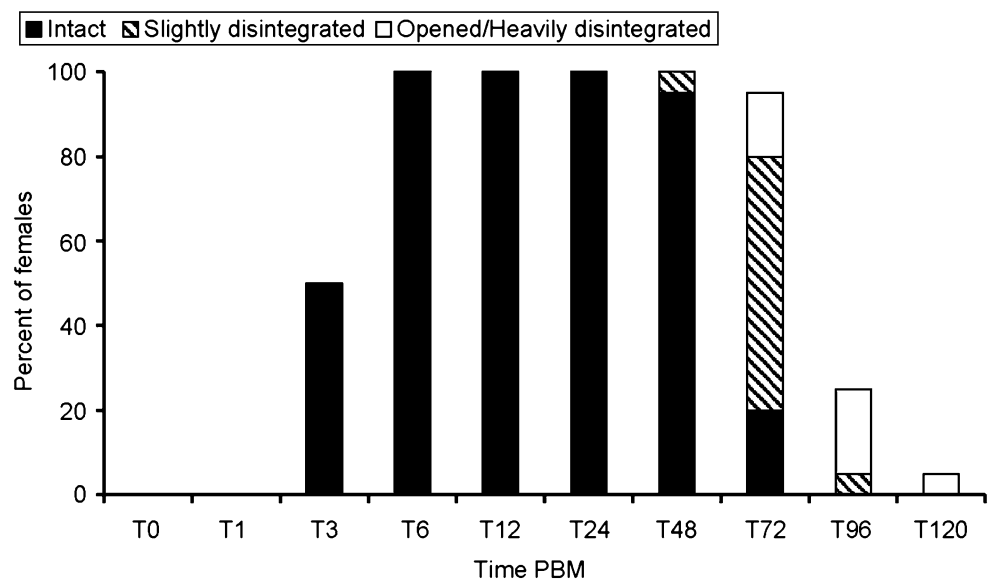
or only scarcely incorporated, showed a strikingly different appearance from other parts of the PM (Fig. 3d, e). As the blood meal decreased in volume, the PM creased (T48, Fig. 3f) and shrank (T72).

#### Development of the PM: electron microscopy

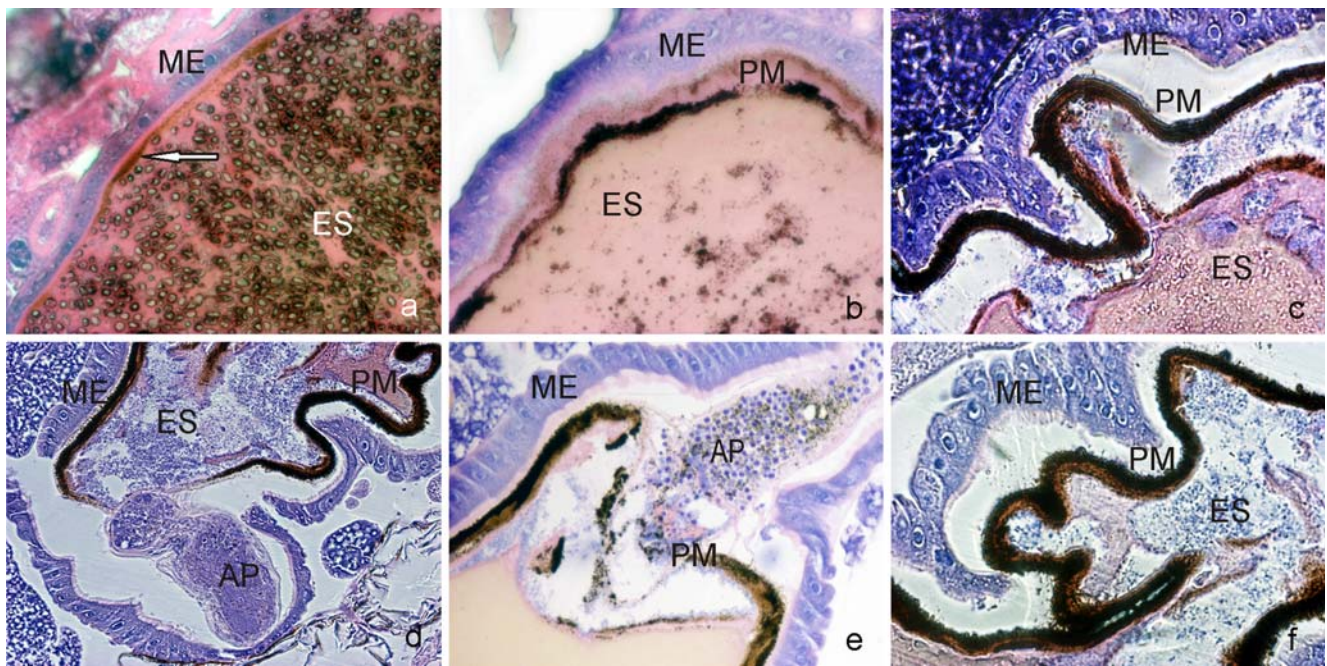
The epithelium of the AMG consisted of a single layer of digestive cells surrounded by the basal lamina. The cells were

characterized with centrally located large nuclei, densely packed apical microvilli, and intricate basal labyrinth (i.e., invaginated basal cell membrane). The cytoplasm was filled with numerous mitochondria, free ribosomes, and rough endoplasmic reticulum (RER). Cells were connected together with a belt of intercellular junctions (*zonula continua*) in the apical region. Invaginations of the basal and baso-lateral cellular membranes together formed an extensive network of channels, viz., the basal labyrinth (Figs. 4, 5).

**Fig. 2** Presence of the peritrophic matrix in *P. dubosqi* females at various time intervals post-blood-meal (*PBM*)







**Fig. 3** Development of the peritrophic matrix (PM) in *P. duboscqi* females observed in histological sections. **a** Heme (arrow) bound to the PM at T6 (ME midgut epithelium, ES endoperitrophic space).  $\times 400$ . **b** Distinct layers of the PM observed at T24.  $\times 400$ . **c**

Multilayered PM with intensive heme incrustation at T48.  $\times 400$ . **d** Transparent anterior plug (AP) without heme incrustation at T48.  $\times 200$ . **e** PM with heme deposits, and AP without heme deposits at T48.  $\times 400$ . **f** Folded PM at T48  $\times 400$

In unfed females, the cells in this region of the gut were columnar in shape; the cytoplasm was either vacuolated with vesicular RER or filled with tubular RER that often formed whorls (Fig. 4a). Distension of the AMG caused by engorgement (T0) resulted in extreme flattening of digestive cells to a squamous form with remote microvilli. Additionally, nuclei were flattened, the basal labyrinth was well formed, and the cytoplasm was densely filled with RER with the occasional appearance of whorls. Abundant secondary lysosomes emerging at the apical cytoplasm indicated the rapid initiation of blood digestion (Fig. 4b). From T1 onward, large lipid spheres appeared within the cytoplasm, and the RER was observed as tubular or vesicular cisternae distributed throughout the cytoplasm (Fig. 4c, d). The epithelium of the AMG returned to its previous height from T12 (Fig. 4d), and the cells regained their original columnar shape by T72 (Fig. 4e). Blood meal remnants among the microvilli and the abundance of large secondary lysosomes in the cytoplasm demonstrated that T72 was the most active period of digestion (Fig. 4f).

Immediately after blood feeding (T0), all the spaces between the epithelium and compact blood cells mass were filled with an electron-dense fine granular material. Simultaneously, the secretion of an electron-lucent fibrillar material was observed along the entire cell surface (data not shown). This secretion continued at T1, when the electron-lucent microfibrils started to form a distinct zone near the microvilli (Fig. 5a). The loosely organized fibrillar network had a

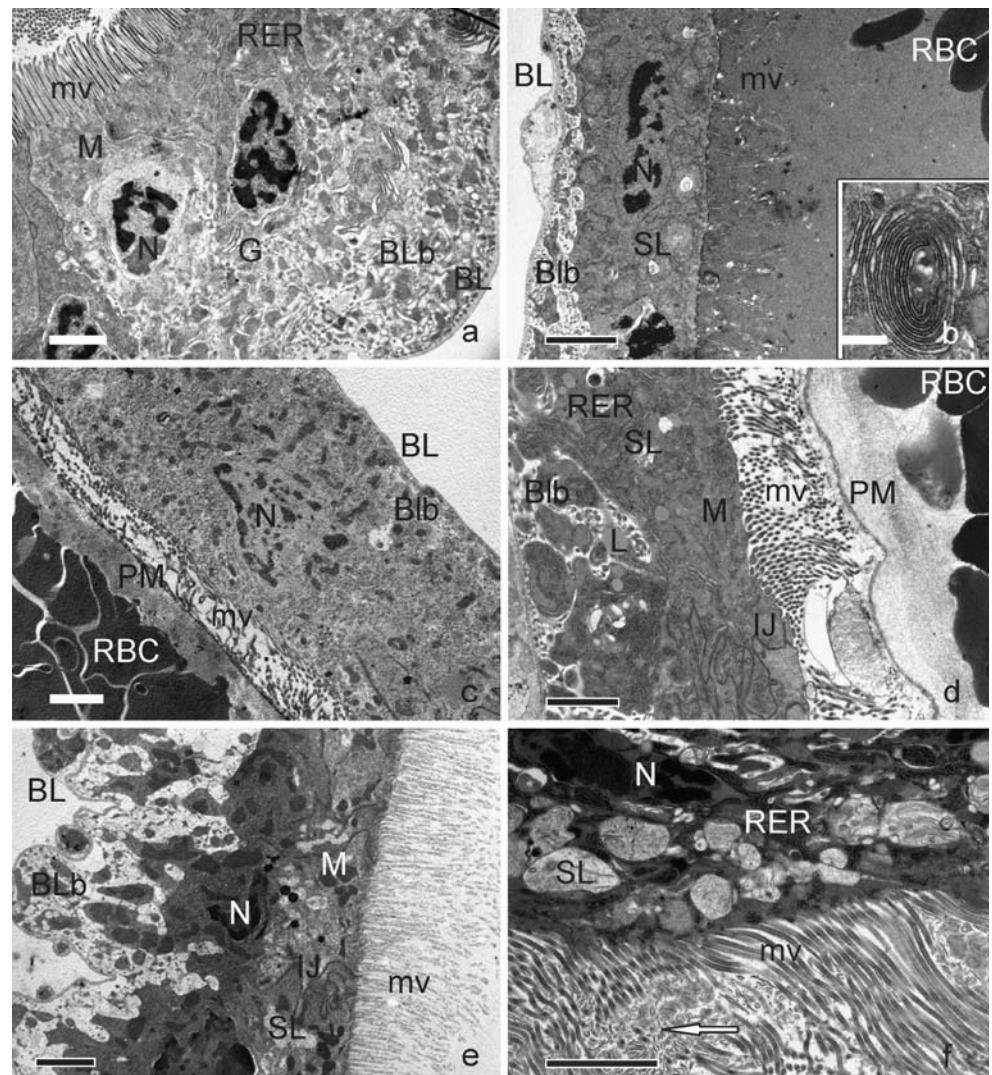
random texture. The electron-lucent secretion continued at T3 when additional electron-dense secretion started. The electron-dense amorphous material fused with the electron-lucent microfibrils by 3 h PBM (Fig. 5b). From T6, the electron-lucent secretion was no longer detectable, whereas the electron-dense secretion was observed until the end of the experiment (T72).

At T6, the first signs of heme encrustation (electron-dense aggregates) were apparent in the immature PM (Fig. 5c). Partially lysed blood cells were often present in the periphery of the alimentary bolus. From T12 to T48, particles from lysed blood cells filled the space between the bolus and PM. The mature but thin (less than 2  $\mu\text{m}$ ) PM was present in all studied females at T12 (Fig. 5d) and consisted of a laminar outer layer and a thick amorphous inner layer. At 1 day PBM (T24), the PM had become thicker (more than 2  $\mu\text{m}$ ) and multilayered with several electron-dense laminae at the midgut site (Fig. 5e). From T48 to T72, the PM became even thicker (about 5  $\mu\text{m}$ ) and was wrinkled (Fig. 5f).

The epithelial cells of the TMG were coated with long dense microvilli that filled almost the whole luminal space. After a blood meal, the TMG epithelial cells were less flattened than epithelial cells in AMG. Regardless of their different morphology, TMG epithelial cells appeared to be active during the processes of secretion (both electron-lucent and electron-dense secretions were observed) and digestion (primary and secondary lysosomes were present), although these processes were not as intensive as those in AMG cells.



**Fig. 4** Electron micrographs of cross sections of the abdominal midgut of *P. duboscqi* females showing the process of digestion. **a** Columnar midgut cells of an unfed female (BL basal lamina, BLb basal labyrinth, G Golgi apparatus, mv microvilli, M mitochondria, N nucleus, RER rough endoplasmic reticulum). Bar 2  $\mu$ m. **b** Flattened midgut cells immediately after blood feeding at T0 (RBC red blood cells, SL secondary lysosomes). Bar 2  $\mu$ m. *Insert*: Whorl of RER. Bar 0.5  $\mu$ m. **c** Midgut epithelium at T6; still flattened and separated from the blood bolus by the peritrophic matrix (PM). Bar 2  $\mu$ m. **d** Digestion of red blood cells (RBC) at T12 (IJ intercellular junctions, L lipidic inclusions). Bar 2  $\mu$ m. **e** Columnar midgut cells at T72. Bar 2  $\mu$ m. **f** Intense digestion at T72. Note the small food particles (arrow) penetrating among the microvilli (mv) and the cytoplasm filled with secondary lysosomes (SL). Bar 2  $\mu$ m



#### Effect of *Leishmania* infections on PM

The intensity of *L. major* infection (number of parasites) increased from T48 to T72 and T96. Then, on T120, it decreased because of defecation. At the latter stage (days 8 and 9 PBM), heavy infections with colonization of the stomodeal valve prevailed (Fig. 6).

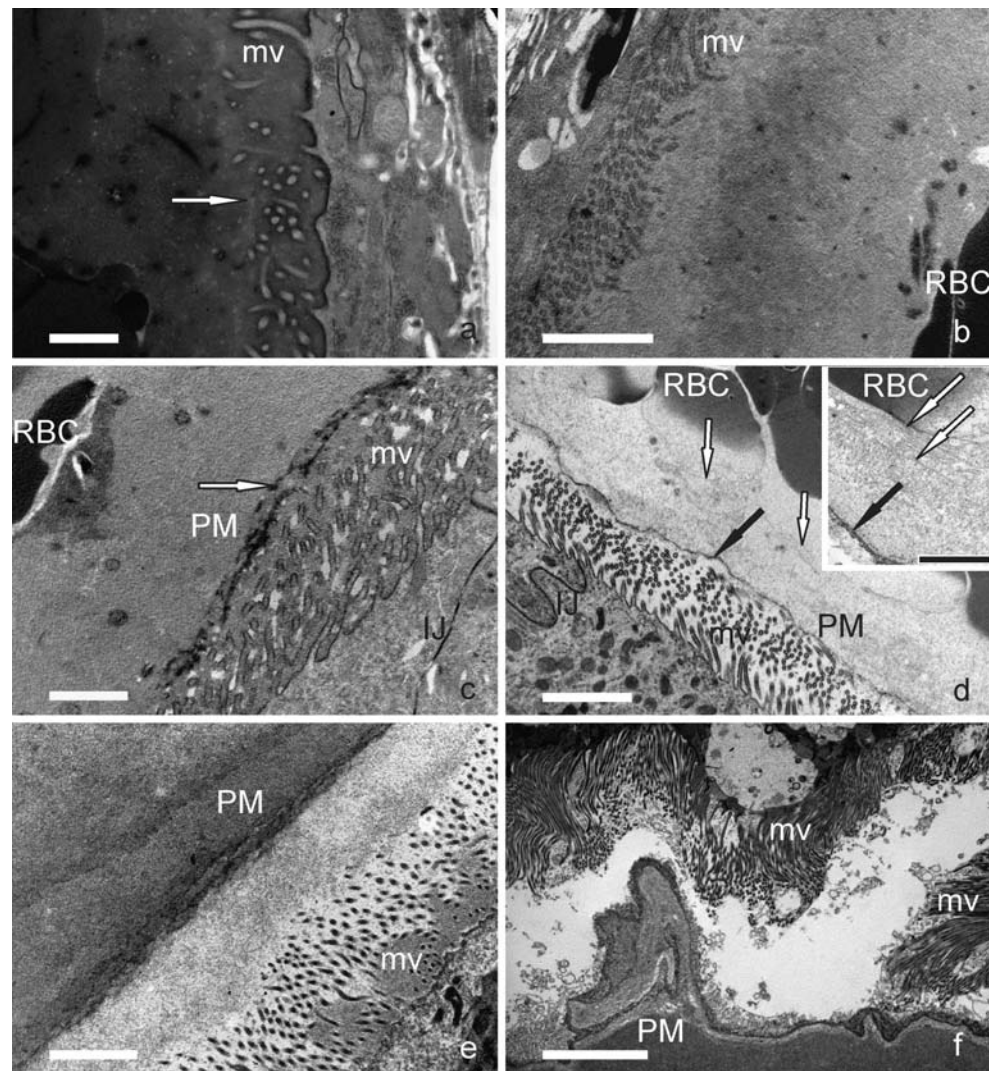
The development of the PM was compared in females infected with *L. major* amastigotes and those fed an uninfected blood meal. Groups of 10 females were dissected at T48 and groups of 30 females at T72, T96, and T120. No significant differences were found in the timing of disintegration of the PM between these two groups (Table 1). Two days after blood feeding (T48), the PM was intact in both groups. One day later (T72), the PM remained mostly intact or was only slightly disintegrated. Parasites were still located inside the PM, but the intensity of the infection rapidly increased (Fig. 6). In some females with an opened PM and disintegrated AP, long nectomo-

nads were found in ectoperitrophic space and even in the TMG (not shown).

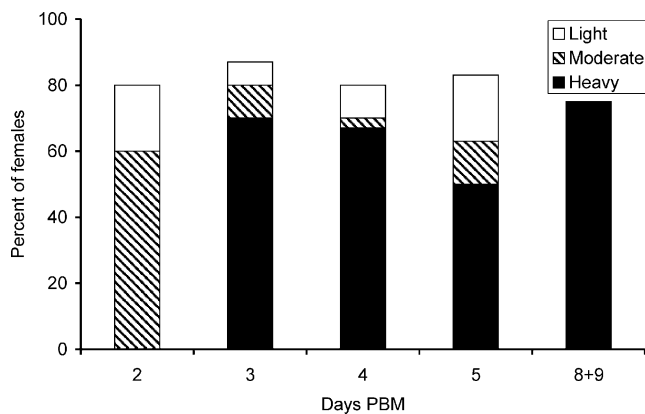
By T96, the pattern of infection was highly diverse. Half of the females had defecated, and parasites colonized the midgut and, in some cases, reached the stomodeal valve. One group of females (21%) showed a heavily disintegrated PM still present in AMG, and parasites had invaded the AMG and TMG; however, when the AP was still fully formed, parasites were restricted to the AMG. In another group of females (25%), parasites were found only inside the slightly disintegrated PM. By T120, most infected females had defecated, and parasites were spread throughout both the AMG and TMG, with colonization of the stomodeal valve being observed in 48% of the flies.

A GFP-expressing line of *L. major* produced a similar pattern of infection of *P. duboscqi*, with an infective dose of  $10^6$  amastigotes/ml, although an infective dose of  $10^7$  amastigotes/ml resulted in greater parasite abundance. By 2 days PBM (T48), promastigotes remained inside the PM,

**Fig. 5** Electron micrographs of cross sections of the abdominal midgut of *P. duboscqi* females showing formation of the peritrophic matrix (PM). **a** Electron-lucid zone (arrow) formed near microvilli (mv) at T1; the electron-dense background originates from blood digestion. Bar 1  $\mu$ m. **b** Electron-dense area between microvilli and the blood bolus at T3 (RBC red blood cells). Bar 2  $\mu$ m. **c** Electron-dense secretion from microvilli and heme incrustation (arrow) of the immature PM at T6 (IJ intercellular junctions). Bar 1  $\mu$ m. **d** Thin PM with solid electron-dense outer layer (black arrow) and diffuse inner layer at T12 (white arrows membranes from digested RBC). Bar 2  $\mu$ m. *Insert:* Higher magnification of PM (white arrows membranes from digested RBC in the endoperitrophic space, black arrow solid electron-dense outer layer). Bar 1  $\mu$ m. **e** Multilayered PM at T24. Bar 2  $\mu$ m. **f** Thick and wrinkled PM at T72. Bar 5  $\mu$ m



and most of the population consisted of short and rounded procyclic forms. At the higher infection dose, the maximal density of parasites was clearly seen in the center and the anterior part of the blood bolus (Fig. 7a, b).

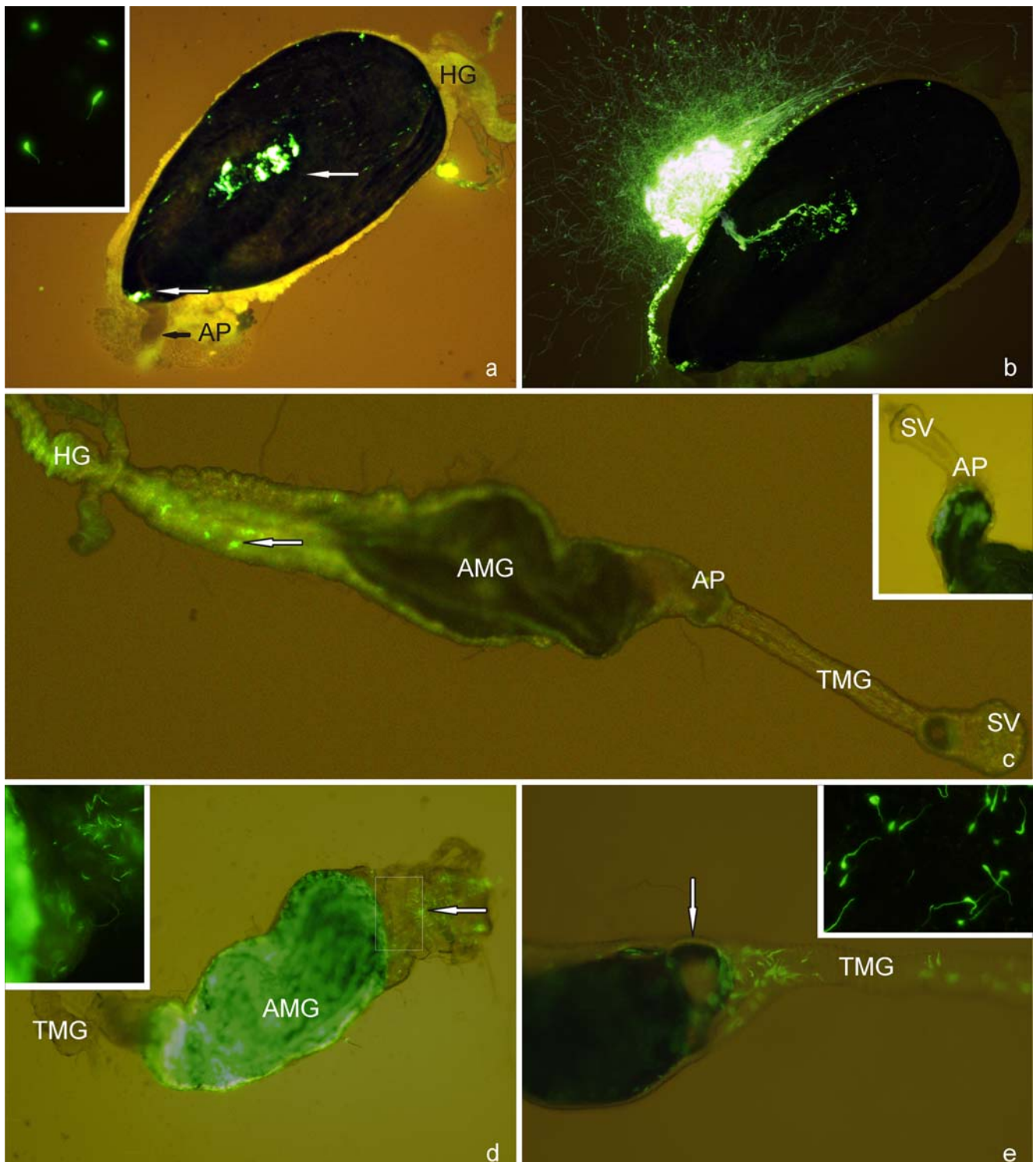


**Fig. 6** Rates and intensities of *L. major* LV561 infections in *P. duboscqi*

Parasites escaping from the posterior end of the PM were often observed by T72, and the posterior PM was open in most (70%) females by T96 (Fig. 7c). Even in sand flies with high infective doses ( $10^7$  amastigotes/ml), parasites escaped only from the posterior end of the PM, despite the extremely high concentration of parasites at the anterior end of the blood meal bolus (Fig. 7d). No escape of promastigotes through the anterior end of the PM was observed. Promastigotes within the ectoperitrophic space migrated anteriorly to the region blocked with the AP (Fig. 7c, d) and, after disintegration of the AP, migrated to the TMG (Fig. 7e).

Histological sections and electron microscopy showed that all promastigotes were located inside the PM by T48, and that a few free-swimming parasites occurred in the ectoperitrophic space by T72. The disintegration of the PM by T96 allowed a massive migration of parasites into the ectoperitrophic space through the posterior opening. Long nectomonads migrated along the disintegrated AP and colonized the TMG (data not shown). In some *P. duboscqi*





**Fig. 7** *P. duboscqi* gut infected by the *L. major* GFP line. **a** Digestive tract dissected at T48 (infective dose:  $10^7$ /ml); promastigotes (arrows) were most abundant in the center and the anterior part of the PM (AP anterior plug, HG hind gut).  $\times 40$ . *Insert*: Short procyclic forms prevail.  $\times 400$ . **b** The same gut after artificial rupture of the PM.  $\times 40$ . **c** *Leishmania* (infective dose  $10^6$ /ml) escaping posteriorly (arrow) from the slightly opened PM at T96 (AMG abdominal midgut, TMG thoracic midgut, SV stomodeal valve).  $\times 40$ . *Insert*: Another midgut

(infective dose  $10^7$ /ml) at T72; parasite migration is blocked by the anterior plug (AP).  $\times 40$ . **d** Posterior escape of parasites (arrow) and blockage of their anterior migration by the AP (infective dose  $10^7$ /ml) at T72.  $\times 42.5$ . *Insert*: Higher magnification of boxed area in **d**.  $\times 200$ . **e** Migration of *Leishmania* (infective dose  $10^7$ /ml) through the region with a disintegrated AP (arrow) to the TMG at T96.  $\times 100$ . *Insert*: Population of parasites consist of various forms with a long flagellum at T96.  $\times 400$



**Table 1** Disintegration of the peritrophic matrix (PM) in females infected with *Leishmania major* amastigotes and in controls fed an uninfected blood meal (PBM post-blood-meal)

Days PBM	Number of infected (INF) and control (C) flies	State of PM					Significance of between-group differences
		Intact	Slightly disintegrated	Opened	Heavily disintegrated/ blood remnants	Defecated	
2	INF 8	8	0	0	0	0	$P=1, \chi^2=0.0000$
	C 10	10	0	0	0	0	
3	INF 26	11	13	2	0	0	$P=0.9881, \chi^2=0.0239$
	C 30	13	15	2	0	0	
4	INF 24	0	6	1	5	12	$P=0.8911, \chi^2=1.1201$
	C 30	1	8	2	5	14	
5	INF 25	0	0	1	1	23	$P=0.3243, \chi^2=2.2520$
	C 30	0	0	1	5	24	

females, an unidentified contaminating yeast was observed in the infected blood meal. Where *Leishmania* promastigotes were never seen burrowing inside the PM, yeast were able to disrupt and penetrate through the PM layers (Fig. 8a, b).

A proposed model of the course of *L. major* early development in *P. duboscqi* is depicted in Fig. 9.

#### Morphological transformations of *Leishmania* in relation to disintegration of the PM

According to the state of the PM, four categories of females were distinguished: females with an intact PM, females with a slightly disintegrated PM, females with a PM that was opened/heavily disintegrated, and females that had defecated. The effect of the disintegration of the PM on the morphological transformation of promastigotes was more pronounced than the effect of the time PBM (Table 2, 3). By

T72, promastigotes from females with a slightly disintegrated PM had a significantly longer ( $P=0.012$ ) and more slender ( $P=0.009$ ) body and longer flagella ( $P=0.002$ ) than the promastigotes from females with an intact PM (Table 2). Parasites from flies with an opened or heavily disintegrated PM had a much longer body and flagellum than those from both the above-mentioned groups ( $P<0.0001$  for all traits). Additionally, the width of the parasite body was smaller than in flies with an intact PM ( $P=0.005$ ). Similarly, by T96, promastigotes from females with a slightly disintegrated PM had significantly shorter flagella and shorter and wider bodies than did parasites from an opened/heavily disintegrated PM and from defecated flies ( $P<0.0001$  in all cases). These morphological changes reflected the transformation from short procyclic promastigotes to elongated nectomonads in correlation with the stages of PM deformation, viz., from an intact PM, to a slightly disintegrated PM, and to an opened/heavily disintegrated PM (Table 3). On the other

**Table 2** Measurements of *Leishmania major* promastigotes: effect of disintegration of PM on *Leishmania* morphological transformation

Days PBM*	State of PM**	Number of measurements	Body length		Body width		Flagellar length	
			Mean (SD) in $\mu\text{m}$	Range in $\mu\text{m}$	Mean (SD) in $\mu\text{m}$	Range in $\mu\text{m}$	Mean (SD) in $\mu\text{m}$	Range in $\mu\text{m}$
3	Intact	120	10.4 (2.0)	6.8–15.3	2.2 (0.8)	0.9–5.1	10.5 (2.9)	3.4–20.4
	Slightly disintegrated	120	11.3 (2.6)	5.1–17.0	1.9 (0.7)	0.9–3.4	11.7 (2.8)	6.8–17.0
	Opened / heavily disintegrated	80	15.6 (2.9)	8.5–23.8	1.8 (0.7)	0.9–3.4	15.8 (2.5)	10.2–20.4
	Total	320	12.0 (3.2)	5.1–23.8	2.0 (0.7)	0.9–5.1	12.3 (3.5)	3.4–20.4
4	Slightly disintegrated	120	12.6 (4.5)	3.4–25.5	1.8 (1.0)	0.9–5.1	13.6 (3.7)	5.1–20.4
	Opened / heavily disintegrated	120	16.4 (3.0)	6.8–25.5	1.4 (0.4)	0.9–1.7	16.4 (2.7)	10.2–25.5
	Defecated	120	17.1 (3.5)	8.5–27.2	1.3 (0.4)	0.9–1.7	16.7 (2.7)	10.2–23.8
	Total	360	15.4 (4.2)	3.4–27.2	1.5 (0.7)	0.9–5.1	15.6 (3.4)	5.1–25.5

\*General effect of the time PBM on morphological transformation of promastigotes:  $F=17.917$ , d.f.=1,  $P<0.0001$

\*\* General effect of the state of the PM on morphological transformation of promastigotes:  $F=80.154$ , d.f.=3,  $P<0.0001$

**Table 3** Representation of *L. major* morphological forms in distinct stages of PM deformation

Days PBM*	Morphological forms	State of PM**			
		Intact	Slightly disintegrated	Opened and heavily disintegrated	Defecated
3	Procyclic promastigotes	88 (73%)	77 (64%)	16 (20%)	-
	Elongated nectomonads	6 (5%)	13 (11%)	53 (66%)	-
	Short nectomonads/leptomonads	26 (22%)	30 (25%)	11 (14%)	-
	Total	120 (100%)	120 (100%)	80 (100%)	-
4	Procyclic promastigotes	-	34 (28%)	12 (10%)	15 (12%)
	Elongated nectomonads	-	40 (33%)	90 (75%)	92 (77%)
	Short nectomonads/leptomonads	-	41 (34%)	18 (15%)	13 (11%)
	Metacyclic promastigotes	-	1 (1%)	0	0
	Paramastigotes	-	4 (4%)	0	0
	Total	-	120 (100%)	120 (100%)	120 (100%)

\*General effect of the time PBM on morphological transformation of promastigotes: Pearson  $\chi^2=139.3$ , d.f.=4,  $P<0.0001$

\*\*General effect of the state of the PM on morphological transformation of promastigotes: Pearson  $\chi^2=261.2$ , d.f.=12,  $P<0.0001$

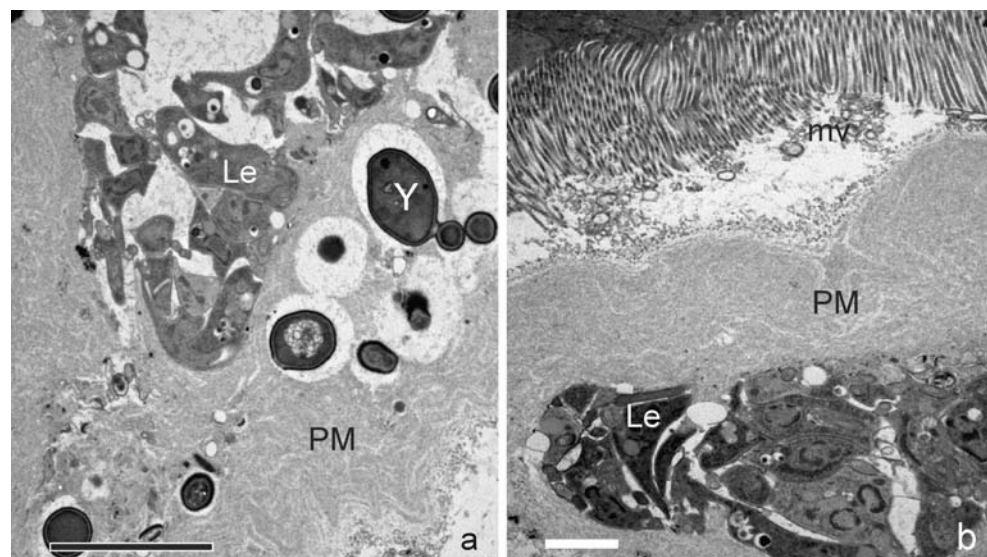
hand, parasites from an opened/heavily disintegrated PM and from defecated flies were morphologically indistinguishable (Tables 2, 3).

## Discussion

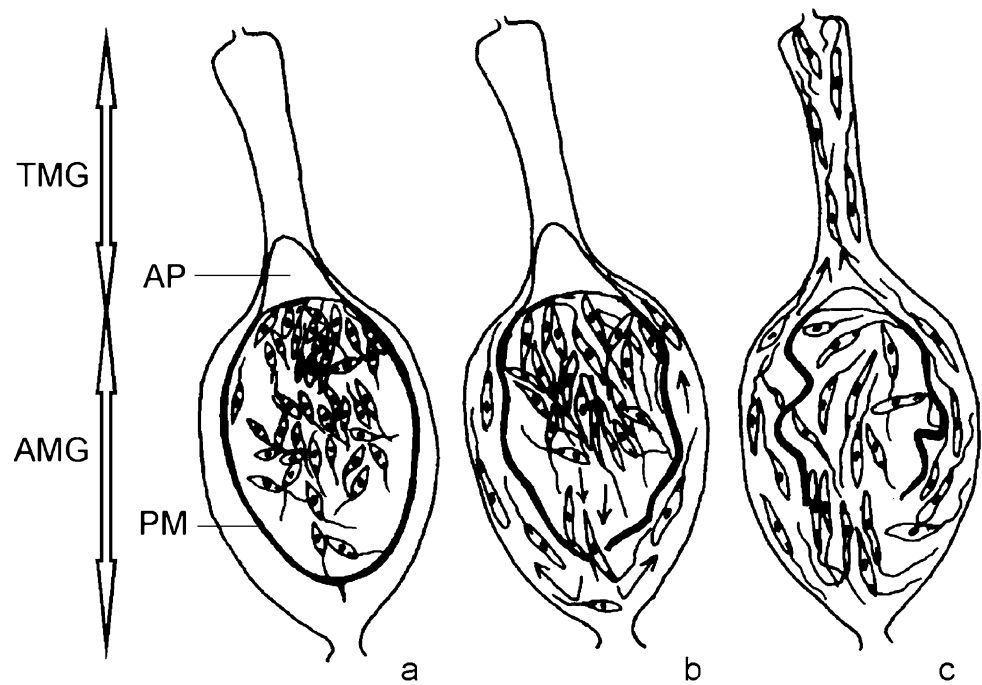
The combination of light microscopy, histology, and electron microscopy has revealed new details concerning PM formation in *P. duboscqi* females. Within 1 h PBM, erythrocytes are concentrated in the AMG forming a compact central bolus surrounded with extruded blood plasma. The volume of blood plasma is apparently much lower than that of blood cells, presumably because of prediuresis of excess liquid by females during feeding (Sádlová et al. 1998).

The formation of the PM has several distinct stages. It starts with the secretion of electron-lucent components having a fibrillar appearance (T0–T3), and at later time intervals, the secretion of electron-dense components prevails. Both electron-dense and electron-lucent components are released into the gut lumen directly from the plasma membrane on the microvillar surface, without the participation of any secretory vesicles. This is in agreement with observations in *P. perniciosus* (Walters et al. 1993), *Lutzomyia spinicrassa* (Walters et al. 1995), or *Culicoides punctatus* and *C. grisescens* (Filimonova 2005). We attribute the electron-lucent fibrils to chitin, whereas the electron-dense amorphous secretion presumably represents proteins and glycoproteins. The later type of secretion continues for 3 days; proteins and glycoproteins coat the chitin framework and are responsible for the electron-dense appearance of the

**Fig. 8** Electron micrographs of the gut of *P. duboscqi* females showing yeast (*Y*) surrounded by a lysed space within the peritrophic matrix (*PM*) layers (**a**) and *L. major* promastigotes (*Le*) filling the endoperitrophic space (**a**, **b**). *mv* Microvilli Bars 3  $\mu\text{m}$  (**a**), 2  $\mu\text{m}$  (**b**)



**Fig. 9** Three phases of development of *L. major* in *P. duboscqi* during blood digestion. **a** Dividing procyclic promastigotes are still enclosed by the PM (T48–T72). **b** PM opens posteriorly and elongated nectomonads escape and migrate forward to the narrow TMG, where their further migration is stopped by the AP (T72–T96). **c** AP and PM disintegrate; parasites can migrate to the TMG thereby resulting in the colonization of the stomodeal valve during late stages of infection (T96–T120)



mature PM. In addition, encrustation with heme contributes to the electron-dense appearance of the PM.

The mature PM of *P. duboscqi* is multilayered and consists of thin laminar outer layers and thick amorphous inner layers. This corresponds to the descriptions of PM in other sand fly species by Walters et al. (1993, 1995) and Secundino et al. (2005). PM width increases from less than 2  $\mu\text{m}$  by 12 h PBM to about 5  $\mu\text{m}$  at 2–3 days PBM. From the second day PBM, the PM is wrinkled and from the third day is strongly folded and then starts to break down. Posterior openings of PM have been found between the third and fifth day PBM.

The rate of formation and the maturity of the PM in blood-feeding Diptera is usually highly species-specific (Lehane 1997). Studied sand fly species we categorize into two groups: a group with rapid PM formation and a group with a slow rate of PM formation. The first group includes *L. spinicrassa* (Walters et al. 1995), *Sergentomyia arpaklensis* (Reznik and Kuznetsova 1987), and *P. caucasicus* (Reznik and Kuznetsova 1983); secretion of PM precursors starts immediately PBM, with the delicate transparent PM forming within 3–6 h and then maturing by about 12 h PBM. The second group includes *P. longipes* (Gemetchu 1974), *P. perniciosus* (Walters et al. 1993), and *L. longipalpis* (Secundino et al. 2005); in these species, the secretion of PM precursors starts later (0.5–4 h PBM), immature PM occurs at 12–18 h, and the PM matures at 24–48 h PBM. Our data show that *P. duboscqi* belongs to the group of Diptera with a rapid PM formation rate.

Surprisingly, in the most closely related species, *P. papatasi*, various authors report diverse results regarding the rate of PM formation. Whereas Blackburn et al. (1988)

have observed the first signs of PM development 4 h PBM, a complete PM at 24 h, and mature multilayered PM at 48 h, Reznik and Kuznetsova (1983) report the start of precursor secretion within 1 h PBM and complete immature PM by 12 h PBM. Similarly, Pimenta et al. (1997) have observed a fully developed PM in *P. papatasi* by 24 h.

The AP, a structure first described in mosquitoes (Freyvogel and Staubli 1965; Richardson and Romoser 1972), has previously been found in some sand fly species (Walters et al. 1993, 1995; Guzman et al. 1994). According to Richardson and Romoser (1972), the AP may serve as a partial barrier between the AMG and TMG, leaving the TMG as a site of digestion and absorption of a sugar meal, without interference with the processes of blood meal digestion in the AMG.

The posterior end of the sand fly PM is mostly depicted as being closed; however, Walters et al. (1993; 1995) have found it opened in *P. perniciosus* and *L. spinicrassa*. In *P. perniciosus*, the opening is outlined by a darkened ring, and in *L. spinicrassa*, it varies from a rough hole to a funnel-shaped opening (Walters et al. 1995). The PT described here for *P. duboscqi* resembles a funnel-shaped opening, but it is a closed structure that does not allow the free escape of red blood cells from the PM. A similarly shaped posterior opening has recently been described by Warburg (2008) in *P. papatasi*.

*Leishmania major* promastigotes undergo a typical suprapylarian development in vector sand flies. After the breakdown of the PM, nectomonads escape into the ectoperitrophic space of the midgut (Shatova et al. 1984; Warburg et al. 1986; Lawyer et al. 1990; Čiháková and Volf 1997). In *P. papatasi*, Schlein et al. (1991) have reported that the



breakdown of the PM differs between uninfected females and those infected by *L. major*; the disintegration of the anterior region has been observed in infected sand flies. In this study of a closely related species, *P. duboscqi*, we have not found any differences in the timing of the degeneration of the PM between infected and uninfected females. Disintegration of the PM starts in both groups by day 3 PBM. Although the maximal density of parasites is present in the central and anterior parts of the blood bolus, *Leishmania* escape only from the posterior opening of the PM, with a similar opening being present in uninfected sand flies.

Schlein et al. (1991) have postulated that the lysis of the chitin layer of the PM is caused by the chitinase of parasite origin and have demonstrated chitinase activity in *L. major*. More recently, the chitinase gene and enzyme activity have been characterized in several *Leishmania* species (Shakarian and Dwyer 1998, 2000). *Phlebotomus papatasi* treated with a chitinase inhibitor allosamidin forms a thicker PM that prevents the escape of *L. major* parasites from the blood meal (Pimenta et al. 1997), whereas over-expression of the parasite chitinase causes the earlier arrival of *Leishmania mexicana* at the stomodeal valve of *Lutzomyia longipalpis* (Rogers et al. 2008). However, the role of *Leishmania* chitinase during promastigote escape to the ectoperitrophic sac remains elusive, as its activity is inhibited by hemoglobin present in the blood meal (Schlein and Jacobson 1994). In *P. duboscqi* females, the PM opens similarly in uninfected and infected females, most likely as a result of the sand-fly-derived chitinase activity described in *P. papatasi* by Ramalho-Ortigao and Traub-Cseko (2003) and Ramalho-Ortigao et al. (2005). Our transmission electron-microscope study has not revealed any signs of PM lysis caused by *Leishmania*, whereas contaminating yeast produces clear lytic plaques (Fig. 8). The above-mentioned findings suggest that *L. major* chitinase does not have an important role in the disintegration of sand fly PM. Parasites “wait” until the PM is broken down by sand fly chitinase (see model in Fig. 9). Rogers et al. (2008) have also suggested that at least some *Leishmania* species rely upon the chitinases of the sand fly.

Nevertheless, we cannot exclude that the process of PM disintegration is species-specific. In a previous study aimed at the development of different *L. major* strains within various sand fly species, Čiháková and Volf (1997) have found a correlation between the timing of escape of a particular strain from the endoperitrophic space and its ability to produce successful late-stage infections in *P. papatasi*. In contrast, in *P. duboscqi*, the escape of various strains from the endoperitrophic space is more uniform and does not correlate with late stage development in the vector.

Although mycoses in populations of sand flies have been reported to reduce the incidence of *Leishmania* in endemic

areas and negatively influence the survival of vectors (Killick-Kendrick 1979; Schlein et al. 1985), we have not observed any impact of yeast contaminating *P. duboscqi* midgut on sand fly mortality or *Leishmania* development.

The disintegration of PM coincides with the transformation of procyclic forms to long nectomonads. Procyclic promastigotes are transformed after the first signs of PM breakdown, perhaps because of contact with salivary components. A similar differentiation of procyclic to nectomonad forms has been demonstrated in vitro by Charlab and Ribeiro (1993) and Charlab et al. (1995) using salivary gland homogenates. In vivo, saliva-derived proteins (Volf et al. 2002) and salivary enzymes such as amylase (Ribeiro et al. 2000) have been demonstrated in the midgut. Accordingly, saliva ingested into the midgut, either with a sugar meal (Bates and Rogers 2004) or alone, can trigger the transformation of parasites.

Finally, our study has revealed an interesting and novel role of the AP during *Leishmania* infection. After initial establishment in the ectoperitrophic space of the AMG, promastigotes move anteriorly. However, this forward migration to the narrowed TMG can be stopped by the AP until the disintegration of the PM. In *P. duboscqi* females with an infective dose of  $10^6$  parasites/ml, *Leishmania* occurrence in the TMG is always connected with the disintegration of the AP. In female sand flies with an opened PM, but complete AP, all the parasites are restricted to the AMG. Thus, the AP can temporarily function as a barrier that prevents the forward migration of nectomonads, as depicted in our model of parasite infection in the sand fly midgut (Fig. 9).

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