

Effect of Blood Components, Abdominal Distension, and Ecdysone Therapy on the Ultrastructural Organization of Posterior Midgut Epithelial Cells and Perimicrovillar Membranes in *Rhodnius prolixus*

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The effects of blood components, nerve-cord severance, and ecdysone therapy on the posterior midgut epithelial cells of 5th-instar Rhodnius prolixus nymphs 10 days after feeding were analyzed by transmission electron microscopy. Cutting the nerve-cord of the blood-fed insects partially reduced the development of microvilli and perimicrovillar membranes (PMM), and produced large vacuoles and small electron-dense granules; insects fed on Ringer's saline diet exhibited well developed microvilli and low PMM production; swolled rough endoplasmatic reticulum and electron-dense granules; Ringer's saline meal with ecdysone led to PMM development, glycogen particles, and several mitochondria in the cytoplasm; epithelial cells of the insects fed on Ringer's saline meal whose nerve-cord was severed showed heterogeneously distributed microvilli with reduced PMM production and a great quantity of mitochondria and glycogen in the cytoplasm; well developed microvilli and PMM were observed in nerve-cord severed insects fed on Ringer's saline meal with ecdysone; Ringer's saline diet containing hemoglobin recovered the release of PMM; and insects fed on human plasma showed slightly reduced PMM production, although the addition of ecdysone in the plasma led to a normal midgut ultrastructural organization. We suggest that the full development of microvilli and PMM in the epithelial cells depends on the abdominal distension in addition to ingestion of hemoglobin, and the release of ecdysone.

Key words: triatomine - midgut - perimicrovillar membranes - hormone - microvilli

Perimicrovillar membranes (PMM), also known as extracellular membrane layers, are structures found on and surrounding the microvilli of epithelial cells of the midgut region of hematophagous bugs (Billingsley & Downe 1983, 1985, 1986a, Terra 1990). These structures are fully developed 10 days after a blood meal. After this time, PMM start to degenerate following excretion by the feces during the next meal of the triatomine (Billingsley & Downe 1983, 1985). It has been suggested that the PMM is in some way involved in the compartmentalization of the digestive process and absorption of blood nutrients (Houseman & Downe 1983, Billingsley 1988, Billingsley & Downe 1986b, 1988, 1989b, Ferreira et al. 1988, Terra 1990, Terra & Ferreira 1994). In *Rhodnius prolixus*, factors

such as diet content (Billingsley & Downe 1989a) and neurohormone secretion (Nogueira et al. 1997, Gonzalez et al. 1998) are apparently involved in the production of PMM. *R. prolixus* in just one feeding ingests a large amount of blood that is stored in the anterior midgut (Wigglesworth 1972). Afterwards, abdominal distension in addition to blood components and humoral factors trigger the activation of the neuroendocrine system, leading to the release of prothoracicotropic hormone (PTTH) from the brain neurosecretory cells. This in turn induces ecdysone (molting hormone) secretion by the prothoracic glands, increasing the epidermal mitosis and, consequently, the deposition of new cuticle (Wigglesworth 1934, 1963, Gilbert et al. 1980, Garcia et al. 1986, Azambuja et al. 1993, Mulye & Davey 1995).

To better understand the influence of the triatomine neuroendocrine system on the midgut ultrastructural organization, more information is required regarding the effect of different blood meal components, abdominal distension and ecdysone on the induction of microvilli and PMM development. Our present observations indicate that PMM depends on the abdominal distension in addition to ingestion of blood components which both result in PTTH release and ecdysone production. We conclude that the structural organization of the midgut epithelium of *R. prolixus* is under neuroendocrine control.

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MATERIALS AND METHODS

Insect rearing and feeding procedures - *R. prolixus* (Hemiptera: Reduviidae) were reared in the laboratory at 28°C and relative humidity of 60-70% (Garcia et al. 1984). Randomly chosen 5th-instar male nymphs, starved for 45 days following ecdysis, were fed using membrane apparatus containing either human blood or other diets (Garcia et al. 1984). Only fully engorged insects were used throughout the experiments.

Preparation of diets - Insects of the control group were fed on citrated human blood. Just before feeding, plasma was prepared by centrifugation of citrated human blood and human erythrocytes were washed 5 times in 0.01 M phosphate-buffered 0.15 M NaCl, pH 7.2 (PBS) 10,000 g for 20 min. The freshly washed red cells were lysed with 40 volumes of cold 50 mM NaCl and then centrifuged at 10,000 g for 30 min to remove debris. Afterwards, the supernatant containing human hemoglobin and other minor erythrocyte components was concentrated by pressure dialysis using Visking tubes (Scientific Instruments Centre Ltd., London), while simultaneously dialyzed against several changes of PBS (Azambuja et al. 1993). Hemoglobin was added to Ringer's solution (129 mM NaCl; 8.6 mM KCl; 8.5 mM CaCl₂; 8.5 mM MgCl₂; 10.2 mM NaHCO₃; 4.3 mM NaH₂PO₄ and 34 mM glucose) (Farmer et al. 1981) to obtain final concentrations of 7 and 14%, which then were administered to the insects as meal (Garcia et al. 1995). Other insects were fed exclusively on plasma or Ringer's solution, both containing 0.1 mM ATP as a phagostimulant (Friend & Smith 1971).

Severance the nerve-cord - To study the role of the central nervous system on the mechanisms of PMM formation, groups of insects were submitted immediately after feeding to a surgical intervention of cutting the nerve-cord, which transmits nervous impulses to the brain, due to abdominal stretching (Van der Kloot 1961). In summary, the prothorax was cut off laterally in front of the first pair of legs with a fine lancet, and the wound was sealed with liquid melted wax at 45°C (Wigglesworth 1934). Mortality of the insects after cutting off the nerve-cord was minimal.

Ecdysone application - a-ecdysone sample (Aldrich) was diluted in ethanol: 0.15 M NaCl (v:v, 1:4) and added to either Ringer's solution or plasma meal in a final concentration of 5 µg/ml immediately before insect feeding. The control group received a meal containing the solvent only.

Transmission electron microscopy - Ten days after insect feeding, the entire digestive tract from 10 insects of each experimental group was removed. Pieces of the posterior midgut were fixed in 2.5% glutaraldehyde (Polyscience, Inc.) and 5 µM CaCl₂ in 0.1M cacodylate buffer (Sigma), pH 7.2 for 2 h at room temperature. After fixation the tissue fragments were washed three times in 0.1M cacodylate buffer, pH 7.2, containing 5 mM CaCl₂, and postfixed in a solution containing 1% osmium tetroxide (Polyscience, Inc.) and 0.8% potassium ferricyanide (Sigma) in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in Epon™. Approximately 100 thin sections of each group were stained with uranyl acetate

and lead citrate. All of the thin sections, containing each one 5 to 10 different cells depending on the used magnification, were then observed with a Zeiss 900 transmission electron microscope. The results were only reported when almost 100% of the insects in each experimental group displayed the same posterior midgut ultrastructural organization. In that observation there was a higher (more than 90%) uniformity of the ultrastructural alterations.

RESULTS

Control insects - The general appearance of *R. prolixus* midgut epithelial cells 10 days after insect feeding on blood meal is shown in Figs 1 and 2, respectively. In all analyzed regions along the posterior midgut, the apical portion of the epithelial cells displays a large number of long densely packed and homogeneously distributed microvilli and full development of PMM which surround the microvilli and reached the midgut lumen (Fig. 1). The mitochondria, vacuoles containing lamellar membranes, and electrondense granules were usually observed in the cytoplasm (Fig. 2).

Blood fed insects and nerve-cord severance - When *R. prolixus* were submitted to nerve-cord severance just after feeding on blood meal some apical regions showed long microvilli homogeneously distributed and well developed PMM (Fig. 3). In other regions, microvilli were disorganized and poor PMM production was observed (Fig. 4). In both cases the cytoplasm had large vacuoles, small electrondense granules and cortical mitochondria (Fig. 3).

Plasma meal - At 10 days in the insects fed on plasma, the microvilli showed either well distributed or disorganized regions although PMM production was apparently slight reduced and limited to some areas (Fig. 5). The cytoplasm exhibited organelles, vacuoles, electrondense granules and vacuoles containing lamellar membranes (Fig. 6). However, following ecdysone application to the plasma meal there was an increase in the quantity of PMM, which completely fill the midgut lumen. Microvilli seemed to be as well distributed and long as in control insects fed on blood. In this group, several large electrondense granules and vacuoles were observed (Figs 7, 8).

Ringer's solution meal, ecdysone therapy and nerve-cord severance - When *R. prolixus* were fed on Ringer's solution, the apical region of intestinal epithelial cells 10 days after feeding demonstrated well developed, densely packed, and homogeneously distributed microvilli with poor PMM production which just lined the top of microvilli and did not reach the midgut lumen (Fig. 9). The cytoplasm had rough endoplasmic reticulum with a lamellar form (inset, Fig. 10), vacuoles and electrondense granules (Fig. 10). However, at 10 days after feeding on Ringer's solution containing ecdysone an increase in the amount of PMM (Fig. 11), and in the cytoplasm, mitochondria, and glycogen were detected (Fig. 12).

The apical region of the midgut epithelial cells of the insects fed on Ringer's saline meal, whose nerve-cord were severed, 10 days after feeding demonstrated heterogeneously distributed microvilli with reduced PMM production (Fig. 13), although a great quantity of mitochondria and glycogen was observed in the cytoplasm (Fig. 14).

As expected, the midgut cells of the nerve-cut insects fed previously on Ringer's solution containing ecdysone exhibited a full developed microvilli and PMM development (Fig. 15), and only few mitochondria in the cytoplasm. However, electrondense granular inclusions were seen in high numbers (Fig. 16).

Ringer's solution meal and hemoglobin - In *R. prolixus* fed on 7% of hemoglobin added to the Ringer's saline meal, the midgut epithelial cells had microvilli and PMM (Fig. 17). The cytoplasm possessed large vacuoles with electrondense granules and particles of glycogen (Fig. 18). Adding 14% of hemoglobin to the Ringer's solution, the ultrastructure of the midgut epithelial cells, i.e, the

PMM had developed (Fig. 19). Electrondense granules, mitochondria, and vacuoles containing lamellar membranes were also detected (Fig. 20).

DISCUSSION

In hematophagous triatomines, abdominal stretch reception is provided by direct stimulation of the abdominal nerves (Van der Kloot 1961, Anwil 1972, Nijhout 1984) inducing the release of PTH produced by the brain neurosecretory cells (Wigglesworth 1934, Gilbert et al. 1980, Garcia et al. 1990). Herein we demonstrate that *R. prolixus* submitted to abdominal distension caused by a non-nourishing diet such as Ringer's solution, shows well devel-

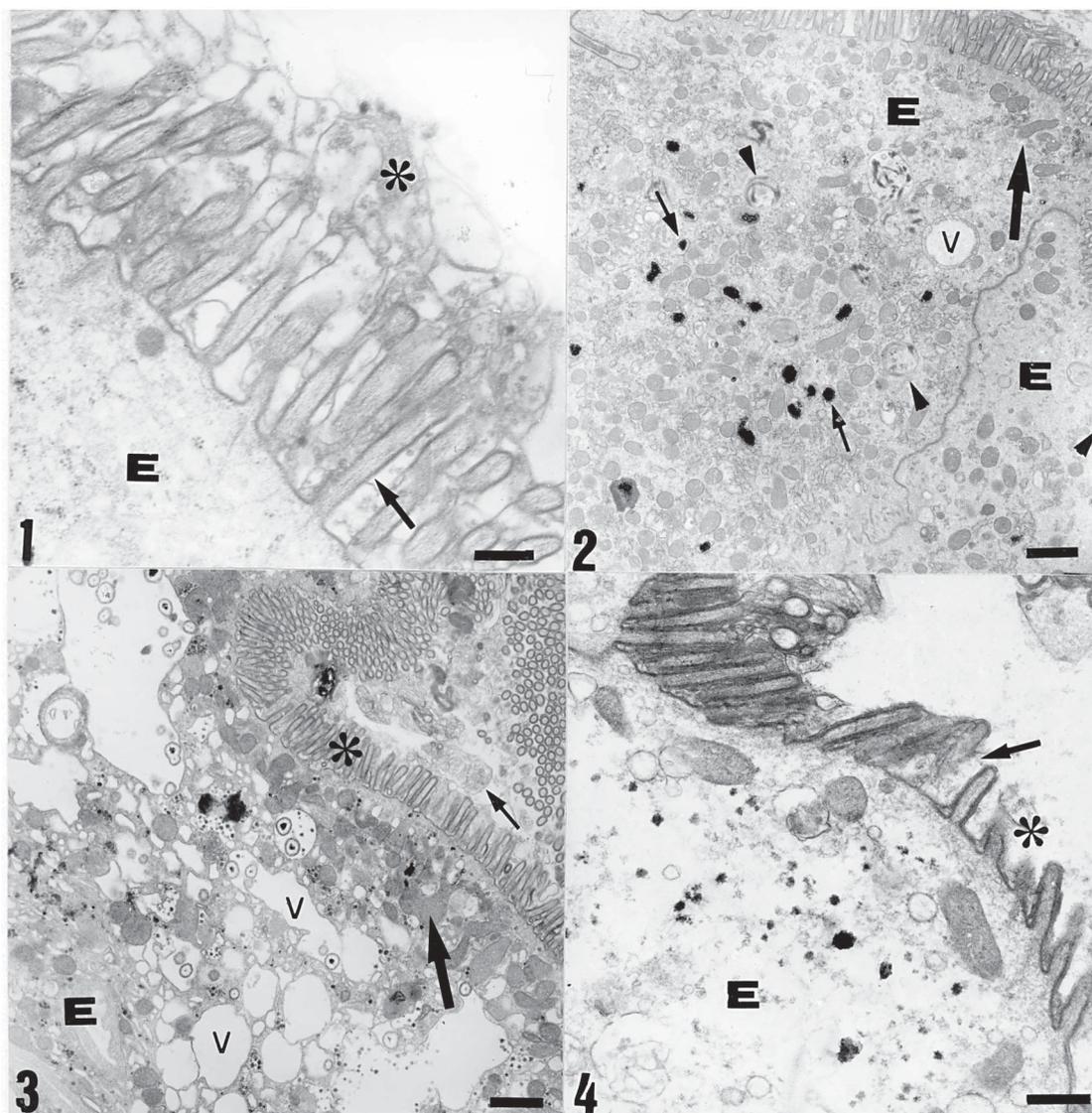


Fig. 1: transmission electron microscopy (TEM) of posterior midgut epithelial cells (E) of 5th-instar nymphs of *Rhodnius prolixus*, 10 days after blood meal. The apical regions of these cells show a large number of a long, densely packed microvilli homogeneously distributed (arrow), and a developed perimicrovillar membranes (PMM) covering the microvilli and reaching the midgut lumen (asterisk). 23,000 \times . Bar: 0.5 μ m. Fig. 2: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after blood meal. The cytoplasm shows a large number of electrondense granules (small arrow), mitochondria (large arrow), vacuole (V), and vacuoles containing lamellar membranes (arrow head). 5200 \times . Bar: 1.8 μ m. Fig. 3: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after blood fed insects and nerve-cord severed. Microvilli (asterisk) and PMM (small arrow) are both well-developed, large vacuoles (V) cortical mitochondria (large arrow). 8000 \times . Bar: 1.2 μ m. Fig. 4: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after blood meal whose nerve-cord was severed. The apical region of the epithelial cells showed poor developed microvilli (asterisk) and in some region poor PMM (arrow) presence. 16,800 \times . Bar: 0.8 μ m.

oped and organized microvilli, however poor PMM production. On the other hand, in this group of insects the cytoplasm was similar to control by presenting mitochondria and several electrondense granules, which suggest that the PMM production was not regulated by abdominal distention.

The role of the nervous system in the process of structural organization of the posterior midgut epithelium was investigated by submitting the insects to a surgical intervention cutting off the nerve-cord and/or by therapy with ecdysone. Wigglesworth (1934) and Van der Kloot (1961) demonstrated that the nerve-cord, as a consequence of abdominal stretching, is responsible for transmitting ner-

vous impulses to the brain, which releases PTTH followed by ecdysone secretion. Moreover, the correct site to cut the correct nerve-cord has been so exact in localization and depth to really get the expected severance. Otherwise, the inhibition of moult is not allowed. Our results show that posterior midgut cells of nerve-cord severed insects exhibit after Ringer's feeding short and clustered microvilli and poor and disorganized PMM production. Neither wrong depth nor incorrect site of the nerve chord severance affected the ultrastructure of the posterior midgut cells or production of PMM in *R. prolixus*. In those sham-operated insects, the midgut cells displayed exactly the same appearance as seen in control insects (not

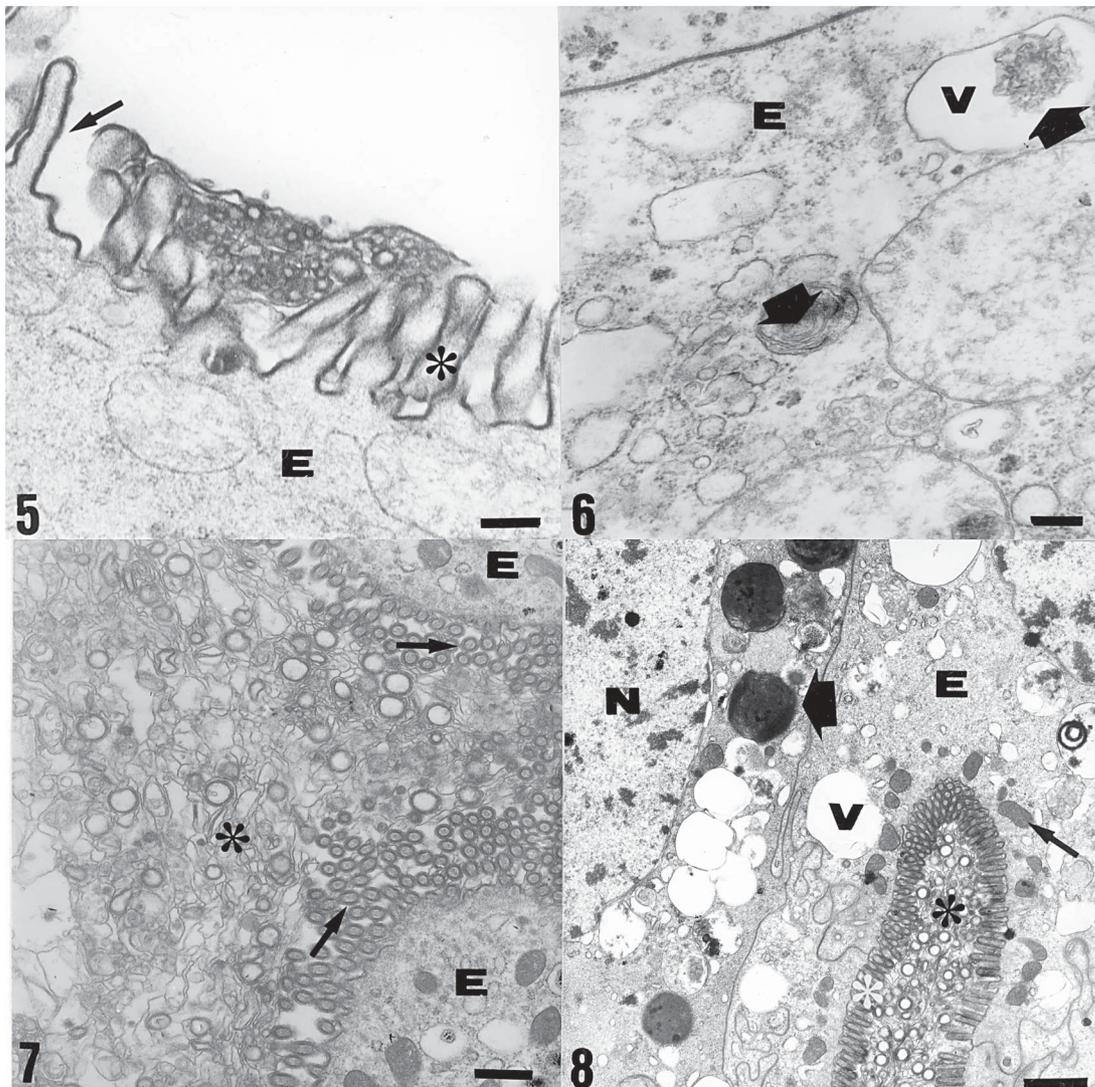


Fig. 5: transmission electron microscopy (TEM) of posterior midgut epithelial cells (E) of 5th-instar nymphs of *Rhodnius prolixus*, 10 days after plasma meal. The apical regions of the cells possess developed heterogeneously distributed microvilli (asterisk). The perimicrovillar membranes (PMM) production is slightly reduced and limited to some areas (arrow). 34,500 \times . Bar: 0.3 μ m. Fig. 6: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after plasma meal. The cytoplasm possesses large vacuoles (V) containing lamellar membranes (large arrow). 42,000 \times . Bar: 0.2 μ m. Fig. 7: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after plasma meal containing ecdysone (5 μ g/ml). The apical regions of the cells show a large number of densely packed microvilli homogeneously distributed (arrow) and fully developed PMM (asterisk). 13,800 \times . Bar: 0.7 μ m. Fig. 8: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after plasma meal containing ecdysone (5 μ g/ml). The apical regions of the cells show a large number of densely packed microvilli homogeneously distributed (white asterisk) and fully developed PMM (black asterisk). The cytoplasm shows a large number of vacuoles (V), large electrondense granules (large arrow), cortical mitochondria (arrow), and nucleus (N). 8050 \times . Bar: 1.4 μ m.

shown). To take into account that microvilli are already present in the posterior midgut cells which have not reached the lumen of the gut even before blood meal (Jensen et al. 1990, Schaub et al. 1992), the reduction in its development after nerve-cord severance led us to suggest that microvilli distribution is related to nerve integrity. In addition, full development of a network of PMM observed when the insects were fed on Ringer's solution containing ecdysone indicates the influence of ecdysone on the morphogenesis of the posterior midgut epithelium cells.

In plasma-fed insects, microvilli were developed and heterogeneously distributed and PMM were apparently poor present. These findings were clear in 5th-instar

nymphs of *R. prolixus* with at least 45 days of starvation, when hemolymph ecdysteroids are detectable in low level after the plasma meal (Azambuja et al. 1993). We then suggest that plasma contains factors, which at a some way interfere with the assembly of microvilli and stimulate synthesis of PMM, confirming the early observations made by Billingsley and Downe (1989a).

As demonstrated by Azambuja et al. (1993), in 5th-instar of *R. prolixus* nymphs a single plasma meal induces a minimal concentration of ecdysteroids in the hemolymph, which is sufficient for producing a low percentage of insects molting. Our results with ecdysone added to plasma feeding showed that complete PMM formation is possible under neuroendocrine control.

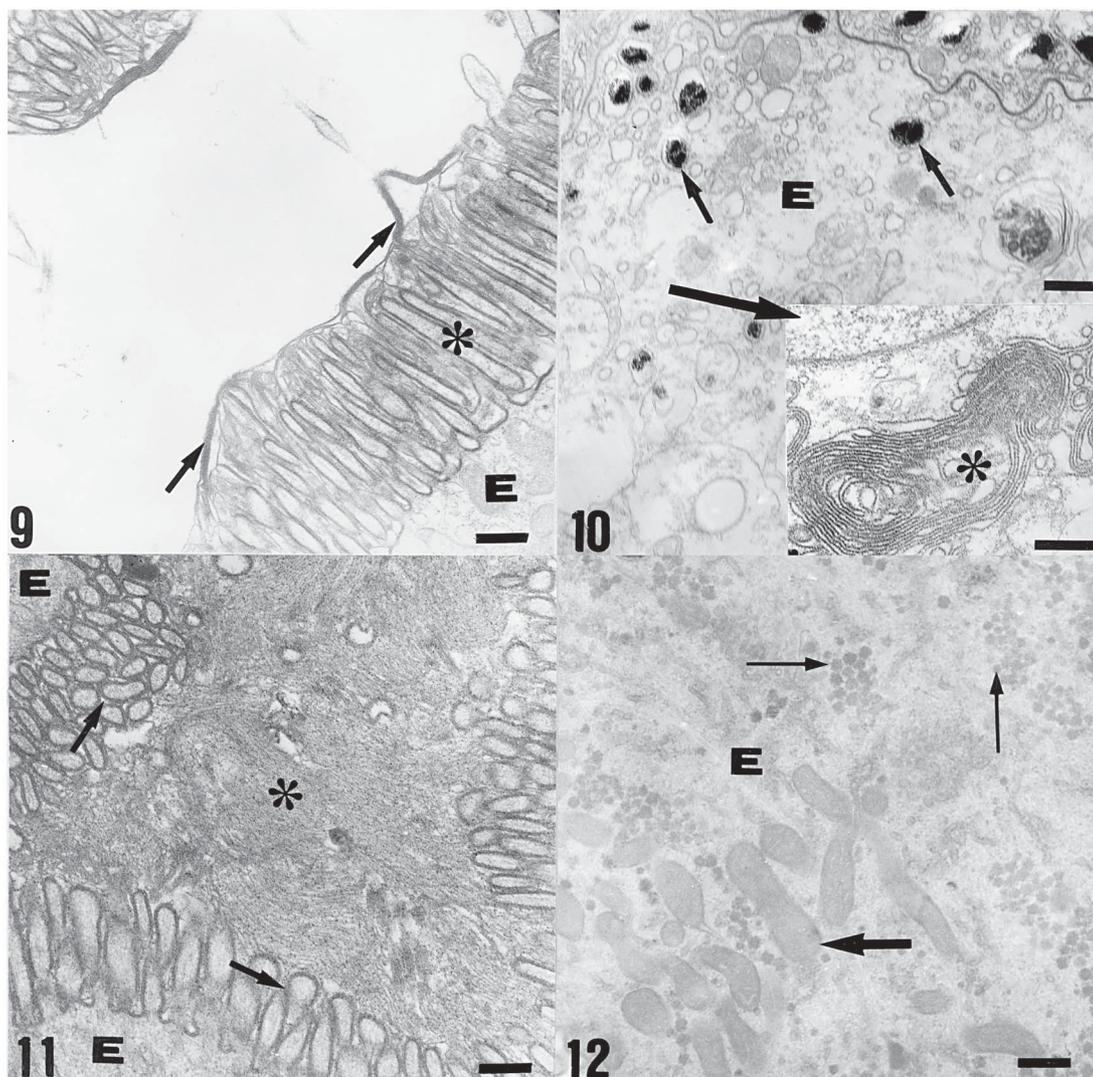


Fig. 9: transmission electron microscopy (TEM) of posterior midgut epithelial cells (E) of 5th-instar nymphs of *Rhodnius prolixus*, 10 days after Ringer's saline meal. The apical regions of the cells present homogeneously distributed and well-developed microvilli (asterisk) and perimicrovillar membranes (PMM) poorly developed just lining the top of microvilli and do not reaching the midgut lumen (arrow). 23,000 \times . Bar: 0.4 μ m. Fig. 10: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal. Electron-dense granules (small arrow). 13,800 \times . Bar: 0.7 μ m. Inset: the rough endoplasmic reticulum is developed (asterisk); nucleus (large arrow). 6800 \times . Bar: 0.9 μ m. Fig. 11: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal containing ecdysone (5 μ g/ml). The apical regions of these cells possess both well-developed microvilli (arrow) and PMM (asterisk). 23,000 \times . Bar: 0.5 μ m. Fig. 12: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal containing ecdysone (5 μ g/ml). The cytoplasm presents glycogen particles (thin arrow) and several mitochondria (large arrow). 34,500 \times . Bar: 0.2 μ m.

Since erythrocyte components are important for inducing ecdysteroids and establishing the ecdysis process in *R. prolixus* nymphs (Azambuja et al. 1993), we analyzed the role of hemoglobin on the morphogenesis of the apical portion of the epithelial midgut cells. In *R. prolixus*, concentration above 7% of hemoglobin was able to induce the development of both microvilli and PMM. In addition, the insects fed on Ringer's solution containing 14% of hemoglobin developed normal microvilli and an extensive network of PMM. Additional support to this hypothesis is provided by early results showing that insects decapitated after a blood meal were incapable of producing ecdysteroids (Steel et al. 1982, Garcia et al.

1990), reaching molt (Wigglesworth 1934), and developing PMM (Gonzalez et al. 1998, 1999). Steel et al. (1982) demonstrated that in *R. prolixus* the regulatory program for controlling the ecdysis is triggered by the blood meal, which stimulates the release of PTTH. Furthermore, Stoka and Noriega (1982) confirmed the presence of ecdysteroids in the midgut tract of triatomines. Thus, the importance of hemoglobin to blood-sucking bugs is evident since it represents about 70% of the total proteins in the blood.

In Hemiptera, perimicrovillar membranes are formed in the Golgi areas of midgut cells, and apparently migrate as the internal membrane of double membrane vesicles that fuse at the apical region of the cell – the outer vesicle mem-

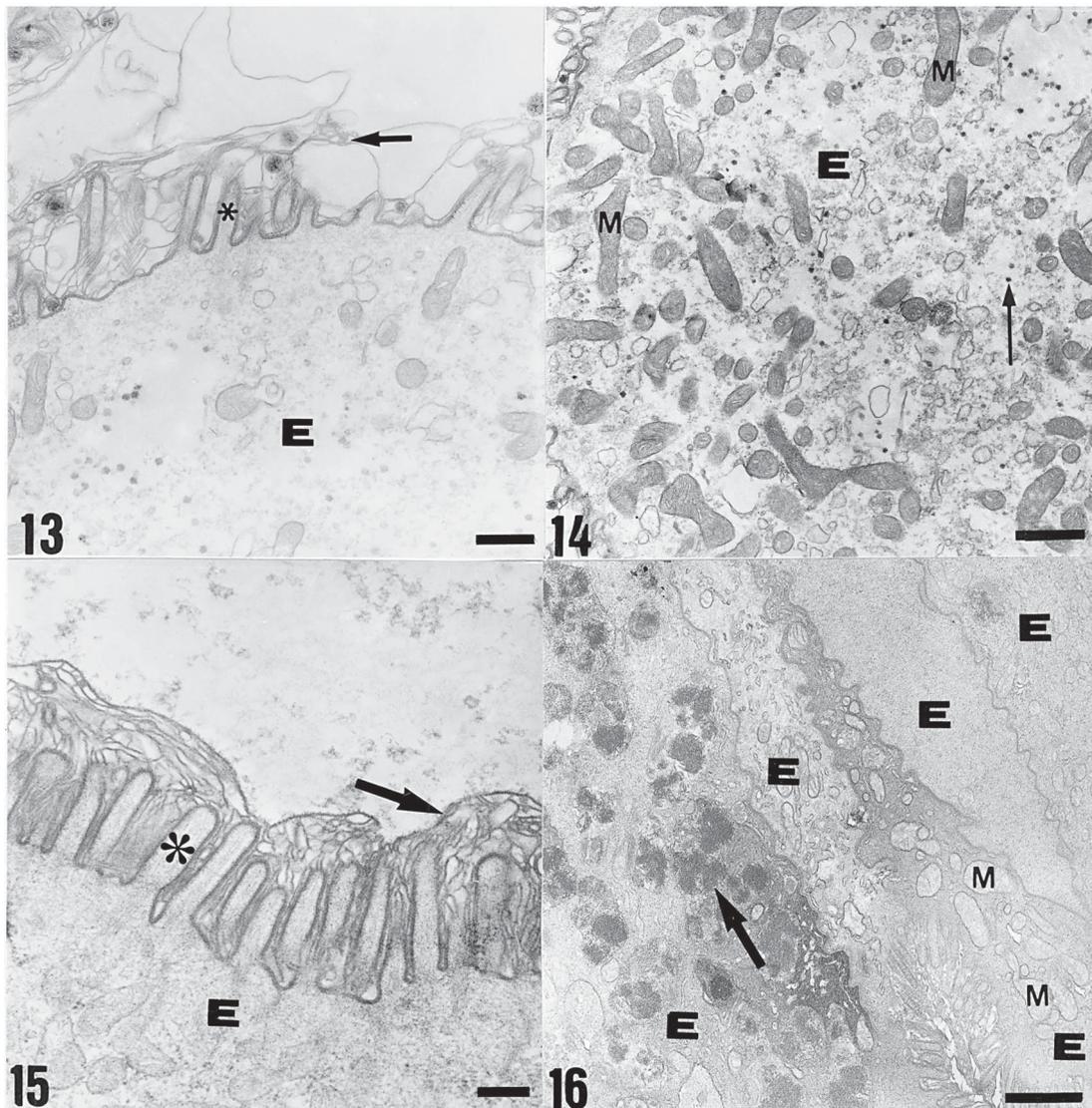


Fig. 13: transmission electron microscopy (TEM) of posterior midgut epithelial cells (E) of 5th-instar nymphs of *Rhodnius prolixus*, 10 days after Ringer's saline meal whose nerve-cord were previously severed. The apical regions of these cells show few microvilli (asterisk) and poor perimicrovillar membranes (PMM) (arrow). 23,000 \times . Bar: 0.4 μ m. Fig. 14: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal whose nerve-cord were previously severed. The cytoplasm was full up of mitochondria (M) and glycogen particles (thin arrow). 13,800 \times . Bar: 0.8 μ m. Fig. 15: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal containing ecdysone (5 μ g/ml), whose nerve-cord were previously severed. The apical regions of these cells show a homogeneously developed microvilli (asterisk) and PMM (arrow). 34,400 \times . Bar: 0.2 μ m. Fig. 16: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal containing ecdysone (5 μ g/ml), whose nerve-cord were previously severed. High number of electron-dense granules (arrow) and mitochondria (M) were observed in the cytoplasm. 8050 \times . Bar: 1.7 μ m.

brane with the microvillar membrane and the inner vesicle membrane with the PMM (Silva et al. 1995, Cristofoleti et al. 2003). Thus, although no measurements have been taken for microvilli length or PMM production our results suggested that in *R. prolixus* the single distension of the abdomen displays apical projection of the epithelial cells, i.e., PMM development. In addition to the abdominal distension, the full development of microvilli and PMM depends on the ingestion of particular components found in the blood, mainly hemoglobin, and consequently on ecdysone. Ringer-fed insects, which present developed rough endoplasmic reticulum, can show inclusions containing membranes even after ecdysone therapy. These structures were observed, in the insects fed on both plasma and

Ringer's solution containing 14 g hemoglobin/ml. Thus, we believe that the PMM production can be regulated by ecdysone and not only to plasma and/or hemoglobin. It is also possible that ecdysone and hemoglobin could be related to the process of exocytosis and organization of PMM playing a role starting the release of the membranes. These hypotheses are in agreement with the theory of perimicrovillar membranes formation described by Silva et al. (1995) and Cristofoletti et al. (2003) for Hemiptera and Aphids, respectively. Further observations using a mathematical approach as morphometric analyses are now in course in our laboratory to deepen the knowledge about the control of posterior midgut ultrastructural arrangement by the insect neuroendocrine factors.

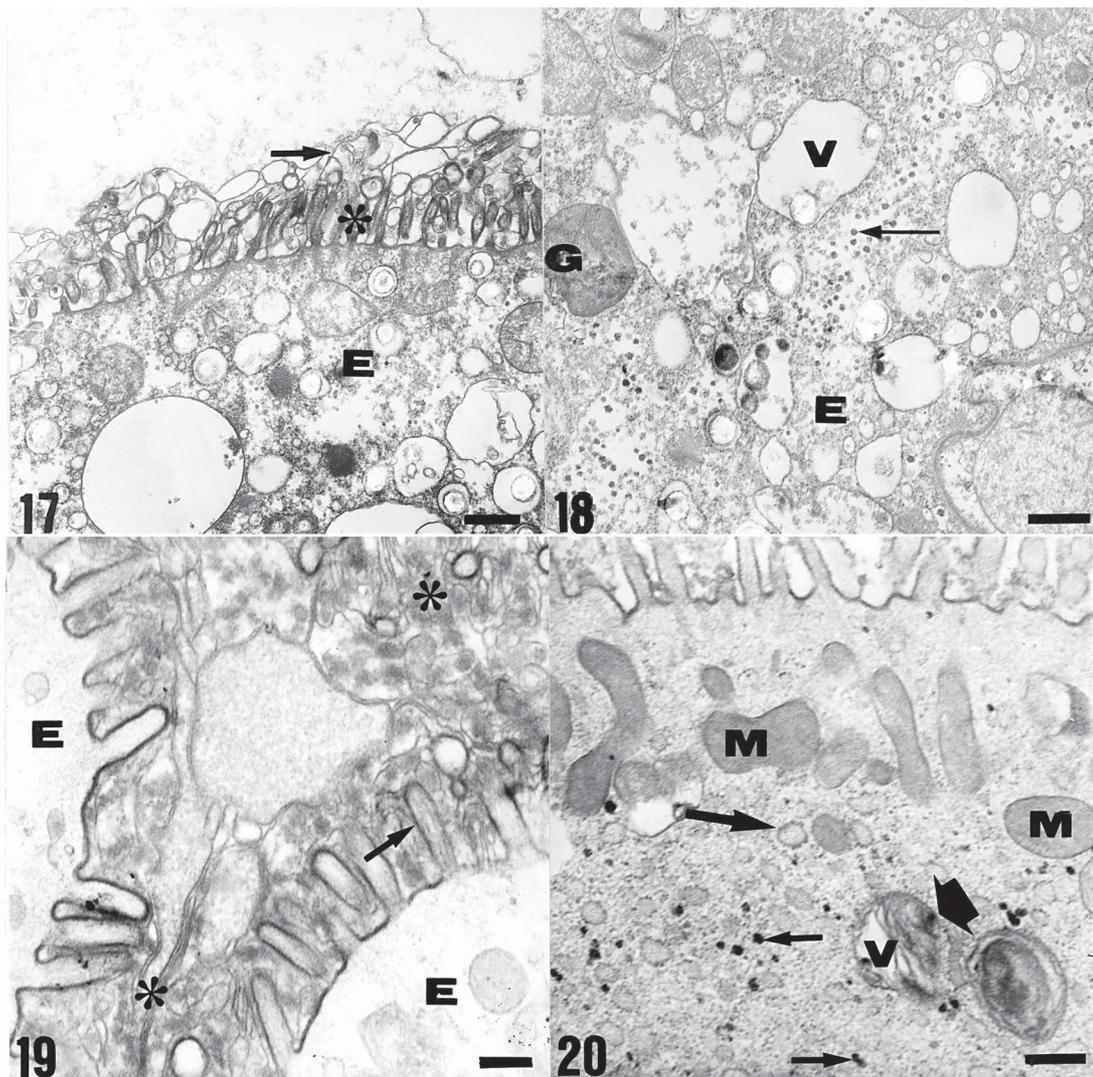


Fig. 17: transmission electron microscopy (TEM) of posterior midgut epithelial cells (E) of 5th-instar nymphs of *Rodnius prolixus*, 10 days after Ringer's meal containing hemoglobin (7 g/100 ml). The apical regions of these cells show both developed microvilli (asterisk) and PMM (arrow). 13,800x. Bar: 0.7 μ m. Fig. 18: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal containing hemoglobin (7 g/100 ml). Electron-dense granules (G) glycogen particles (arrow) and vacuoles (V) were observed in the cytoplasm. 13,800x. Bar: 0.8 μ m. Fig. 19: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal containing hemoglobin (14 g/100 ml). The apical portion presents microvilli (arrow) and largely developed PMM (asterisk). 34,500x. Bar: 0.2 μ m. Fig. 20: TEM of posterior midgut epithelial cells (E) of 5th-instar nymphs of *R. prolixus*, 10 days after Ringer's saline meal containing hemoglobin (14 g/100 ml). Cortical mitochondria (M), electron-dense granules (small arrow), vacuoles (V) containing lamellar membranes (large arrow), and rough endoplasmic reticulum (arrow) were observed in the cytoplasm. 34,500x. Bar: 0.3 μ m.

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