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Aedes aegypti midgut remodeling during metamorphosis

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ABSTRACT

The *Aedes aegypti* midgut is restructured during metamorphosis; its epithelium is renewed by replacing the digestive and endocrine cells through stem or regenerative cell differentiation. Shortly after pupation (white pupae) begins, the larval digestive cells are histolized and show signs of degeneration, such as autophagic vacuoles and disintegrating microvilli. Simultaneously, differentiating cells derived from larval stem cells form an electron-dense layer that is visible 24 h after pupation begins. Forty-eight hours after pupation onset, the differentiating cells yield an electron-lucent cytoplasm rich in microvilli and organelles. Dividing stem cells were observed in the fourth instar larvae and during the first 24 h of pupation, which suggests that stem cells proliferate at the end of the larval period and during pupation. This study discusses various aspects of the changes during midgut remodeling for pupating *A. aegypti*.

1. Introduction

Midgut morphology varies with development stage in *Aedes aegypti*. In the larval stage, it is composed of a tube with its posterior region slightly wider than the anterior, but in the adult stage, the anterior region is thinner and the posterior has the shape of an expandable sac, which accommodates the blood after feeding. Despite such differences, larval and adult *A. aegypti* midguts are composed of a single multifunctional epithelial layer externally coated by muscle fibers [1,2].

The *A. aegypti* midgut epithelium consists of three cell types: digestive, stem (also known as regenerative) and enteroendocrine. The digestive cells have microvilli, produce digestive enzymes and absorb digestion products. Among such cells are small, undifferentiated, stem cells that may be isolated, in pairs or in small clusters, throughout the *A. aegypti* midgut [3–5]. Enteroendocrine cells presumably regulate such functions as digestive epithelium, intestinal muscle cell and nervous system functions by secreting substances into the hemolymph [3,6,7].

During metamorphosis, the *A. aegypti* midgut epithelium is histolized and reconstructed through the differentiation and proliferation of stem cells [8,9]. Although such cells differentiate and proliferate during development for different insects [9–14], including adult *Drosophila melanogaster* [15,16], in *A. aegypti*, such cells likely only divide until the initial pupation stage [3,5,8].

A. aegypti midgut histolysis begins shortly before ecdysis, when the larvae develop into pupae, and is described as "programmed cell death" (caspase-dependent apoptosis) [8,12]. However, such apoptosis includes factors related to caspase activation, which might not be the mechanism in an insect's midgut because observations from *D. melanogaster* development suggest that such enzymes are not involved in digestive cell death during ecdysis (larva-pupa), even when such enzymes are highly active [17–19].

The midgut is a well-studied organ in adult *A. aegypti*, the site for blood digestion and the "gateway" for various pathogens through the ingestion of contaminated blood. Such pathogens include arboviruses (such as dengue and yellow fever arboviruses) and the avian malaria protozoa *Plasmodium gallinaceum* [20–22]. Therefore, studies on this organ are important to establish strategies for blocking such pathogens' transmissions. Accordingly, interference with normal midgut development may reduce nutrient absorption and storage in larvae, which may reduce adult fertility. Thus, detailed knowledge on *A. aegypti* midgut development may yield targets that negatively affect fertility and facilitate population control.

Although the *A. aegypti* midgut has been well studied, several aspects of its changes during pupation have not been well explored. Herein, we describe certain changes to the *A. aegypti* midgut epithelium during pupation, including larval digestive cell replacement in adults.

2. Materials and methods

2.1. Mosquitoes

The *A. aegypti* specimens (PP-Campos strain, Campos dos Goytacazes) were collected from a colony maintained in the insectary of the Department of General Biology, Federal University of Viçosa (Universidade

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Federal de Viçosa — UFV), which is raised in plastic trays with dechlorinated water and is provided with turtle feed (Reptolife).

Fifty fourth instar larvae (L4), 150 female pupae in different stages [white pupa (WP) collected immediately after ecdysis and pupae 24 and 48 h (P24 and P48, respectively) after ecdysis] and 50 newly emerged (NE) adult females were used.

2.2. Histology

The midguts were dissected in a saline solution for insects (0.1 M NaCl, 20 mM KH_2PO_4 or 20 mM Na_2HP_4) and fixed in Zamboni's fixative [23] for 2 h. After fixation, ten midguts from each phase were separated and washed with distilled water, dehydrated in a graded series of increasing ethanol (70–100%), embedded in historesin (Historesin, Leica), cut into 3-μm sections, stained with hematoxylin–eosin (HE) or Xilidine Pounceau (XP), mounted in Eukitt medium (Fluka) and photographed using an optical Olympus BX60 microscope coupled with digital Olympus Q-Color3 camera.

2.3. Transmission electron microscopy (TEM)

The samples were separated and dissected in 0.1 M sodium cacodylate (pH 7.2) and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) for 2 h. After fixation, the midgut fragments were washed in the same buffer and postfixed in 1% osmium tetroxide for 2 h in the dark. Following postfixation, the specimens were washed twice in 0.1 M PBS, dehydrated in an ascending ethanol dilution series (70–100%) and pre-infiltrated in LR White resin solution and 100% ethanol (2:1) for 1 h. Subsequently, the samples were embedded in pure resin and maintained at room temperature for 16 h, followed by polymerization in gelatin capsules (Electron Microscopy Sciences) at 60 °C for 24 h.

Ultrathin sections were placed on copper grids and contrasted for 20 min in 1% aqueous uranyl acetate and lead citrate. The samples were observed and photographed using a SEM Zeiss EM 109 at the Microscopy and Microanalysis Unit of the Federal University of Viçosa (Núcleo de Microscopia e Microanálise da Universidade Federal de Viçosa — NMM-UFV).

2.4. Immunofluorescence

After fixation in Zamboni's fixative, the midguts were washed three times for 30 min each in PBS with 1% Triton X-100 (PBST) and then incubated for 24 h at 4 °C in anti-FMRFamide primary antibody solution (Sigma) (1:400) with 1% PBST. Thereafter, the samples were washed with PBS three times (5 min each) and incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma) (1:500) in PBS for 24 h at 4 °C followed by three washes with PBS. The nuclei were stained with DAPI (diamidino-2-phenylindole) for 30 min and mounted in anti-fading solution Mowiol (Fluka). The slides were analyzed and photographed using an Olympus BX60 epifluorescence microscope with a WB filter, as previously described.

The proliferating cell observations were validated using antibodies that identify the nuclear protein phospho-histone H3 *in situ* [24]. Thus, the midguts were previously washed and fixed as described above then incubated for 24 h at 4 °C in an anti-phospho-histone H3 primary antibody solution (Cell Signaling) (1:100) in 1% PBST. After this step, the samples were washed three times with PBS and incubated with an FITC-conjugated secondary antibody (Sigma) (1:500) in PBS for 24 h at 4 °C, followed by three 10-min washes with PBS [25]. The slides were mounted in Mowiol, analyzed and photographed using an epifluorescence microscope or a Zeiss LSM 510 confocal microscope at the NMM-UFV.

For the control, midguts from each mosquito developmental stage were used and treated as described above, except for incubation with

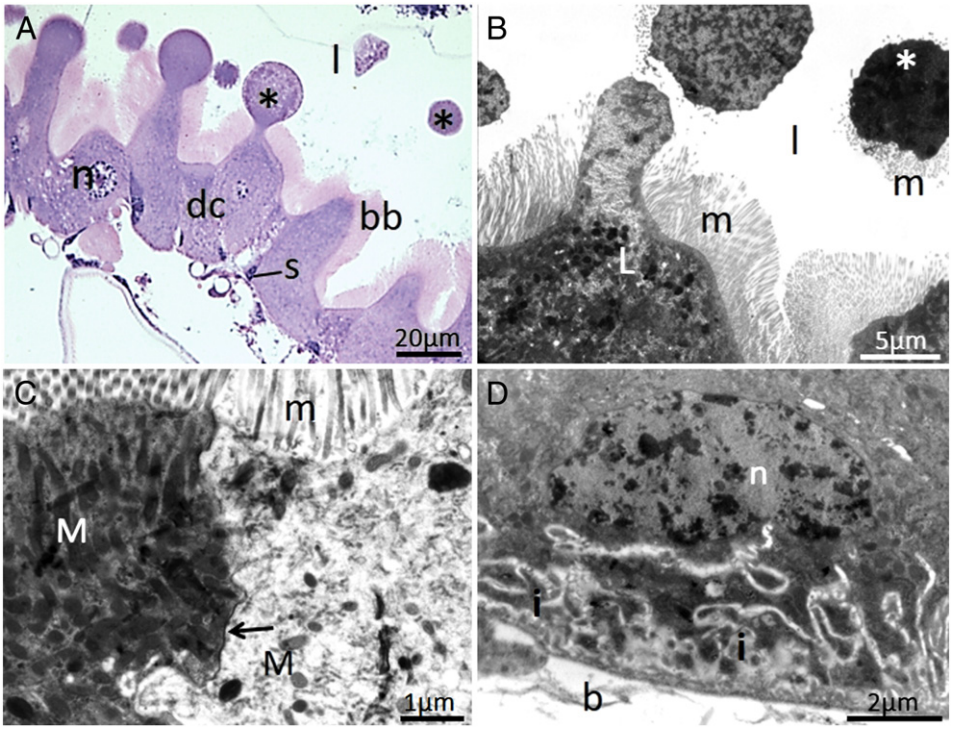

Fig. 1. *A. aegypti* L4 midgut. [A] Digestive cells (dc) stained with HE, which include developed brush borders (bb) and apocrine secretions released as bubbles (*). [B] Digestive cell apical region with developed microvilli (m). Notably, certain cell parts (*) that detach in the lumen (l) direction are lined by microvilli. [C] Lateral contact between two digestive cell apical regions; one is electron-lucent, the other is electron-dense. They are joined by septate junctions (arrow). Several mitochondria (M) are shown in the cell apices; they are more abundant in the electron-dense cell. [D] Digestive cell basal region with cell membrane invaginations (i). b—basal lamina; L—lysosome-like structures; n—digestive cell nucleus; s—stem cell.