WAGNER GONZAGA GONÇALVES

MUDANÇAS NOS ÓRGÃOS EXCRETORES DA ABELHA *Apis mellifera* DURANTE A METAMORFOSE: MORFOGÊNESE, REMODELAÇÃO, MORTE E PROLIFERAÇÃO CELULAR

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

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Dedico

A Deus, por sempre iluminar o meu caminho.

Aos meus pais, Ernesto e Tereza por dedicarem suas vidas refletindo os verdadeiros valores da vida.

Ao meu irmão Danilo, por trilhar com sabedoria as fases da vida, sendo um grande filho e irmão.

À minha esposa Priscylla, por seu companheirismo em todos os momentos de nossas vidas.

A todos que com seu amor me fazem buscar o melhor a cada dia.

SUMÁRIO

RESUMO

GONÇALVES, Wagner Gonzaga, D.Sc., Universidade Federal de Viçosa, julho de 2017. **Mudanças nos órgãos excretores da abelha *Apis mellifera* durante a metamorfose: morfogênese, remodelação, morte e proliferação celular**. Orientador: José Eduardo Serrão.

A abelha *Apis mellifera* tem importância ecológica e econômica, no entanto, sofre um declínio populacional, talvez devido à exposição a compostos tóxicos, que são excretados pelos túbulos de Malpigui. Durante a metamorfose de *A. mellifera*, os túbulos de Malpigui degeneram e são formados *de novo*. O objetivo deste trabalho foi verificar os eventos celulares na renovação dos túbulos Malpighi, acompanhando na metamorfose, quais as etapas da remodelação celular, determinando os tipos celulares e seus papéis na atividade excretora e inicio do controle homeostático em *A. mellifera*. As análises ultraestruturais e de imunofluorescência mostraram que as células dos túbulos de Malpighi de larvas degeneraram por apoptose e autofagia (larva L5S e pré-pupa) e novos túbulos de Malpigui são formados por proliferação celular. (larva L5S e pupa de olhos castanhos). A ultraestrutura das células dos túbulos de Malpigui sugere que uma remodelação celular ocorra a partir de pupa de olhos marrons, indicando o inicio de uma atividade de excreção nos túbulos de Malpigui pupais. Em abelhas adultas (recém-emergida e forrageira), dois tipos celulares ocorrem nos túbulos de Malpigui, um com características ultraestruturais de produção da urina primária e outro tipo de célula com características que sugerem um papel na reabsorção da urina primária. Este estudo sugere que, durante a metamorfose, os túbulos de Malpigui não são funcionais até pupa de olhos castanhos, indicando que *A. mellifera* pode ser vulnerável a compostos tóxicos nas primeiras nas fases pupais. Além disso, a ultraestrutura celular sugere que os túbulos Malpigui podem ser funcionais a partir de pupa de olhos marrons e adquirem maior complexidade na abelha operária forrageira. Nos insetos, o intestino posterior é um órgão homeostático, sendo este dividido em piloro, íleo e reto, que reabsorvem água, íons e pequenas moléculas produzidas na filtragem da hemolinfa e nas fezes. Esse estudo reporta as mudanças morfológicas e os eventos celulares que ocorrem no intestino posterior durante a metamorfose da abelha *A. mellifera*. No intestino posterior, a imunolocalização de autofagossomos e a ultraestrutura as células epiteliais e do revestimento cuticular sugerem que em pré-pupa tem início a degradação cuticular, que em pupas de olhos brancos e rosas é reabsorvida e reciclada por autofagossomos, sendo a deposição da nova cutícula em pupa de olhos castanhos. Em larva L5S e pré-pupa, o intestino posterior apresenta proliferação celular em suas extremidades anterior e posterior. Na pupa, as regiões do piloro, íleo e reto estão evidentes e com proliferações celulares que cessam a partir de pupa de olhos marrons. Apoptose ocorre de larva L5S até pupa de olhos rosas. Em pupas de olhos

castanhos e marrons, o epitélio do íleo muda de pseudoestratificado para simples somente após a produção da lâmina basal e o epitélio retal é achatado. Nas células do íleo de pupa de olhos pretos ocorrem grandes vacúolos e espaços subcuticulares, enquanto que na operária adulta forrageira, ocorrem invaginações apicais longas e muitas mitocôndrias, sugerindo uma atividade no transporte de compostos. Os resultados mostram que a morfogênese do intestino posterior é dinâmica, com remodelações teciduais e eventos celulares para a formação de diferentes regiões do órgão, reconstrução de uma nova cutícula e remodelação dos músculos viscerais.

ABSTRACT

GONÇALVES, Wagner Gonzaga, D.Sc., Universidade Federal de Viçosa, July, 2017. **Changes in the excretory organs of the bee *Apis mellifera* during the metamorphosis: morphogenesis, remodeling, death and cell proliferation**. Adviser: José Eduardo Serrão.

The honeybee *Apis mellifera* has ecological and economic importance, however, experience a population decline, perhaps due to exposure to toxic compounds, which are excreted by Malpighian tubules. During metamorphosis of *A. mellifera*, the Malpighian tubules degenerate and are formed *de novo*. The objective of this work was to verify the cellular events of the Malpighian tubules renewal and accompany in the metamorphosis, which are the gradual steps of cell remodeling, determining different cell types and their roles in the excretory activity and onset of homeostatic control in *A. mellifera*. Immunofluorescence and ultrastructural analyses showed that the cells of the larval Malpighian tubules degenerate by apoptosis and autophagy (larvae instar L5S and prepupae) and the new Malpighian tubules are formed by cell proliferation (L5S larvae until light-brown eyed pupae). The ultrastructure of the cells in the Malpighian tubules suggest that cellular remodeling only occurs from dark-brown eyed pupae, indicating the onset of excretion activity in pupal Malpighian tubules. In adult bees (newly emerged and forager), two cell types occur in the Malpighian tubules, one with ultrastructural features of primary urine production and another cell type with characteristics that suggest a role in primary urine reabsorpotion. This study suggest that during the metamorphosis, Malpighian tubules are non-functional until the light-brown eyed pupae, indicating that *A. mellifera* may be more vulnerable to toxic compounds at early pupal stages. In addition, cell ultrastructure suggests that the Malpighian tubules may be functional from dark-brown eyed pupae and acquire greater complexity in the forager worker bee. 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.

INTRODUÇÃO GERAL

As abelhas exploram longas distâncias na obtenção de recursos nutricionais (néctar e pólen) e água (Haydark, 1970; Winston, 1987). Portanto, as abelhas forrageiras podem ser expostas ou levar para colônia compostos tóxicos (Porrini et al., 2003), dado isso, tem sido reportada a presença de resíduos tóxicos no interior de colônias de abelhas, sendo esses resíduos encontrados no mel, pólen e cera (Fakhimzadeh and Lodenius, 2000; Korta et al., 2003; Chauzat et al., 2006;). Atualmente, a exposição das abelhas a inseticidas, acaricidas, metais pesados, patógenos e mudanças climáticas é associada com o declínio populacional das abelhas, principalmente *Apis mellifera* (Oldroyd,2007; vanEngelsdorp et al., 2009; Moroń et al., 2012; Lima et al., 2016).

Como a causa da redução populacional das abelhas *A. mellifera* não é compreendida e diferentes estresses ambientais são associados com a extinção das abelhas, o estudo dos órgãos excretores é importante para compreensão do quanto esses órgãos regulam a hoemeostase corporal nas diferentes fases do desenvolvimento das abelhas; assegurando a desintoxicação e a sobrevivência desses indispensáveis polinizadores.

Em insetos adultos, o sistema excretor é formado pelos túbulos de Malpighi e intestino posterior, sendo esses órgãos banhados pela hemolinfa, fluido que nutre todos os órgãos internos (Dobrovsky, 1951). Em geral, os túbulos de Malpighi são responsáveis pela filtragem da hemolifa, eliminando compostos tóxicos ou em excesso (Gaertner et al., 1998; Beyenbach et al., 2010; Chahine and O'Donnell, 2011). Em seguida, o intestino posterior recebe esse filtrado dos túbulos de Malpighi e reabsorve os compostos úteis, principalmente água e íons (Phillips, 1986). Deste modo, o sistema excretor mantém o equilíbrio homeostático da hemolinfa.

Embora esses órgãos sejam fundamentais para as abelhas, há poucos estudos sobre os eventos celulares e as propriedades morfofuncionais dos órgãos excretores

durante a metamorfose. Durante esse período do desenvolvimento, os órgãos internos dos insetos são totalmente modificados para desempenhar suas funções no individuo adulto (Cruz-Landim, 2009). Desse modo, é comum que no desenvolvimento pós-embrionários desses órgãos ocorram intensas proliferações celulares, assim como reportado para o intestino médio, cérebro e intestino posterior de diferentes espécies de insetos (Takashima et al., 2008; Cruz et al., 2013; Fernandes et al., 2014; Ganeshina et al., 2000; Franzetti et al., 2016).

As proliferações celulares durante a metamorfose dos insetos promovem o crescimento de órgãos e tecidos, mas, juntamente com mortes celulares programadas, garantem a renovação celular e a reestruturação de órgãos em desenvolvimento (Robertison, 1936; Ganeshina et al., 2000; Harvey er al., 2003; Hakim et al., 2010). Como observado na metamorfose de *Melipona quadrifasciata* (Hymenoptera) e *Bombyx mori* (Lepidoptera), o intestino médio tem seu epitélio renovado por proliferações celulares e apoptoses (Cruz et al., 2013; Franzetti et al., 2012), enquanto que as papilas retais de *M. quadrifasciata*, localizadas no final do intestino posterior, surgem por proliferações celulares e são moldadas por mortes celulares programadas (Santos et al., 2009).

Na degeneração dos órgãos internos dos insetos, mortes celulares por apoptoses podem ocorrer junto ou após a autofagia, evento celular que também promove a degradação de conteúdo intracelular. Eventos apoptóticos e autofágicos atuando em conjunto são reportados no desenvolvimento do intestino médio e corpo gorduroso de insetos (Cruz et al., 2013; Santos et al., 2015). Entretanto, na metamorfose de *Drosophila melanogaster* (Diptera) somente a autofagia é essencial na degeneração do intestino médio, enquanto que nas glândulas salivares a degeneração celular é inibida com o bloqueio de vias autofágicas (Berry and Baehrecke, 2007; Denton et al., 2009).

Embora a apoptose, autofagia e proliferações celulares sejam importantes, pouco é sabido sobre a ocorrência desses eventos nos órgãos excretores durante a metamorfose dos insetos. Na abelha *Apis mellifera*, o desenvolvimento dos túbulos de Malpighi e intestino posterior foram evidenciados somente por técnicas histológicas, demonstrando o crescimento e a diferenciação anatômica desses órgãos ocasionada por mitoses (Dobrovsky, 1951; Cruz-Landim and Silva-Mello, 1970). Além disso, na metamorfose das abelhas os túbulos de Malpighi são degenerados e formados novamente, fato que não ocorre em *D. melanogaster*, o principal modelo utilizado no entendimento da morfogênese e fisiologia dos órgãos excretores (Cruz-landim, 2000; Shukla and Tapadia, 2011).

Apesar dos túbulos de Malpighi de *D. melanogater* não serem degenerados durante a metamorfose, proteínas apoptóticas e anti-apoptóticas foram encontradas no interior dos núcleos e nas proximidades da membrana basal das células (Tapadia and Gautam 2011; Shukla and Tapadia, 2011). Quanto ao intestino posterior desses insetos, foi reportada uma renovação epitelial iniciada na fase larval, quando células-tronco localizadas na extremidade anterior do órgão são ativadas e uma zona de proliferação celular é formada (Takashima et al., 2008). Nesse processo, o epitélio do intestino posterior é renovado a partir de sua extremidade anterior, enquanto que as células mais velhas entram em morte celular apoptótica (Robertson, 1936; Takashima et al., 2008)

Os aspectos morfofuncionais dos órgãos excretores no decorrer da metamorfose das abelhas também foram analisados por métodos histológicos. No entanto, em *A. mellifera* e *M. quadrifasciata* não foram sugeridas uma atividade funcional para aos túbulos de Malpighi e intestino posterior no decorrer da metamorfose (Dobrovsky, 1951; Cruz-Landim and Silva-Mello, 1970; Cruz-landim, 2000).

De modo geral, correlações entre a morfologia celular e a atividade de excreção durante a metamorfose dos insetos foram realizadas somente para túbulos de Malpighi

do holometábolo *Calpodes ethlius* (Lepidoptera) e do hemimetábolo *Rhodnius prolixus* (Hemiptera) (Ryerse, 1979; Skaer et al., 1990). Os túbulos de Malpighi desses insetos desenvolvem-se de maneiras diferentes durante a metamorfose, pois na fase larval do inseto holometábolo ocorre uma alta atividade de excreção, que posteriormente é inibida na maior parte da fase pupal. Contudo, durante a metamorfose do inseto hemimetábolo a atividade de excreção não é inibida nas fases ninfais. Apesar das diferenças, a taxa de excreção dos túbulos de Malpighi desses insetos está correlacionada com a morfologia celular, sendo proporcional ao numero e tamanho das microvilosidades e labirintos basais das células no decorrer da metamorfose (Ryerse, 1979; Skaer et al., 1990).

Portanto, trabalhos que reportam parâmetros morfofuncionais dos túbulos de Malpighi e do intestino posterior durante a metamorfose dos insetos são raros.

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OBJETIVOS

Descrever a morfofisiologia e caracterizar os eventos celulares que atuam na formação e remodelação dos órgãos excretores no decorrer das diferentes fases do desenvolvimento pós-embrionário da abelha *A. mellifera*.

CAPÍTULO 1

Post-embryonic development of the Malpighian tubules in *Apis mellifera* (Hymenoptera) workers: morphology, remodeling, apoptosis and cell proliferation

Abstract

The honeybee *Apis mellifera* has ecological and economic importance, however, experience a population decline, perhaps due to exposure to toxic compounds, which are excreted by Malpighian tubules. During metamorphosis of *A. mellifera*, the Malpighian tubules degenerate and are formed *de novo*. The objective of this work was to verify the cellular events of the Malpighian tubules renewal and accompany in the metamorphosis, which are the gradual steps of cell remodeling, determining different cell types and their roles in the excretory activity and onset of homeostatic control in *A. mellifera*. Immunofluorescence and ultrastructural analyses showed that the cells of the larval Malpighian tubules degenerate by apoptosis and autophagy (larvae instar L5S and prepupae) and the new Malpighian tubules are formed by cell proliferation (L5S larvae until light-brown eyed pupae). The ultrastructure of the cells in the Malpighian tubules suggest that cellular remodeling only occurs from dark-brown eyed pupae, indicating the onset of excretion activity in pupal Malpighian tubules. In adult bees (newly emerged and forager), two cell types occur in the Malpighian tubules, one with ultrastructural features of primary urine production and another cell type with characteristics that suggest a role in primary urine reabsorpotion. This study suggest that during the metamorphosis, Malpighian tubules are non-functional until the light-brown eyed pupae, indicating that *A. mellifera* may be more vulnerable to toxic compounds at early pupal stages. In addition, cell ultrastructure suggests that the Malpighian tubules may be functional from dark-brown eyed pupae and acquire greater complexity in the forager worker bee.

Keywords: Morphology of apoptosis, Autophagy, Excretion, Vulnerability.

Introduction

The ecological and economic importance of bees is due to their role in pollination, production of honey, pollen, wax, propolis, and agricultural pollination (Klein et al. 2007; Kerr et al. 2010; Potts et al. 2010; Oliveira et al. 2015). In fact c.a. 75% of the plant species cultivated worldwide are pollinated by bees (Klein et al. 2007; Potts et al. 2010). Among bees, *Apis mellifera* is the most used in many agricultural crops (Bauer and Wing 2010; Breeze et al 2011). Despite the ecological and economic importance, bee populations have shown a strong decline (Oldroyd 2017; vanEngelsdorp et al. 2009). Multiple factors may explain this reduction, such as pathogens, pesticides, fungicides and heavy metals (Oldroyd 2017; vanEngelsdorp et al. 2009; Moroń et al. 2012; Rodrigues et al. 2016). Thus, the study of homeostatic organs is fundamental, because in environmental stress these organs ensure the survival of the bees or are damaged, as is the case of adult *Apis mellifera* Malpighian tubules exposed to sublethal doses of pesticides (Almeida et al. 2013; Catae et al. 2014).

Malpighian tubules are the main regulators of homeostasis in insects, excreting toxic or excess compounds through the formation of primary urine (Dow et al. 1998; Klowden 2007; Cruz-Landim 2009; Beyenbach et al. 2010). In bees, the Malpighian tubules are long free tubular filaments in the hemocele opening at the mid-hindgut transition (Cruz-Landim 2000).

Apis mellifera Malpighian tubules emerge after around 43-46 hours of embryonic development and 10-13 hours after midgut formation (Fleig 1986). However, during post-embryonic development many organs undergo modifications to perform their functions in the adult insect (Dobrovsky 1951; Jiang et al. 1997; Chapman 2013). During insect metamorphosis, many organs are modified by the balance between cell death and proliferation (Berry and Baehrecke 2007; Cruz et al. 2013; Santos et al. 2015; Franzetti et al. 2016).

In different bee species, larvae have from 4 to 13 Malpighian tubules (Cruz-Landim 2009; Barbosa-Costa et al. 2012), which degenerate in the transition to pupae (Cruz-Landim 2000). In *Apis mellifera* and *Melipona quadrifasciata anthidioides*, degeneration in the Malpighian tubules occurs by programmed cell death and concomitantly new Malpighian tubules begin to be formed (Oertel 1930; Cruz-Landim 2000). In adult bees, the number of Malpighian tubules varies from 25 to 94 (Cruz-Landim 2009; Barbosa-Costa et al. 2012).

In general, Malpighian tubules of bees have a single layered epithelium with cubic cells, with long microvilli and long and narrow basal labyrinths (Oertel 1930; Cruz-Landim and Silva-Mello 1970; Silva-de-Moraes and Cruz-Landim 1976; Cruz-Landim 2000). Variations in the size of microvilli, size and dilations in the basal labyrinths and presence of spherocrystals or vesicles in the cytoplasm, indicate whether the cells along the Malpighian tubules release or absorb compounds from the lumen (Cruz-Landim 1998). In addition, muscles fibers are found in the outer surface of the Malpighian tubules that arise after the larval phase (Oertel 1930; Cruz-Landim 2000).

This study presents new perspectives on post-embryonic development of the Malpighian tubules in *A. mellifera* workers, describing the cellular events necessary for their degeneration and formation, as well as cellular remodeling, providing evidence of onset of excretory activity during metamorphosis.

Materials and Methods

Bees

Apis mellifera workers were collected directly from hives in colonies in the Central Apiary at the Universidade Federal de Viçosa. The bees were collected in the following 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).

Histology

Five bees from each developmental phase were dissected in insect physiological solution (0.1 M NaCl, 20 mM KH_2PO_4, 20 mM Na_2HPO_4), the whole alimentary canal with attached Malpighian tubules was removed and transferred to Zamboni's fixative solution (Stefanini 1967) for 12 hours. Subsequently, the samples were dehydrated in a graded ethanol series (70, 80, 90, 95%) and embedded in JB4 resin. Sections 3 μm thick were stained with hematoxylin and eosin, and analyzed and photographed in a light microscope (Olympus BX-60) with digital camera (Q-Color, 3 Olympus).

Immunofluorescence

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

F0382, Antibody Registry: AB_259384). Afterward, the samples were washed and incubated for 30 min with nuclear marker TO-PRO-3 Iodide (1:1000) (Life Technologies). The samples were washed and mounted in Mowiol (Sigma-Aldrich, USA) and analyzed with a Zeiss LSM-META (Carl Zeiss AG, Oberkochen, Germany) confocal microscopy at the Center for Microscope and Microanalysis at the Universidade Federal de Viçosa. For negative controls, incubations with primary antibodies were omitted.

Transmission electronic microscopy

Five bees from each developmental phase were dissected in insect physiological solution. The whole alimentary canal with attached Malpighian tubules was removed rapidly and transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 with 0.2 M sucrose. Following this, fragments of the proximal and distal portion of the Malpighian tubules were dissected and 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, 99%) and embedded in LR White resin (Sigma-Aldrich, USA). Ultrathin sections were stained for 20 minutes with 1% aqueous uranyl acetate and lead citrate (Reynolds 1963) for 10 minutes. The samples were analyzed and photographed in a Zeiss EM 109 transmission electron microscope at the Center for Microscopy and Microanalysis at the Universidade Federal de Viçosa.

Results

Degeneration of the Malpighian Tubules: Morphology

To better understand the cellular events that occur during the morphogenesis of the new Malpighi tubules, we first characterize the histolysis of Malpighian tubules in the larval and prepupal phases.

In the larval phase L5 of *A. mellifera*, the Malpighian tubules showed an enlarged lumen and wall formed by a single layer of flattened cells with great

cytoplasmic spaces at the apical and lateral regions and nucleus with some nucleoli (Fig. 1a). The cytoplasm of these cells showed few mitochondria, extensive rough endoplasmic reticulum in the perinuclear region and cytoplasmic spaces similar to "glycogen islands" (Fig. 1b, c). The apical region of the Malpighian tubule cells showed non-compact and long microvilli (Fig. 1c) and in the cell basal region plasma membrane invaginations occurred, forming short and dilated labyrinths (Fig. 1d). These cells were fixed onto a thin basal lamina (Fig. 1d).

The Malpighian tubules of larvae L5S showed cells with little evident microvilli and cellular fragments containing "glycogen islands" thrown into the lumen (Fig. 2a). In the cytoplasm of these cells vesicles similar to autophagosomes occurred and the endoplasmic reticulum was fragmented into vesicles (Fig. 2b, c). The basal region of the cells showed mitochondria and a well-developed basal labyrinth (Fig. 2d). Vesicles were abundant between the basal labyrinths and scarce in the apical region of the cell (Fig. 2a, d).

In prepupae, the Malpighian tubules showed histolysis, characterized by loss of striated border, irregular nucleus and decrease of epithelial volume and lumen (Fig. 2e, f). The cytoplasm of these cells showed Irregular nucleus, many vacuoles and autophagosomes (Fig. 2g, h). Subsequently, the cytoplasm showed only fragmented mitochondria (Fig. 2h) and accumulation of electron-dense material between the basal surface of the cell and the basal lamina (Fig. 2i). In this phase the cell debris were surrounded by a basal lamina not degraded (Fig. 2i).

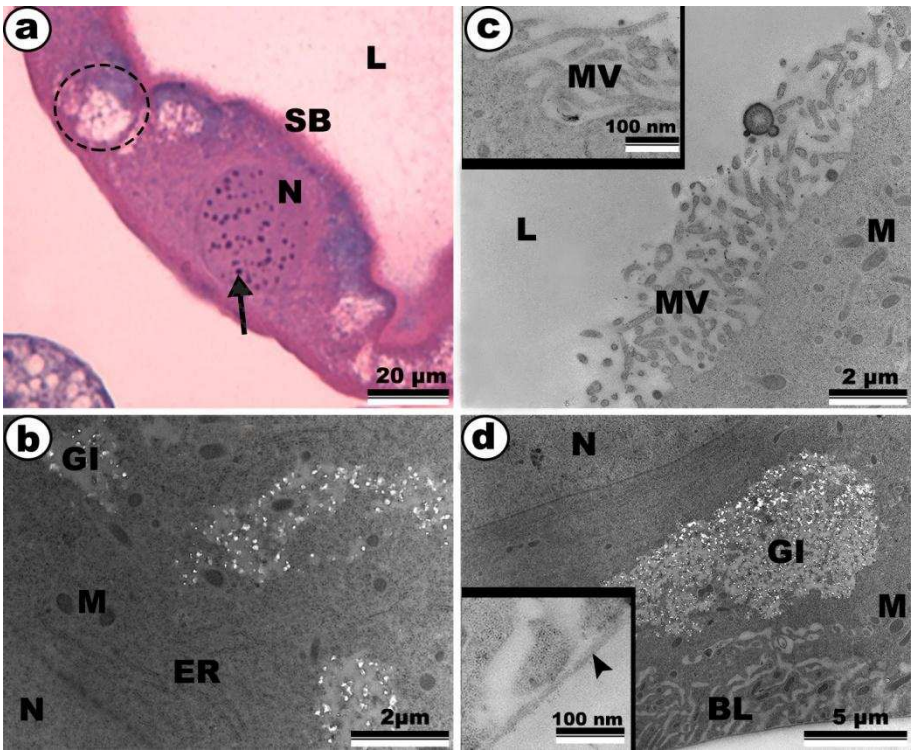

Fig. 1 Micrographs of Malpighian tubules cells of *Apis mellifera* in last larval instar (L5). [**a]** L5 larval cell showing partially acidic cytoplasmic compartments (dashed circumference), striated border (SB), nucleus (N) and nucleolus (arrow). Note the lumen (L). [**b]** Middle cell region with nucleus (N), rough endoplasmic reticulum (ER), "glycogen islands" (GI) and mitochondria (M). [**c]** Apical cell region showing microvilli (MV) and mitochondria (M). [**insert**] Elongated microvilli (MV). [**d]** Basal cell region with short basal labyrinths (BL), mitochondria (M), "glycogen islands" (GI) and nucleus (N). [**insert**] Thin basal lamina (arrowhead).

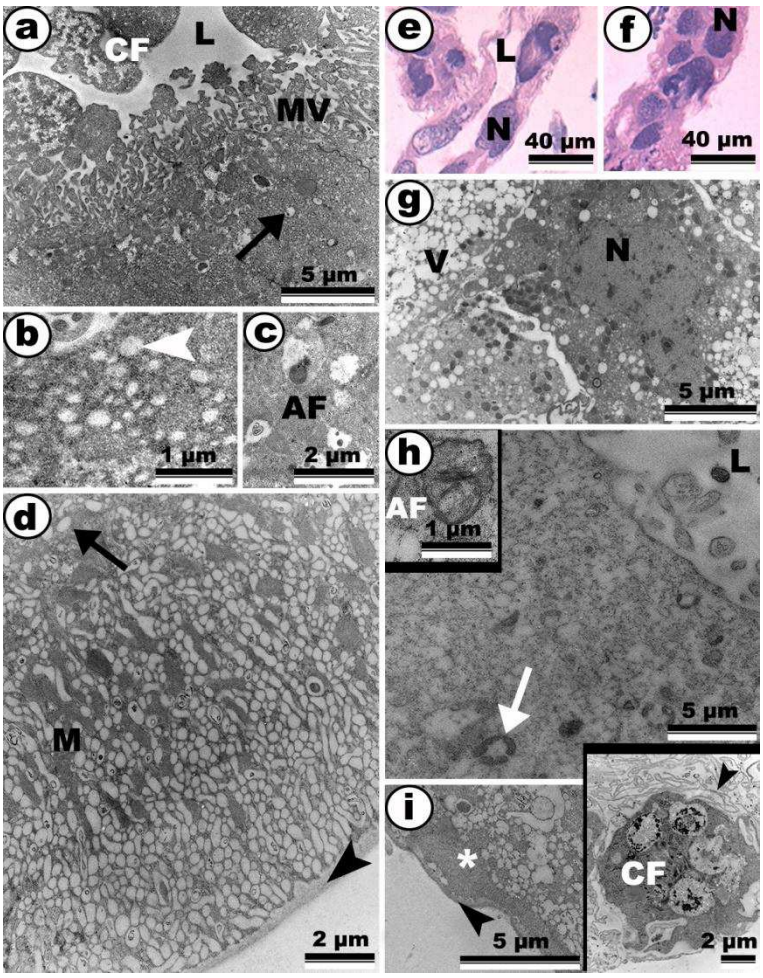

Fig. 2 Micrographs of Malpighian tubules cells of *Apis mellifera* in last larval instar (L5S) and prepupae. [**a-d]** Malpighian tubules of larval L5S with cellular fragments (CF) in the lumen (L) and cells showing irregular microvilli (MV), vesicles (arrow), endoplasmic reticulum collapsed in small vesicles (white arrowhead), mitochondria (M) and autophagosomes (AF). [**e-i**] Malpighian tubules of prepupae with narrow lumen (L) and cells with irregular nuclei (N), many vacuoles (V) and afterward absence of microvilli on the luminal surface (L), fragmented mitochondria (white arrow) and basal cell region with electron-dense material (asterisk) above the basal lamina (arrowhead). [**Inserts]** Note the autophagosomes in the cytoplasm (AF) and cellular fragments (CF) always contained by the basal lamina (arrowhead).

Degeneration of the Malpighian tubules: Cell Death

As in L5, L5S larvae and prepupae we report the morphology of Malpighian tubules in degeneration, analyzed by immunolocalization in which phases events of cell death occur. Thus, in the degeneration of the Malpighian tubules verified the occurrence of apoptosis and authophagy in L5S larvae (Fig. 3a, c) and prepupae (Fig. 3b, d).

Formation of the new Malpighian tubules: Cell Proliferation

Concomitant with degeneration of Malpighian tubules, we analyze in which phase the formation of new Malpighian tubules occurs and the resulting cellular events. The formation of new Malpighian tubules was initiated in L5S with intense cell proliferation (Fig. 3e), followed by a reduction until light-brown eyed pupae (Fig. 3f, g, h).

Myoepithelial cells were associated with Malpighian tubules from their budding, with proliferation in L5S (Fig. 3i) and prepupae (Fig. 3f). However, in prepupae some myoepithelial cells in apoptosis also occurred (Fig. 3j). In addition, the Malpighian tubules showed autophagy from prepupae up until newly emerged adults (Fig. 3k).

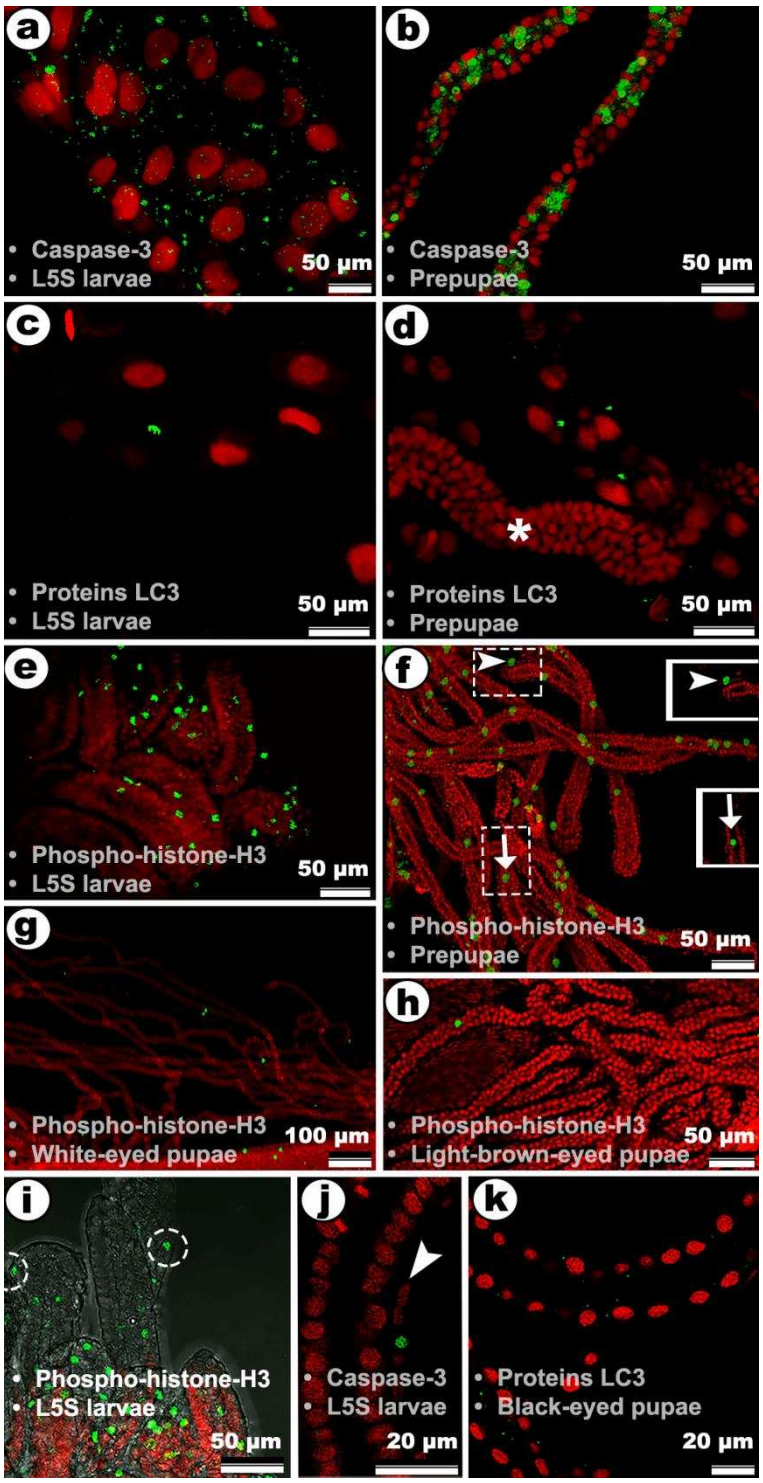

Fig. 3 Micrographs of Malpighian tubule cells of *Apis mellifera* positive for cleaved caspase-3 (indicating apoptotic cells), phospho-histone-H3 (indicating cell proliferation) and proteins LC3 (indicating the presence of autophagosomes). [**a]** L5S larvae and [**b]** prepupae with Malpighian tubules cells showing small and irregular nuclei (red) and positivity for cleaved caspase-3 (green). [**c]** L5S larvae and [**d]** prepupae with Malpighian tubules cells showing irregular nuclei (red) and positivity for LC3 (green). Note Newly formed Malpighian tubules (asterisk). [**e]** Malpighian tubules in formation

of L5S larvae, [**f**] prepupae, [**g**] white-eyed pupae and [**h**] light-brown eyed showing cells with small nuclei (red) and positivity for phospho-histone-H3 (green). [**inserts**] Longitudinal plane of the Malpighian tubules of the dashed region with cell proliferation in cells that arisen the tubules (arrow) and myoepithelial cells (arrowhead). [**i]** Myoepithelial cells (dashed region) showing positivity for phosphorylated histone-H3 (green) in L5S larval tubules. [**j]** Malpighian tubules of prepupae with myoepithelial cells showing positivity for cleaved caspase-3 (green). Note the flattened nucleus of the myoepithelial cell (arrowhead). [**k]** Malpighian tubules of black-eyed pupae with their cells (arrow) and myoepithelial cells (arrowhead) positive for LC3 (green).

Cellular Remodeling in the Malpighian Tubules

Because we found Malpighian tubules degeneration in the final larval stage followed by intense cell proliferation in these organs, we evaluated the morphological features during *de novo* remodeling of the Malpighian tubules with light and transmission electron microscopies.

In the prepupae, the new Malpighian tubules showed enlarged lumen and wall formed by cells with absent microvilli and long intercellular spaces (Fig. 4a). In the cytoplasm of these cells there were few mitochondria, electron-lucent vesicles and "glycogen islands" (Fig. 4a, b). The Malpighian tubules were lined externally by a thin basal lamina and myoepithelial cells (Fig. 4c).

In pupae of white-eyed until pupae of light-brown eyed, the Malpighian tubules showed a closed or evident lumen (Fig. 5a, b), while the microvilli of the cells were absent or irregular (Fig. 5c-d, f-h). In these phases the cytoplasm showed few mitochondria, vesicles and the intercellular spaces reached only until the median region of the cell (Fig. 5c, e), being that in light-brown eyed pupae larger mitochondrial number occurred (Fig. 5f).

The diameter of the Malpighian tubules increase from dark-brown eyed pupae, but the lumen was little evident (Fig. 6a). The cells showed long microvilli and a

cytoplasm with many mitochondria, vesicles, autophagosomes and spaces similar to the "glycogen islands" (Fig. 6b, c). In black-eyed pupae the Malpighian tubules were similar (Fig. 6e, f), however the lumen was more evident (Fig. 6d), the basal labyrinths were abundant and the presence of spherocrystals occurred (Fig. 6f). From of dark-brown and black eyed pupae, tracheoles (Fig. 6f) and muscle cells with many organized myofibrils (Fig. 7a) were attached to the basal lamina.

In the newly emerged adult workers of *A. mellifera*, the Malpighian tubules had large lumen and the epithelium with well-developed striated border (Fig. 7b). In addition, there were cells with different electron-densities (Fig. 7c). Electron-dense cells had many mitochondria, electron-lucent vesicles, spherocrystals, short microvilli and enlarged basal labyrinth (Fig. 8a-c). The electron-lucid cells were similar (Fig. 8d-f), but with longer microvilli (Fig. 8d), long and narrow basal labyrinths and few spherocrystals (Fig. 8e, f).

Two cell types also occurred in the Malpighian tubules of forager workers. The electron-dense ones in the proximal region of the tubules showed many mitochondria, long microvilli and a long, narrow basal labyrinth (Fig. 9a-c). However, electron-dense cells in the distal end of the Malpighian tubules showed many vacuoles and shorter and less compact microvilli (Fig. 9d-f). Independently of location in the Malpighian tubules, the electron-dense cells showed spherocrystals, autophagosomes and a rich rough endoplasmic reticulum (Fig. 9g-j).

Electron-lucent cells occurred along the Malpighian tubules of forager workers. These cells had many mitochondria, long microvilli, and a long, irregular and enlarged basal labyrinth (Fig. 10a-c). The basal lamina was thick, followed by muscle cells and tracheoles (Fig. 10c).

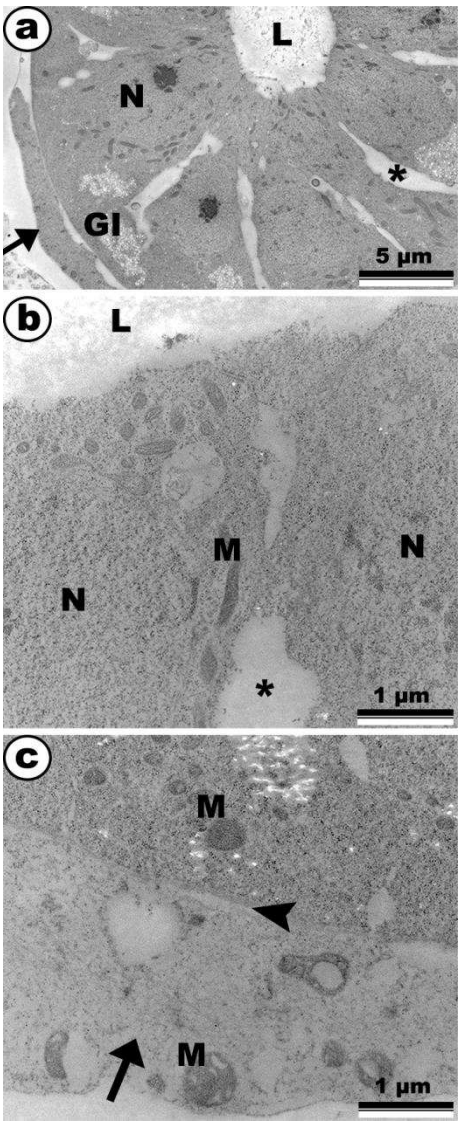

Fig. 4 Micrographs of newly formed Malpighian tubules of *Apis mellifera* in prepupae. [**a]** overview new Malpighian tubules with enlarged lumen (L), long intercellular spaces (asterisk), outer coating of myoepithelial cells (arrow) and showing small cells and nuclei with cytoplasmic "glycogen islands (GI). [**b]** Apical cell region without microvilli and with mitochondria (M). Note the intercellular spaces (asterisk) and lumen (L). [**c**] Basal cell region with mitochondria (M). Note a thin basal lamina (arrowhead) and myoepithelial cell (arrow) with mitochondria (M).

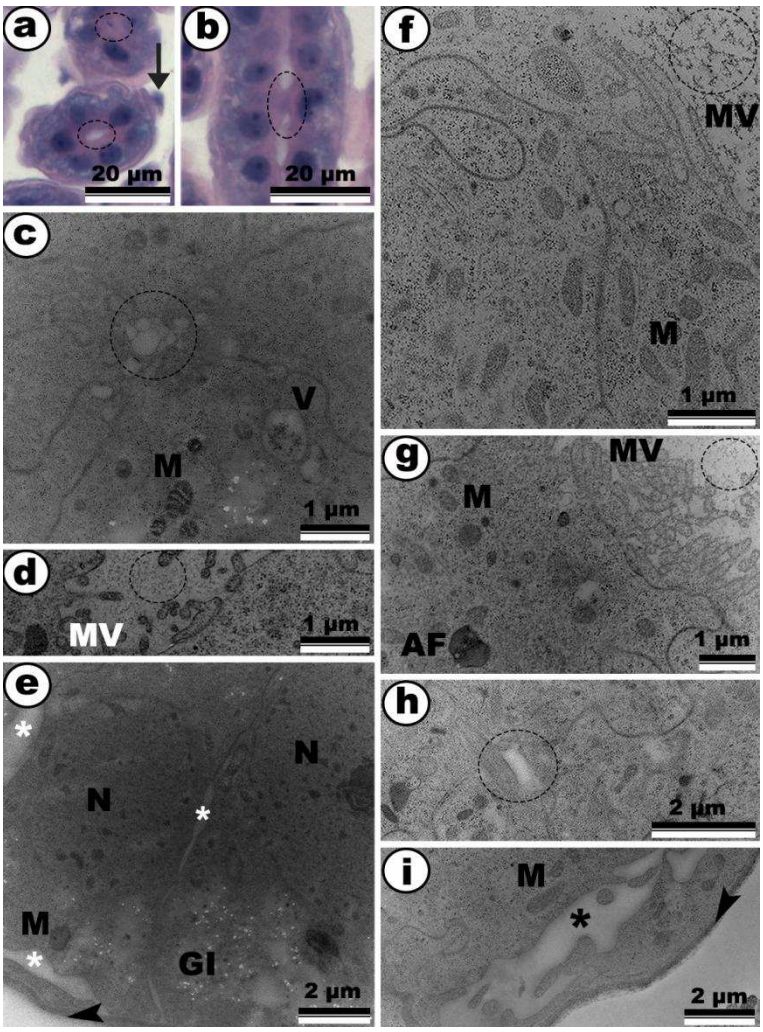

Fig. 5 Micrographs of Malpighian tubules of *Apis mellifera* in pink-eyed pupae (PEP) and light-brown-eyed pupae.(LBEP) [**a-b]** Malpighian tubules of PEP with closed or evident lumen (dashed circumference) and, cell condensed nucleus (N). Note myoepithelial cells (arrowhead). [c-d] Malpighian tubules of PEP with luminal region closed or open (dashed circumference) and cells with irregular microvilli (MV), mitochondria (M) and vesicles (V). **e** Malpighian tubules of PEP with basolateral cell region with nucleus rich in decondensed chromatin (N), "glycogen islands" (GI) and few mitochondria (M). Note basal lamina (arrowhead) and intercellular spaces (asterisks). [**f-h**] Malpighian tubules of LBEP with luminal region closed or open (dashed circumference) and cells with irregular microvilli (MV), mitochondria (M) and autophagosomes (AF). Note the basal lamina (arrowhead) and intercellular spaces (asterisks).

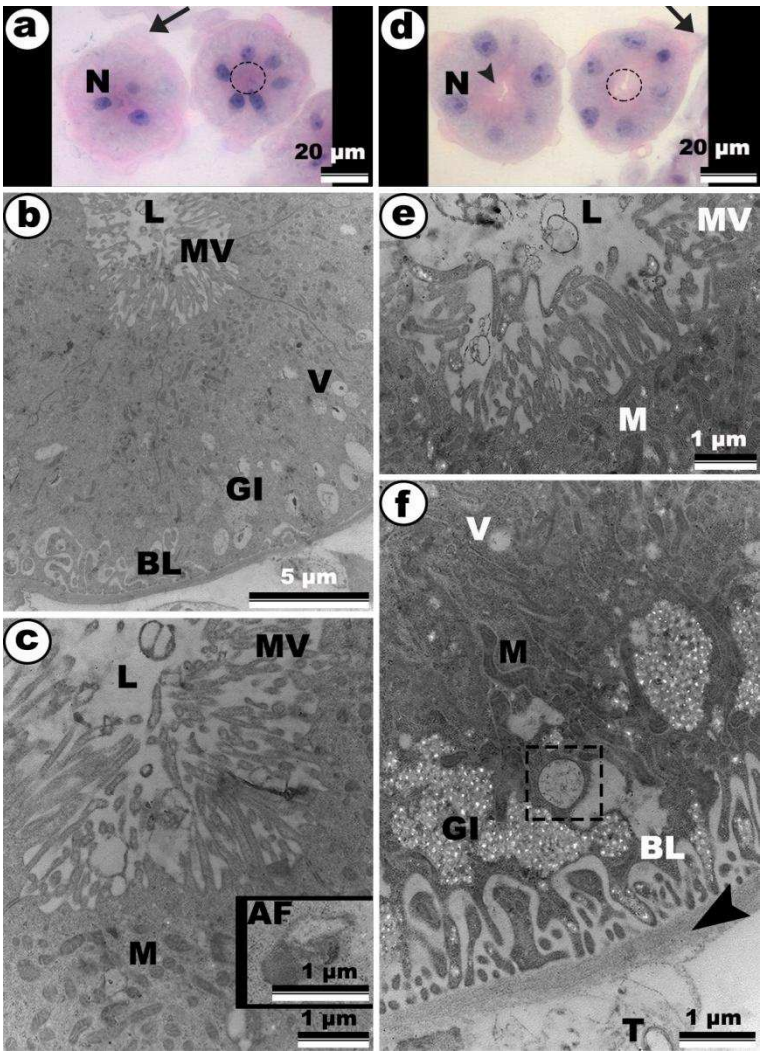

Fig. 6 Micrographs of Malpighian tubules of *Apis mellifera* in dark-brown-eyed pupae (DBEP) and black-eyed pupa (BEP). [**a]** Malpighian tubules of DBEP with non-evident lumen (dashed circumference) and cells with decondensed nucleus (N). Note muscle cells (arrows). [**b**] Overview cells of DBEP with microvilli (MV), "glycogen islands" (GI) and vesicles (V). Note the lumen (L) and some basal labyrinths (BL). [**c**] Apical cell region with long microvilli (M) and many mitochondria (M). [**d**] Malpighian tubules of BEP with evident lumen (dashed circumference), striated border (arrowhead) and cells with decondensed nucleus (N). Note muscle cells (arrows). [**e]** BEP showing apical region of the cell with long microvilli (M) and many mitochondria (M). Note the lumen (L). [**f]** BEP showing basal region of the cell with mitochondria (M), vesicles (V), "glycogen islands" (GI) and spherocrystials (dashed region). Note many sinuous intercellular spaces (BL), basal lamina (arrowhead) and tracheoles (T).

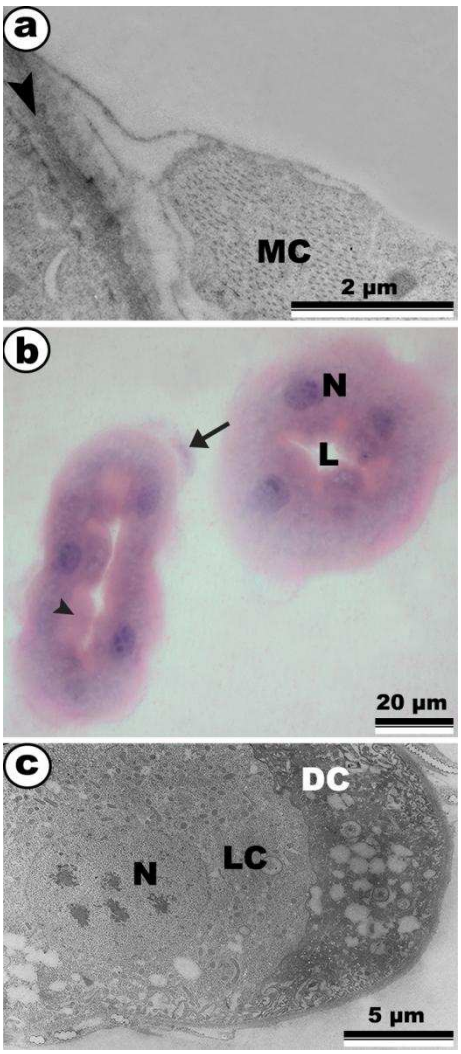

Fig. 7 Micrographs of Malpighian tubules of *Apis mellifera* in black-eyed puape (DBEP) and adult newly emerged (AE). [**a]** DBEP with Malpighian tubules showing muscle cells (MC) with many organized myofibrils. [**b**] Adult newly emerged with Malpighian tubules showing cells of decondensed nuclei (N) and striated border (arrowhead). Note: Lumen (L) dilated and muscle cells (arrow). [**c]** Cell of the Malpighian tubules of AE showing electron-dense cell (DC) and a more electron-lucid cell (LC) with its nucleus rich in decondensed chromatin. Note the basal lamina (arrowhead).

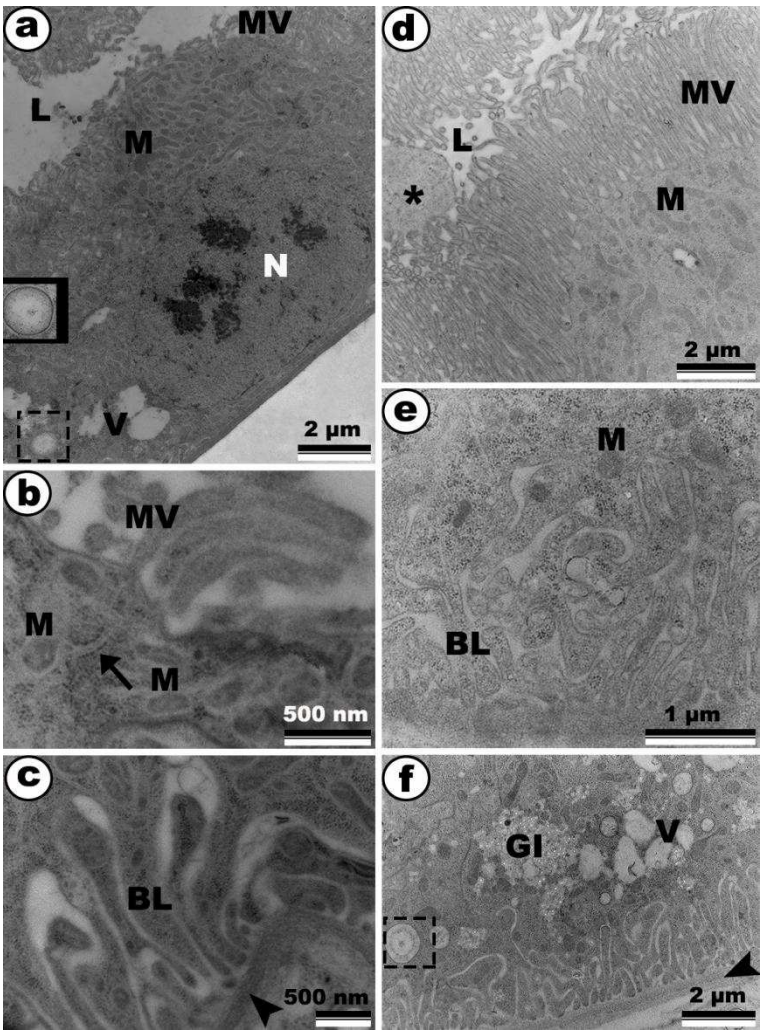

Fig. 8 Micrographs of electron-dense cells (DC) and electron-lucid cells (LC) of the Malpighian tubules of *Apis mellifera* newly emerged adult. [**a]** DC showing nucleus with decondensed chromatin (N), many mitochondria (M), vesicles (V) and microvilli (MV). [**insert**] Spherocrystal of the dashed region. Note the lumen (L). [**b]** Apical cell region with short microvilli (MV), rough endoplasmic reticulum (arrowhead) and mitochondria (M). [**c]** Basal cell region with dilated basal labyrinths (BL). Note the basal lamina (arrowhead). [**d**] LC showing apical region with long microvilli (MV) and many mitochondria (M). Note the lumen (L) with electron-lucid material (asterisk). [**e-f**] LC showing basal region with "glycogen islands" (GI), spherocrystals (dashed region), vesicles (V) and mitochondria (M). Note the basal lamina (arrowhead).

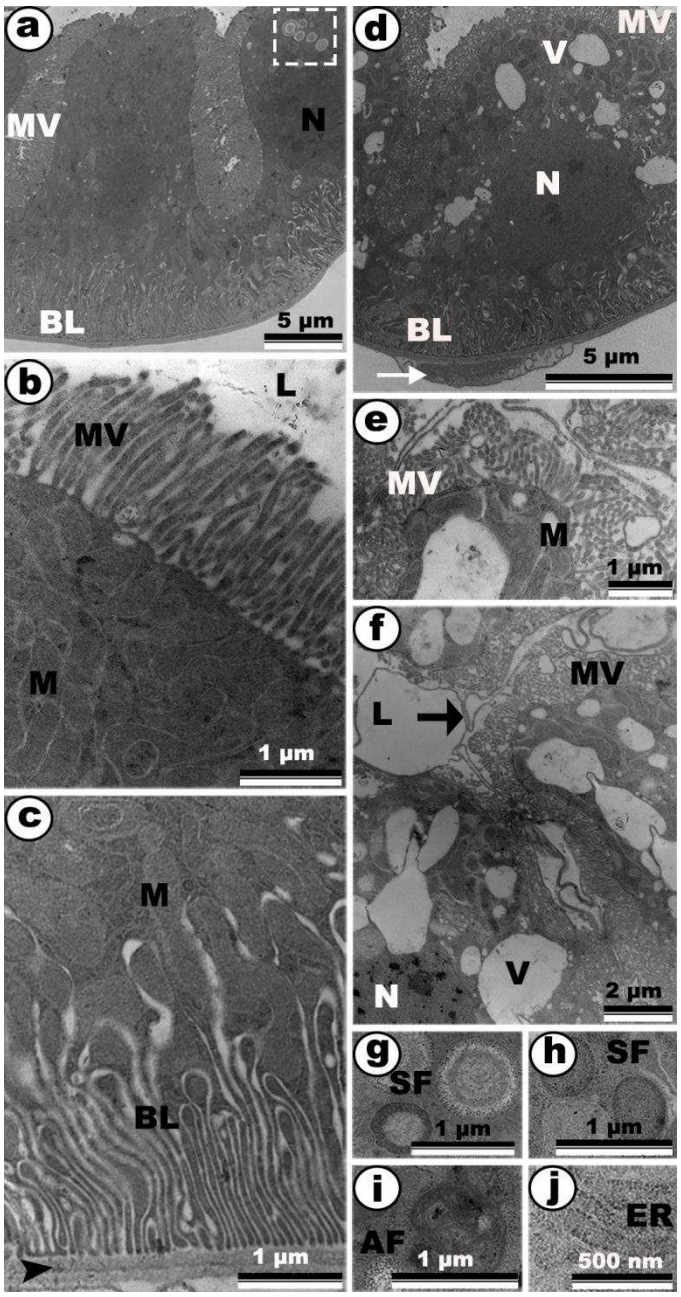

Fig. 9 Micrographs of electron-dense cells in the proximal region and electron-dense cells in the distal region of the tubule Malpighian of *Apis mellifera* foragers. [**a-c]** The electron-dense cells in the proximal showing electron-dense nucleus (N), spherocrystals (dashed region), microvilli (MV) and many mitochondria (M). Note the lumen (L), basal lamina (arrowhead) and long basal labyrinths (BL). [**d-f**] electron-dense cells in the distal region showing electron-dense nucleus (N), short microvilli (MV), mitochondria (M) and many vacuoles (V). Note muscle cells (white arrow) and membranous material (black arrow) in the lumen (L). [**g-j**] All cells showed different aspects of spherocrystals (SF) in the cytoplasm, autophagosome (AF) and a rich in endoplasmic reticulum (ER).

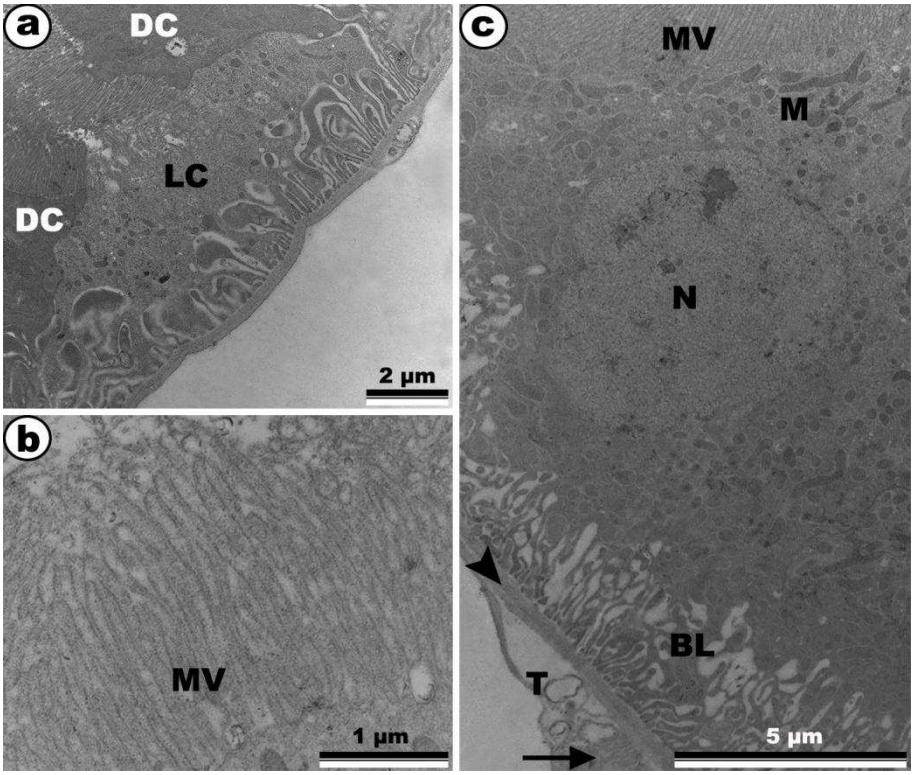

Fig. 10 Micrographs of electron-lucid cells in the Malpighian tubules of *Apis mellifera* forager. [**a]** Part of the electron-lucid cell (LC) between two electron-dense cells (DC). [**b-c]** Cell with nucleus with decondensed chromatin (N), long microvilli (MV), many mitochondria (M) and basal labyrinths (BL). Note muscle cells (arrow), tracheoles (T) and basal lamina (arrowhead).

Discussion

In the Malpighian tubules of insects, highly active cells show abundance of mitochondria, long and numerous microvilli, extensive basal labyrinth, spherocrystals and thick basal lamina (Berridge and Oschman 1969; Meyran 1982; Skaer et al. 1990). Considering that in *A. mellifera* L5 larvae these features are absent, we suggest that in their Malpighian tubules the excretion may occur at a reduced rate, similar to that reported in the Malpighian tubules of the hemimetabolous *Rhodnius prolixus* and holometabolous *Calpodes ethlius*, with progressive increase in the excretory activity during metamorphosis according the microvilli and basal labyrinths become more developed in size and number (Ryerse 1979; Skaer et al. 1990). The low excretory

activity in the larval Malpighian tubules of *A. mellifera* occur because all the products of excretion are stored in the organ lumen until the last instar larval, when the passage between the midgut and hindgut opens (Oertel 1930; Dobrovsky 1951; Cruz-Landim 2000).

In *A. mellifera* L5S larvae, many cell fragments containing "glycogen islands" are released into the Malpighian tubules lumen, as reported for larvae of the stingless bee *M. quadrifasciata anthidioides* (Silva-de-Moraes and Cruz-Landim 1976). In this larval phase, the hindgut of *A. mellifera* loses the cuticular coating and subsequently exhibits absorptive characteristics (unpublished data); therefore, as the basal lamina is not degraded, cellular fragments in the Malpighian tubule lumen may be transported to the hindgut to be used as an additional source of nutrients, similar to reports regarding the midgut metamorphosis of *M. quadrifasciata anthidioides* (Neves et al. 2003) and *Bombyx mori* (Lepidoptera) (Franzetti et al. 2015).

The Malpighian tubules of bee larvae are fully histolyzed during metamorphosis (Oertel 1930; Cruz-Landim 2000), which occurs through cell death via apoptosis and autophagy as shown in this study. However, in *Drosophila melanogaster* (Diptera), the Malpighian tubules do not undergo histolysis (Shukla and Tapadia 2011; Tapadia and Gautam 2011), suggesting different mechanisms among different insects.

This is the first report of the occurrence of apoptosis in the Malpighian tubules of L5S larvae and prepupae of *A. mellifera*, with apoptotic cells showing nuclear chromatin condensation, increased vacuolization, endoplasmic reticulum collapse, mitochondrial fragmentation and apical cell fragments cast off to the lumen as reported for other organisms (Karbowski and Youle 2003; Skulachev et al. 2004; Houwerzijl et al. 2004; Arbustini et al. 2008; Kroemer et al. 2009; Cruz et al. 2013; Carvalho et al. 2015).

Apoptosis in the Malpighian tubules studied here is stimulated by the presence of cells containing cleaved caspase-3. Apoptosis can be triggered by different biochemical pathways with initiator caspases stimulating cleavage of executor ones such as caspase-3 (Fuchs and Steller 2011). In *D. melanogaster*, expression of apoptotic proteins in the Malpighian tubules during metamorphosis does not trigger cell death, but the imbalance in the expression of these proteins results in malformed Malpighi tubules (Shukla and Tapadia 2011; Tapadia and Gautam 2011).

Besides cell death via apoptosis, in *A. mellifera*, the Malpighian tubules of L5S larvae show autophagy, evidenced by the presence of LC3 positive cells. However, the ultrastructural analyses show few autophagosomes in these cells. Thus, we suggest that in *A. mellifera*, apoptosis is the main mechanism of cell death in the Malpighian tubules, although autophagy occurs at a low rate, similar to reported for the midgut (Cruz et al. 2013) and fat body (Santos et al. 2015) of bees.

Together with degeneration of the Malpighian tubules, the formation of new Malpighian tubules occurs in the L5S larvae of *A. mellifera.* The intense cell proliferations contribute to the formation and growth of the new Malpighian tubules, which remain with their free distal ends in the hemocoel. In *D. melanogaster* and *Tribolium castaneum* (Coleoptera), the distal end of the Malpighian tubules has the *Tip cell*, which anchors this organ in the gut, releasing a cell proliferation factor that extends the tubules from their distal end (Skaer 1989; Denholm 2013). In the *A. mellifera* studied here, there is no *Tip cell* and cell proliferation occurs along the entire length of the Malpighian tubules, suggesting that an extrinsic factor may regulate cell proliferation, unlike what occurs in the Malpighian tubules of *D. melanogaster* (Denholm 2013).

The new Malpighian tubules initiate their formation in L5S larvae and undergo morphological changes until the *A. mellifera* adult phase. In prepupae, new Malpighian

tubules have enlarged lumen and widened intercellular spaces up to the apical cell region, whereas until light-brown-eyed pupae narrowing of the lumen occurs, the intercellular spaces are shortened, and the cells have few mitochondria, microvilli and an absence of spherocrystals in the cytoplasm. Similar features have been reported in the Malpighian tubules during the post-embryonic development of *R. prolixus* and *Calpodes ethlius* (Lepidoptera) (Ryerse 1979; Skaer et al. 1990). Thus, we suggest that new Malpighian tubules, from prepupae until light-brown-eyed pupae of *A. mellifera* have low metabolic activity or may be non-functional.

The Malpighian tubules of *A. mellifera* increase in diameter as they progress in their development, with the diameter of the Malpighian tubules increasing from dark-brown and black-eyed pupa. Furthermore, there is the microvilli elongation, an increase in the mitochondrial population, presence of spherocrystals and the formation of the basal labyrinths. During these developmental phases, the Malpighian tubules show contact with tracheoles on their outer surface. These features are present in insects with Malpighian tubules active in excretion (Berridge and Oschman 1969; Ryerse 1979; O'donnell et al. 1985; Skaer et al. 1990; Cruz-Landim 1998; King and Denholm 2014; Gonçalves et al. 2014), suggesting that in *A. mellifera* the onset of the excretion process during metamorphosis occurs in the dark-brown-eyed pupae, c.a. five days after molt from larvae to pupae (Jay 1962; Michelette and Soares 1993; Eichmüller 1994).

In the Malpighian tubules of newly emerged *A. mellifera* adult workers, there are cells of different electron-densities with differences in the basal labyrinth and microvilli. In *D. melanogaster* the invasion of mesodermal cells that differentiate into stellate cells in the Malpighian tubules has been reported (Denholm et al. 2003; Denholm 2013). However, in *A. mellifera* there is no evidence of this cellular invasion. Although the cells of the Malpighian tubules of the newly emerged *A. mellifera* adults have variations in their microvilli, they have many mitochondria and spherocrystals, characteristics of

cells with excretion activity, which may store or transport to the lumen compounds from the hemolymph (Wigglesworth and Salpeter 1962; Berridge and Oschman 1969; Cruz-Landim 1998; Gonçalves et al. 2014).

From the beginning of their formation in L5S to the newly emerged adult, the cells of the *A. mellifera* Malpighian tubules have some "glycogen islands" in the cytoplasm. This feature was reported in the stingless bees *M. quadrifasciata anthidioides* and *Trigona spinipes* (Silva-de-Moraes and Cruz-Landim 1976; Serrão and Cruz-Landim 2000) and insects undergoing metamorphosis (Dean et al. 1985; Conti et al. 2010; Carvalho et al. 2013; Franzetti et al. 2016). Thus, in *A. mellifera* these "glycogen islands" may be a necessary energy reserve (Arrese and Soulages 2010) for intense cellular remodeling and the onset of excretory activity.

In *A. mellifera* adult forager workers, we also found two cell types in the Malpighian tubules. However, only electron-dense cells have morphology similar to that reported as principal cells in other insects (Palmer et al. 1986; Hanrahan and Nicolson 1987; Wessing et al. 1999; Beyenbach 2003). In addition, *A. mellifera* electron-lucent cells are not similar to stellate cells (Beyenbach 2003; Beyenbach et al. 2010).

The electron-dense cells of *A. mellifera* have many spherocrystals and mitochondria, a long and narrow basal labyrinth and well-developed microvilli, features of cells that actively store and transport solutes from the hemolymph (Berridge and Oschman 1969; Bell and Anstee 1967; Beyenbach et al. 2003; Beyenbach et al. 2010). The architecture of the basal labyrinth and microvilli also suggests the formation of an osmotic gradient for the water flow towards the organ lumen (Berridge and Oschman 1969).

At the distal end of the Malpighian tubules of *A. mellifera* forager workers, the electron-dense cells have many vacuoles, similar to that reported for *Solenopsis saevissima* (Hymenoptera), *Acheta domesticus* (Orthoptera) and *Macrosteles fascifrons*

(Hemiptera) (Smith and Littau 1960; Hazelton et al. 2001; Arab and Caetano 2002). In the *A. mellifera* studied here, these characteristics suggest a rapid release of vacuolar content to the lumen; promoting flow from the distal to the proximal end of the Malpighian tubules as supposed to occur in other insects (Smith and Littau 1960; Hazelton et al. 2001; Klowden, 2007).

The presence of electron-lucent cells is present in newly emerged adult bees onward, which have few cytoplasm vesicles and spherocrystals. However, only in forager bees the spherocrystals are absent. The absence of spherocrystals has also been reported in the stellate cells of *D. melanogaster* and *Calliphora erythrocephala* (Diptera), which has been associated with low activity in the transport of solutes to the lumen (Berridge and Oschman 1969, Wessing et al. 1999; Beyenbach 2003). In *R. prolixus*, cells without spherocrystals play a role in reabsorption of compounds from the primary urine in the proximal region of the Malpighian tubules (Wigglesworth and Salpeter, 1962; Maddrell 1991).

In *A. mellifera* electron-lucent cells have long microvilli and an extensive and enlarged basal labyrinth. Although it is difficult to determine the direction of fluid transport, the ultrastructural characteristics of these were similar to those reported for the midgut of *B. mori* and the Malpighian tubules of *M. quadrifasciata anthidioides*, *R. prolixus*, *Musca domestica* (Diptera*)* and *Macrosteles fascifrons* (Hemiprera), which function in the absorption of substances from the organ lumen (Smith and Littau 1960; Sohal et al. 1974; O'donnell et al. 1985; Cruz-Landim 1998; Gonçalves et al. 2014).

Our findings during the metamorphosis of *A. mellifera* show the occurrence of apoptosis and autophagy in the degeneration of the Malpighian tubules. Concomitant with this degeneration, new Malpighian tubules are formed with cells showing ultrastructural characteristics of absorption even during the metamorphosis, perhaps due to the need to eliminate metabolites during development (Skaer et al. 1990). However,

from white-eyed to light-brown eyed pupae, *A. mellifera* Malpighian tubules seem inactive, suggesting that in these early stages of metamorphosis, bees may be more susceptible to the action of toxic compounds, since *A. mellifera* larvae exposed to sublethal doses of insecticides die when they are in the pupal phase or after defecation of the larvae when exposed to fungicides (Mussen et al. 2004; Xavier et al. 2015).

This work provides temporal and morphological parameters of the Malpighian tubules of bees, becoming a resource for understanding of its formation and of necessary mechanisms for the process of excretion insects.

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CAPÍTULO 2

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

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.

Keywords: Ileum, Rectum, Remodeling, Cuticular degradation, Degradation of the basal lamina.

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 a 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 in *Rhodnius prolixus* (Casida & Maddrell, 1971; Singh & 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, 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 midgut-hindgut passage in the prepupae, the hindgut epithelium is disorganized, and the lining cuticle and visceral muscles are lost (Dobrovsky, 1951).

During bee metamorphosis, mitoses promote the 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 & Silveira-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 & Silveira-Mello, 1970; Cruz-Landim & Calvalcante, 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.

The objective of this study was to describe the morphological changes that take place in the hindgut of *A. mellifera* during metamorphosis and the cellular events associated with these modifications.

Materials and Methods

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).

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 hours. 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. 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. 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).

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 intestine near the pylorus) and descending region (final prolongation of the intestine) were isolated. 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. 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 minutes with 1% aqueous uranyl acetate and lead citrate for 10 minutes (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.

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 hours. Samples were then washed in 0.1 M sodium phosphate buffer pH 7.2 plus 1% Tween-20 (PBST) for 2 hours. 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 incubated for 30 min with the nuclear marker TO-PRO-3 Iodide (1:1000) (Life Technologies). The samples were washed and mounted in Mowiol (Sigma-Aldrich, USA) and analyzed with a Zeiss LSM-META (Carl Zeiss AG, Oberkochen, Germany) confocal microscope at the Center for Microscopy and Microanalysis of the Federal University of Viçosa. Cell proliferations along the hindgut were counted and statistical data were obtained from the computer program GraphPad Prism 5 (GraphPad Software, Inc.), being analyzed through Tukey's test (Significance, $P < 0.05$).

For negative controls, incubation with primary antibodies was omitted.

Results

Morphology

In L5 and L5S larvae, the epithelia of the ascending regions (precursor of the ileum) and descending regions (precursor of the rectum) were similar, consisting of columnar cells, a thin cuticle, and circular muscles (Fig. 1a). Epithelial cells had many mitochondria and invaginations of the apical plasma membrane, which were more developed in cells of the ascending region (Fig. 1b, c). The nucleus of these cells had a predominance of decondensed chromatin and an evident nucleolus (Fig. 1d). The perinuclear region showed a rough endoplasmic reticulum and cytoplasmic spaces similar to "glycogen islands" (Fig. 1d, e). In addition, this epithelium had regular intercellular channels and a thick basal lamina (Fig. 1d).

The hindgut epithelium of prepupae had cells with nuclei at different heights and many vesicles in the apical cytoplasm (Fig. 1f). The cuticle and muscle layer were disorganized (Fig. 1f), with the cuticle containing electron-lucid regions (Fig. 2b, 2c). The cytoplasm was rich in mitochondria, electron-dense vesicle content, and autophagosomes (Fig. 1g, h), which contained mitochondrial debris inside them (Fig. 1i, j). The intercellular spaces were large and branched, and the basal lamina disorganized (Fig. 1k).

At the beginning of the pupal stage, in white- and pink-eyed pupae, the hindgut epithelium of the precursor region of the ileum (ascending region) had cells with nuclei at different heights, characterizing a pseudostratified epithelium, whereas the precursor region of the rectum (descending region) had inclined cells with aligned nuclei (Fig. 2a, b). In both ascending and descending regions, the cuticle was disorganized with an acidophilic epicuticle and a basophilic endocuticle (Fig. 2a), which under high resolution showed only an organized epicuticle (Fig. 2c, d). Epithelial cells showed an apical plasma membrane with short projections to the lumen and coated vesicles (Fig. 2e). The pseudostratified epithelium had enlarged intercellular spaces with some narrowed regions (Fig. 2d, e), and the basal lamina was absent (Fig. 2d). In the middle-apical cytoplasm, there were many mitochondria (Fig. 2c), vesicles (Fig. 2e), microtubules, and autophagosomes (Fig. 2d, g, h).

In light-brown-eyed pupae, the ileum epithelium had cells with nuclei in regular positions, large cytoplasmic spaces, and many large vesicles (Fig. 3a). However, the rectum epithelium showed flattened cells and nuclei (Fig. 3b). Throughout the hindgut epithelium, the apical cell surface was weakly stained (Fig. 3a), and the muscles were arranged in circular and longitudinal layers (Fig. 3c). The new cuticle deposition started with the epicuticle (Fig. 3d, e, f), and ileum cells were rich in large vesicles with electron-dense content (Fig. 3g). The formation of the basal lamina began at this

developmental stage (Fig. 3h). In a more advanced stage of the cuticle deposition, the ileum cells had few vesicles and narrow and sinuous intercellular spaces (Fig. 3i, j); rectum cells were flattened, with wide and sinuous intercellular spaces (Fig. 3k, l).

The ileum epithelium of dark-brown-eyed pupae showed a folded lining cuticle (Fig. 4a) and a basal lamina invading the intercellular spaces (Fig. 4b). In the basal cell region, there were large mitochondria, whereas in the apical cell region there were few vesicles and small mitochondria (Fig. 4c, d). The rectal epithelium was flattened (Fig. 4e), with few mitochondria and profiles of rough endoplasmic reticulum (Fig. 4f, g). These cells rested on a thin basal lamina (Fig. 4h), followed by muscle cells similar to those of light-brown-eyed pupae. Until adult emergence, the rectum did not display other morphological changes.

In the black-eyed pupae, the ileum cells showed many cytoplasmic vacuoles and an acidophilic apical portion (Fig. 5a, b, c), mainly in the anterior region of the organ (Fig. 5a, b), in which the subcuticular spaces were larger (Fig. 5d, e) and the basal lamina thicker (Fig. 5f, g) than in the other regions of the ileum. Tracheoles began to appear in the intercellular spaces (Fig. 5b, f).

In newly emerged adult bees, cells of the ileum had a nucleus with partially condensed chromatin and a more electron-dense cytoplasm than those found during the metamorphosis, with few vesicles, "glycogen islands", and large mitochondria (Sup. 1a, b). The intercellular spaces were dilated and sinuous (Sup. 1a).

Forager workers had the ileum epithelium with different cell types. Those in the anterior region showed narrower invaginations in the apical plasma membrane that reached the median cell portion (Fig. 6a, b, c), associated with larger mitochondria (Fig. 6b), than those in the other cytoplasmic portions (Fig. 6c). The intercellular spaces were sinuous, and the basal lamina thick (Fig. 6d, e). The cells of the posterior region of the ileum had longer, narrower, and more irregular apical membrane invaginations,

associated with small mitochondria (Fig. 7a, b, c), than cells of the anterior region. The basal lamina was thin, and the intercellular spaces regular and narrower (Fig. 7d, f) than those of the anterior region. Both cell types had some autophagosomes (Fig. 6f, 7e).

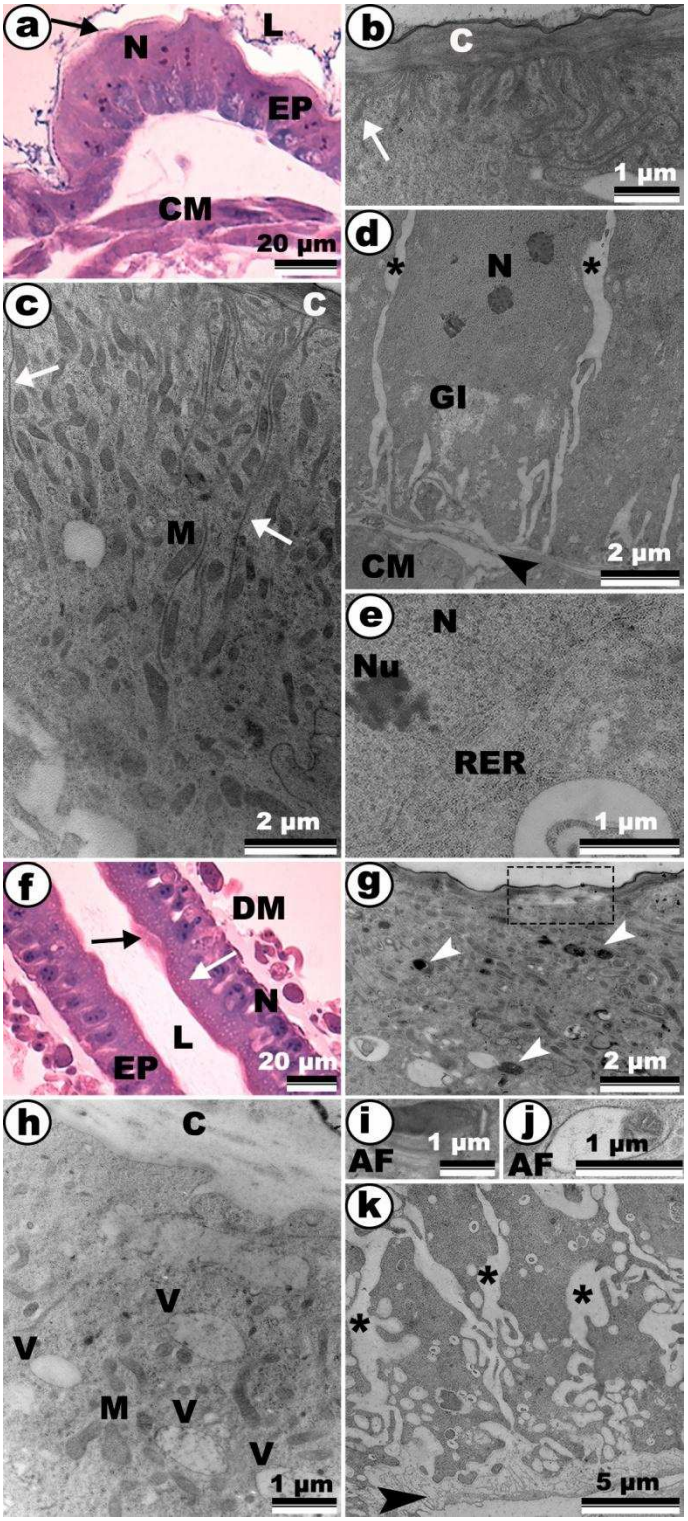


Fig. 1: Micrographs of the hindgut of *Apis mellifera* in the last larval instar (L5 and L5S) and prepupae. [**a**] Epithelium (EP) of the L5S hindgut showing columnar cells and nucleus (N). Note the thin cuticular lining (black arrow), circular musculature (CM) and

intestinal lumen (L). [**b-c**] Larva L5S showing cells of the intestine with short invaginations of the apical plasma membrane (white arrows) and cells from the posterior region with long invaginations (white arrows) and mitochondria (M). Note the cuticular lining (C). **[d-e]** Mean-basal position of L5S larvae showing nuclei (N), nucleoli (Nu), rough endoplasmic reticulum (RER) and "glycogen islands" (GI). Note the intercellular spaces (asterisk), basal lamina (arrowhead) and circular muscle (CM). [**f**] Epithelium of the hindgut (EP) of prepupae showing columnar cells with nuclei (N) in different heights and many vesicles (white arrow). Note the cuticular lining (black arrow) and disorganized musculature (DM). **[g-h]** Prepupae with the apical portion of an intestinal cell showing many autophagosomes (white arrowhead), mitochondria (M) and vesicles of electron-dense content (V). Note the cuticles (C) with electron-lucid sites (dashed square). **[i-j]** Detail of autophagosomes (AF). **[k]** Prepupae with the basal region of the intestinal epithelium showing a network of intercellular spaces (asterisks) and disarranged basal lamina (black arrowhead).

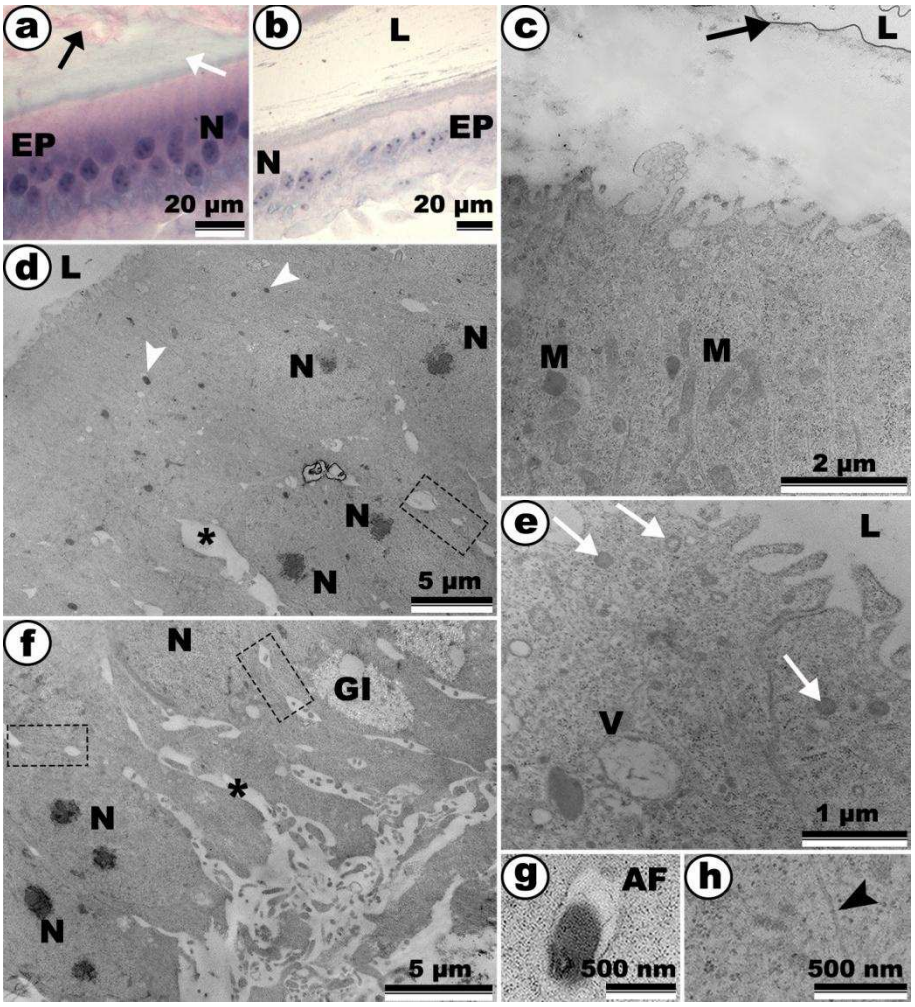


Fig. 2: Micrographs of the posterior intestine of *Apis mellifera* in the pupae phases of white and pink eyed. [**a**] White-eyed pupae with precursor intestinal epithelium (EP) of the ileum presenting cells with many nuclei (N) in various heights. Note the cuticular lining with epicuticle (black arrow) and procuticle (white arrow) irregular. [**b**] Pink-eyed pupae with epithelial (EP) precursor of rectum showing inclined cells of nuclei (N) aligned. Note the luminal region (L). **[c-f]** White-eyed pupa with the apical-basal region of its cells showing nuclei (N) at various heights, mitochondria (M), many autophagosomes (white arrowhead), vesicle coated (white arrows), few vesicles and "glycogen islands" (GI). Note the cuticle only with a trace of the epicuticle (black arrow) in the lumen (L) and the intercellular spaces (asterisk) long and interrupted by cellular contacts (dashed rectangles). **[g-h]** Detail of autophagosomes (AF) and abundant microtubules (black arrowhead) in the cytoplasm.

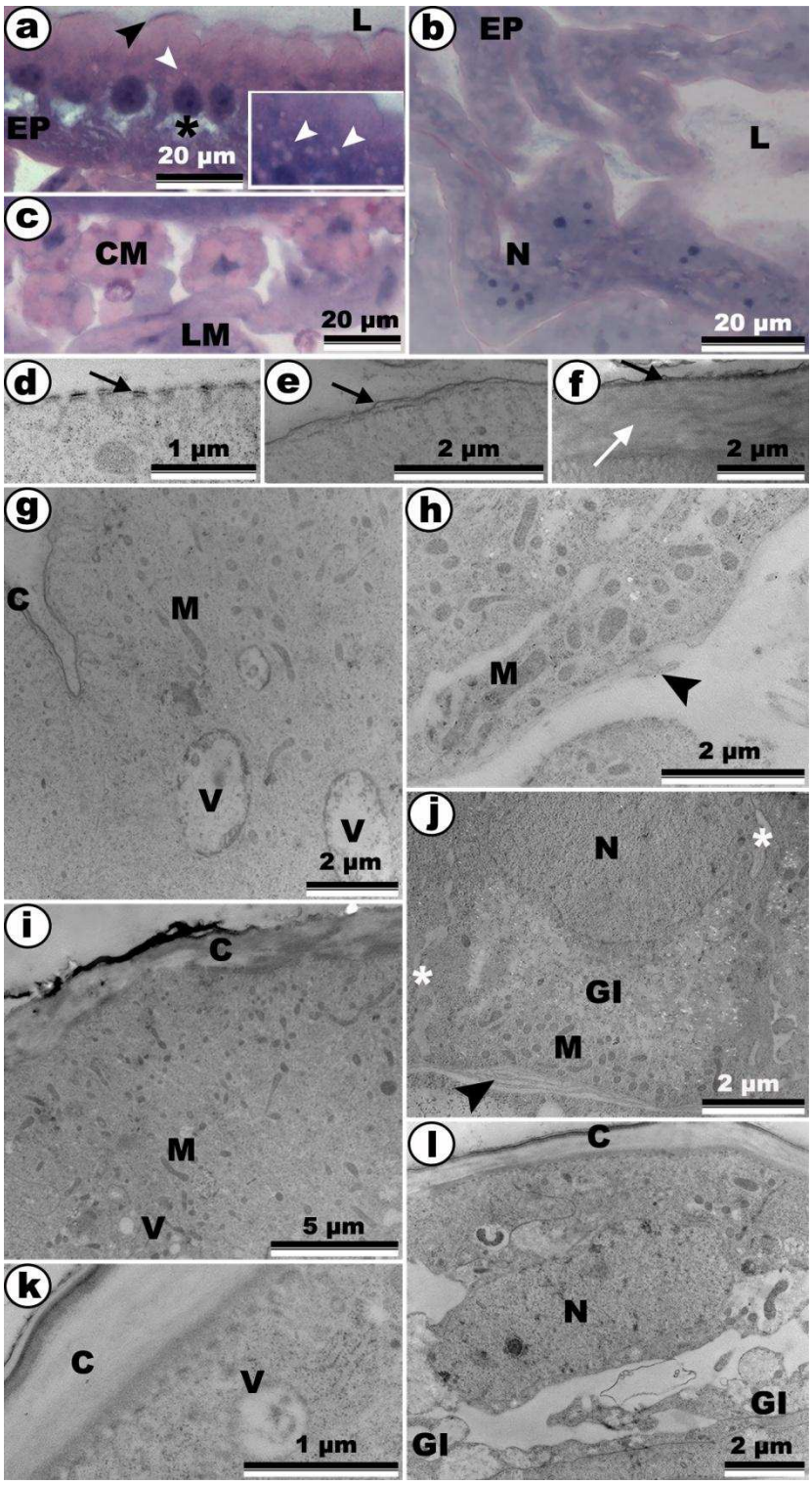


Fig. 3: Micrographs of the hindgut of *Apis mellifera* in the light-brown-eyed pupae phase. **[a]** Intestinal epithelium (EP) precursor of the ileum showing columnar cells, nuclei aligned (N), many vesicles (arrowhead) and cytoplasmic spaces (asterisk). [**Insert**] Detail of the apical pole of a cell with many vesicles (arrowhead). [**b**] Intestinal epithelium (EP) precursor of the rectum showing lower cells and nuclei (N). Note the lumen (L). **[c]** New muscle lining with circular fibers (C) and longitudinal fibers (LM). **[d-f]** Deposition of the cuticular layer with the epicuticle (black arrow) synthesized

before the procuticle (white arrow). **[g-h]** Epithelium of the ileum with a thin cuticle layer (C), return of the basal lamina (black arrowhead) and with cells showing many bulky vesicles (V) and mitochondria (M). **[i-j]** Epithelium of the ileum with a thick cuticle layer (C), thick basal lamina (black arrowhead), regular intercellular spaces (asterisks) and with cells showing mitochondria (M), "glycogen islands" (GI) and few vesicles (V). **[k-l]** Rectal epithelium with thick cuticle layers showed cells with flat (N) nuclei and many "glycogen islands" (GI).

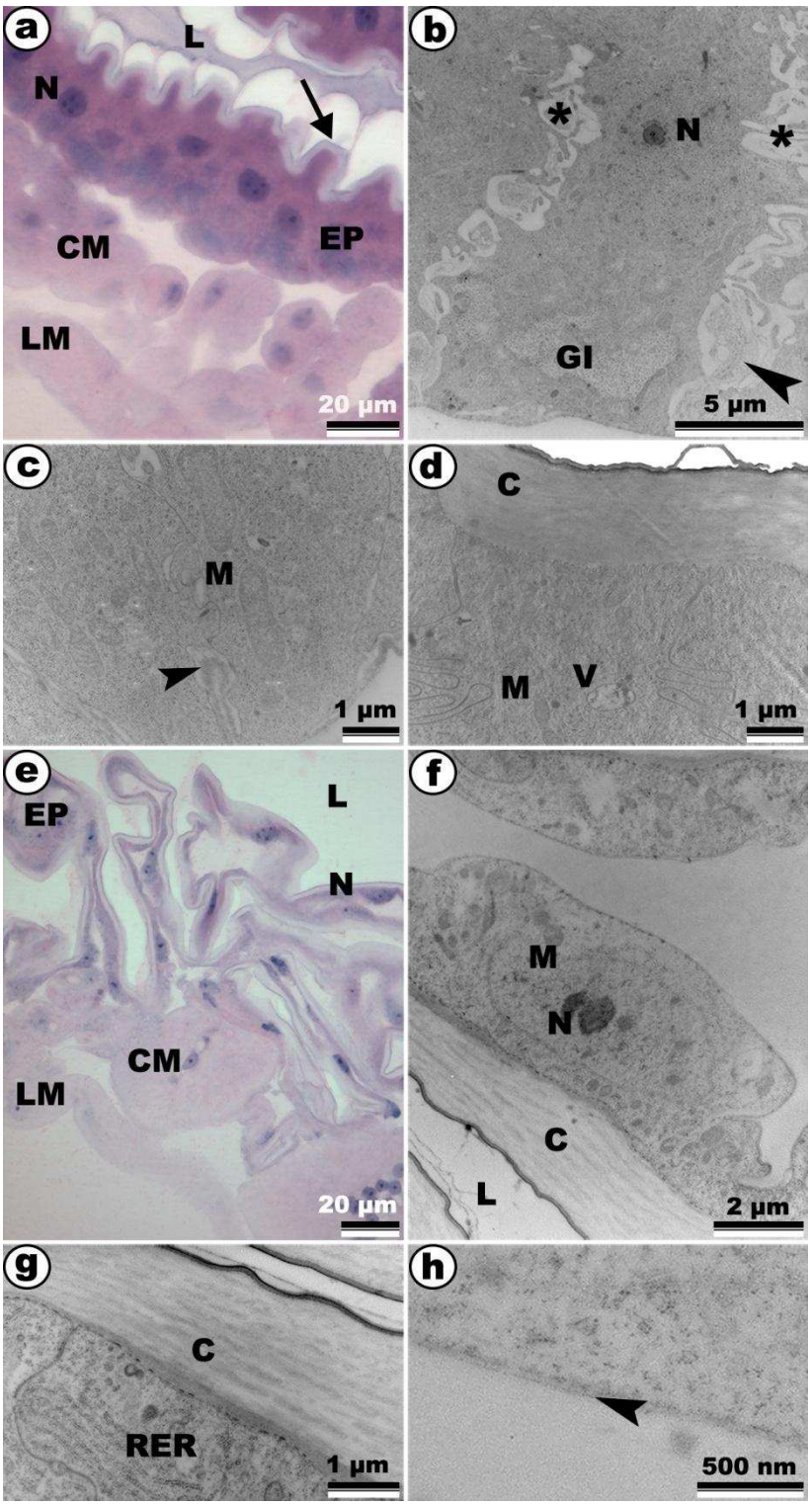


Fig. 4: Micrographs of the epithelium of the ileum and rectum of *Apis mellifera* in the phase of dark-brown-eyed pupae. [**a**] Epithelium (EP) of the ileum with columnar cells and aligned nuclei (N). Note the luminal region (L), the corrugated cuticular lining (black arrow) and the circular (CM) and longitudinal (LM) muscle fibers. **[b]** Overview of the intestinal cell of the ileum showing nucleus with predominance of decondensed chromatin and "glycogen islands" (GI). [**c**] Basal pole of cell of the ileum showing large mitochondria (M). Note the intercellular space (asterisks) and basal lamina (black

arrowhead). [**d**] Apical pole of the cell with few mitochondria (M) and vesicles (V). Note the cuticle (C). [**e**] Epithelium (EP) of the rectum with cells and nuclei (N) very flattened. Note the lumen (L) and circular (CM) and longitudinal (LM) muscle fibers. **[f-h]** In general, the squamous cells of the rectal epitheliumshowed nuclei with predominance of decondensed chromatin (N), few mitochondria (M) and rough endoplasmic reticulum (RER). Note the cuticle (C) and the return of the basal lamina (black arrowhead).

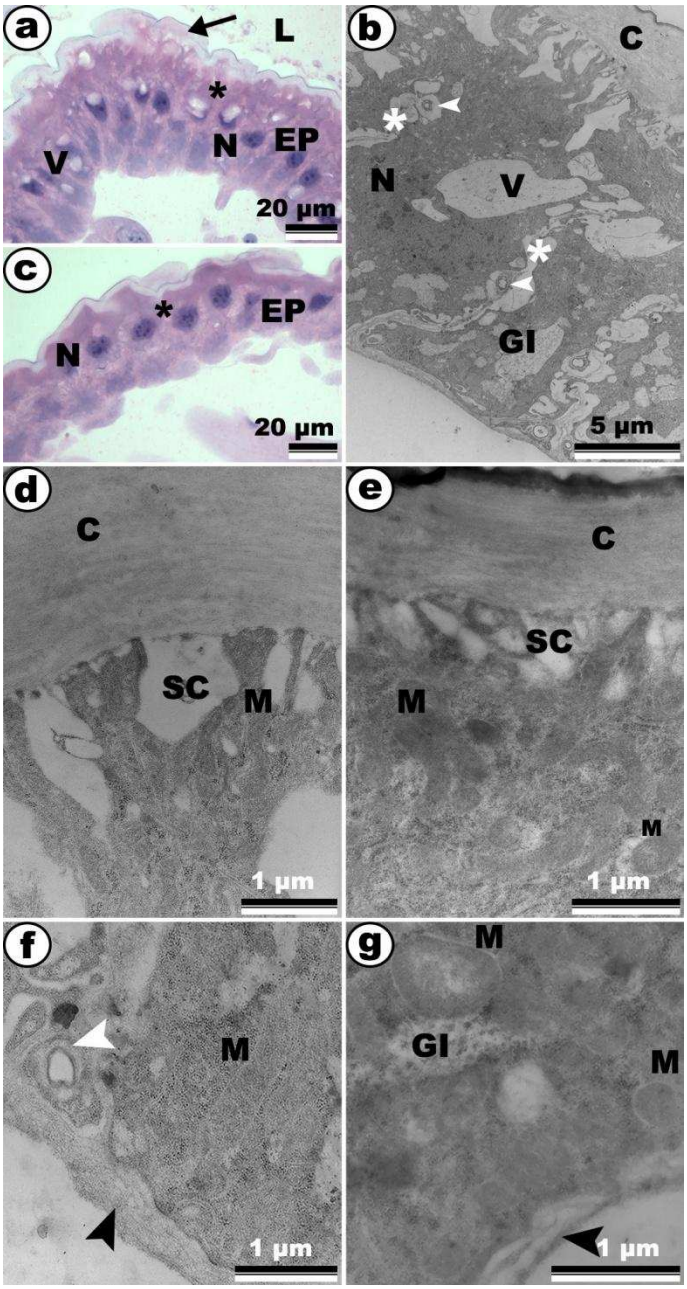
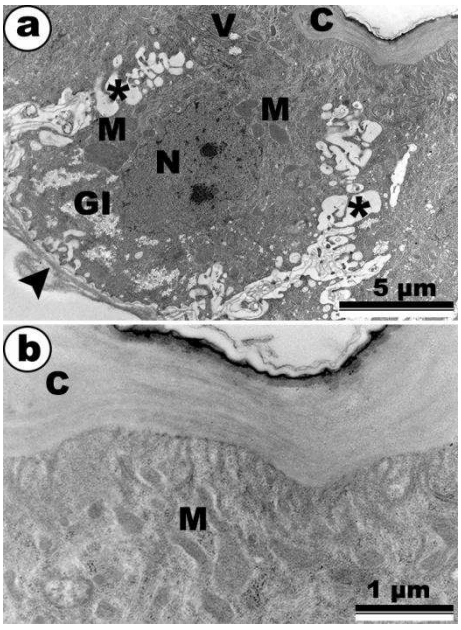


Fig. 5: Micrographs of the ileal epithelium of *Apis mellifera* in the phase of black-eyed pupae. [**a**] Epithelium (EP) of the anterior region of the ileum with columnar cells showing an eosinophilic zone at the apical pole (asterisk) and vacuoles (V) near the

nuclei (N). Notice the cuticle (black arrow) and the lumen (L). **[b]** Cells of the anterior region of the ileum with cytoplasm and electron-dense nuclei (N), bulky vacuoles (V) and "glycogen islands" (GI). Note the cuticle (C) and tracheoles (arrowhead) within the intercellular spaces (asterisks). **[c]** Epithelium (EP) of the posterior region of the ileum showing columnar cells with nuclei (N) and an eosinophilic zone at the apical pole (asterisk). **[d-e]** Cell of the anterior region of the ileum forming voluminous subcuticular spaces (SC) and cell of the posterior region with small subcuticular spaces (SC) and mitochondria (M). Note the cuticular lining (C). **[f-g]** Cell of the anterior region of the ileum lined with a thick basal lamina (black arrowhead) and tracheiolas (white arrowhead) and cell of the posterior region of the ileum with mitochondria (M), "glycogen islands" (GI) and lined by thin basal lamina (black arrowhead).



Sup. 1: Micrographs of the epithelium of the ileum of *Apis mellifera* newly emerged to adult phase. **[a]** Cell showing electron-dense nucleus (N), large mitochondria (M), rare vesicles (V) and "glycogen islands" (GI). **[b]** Apical pole of the cell with small mitochondria (M). Note the cuticular lining (C), basal lamina (black arrowhead) and intercellular spaces (asterisk).

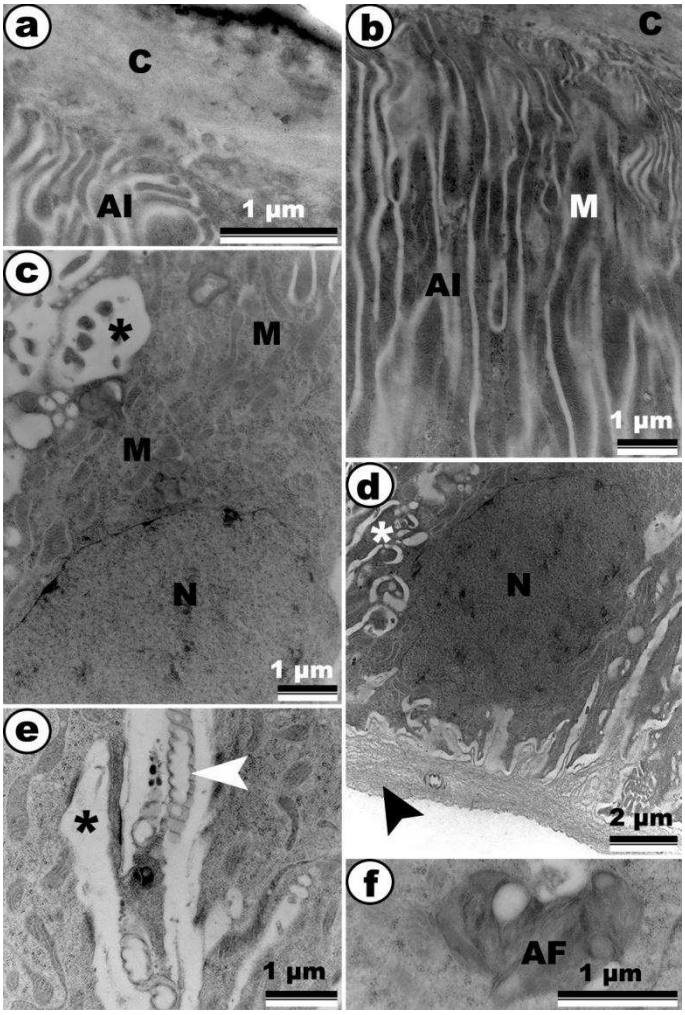


Fig. 6: Cell micrographs of the anterior region of the ileum of forager *Apis mellifera*. [**a-b**] Apical region of the cell showing the apical plasma membrane with long invaginations and (AI) associated with elongated mitochondria (M). **[c-e]** Middle-basal region of the cell exhibiting electron-dense nucleus (N) and many small mitochondria (M). **[f]** Detail of an autophagosome (AF) in the cytoplasm. Note the cuticle (C), bulky and bifurcated intercellular spaces (asterisks), tracheoles (white arrowhead) and thick basal lamina (black arrowhead).

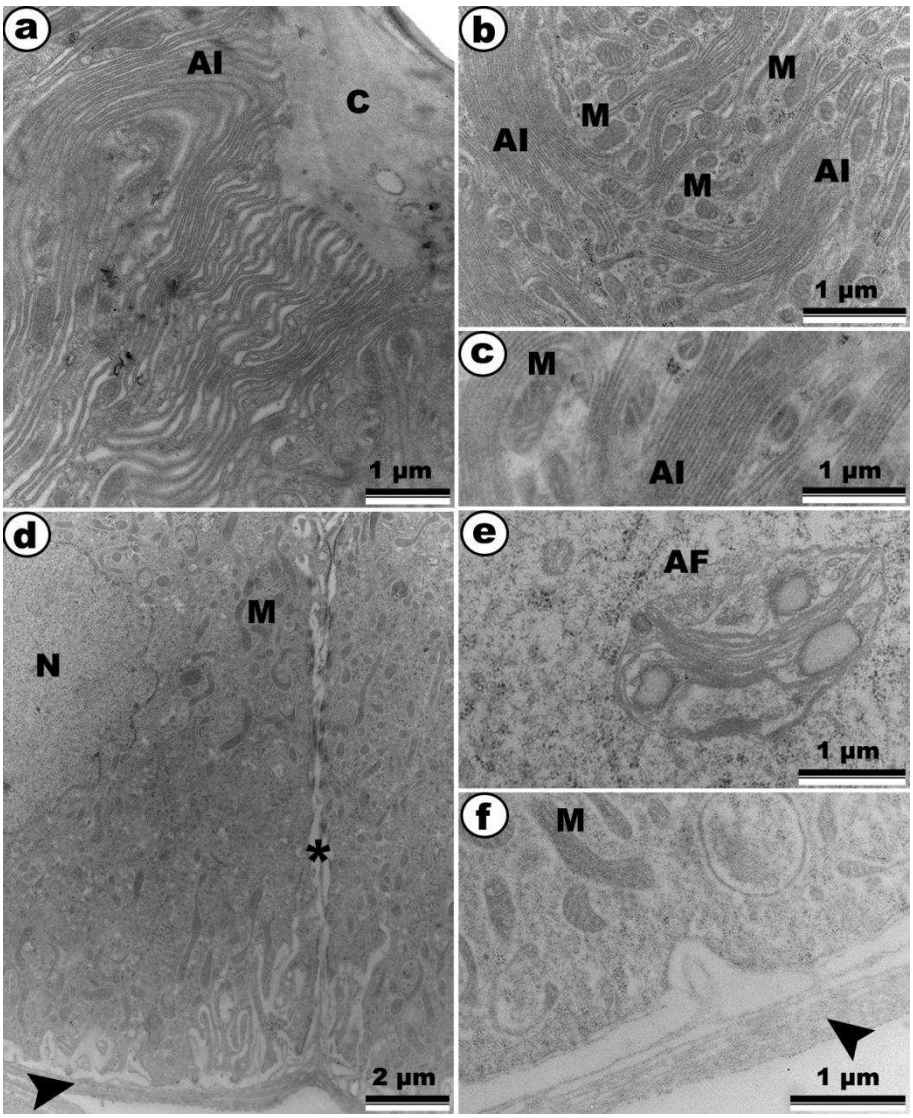


Fig. 7: Cell micrographs of the posterior region of the ileum of forager *Apis mellifera*. **[a-c]** Apical region of the cell showing the apical plasma membrane with invaginations (AI) very sinuous and associated with many small mitochondria (M). Middle-basal region of the cell exhibiting nucleus (N) electron-lucid, mitochondria (M) and autophagosomes (FA). Note the cuticular lining (C), the thin basal lamina (black arrowhead) and the narrow intercellular spaces (asterisk).

Cellular events: proliferation, apoptosis, and autophagy

In the hindgut epithelium, cell proliferation started in L5S larvae and stopped in dark-brown-eyed pupae (Fig. 8).

In L5S larvae, cell proliferation was abundant in the pylorus and in the descending region (precursor of the rectum) (Fig. 9a, b). In prepupae, cell proliferation

increased in these regions and also occurred in the ascending region (precursor of the ileum) (Fig. 9c, d). In white-eyed pupae, cell proliferation occurred throughout the hindgut epithelium (Fig. 9e, f, g), but from pink-eyed to brown-eyed pupal stages, cell proliferation was gradually reduced, with the anterior and posterior regions of the ileum showing less cell proliferation (Fig. 9i, l) than the pylorus (Fig. 9h, k, n) and rectum regions (Fig. 9j, m, o). Muscle cell proliferation occurred only in pink- and light-brown-eyed pupae (Fig. 9p).

Apoptosis occurred throughout the hindgut epithelium from L5S larval to white-eyed pupal stages (Fig. 10a, b), whereas in muscle cells, apoptosis occurred from prepupae (Fig. 10c) to pink-eyed pupae (Fig. 9p).

In the rectum, the epithelial cells showed many apoptotic cells from the stage of L5S larvae (Fig. 10e) to the pink-eyed pupae (Fig. 10i, j). In prepupae, there were many apoptotic muscle cells (Fig. 10f), decreasing in number in the white (Fig. 10g) and pink-eyed pupal stages (Fig. 10h, i).

Autophagy was detected in the muscle cells of L5S larvae (Fig. 11a), white- and pink-eyed pupae (Fig. 11d), and in the epithelial cells of prepupae (Fig. 11b, c). Autophagy was scarce in the later pupal stages and in adult bees (Fig. 11e).

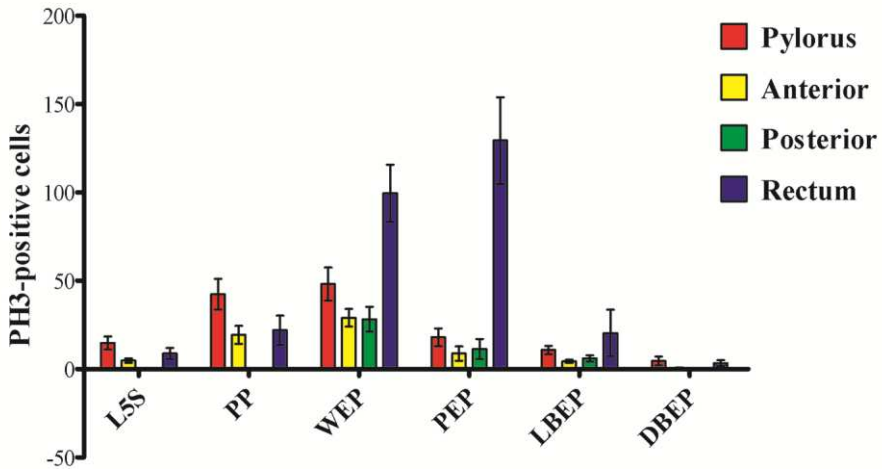


Fig. 8: Graphic with cells of *Apis mellifera* positive for phosphorylated histone-H3 (showing cell proliferation) in segments of the hindgut precursors of the pylorus, of the

anterior and posterior regions of the ileum and the rectal region. Larvae (L5S), prepupae (PP) and pupae of eyed white (WEP), pink (PEP), light-brown (LBEP) and dark-brown (DBOP) were analyzed.

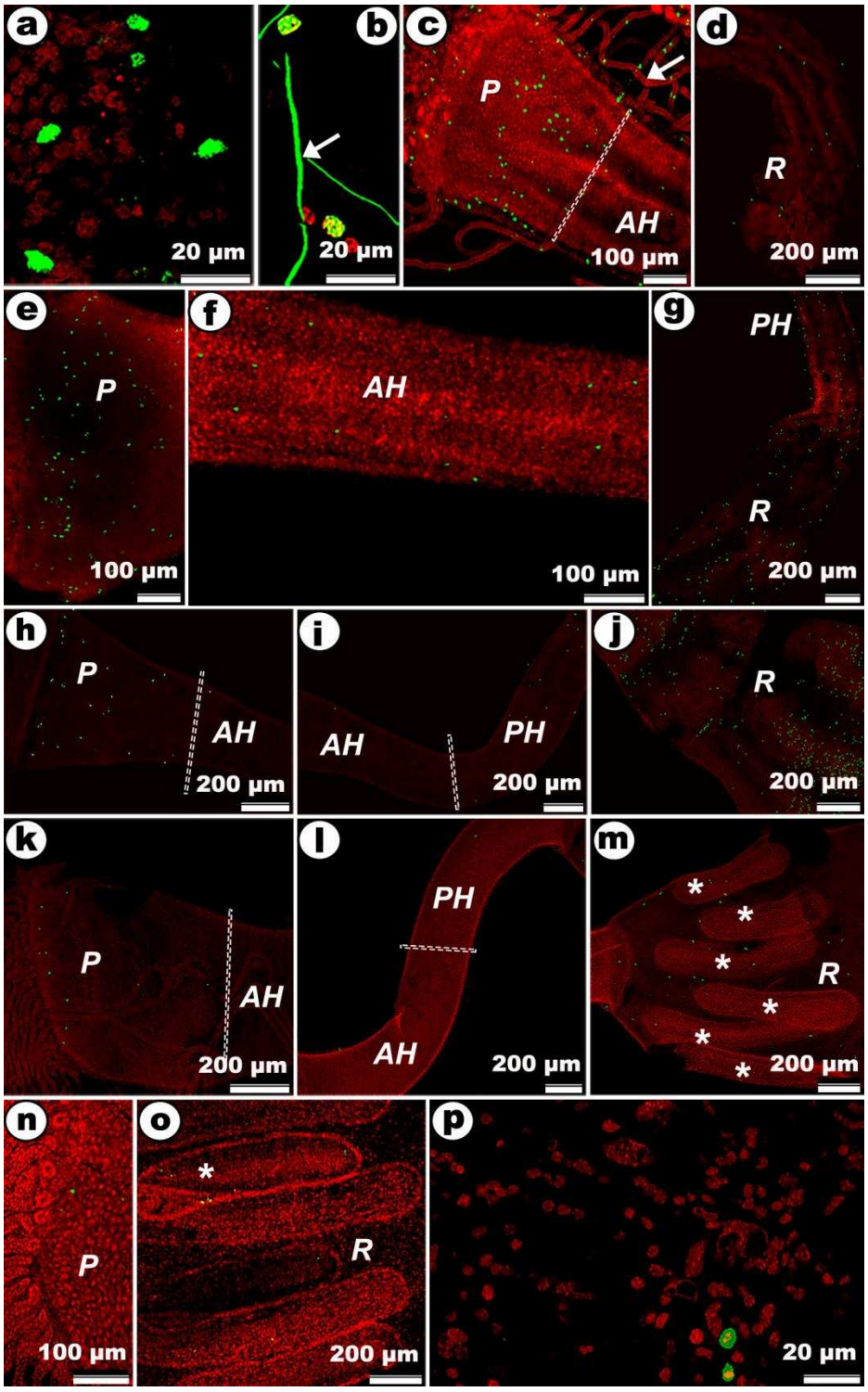


Fig. 9: Micrographs of cells of the hindgut of *Apis mellifera* positive for phosphorylated histone-H3 (showing cell proliferation). [**a**] L5S larvae with cells in the precursor region

of the pylorus positive for phosphorylated histone-H3 (green). [**b**] Larvae L5S with cells of the precursor region of the rectum positive for phosphorylated histone-H3 (green). Note small nuclei (red) of tracheoles (arrow) autoflowering. **[c-d]** Prepupae with cells of the pylorus (P) and cells of the gut regions precursor of the anterior half of the ileum (AH) and rectum (R) positive for phosphorylated histone-H3 (green). Note a line delimiting regions, nuclei (red) and Malpighian tubules positive for phosphorylated histone-H3 (arrow). **[e-g]** White-eyed pupae with cells of the pylorus (P) and cells of the gut of regions precursors of the anterior half of the ileum (AH), posterior half of the ileum (PH) and the rectum (R) positive for phosphorylated histone-H3 (green). **[h-j]** Pink-eyed pupae and **[k-m]** light-brown-eyed pupae with many phosphorylated histone-H3 positive cells (green) in the pyloric (P) and rectal (R) regions, while in the anterior (AH) and posterior (PH) halves of the ileum there is a reduction in this positivity. Note the rectal papillae (asterisks), a line delimiting regions and nuclei (red). **[n-o]** Dark-brown-eyed pupae with rare phosphorylated histone-H3 (green) positive cells in the pylorus (P) and rectal papillae (asterisk) of the rectum (R). [**p**] Light-brown-eyed pupae with muscular lining showing cell positive for phosphorylated histone-H3 (green).

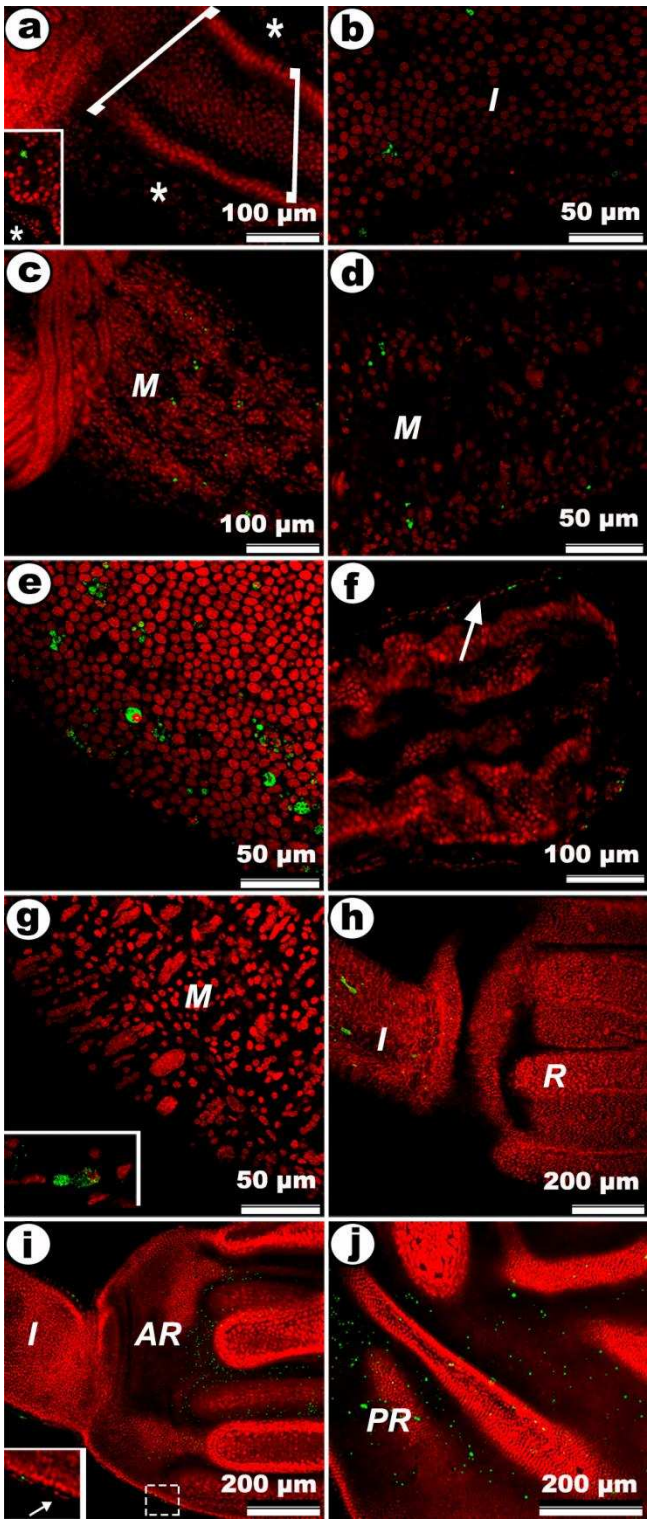


Fig. 10: Micrographs of cells of the ileum and rectum of *Apis mellifera* positive for cleaved caspase-3 (indicating apoptotic cells). [**a**] Prepupae with the epithelium of the ileum (bracket) and muscular lining (asterisks). [**Insert**] Detail of cell of the ileum (I) positive for caspase-3 cleaved (green). [**b**] White-eyed pupae with ileal epithelium showing positivity to cleaved caspase-3 (green). [**c**] Prepupae with muscle lining cells (M) of the ileum positive for cleaved caspase-3 (green). [**d**] White-eyed pupae with muscle lining cells (M) of the ileum positive for cleaved caspase-3 (green). Notice the

nucleus (red). [**e**] White-eyed pupae with epithelium of the rectum positive for cleaved caspase-3 (green). [**f**] Prepupae with the precursor region of the rectum with its muscular lining (arrow) positive for caspase-3 cleaved (green). [**g**] White-eyed pupae with its muscular lining (M) of the precursor region of the rectum. [**Insert]** Nucleus (red) of muscle cell with rare positivity for cleaved caspase-3 (green). [**h**] Pink-eyed pupae and its external surface of the ileum (I) and rectum (R), with positivity for cleaved caspase-3 (green) only in the muscular lining of the ileum. **[i-j]** Pink-eyed pupae with epithelium of ileum without positivity (I) and the epithelia of the anterior (RA) and posterior (PR) regions of the rectum with very positivity for cleaved caspase-3 (green). [**Insert**] Detail of the dashed region showing a rare positivity to cleaved caspase-3 (green) in the anterior (AR) muscle lining of the rectum.

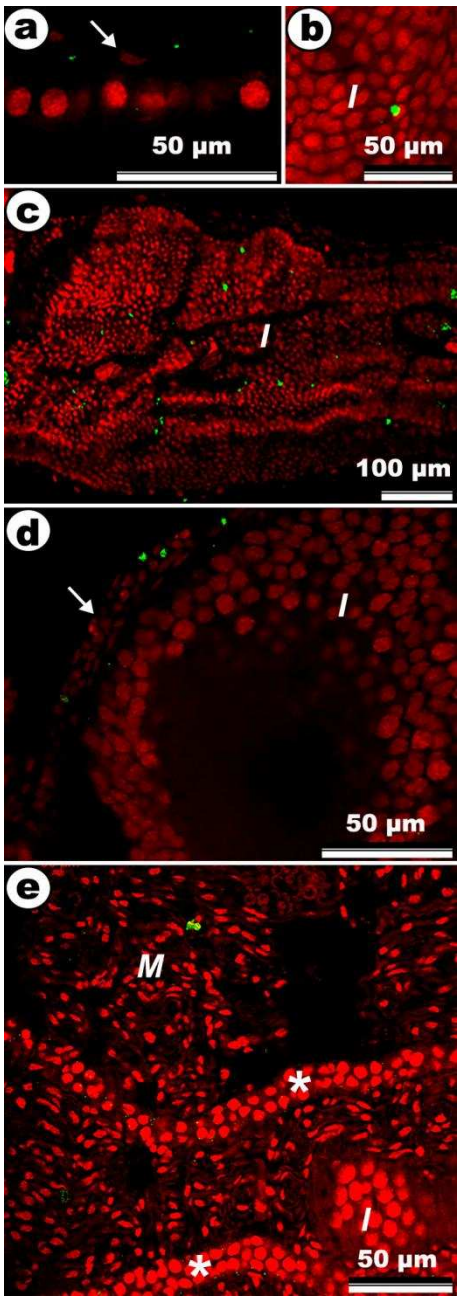


Fig. 11: Micrographs of cells of the ileum of *Apis mellifera* positive for proteins-LC3 (indicating autophagosomes). **[a]** Larvae L5S with muscular lining of ileum positive for proteins-LC3 (green). **[b-c]** Prepupae with ileal epithelium (I) showing high positivity for proteins-LC3 (green). **[d]** Pink-eyed pupae with the muscular lining (arrow) of the ileum (I) with high positivety for proteins-LC3 (green). **[e]** Dark-brown eyed pupae with muscular linnig (M) of ileum (I) with low positivity for proteins-LC3 (green). Note nuclei (red) of the muscle lining cells (M) and Malpighian tubules (asterisk).

Discussion

Our results show that during the metamorphosis of *A. mellifera*, the hindgut has a dynamic development, with the remodeling of epithelial and muscle cells and the deposition of new cuticle. Cell proliferation, apoptosis, and autophagy mediate these events.

The L5 and L5S larvae of *A. mellifera* have hindgut epithelial cells with many mitochondria and invaginations of the apical plasma membrane, which are longer in the posterior region of the organ. A regional morphological difference in the hindgut has been reported in the fruit fly *Drosophila melanogaster* larvae (Murakami & Shiotsuki 2001). In the insect ileum, cells with long invaginations of the apical plasma membrane are specialized in the absorption of compounds from the gut content (Cruz-Landim, 1994; Villaro et al., 1999). Thus, this characteristic of the descending region of the hindgut of *A. mellifera* larvae suggests a high activity in the transport of compounds, which may occur in L5S larvae, when the mid-hindgut transition is opened for excretion before the pupal molt (Dobrovsky, 1951; Cruz-Landim, 2000).

In prepupae of *A. mellifera*, the hindgut epithelium has a cuticle with electron-lucid regions and cells with many vesicles. The high number of vesicles may be due to enzyme secretion for the digestion of the larval cuticle, similar to what occurs in the body cuticle apolysis during insect molting (Reynolds & Samuels, 1996; Qu et al., 2014). The epithelial cell degradation is also suggested to occur by the presence of mitochondrial debris in autophagosomes (Heynen et al., 1985).

In the ileum of white- and pink-eyed pupae, the cuticle is almost completely absent, with only the presence of the epicuticle. In these stages of bee development, the epithelial cells have apical plasma membrane invaginations, a large number of coated vesicles and microtubules. These characteristics suggest that in the early stages of *A. mellifera* pupae, a mediated endocytosis of the gut lumen occurs, possibly of organic compounds of cuticular degradation, similar to that in the hindgut of *Locusta migratoria*

(Orthoptera) and the epidermis of *Calpodes ethlius* (Lepidoptera) during insect molting (Locke & Huie, 1979; Peacock, 1986). The reabsorbed compounds from the degraded cuticle are reused during the production of the new cuticle (Surholt, 1975; Kaznowsk et al., 1986; Reynolds & Samuels, 1996). Our findings show the existence of many autophagosomes after cuticle degradation, suggesting that this organelle may play a role in the recycling of absorbed cuticular compounds, since the fusion of autophagosomes with vesicles from the endocytic pathway has already been reported (Locke & Huie, 1979, Lamb et al., 2012; Alberts et al., 2015).

In the hindgut epithelium of prepupae, autophagosomes were evidenced by cell ultrastructure and the immunolocalization of LC3-proteins. However, in white- and pink-eyed pupae, these organelles were identified only through transmission electron microscopy, indicating that the prepupal stage is the principal phase for autophagosome formation, as the LC3-proteins (LC3A and LC3B) are degraded after autophagosome formation (Kabeya et al., 2000; Wu et al., 2006). Thus, in white- and pink-eyed pupae, these LC3-proteins are degraded.

The light-brown-eyed pupal phase of *A. mellifera* honeybees may be referred to as a transitional period, when the deposition of the new hindgut cuticle begins, being characterized by the presence of the epicuticle. This is similar to that reported during insect molting (Reynolds & Samuels, 1996; Chapman, 2013), with the epicuticle protecting the new cuticle from the enzymes in the molt-mutated fluid that degrades the old cuticle (Merzendorfer & Zimoch, 2003, Kloden, 2007). The ileum cells, in this developmental stage of *A. mellifera*, have many vesicles in the apical cytoplasm that have been suggested to participate in the release of cuticle compounds, as reported to occur in *C. ethlius* (Lepidoptera) (Locke, 1969), *Balanus balanoides* (Crustacea) (Koulish & Klepal, 1981) and *Oniscus asellus* (Isopoda) (Price & Holdich, 1980). The deposition of the new hindgut cuticle is complete in the dark-brown-eyed pupal stage.

In dark-brown-eyed pupae, the basal lamina invades the intercellular spaces of the ileum cells, suggesting that it has a function in the formation of the tracheoles found in this pupal stage. In *Manduca sexta* larvae (Lepidoptera), an extracellular matrix, precursor of the basal lamina, is associated with the expansion of tracheoles (Nardi, 1984; Nardi et al., 1985). In addition, the basal lamina plays an important role in the attachment of migratory cells during morphogenesis (Amemiya, 1989; Martinek et al., 2008).

In black-eyed pupae of *A. mellifera*, the epithelial cells have large vacuoles and long apical plasma membrane invaginations. Vacuoles are common in epithelia that rapidly transport great amounts of fluids, as in the Malpighian tubules of *Acheta domesticus* (Orthoptera) (Hazelton et al., 2001), rectal papillae of *Poecilimon cervus* (Orthoptera) (Polat et al., 2016), and proximal renal tubule of mouse (Grahan & Karnovsky, 1966). The presence of large vacuoles, basal lamina, and large subcuticular spaces in the anterior ileum region of black-eyed pupae suggests that this region may be specialized in fluid transport (Cruz-Landim, 1994; Cruz-Landim, 1996; Villaro et al., 1999). However, a concomitant cellular expansion cannot be ruled out (Hazelton et al., 2001), as there is a reduction in cuticular folds and an increase in cell height. During this stage, which is ca. four days long (Michelette & Soares, 1993), the ileum epithelial cells gradually reduce the length of the apical plasma membrane invaginations and the number of vacuoles, so that in the newly emerged adult worker of *A. mellifera*, these cells have short membrane invaginations, few vesicles, and large mitochondria, characteristics that suggest a low transportation of fluid, such as that reported in the ileum of *L. migratoria* (Peacock, 1986).

Forager workers of *A. mellifera* are older and have the anterior region of ileum cells with long apical plasma membrane invaginations reaching the middle cell region associated with mitochondria, suggesting that this region may be highly active in the

absorption of water and ions from the gut lumen, as reported in the aphid *Philaenus spumarius* (Marshall & Cheung, 1973), in the stingless bee *Melipona quadrifasciata anthidioides* (Cruz-Landim, 1994), and in the bumblebee *Bombus morio* (Gonçalves et al., 2014).

The posterior region of the ileum of *A. mellifera* forager workers has a greater number of mitochondria and apical plasma membrane invaginations, both longer and more irregular, when compared to younger bees, suggesting the formation of a permanent osmotic gradient provided by the active transport of ions into the cell and the consequent influx of water from the ileum lumen, as reported in other insects (Diamond & Bossert, 1967; Villaro et al., 1999; Gonçalves et al., 2014).

During the hindgut metamorphosis of *A. mellifera*, cell proliferation in the ileum occurs in a given sequence and, in the course of these events, the anterior region has a greater number of proliferating cells, suggesting that the growth of the ileum occurs preferentially from the anterior end, similar to that suggested in *D. melanogaster* (Robertson, 1936) and *M. quadrifasciata anthidioides* (Cruz-Landim & Silveira-Mello, 1970). Although the role of the anterior region of the ileum is not well understood, hindgut cells of *D. melanogaster* are derived from stem cells of the pylorus (Takashima et al., 2008; Fox & Spradling, 2009).

Our results suggest that the differentiation of the rectum occurs independently of the ileum, because cell proliferation is intense during the development of the rectal epithelium of *A. mellifera*, while, in the ileum, this process occurs at a low rate. The high number of proliferating cells in the precursor region of the rectum results both in epithelial expansion and in rectal pad differentiation, which are formed in the anterior region of the rectum of white-eyed pupae (Dobrovsky, 1951; Santos et al., 2009).

In addition to cell proliferation, the remodeling of the hindgut of *A. mellifera* also involves apoptosis, which is more intense in the visceral muscle than in the

epithelium of the ileum, whereas in the rectum, during metamorphosis, apoptosis occurs mainly in the epithelium, suggesting an important role in the formation of organ anatomy and rectal pads (Dobrovsky, 1951; Santos et al., 2009).

Overall, our results show that the morphogenesis of the *A. mellifera* hindgut is dynamic, with tissue remodeling and cellular events taking place for the differentiation of the ileum and rectum, organs with different anatomical and ultrastructural organizations in adult bees. In addition, we report for the first time the formation of a new cuticle and the reorganization of visceral muscles during the hindgut metamorphosis of *A. mellifera.*

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CONCLUSÕES GERAIS

Esse estudo trouxe dados inovadores sobre o desenvolvimento dos órgãos excretores dos insetos. Na metamorfose de *A. mellifera*, a fase larval apresentou as células dos túbulos de Malpighi com características ultraestruturais de baixa atividade de excreção, período que toda urina primaria é estocada em seu lúmen. A subsequente degeneração desses túbulos de Malpighi pode ser necessária para o desenvolvimento, uma vez que os fragmentos celulares são uma rica fonte nutricional durante períodos em que o conteúdo intestinal foi defecado e as pupas são privadas de alimento. Além disso, os novos túbulos de Malpighi apresentaram diferentes atividades funcionais. Até a fase de pupa de olhos castanhos, os túbulos de Malpighi não apresentaram características ultraestruturais de atividade de excreção, sugerindo um período de susceptibilidade a compostos tóxicos. Entretanto, posteriormente esses túbulos apresentam características de alta atividade excretora, sugerindo que durante a metamorfose metabolitos são eliminados. Em abelhas operárias forrageiras, expostas a diferentes fatores ambientais, os túbulos de Malpighi são complexos, com regiões de atividades distintas e dois tipos celulares com funções opostas e alta atividade metabólica.

O intestino posterior de *A. mellifera* é remodelado a partir do último instar larval até pupa de olhos castanhos. Nessas fases do desenvolvimento, a imunolocalização de autofagossomos e a ultramorfologia epitelial e cuticular sugerem a atuação das células epiteliais na degradação cuticular, reciclagem da cutícula degradada e deposição do novo revestimento cuticular. Além disso, a participação da lâmina basal na remodelação epitelial é sugerida, pois após a formação da lâmina basal o epitélio mudou de pseudoestratificado para estratificado, seguida por invasão de traqueíolas em pupa de olhos marrom. Em pupa de olhos pretos, as regiões do intestino posterior (piloro, íleo e reto) estão anatomicamente diferenciadas devido as proliferações celulares e apoptoses ocorridas nas células epiteliais e musculares. Nessa fase, as células do epitélio retal mostra-se achatada, enquanto que as células do epitélio do íleo tem grandes vacúolos

citoplasmáticos, sugerindo uma atividade no transporte de fluidos. Posteriormente, na abelha forrageira, as células do ileo contem muitas mitocôndrias e a membrana plasmatica apical com longas invaginacoes, caracteríscas de epitélio especializado no transporte de compostos.

Os resultados aqui obtidos mostram que na metamorfose de *A. mellifera* os órgãos excretores são dinâmicos, com as células dos túbulos de Malpighi e intestino posterior passando por apoptose, proliferações e remodelações, levando a órgãos diferenciados anatomicamente e com características de atividade de excreção ainda na fase de pupa. Ademais, os órgãos excretores tornam-se mais especializados na atividade de excreção na abelha adulta forrageira que está exposta constantemente a fatores de estresse homeostático.