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Original research article

# Post-embryonic changes in the hindgut of honeybee *Apis mellifera* workers: Morphology, cuticle deposition, apoptosis, and cell proliferation

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ABSTRACT

In insects, the hindgut is a homeostatic region of the digestive tract, divided into pylorus, ileum, and rectum, that reabsorbs water, ions, and small molecules produced during hemolymph filtration. The hindgut anatomy in bee larvae is different from that of adult workers. This study reports the morphological changes and cellular events that occur in the hindgut during the metamorphosis of the honeybee *Apis mellifera*. We describe the occurrence of autophagosomes and the ultrastructure of the epithelial cells and cuticle, suggesting that cuticular degradation begins in prepupae, with the cuticle being reabsorbed and recycled by autophagosomes in white- and pink-eyed pupae, followed by the deposition of new cuticle in light-brown-eyed pupae. In L5S larvae and prepupae, the hindgut undergoes cell proliferation in the anterior and posterior ends. In the pupae, the pylorus, ileum, and rectum regions are differentiated, and cell proliferation ceases in dark-brown-eyed pupae. Apoptosis occurs in the hindgut from the L5S larval to the pink-eyed pupal stage. In light-brown- and dark-brown-eyed pupae, the ileum epithelium changes from pseudostratified to simple only after the production of the basal lamina, whereas the rectal epithelium is always flattened. In black-eyed pupae, ileum epithelial cells have large vacuoles and subcuticular spaces, while in adult forager workers these cells have long invaginations in the cell apex and many mitochondria, indicating a role in the transport of compounds. Our findings show that hindgut morphogenesis is a dynamic process, with tissue remodeling and cellular events taking place for the formation of different regions of the organ, the reconstruction of a new cuticle, and the remodeling of visceral muscles.

## 1. Introduction

The honeybee *Apis mellifera* has great ecological and economic importance, benefiting nature and humans with ecosystem services, such as pollination, and providing food and raw materials for pharmacological products. Unfortunately, this species suffers a phenomenon called "Colony Collapse Disorder" characterized by the increase in honeybee colony losses (Moroń et al., 2012). The stressing agents that may cause this phenomenon are pathogens, habitat loss, pesticide exposure, and climate changes, each exerting different effects (Oldroyd, 2007; Gallai et al., 2009; Moroń et al., 2012; Lima et al., 2016).

Pesticides affect different biochemical pathways of honeybees, causing, for instance, ATPase inhibition and decrease in the levels of circulating carbohydrates in the hemolymph (Bendahou et al., 1999; Rabea et al., 2010). Insecticide effects on the excitation of neuroendocrine cells and the release of diuretic hormones have been reported (Casida and Maddrell, 1971; Singh and Orchard, 1982). All these factors may affect hemolymph homeostasis, indicating that the study of homeostatic organs is important for the comprehension of pesticide effects on insect homeostasis and survival.

The insect hindgut is divided into pylorus, ileum, and rectum, regions that play a role in hemolymph homeostasis (Phillips et al., 1987). This organ receives the primary urine produced in the Malpighian tubules and selectively reabsorbs water and ions, maintaining the bee's osmotic balance (Phillips et al., 1987; Nicolson, 1990; Villaro et al., 1999). In addition, small molecules may also be absorbed from the primary urine and the food bolus (Phillips et al., 1987).

Despite the hindgut's physiological importance, its morphogenesis during the metamorphosis of holometabolous insects is poorly studied. In honeybee larvae, the passage from the midgut to the hindgut remains closed until the end of larval development, before the pupal molting, being a simple and non-functional tube (Snodgrass, 1956). During bee metamorphosis, the posterior intestine undergoes intense remodeling, forming the ileum and rectum in adult bees (Dobrovsky,

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1951; Santos et al., 2009). In the honeybee *A. mellifera*, the larval hindgut begins in the pyloric region, having in sequence anterior-ventrally (ascending) and posterior-ventrally (descending) curved tubular segments (Dobrovsky, 1951). After the opening of the mid-gut-hindgut passage in the prepupae, the hindgut epithelium is disorganized, and the lining cuticle and visceral muscles are lost (Dobrovsky, 1951).

During bee metamorphosis, cell division promotes the anatomical differentiation of the hindgut into the ileum, rectum, and rectal papillae, the epithelial cells have many vacuoles and dense granules, and a new cuticle and visceral muscles are produced (Dobrovsky, 1951; Cruz-Landim and Silva-Mello, 1970). Although these studies have provided important data on the changes that occur in the hindgut during bee metamorphosis, their analyses are restricted to histological aspects and more detailed information on the events of programmed cell death and cell proliferation are necessary.

In insect metamorphosis, there is a balance between cell proliferation and cell death during the remodeling of the alimentary canal (Dobrovsky, 1951; Cruz-Landim and Silva-Mello, 1970; Cruz-Landim and Cavalcante, 2003; Cruz et al., 2013). In the stingless bee *Melipona quadrifasciada*, apoptotic and autophagic activities are reported to occur during the metamorphosis of the midgut (Cruz et al., 2013). During the metamorphosis of the silkworm *Bombyx mori,* autophagy precedes apoptosis in the midgut (Franzetti et al., 2012), while in *Drosophila melanogaster* only autophagy is essential for the formation of this organ (Denton et al., 2009). Thus, different cellular events occur in the remodeling of the alimentary canal in different insect species.

In the metamorphosis of *A. mellifera*, the hindgut undergoes anatomical modifications and its cuticular lining is totally renewed, suggesting the hypothesis that the remodeling of hindgut is regulated by cell death and proliferation, whereas epithelial cells perform cuticle degradation and deposition. Thus, the objective of this study was to describe the morphological changes that take place in the hindgut of *A. mellifera* during metamorphosis to verify the cellular events associated with these modifications in this insect that is model for social behavior studies and essential for global ecology and agriculture through pollination.

## 2. Materials and methods

### 2.1. Bees

*Apis mellifera* workers were randomly collected directly from seven colonies in the Central Apiary of the Federal University of Viçosa, Minas Gerais, Brazil. The bees were collected in different developmental phases: fifth instar larvae without sealed brood cell (**L5**), fifth instar larvae with sealed brood cell and empty midgut (**L5S**), prepupae (**PP**) (Myser, 1954), white-eyed pupae (**WEP**), pink-eyed pupae (**PEP**), light-brown-eyed pupae (**LBEP**), dark-brown-eyed pupae (**DBEP**), black-eyed pupae (**BEP**), newly emerged workers that did not feed yet (**EA**) and adult workers that returned from foraging activity (**FA**) (Jay, 1962; Eichmüller, 1994) (Sup. 1).

### 2.2. Histology

Five bees from each developmental stage were dissected in insect saline solution (0.1 M NaCl, 20 mM $KH_2PO_4$, 20 mM $Na_2HPO_4$), and their hindguts were transferred and kept in a Zamboni's fixative solution (Stefanini et al., 1967) for 12 h. In larvae and prepupae, fragments of the ascending region (prolongation of the gut near the pylorus) and descending region (final prolongation of the gut) were isolated (Fig. 1). In the pupal phases, after the anatomical differentiation, fragments of the rectal region and of the anterior and posterior regions of the ileum were separated for analysis (Fig. 1). Then, the samples were dehydrated in a graded ethanol series (70%, 80%, 90%, and 95%) and embedded in JB4 resin. Subsequently, 3 μm thick sections were stained with hematoxylin and eosin and analyzed and photographed under a light microscope (Olympus BX-60) using a digital camera (Q-Color, 3 Olympus).

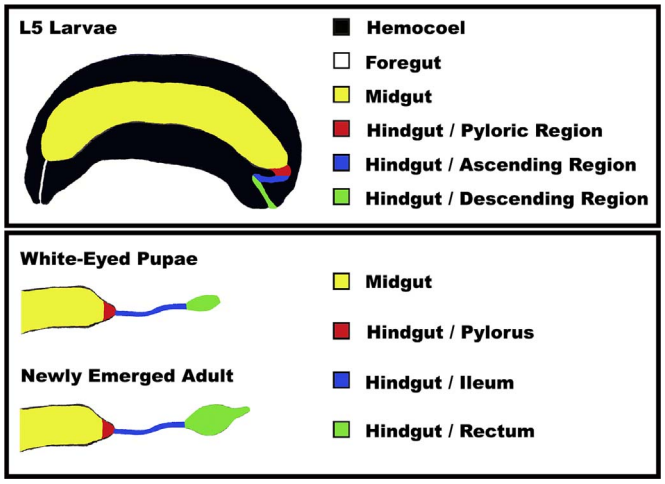


**Fig. 1.** Schematic draw showing the hindgut regions and the anatomical changes during the metamorphosis of *Apis mellifera*. The hindgut of L5 larvae, white-eyed pupae and newly emerged adult were illustrated.

### 2.3. Transmission electron microscopy

Five bees from each developmental phase were dissected in insect physiological solution. The hindgut was removed and transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 with 0.2 M sucrose. In larvae and prepupae, fragments of the ascending region (prolongation of the gut near the pylorus) and descending region (final prolongation of the gut) were isolated (Fig. 1). In the pupal phases, after the anatomical differentiation, fragments of the rectal region and the anterior and posterior regions of the ileum were separated for analysis (Fig. 1). These fragments were post-fixed in 1% osmium tetroxide in the same buffer for two hours. The samples were dehydrated in a graded ethanol series (70%, 80%, 90%, and 99%) and embedded in LR White resin (Sigma-Aldrich, USA). Ultrathin sections were stained for 20 min with 1% aqueous uranyl acetate and lead citrate for 10 min (Reynolds, 1963). The samples were analyzed and photographed in a Zeiss EM 109 transmission electron microscope at the Center for Microscopy and Microanalysis of the Federal University of Viçosa.

### 2.4. Immunofluorescence

*Apis mellifera* individuals from each developmental phase were dissected in insect physiological solution and their hindgut was removed to identify cell proliferation, apoptosis, and autophagy. Seven individuals were used for each analysis. After dissection, the alimentary canal was transferred and kept in a Zamboni's fixative solution for 2 h. Samples were then washed in 0.1 M sodium phosphate buffer pH 7.2 plus 1% Tween-20 (PBST) for 2 h. Following this, the samples were incubated for 12 h with the following primary antibodies diluted in PBST: (1) anti-phospho-histone H3 (1:100) (Cell Signaling Technology Cat# 9701S, Antibody Registry: AB_331534) for the detection of cell proliferation, (2) anti-cleaved caspase-3 (1:500) (Trevigen Cat# 2305-PC-100, Temporary Antibody Registry: AB_2665453) for apoptosis detection, and (3) anti-LC3A/B (1:500) (Abcam Cat# ab128025, Antibody Registry: AB_11143008) for the identification of autophagy. The samples were then washed in PBST and incubated for 12 h with an FITC-conjugated anti-rabbit IgG secondary antibody (1:500) (Sigma-Aldrich Cat# F0382, Antibody Registry: AB_259384). Afterward, the samples were washed and