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Original article

Proliferation and cell death in the midgut of the stingless bee *Melipona quadrifasciata anthidioides* (Apidae, Meliponini) during metamorphosis

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Abstract – This study quantitatively compared proliferation and cell death in the remodeling of the midgut epithelium in *Melipona quadrifasciata anthidioides* during metamorphosis to elucidate the renewal mechanism of the midgut in bees during postembryonic development. An anti-phosphohistone H3 antibody was used to mark mitotic cells. An apoptotic cell marking kit was used (Apo-TRACE®) to identify cells undergoing the process of cell death. The ultrastructural aspects of cell death were also analyzed. The highest proliferative and apoptotic rates were observed in pink-eyed pupae. An increased number of cells and rebuilding of regenerative cell nests at the end of metamorphosis is a consequence of cell division in black-eyed pupae because cell division occurs at this stage in the absence of apoptosis. During metamorphosis, midgut epithelial cell death in *M. quadrifasciata anthidioides* occurs as a result of apoptosis and autophagy.

midgut / regenerative cell / cell death / metamorphosis / Hymenoptera

1. INTRODUCTION

Holometabolous insects have a well-defined metamorphosis characterized by a series of morphophysiological changes between the larval and adult stages. Metamorphosis in holometabolous insects involves drastic changes in larval organization, including the substitution of various tissues with key regulatory processes, such as programmed cell death, cell proliferation, and differentiation (Illa-Bochaca and Montuenga 2006).

In bees, changes in the digestive tract are related in part to changes in feeding behaviors, which is different in the larval and adult stages (Gama and Cruz-Landim 1984). The digestive tract of insects is divided into the foregut, the midgut, and the hindgut (Chapman 1998; Cruz-Landim 2009). The midgut epithelium in bees consists of three cell types: digestive cells that are responsible for enzyme secretion and nutrient absorption, endocrine cells that produce hormones, and regenerative cells that play a role in cell renewal (Serrão and Cruz-Landim 1996; Martins et al. 2006). During metamorphosis, the digestive epithelium in insects undergoes rapid changes with continuous cell proliferation and death (Uwo et al. 2002; Corley

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and Lavine 2006; Tettamanti et al. 2007; Hakim et al. 2010; Nardi et al. 2010; Franzetti et al. 2012). In bees, the larval digestive cells die and reorganization of the gut epithelium occurs through regenerative cells present in the larval epithelium (Martins et al. 2006). The production of new cells occurs due to regenerative cell proliferation and differentiation in digestive or endocrine. Regenerative cells are consequently referred to as stem cells (Baldwin and Hakim 1991; Illa-Bochaca and Montuenga 2006; Corley and Lavine 2006; Hakim et al. 2010).

Cruz-Landim et al. (1996) reported that mitosis is common in the adult regenerative cells in several orders of insects, but that does not occur in adult Hymenoptera. The number of regenerative cells in adult hymenopterans is expected to be defined during the pupal stage (Cruz-Landim and Silva-de-Moraes 2000). However, the number of proliferating cells is not sufficient to explain the large increase in the number of regenerative cell nests that occur from larvae to adult, even during metamorphosis (Neves et al. 2003; Martins et al. 2006; Cruz et al. 2011).

The renewal mechanisms in the gut of bees during metamorphosis have been studied (Cruz-Landim and Cavalcante 2003; Martins et al. 2006; Cruz et al. 2011). The classical method of counting mitotic cells in stained histological sections underestimates the true proliferative fraction of cells in tissues (Neves et al. 2003). Immunodetection of phosphorylated histone H3 is commonly used to estimate the mitotic index in tissues. Phosphorylation of histone H3 is spatially and temporally correlated with chromatin condensation and occurs almost exclusively during mitosis (Hendzel et al. 1997).

In the remodeling of the midgut epithelium during metamorphosis, a highly regulated series of cell death occurs. Current knowledge of programmed cell death in some insect tissues is controversial regarding classification because the typical features of apoptosis in vertebrates are not unconditionally present in insect cells (Ferreira and Malaspina 2009). Cell death as a result of apoptosis, autophagy, and necrosis has been reported in insect tissues (Rost 2006; Tettamanti et al. 2007; Rost-Roszkowska 2008; Santos et al. 2009). The morphological aspects of cell death can be visualized using transmission electron microscopy (TEM). These cells can be quantitatively identified using cell death detection kits, such as the Apo-TRACE (Sigma). Molecules in the Apo-TRACE kit respond to changes in the plasma membrane potential and phospholipid distribution, which are characteristics of apoptotic cells. Apo-TRACE molecule selectively crosses the plasma membrane of apoptotic cells in the early stages of cell death and accumulates in the cytoplasm (Aloya et al. 2006).

The objective of this study was to quantitatively evaluate cell death and proliferation in the midgut of *Melipona quadrifasciata anthidioides* during metamorphosis.

2. MATERIAL AND METHODS

2.1. Bees

Stingless worker bees (*M. quadrifasciata anthidioides*) were obtained in the following developmental stages: prepupae (pharate pupae), white-eyed pupae, pink-eyed pupae, black-eyed pupae, and adults. The degree of eye pigmentation has been widely used as an indicator of pupal development. The current classification of the developmental stages in social bees is detailed by Pinto et al. (2002). Four individuals from each developmental stage were used in each procedure. All of the specimens were obtained from the colonies maintained at the Central Apiary of the Federal University of Viçosa (UFV) in the state of Minas Gerais, Brazil.

2.2. Immunofluorescence—phosphorylated histone H3

Individuals were cold anesthetized and the midgut was dissected under a stereomicroscope. The midgut was divided into anterior, middle, and posterior regions and transferred to Zamboni's fixative solution (Stefanini et al. 1967) for 30 min at room temperature. The samples were washed three times in 0.1 M sodium phosphate buffer with 1 % Tween 20 (PBST) for 5 min at 37 °C. The samples were incubated in

0.1 % bovine serum albumin (BSA) at room temperature for 10 min, washed in PBST for 5 min, and transferred to a 1:30 dilution of normal goat serum in PBST for 10 min. The samples were washed three times in PBST and incubated with anti-phosphohistone H3 (Cell Signaling) developed in rabbits at a dilution of 1:100 in PBST for 2 h at room temperature. The samples were washed three times in PBST and incubated with anti-rabbit IgG-FITC conjugated secondary antibody (Sigma) at 1:500 for 2 h at room temperature, followed by washing in PBST for 5 min. The midguts were incubated in 4,6-diamidino-2-phenylindole (DAPI; 100 ng/mL) for 30 min for nuclear staining. As a negative control, incubation with anti-phosphohistone H3 was omitted, and the samples were treated with PBS containing BSA. The proventriculus was used as a positive control because this foregut region is formed during the pupal stage (Snodgrass 1956). Samples were mounted onto a slide with a coverslip in a 50 % sucrose solution and examined under a fluorescence microscope (Olympus BX-60).

2.3. Apoptosis

The midguts were dissected as described and incubated in Apo-TRACE® (Sigma) 5 μL/mL for 1 h at room temperature. The samples were washed in 0.1 M PBS and transferred to Zamboni's fixative solution for 1 h at room temperature. The samples were washed in PBS and counterstained with 0.5 % propidium iodide for 30 min, washed again with PBS, and mounted in a 50 % sucrose solution for examination under a Zeiss LSM 510 Meta laser confocal microscope.

2.4. Transmission electron microscopy

The bees were dissected as described in the presence of 0.1 M sodium cacodylate buffer (pH 7.2) containing 0.2 M sucrose. The anterior, middle, and posterior midgut regions were transferred to Karnovsky's solution prepared with 0.1 M cacodylate buffer (pH7.2) for 4 h at room temperature. The samples were postfixed in 1 % osmium tetroxide in the same buffer, dehydrated in a graded ethanol series, and embedded in LR White resin (Electron Microscopy Sciences). Ultrathin sections were stained with lead citrate and 1 % aqueous uranyl acetate and observed under TEM (Zeiss EM 109) in the Center of Microscopy and Microanalysis at the UFV.

2.5. Quantification of cell proliferation and death

The anterior, middle, and posterior midgut regions submitted to detection of mitotic and apoptotic cells were used to counting the number of cells undergoing processes of the proliferation and death with the computer program Image-Pro Plus 4.0 (Media Cybernetics). In each midgut region, five 0.18 mm^2 areas were randomly selected. Counting was performed in four bees at each developmental stage.

2.6. Statistics

The number of mitotic and apoptotic cells in the entire midgut at different stages was compared using one-way analysis of variance test with comparisons with the Dunn test, at 5 % of significant level. The *t* test was used to compare the number of mitotic and apoptotic cells in the entire midgut within each developmental stage.

3. RESULTS

3.1. Cell division

Many phosphohistone H3-positive cells were observed in the midgut of the bees (Figure 1A–C). The highest number of mitotic cells was shown in the prepupae and pink-eyed pupae (Figure 2), followed by the white-eyed pupae, and the black-eyed pupae, which had the lowest number of mitotic cells. Mitotic cells were not detected in adult bees.

Comparing the number of mitotic cells between the anterior, middle, and posterior midgut regions within the same developmental stage showed that the anterior midgut had a higher number of mitotic cells than the middle and posterior midgut regions only in the black-eyed pupae.

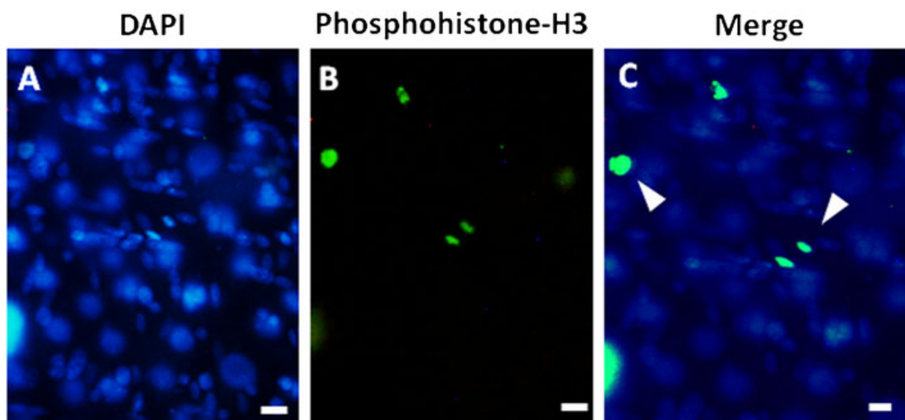

Figure 1. Midgut of white-eyed pupae of *M. quadrifasciata anthidioides*. **A** Nuclei stained with DAPI (*blue*). **B** The same region (**a**) with anti-phosphohistone H3 FITC conjugated (*green*). **C** Merged. Anti-phosphohistone-H3 (*arrowhead*). *Bars*=10 µm.

3.2. Apoptosis

Cell death was observed in the prepupae and the white-eyed and pink-eyed *M. quadrifasciata anthidioides* pupae (Figure 3A, B), with no significant differences between them. Apoptotic cells were not found in the black-eyed pupae and adult stingless bees (Figure 4).

The anterior midgut region of the white-eyed pupae had the highest number of apoptotic cells, while there were no differences in the number of apoptotic cells in the midgut regions at the other stages of development.

There was a similar number of apoptotic and mitotic cells in the prepupae and pink-eyed pupae. In the white-eyed pupae, there were more mitotic than apoptotic cells, and in the black-eyed pupae, mitotic cells were observed, but apoptotic cells were not present (Figure 5).

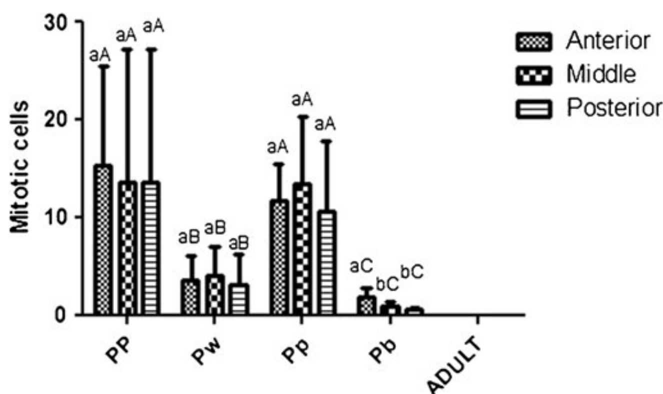

Figure 2. Analysis of variance (ANOVA) relating the number of cells marked by phosphohistone H3 between different regions of the midgut at each stage of development and between stages. *PP* prepupae, *Pw* white-eyed pupae, *Pp* pink-eyed pupae, *Pb* black-eyed pupae. *Different lowercase letters above the bars* indicate significant differences in different regions of the midgut for the same development stage. *Different capital letters* indicate significant differences between stages of development.

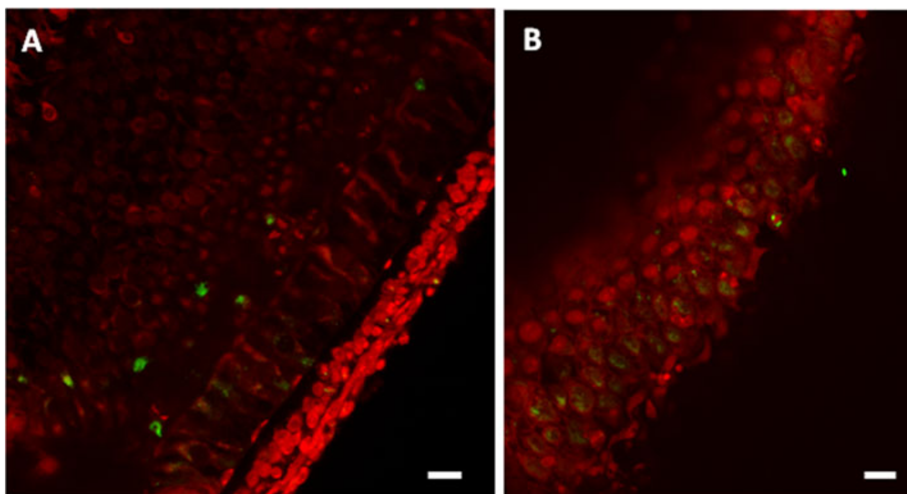

Figure 3. Deaths cell Apo-TRACE positives (*green*) in the midgut of *M. quadrifasciata anthidioides*. Nuclei stained with propidium iodide (*red*). **A** Prepupae. **B** Pink-eyed pupae. *Bars*=20 µm.

Ultrastructural features of apoptosis, such as changes in the nuclear shape with fragments surrounded by the nuclear envelope (Figure 6A–C) and changes in mitochondrial morphology, which had fragmented and disorganized cristae (Figure 6B), were shown in the developmental stages of *M. quadrifasciata anthidioides* with Apo-TRACE-positive cells. In addition, autophagic double membrane bound vacuoles were shown in the in pink-eyed pupae (Figure 6D).

4. DISCUSSION

Renewal of the midgut epithelium in insects has been widely described (Baldwin and Hakim 1991; Jiang et al. 1997; Uwo et al. 2002; Tettamanti et al. 2007; Franzetti et al. 2012). Several researchers

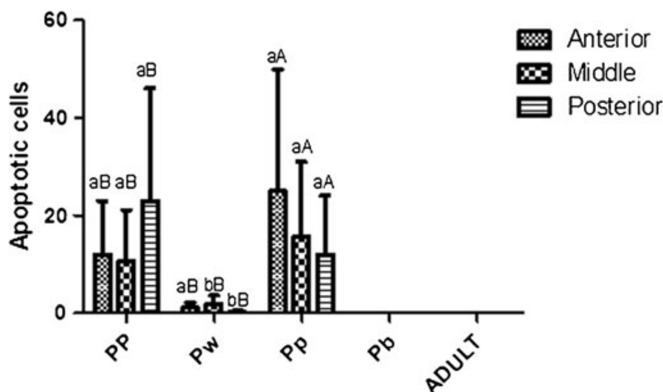

Figure 4. Analysis of variance (ANOVA) describing the stages of development with the number of cells marked by Apo-TRACE® and the regions of the midgut. *PP* prepupae, *Pw* white-eyed pupae, *Pp* pink-eyed pupae, *Pb* black-eyed pupae. *Different lowercase letters above the bars* indicate significant differences in different regions of the midgut for the same stage of development. *Different capital letters* indicate significant differences between stages of development.

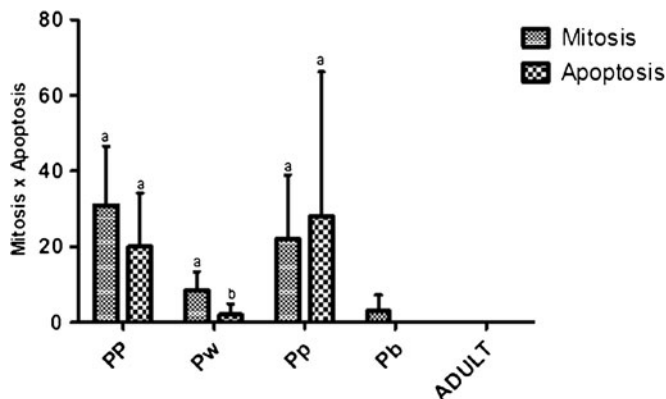

Figure 5. Analysis of variance (ANOVA) relating the proliferation rate with the number of cells marked by Apo-TRACE® in the midgut. *PP* prepupae, *Pw* white-eyed pupae, *Pp* pink-eyed pupae, *Pb* black-eyed pupae. *Different lowercase letters above the bars* indicate significant differences between mitosis and apoptosis at the same stage of development.

have focused on the reorganization of the midgut in bees during metamorphosis (Cruz-Landim and Cavalcante 2003; Neves et al. 2003; Martins et al. 2006). Morphometric data have suggested the need to form new digestive cells to assure an increase in the size of the midgut during the metamorphosis of *M. quadrifasciata anthidioides* (Cruz et al. 2011).

Immunodetection of phosphorylated histone H3 may be a useful tool for assessing and quantifying cell proliferation of the midgut cells in bees given that classical staining procedures to detect mitotic cells have shown poor results (Martins et al. 2006; Cruz et al. 2011). Marking apoptotic cells with Apo-TRACE® aids in the quantification of cells undergoing the process of cell death.

We observed a high number of mitotic and apoptotic cells in the prepupal stage with low variance. During the prepupal stage, release of some larval cells into the midgut lumen occurs in conjunction with growth and differentiation of the pupal organs (Martins et al. 2006). The prepupal stage represents the beginning of metamorphosis (Pinto et al. 2002) and the transition from a larva to an adult, during which time a high level of cell reorganization is expected.

In the white-eyed pupae, a few mitotic cells were detected. There were more mitotic cells than apoptotic cells. The first transformation of the midgut in bees during metamorphosis is completed in white-eyed pupae (Cruz-Landim 2009). Cruz et al. (2011) reported that increase in the midgut size occurs due to increase in the digestive cell volume. All together, we suggest that in *M. quadrifasciata anthidioides* white-eyed pupae, there is not a need for a substantial increase in cell proliferation rate because the increase in cell volume combined with the low rate of cell death appears to be sufficient to overcome the increase in the midgut.

In pink-eyed *M. quadrifasciata anthidioides* pupae, we observed extensive changes in transformation of the midgut due to a large number of mitotic and apoptotic cells. In bees, new midgut transformation begins in pink-eyed pupae during metamorphosis (Cruz-Landim and Mello 1970; Cruz et al. 2011). This midgut transformation coincides with a rise in ecdysteroid concentrations in *M. quadrifasciata anthidioides* (Pinto et al. 2002). An increase in the cell proliferation rate in insect tissues has been correlated with a high ecdysteroid titer (Champlin and Truman 1998). In cultured insect cells, ecdysteroid hormones stimulate proliferation, although high concentrations of the hormone may induce apoptosis (Champlin and Truman 1998).

Cruz et al. (2011) reported an increase in the total volume of the midgut without any increase in cell volume in black-eyed *M. quadrifasciata*

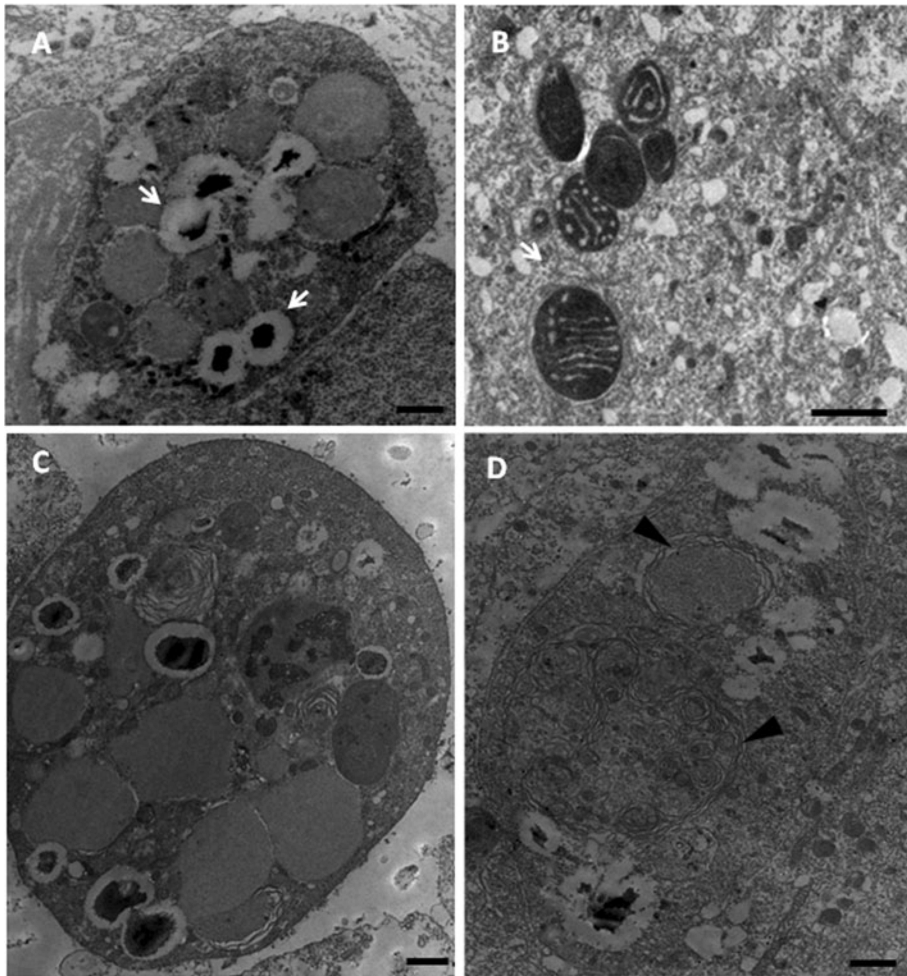

Figure 6. Cell death in the midgut of *M. quadrifasciata anthidioides*. **A** Prepupae nuclear fragmentation (*arrow*) is noted in the apoptotic cell. *Bar* 2 μm. **B** White-eyed pupae with mitochondria (*arrow*) characteristic of apoptosis. *Bar*=5 μm. **C**, **D** In pink-eyed pupae both nuclear fragmentation characteristic of apoptosis and also the presence of multilamellar bodies characteristic of autophagy are observed (*arrowhead*). *Bars*=2 μm.

anthidioides pupae, suggesting an increase in the number of digestive cells. Regenerative cell nests were observed during this period, but mitosis was not detected. In the present study, mitotic cells were detected in black-eyed pupae, but apoptotic cells were not. Thus, the increased number of digestive cells and the presence of regenerative cell nests may be due to an increased cell proliferation rate that occurs in this developmental stage combined with the peak ecdysteroid titer described by Pinto et al. (2002).

In *M. quadrifasciata anthidioides* adults, mitotic or apoptotic cells were not detected, suggesting that there is no need for extensive substitution of digestive cells in adulthood. Martins et al. (2006) stated that proliferation of regenerative cells does not occur during metamorphosis of *M. quadrifasciata anthidioides* because regenerative cells in the larva are sufficient to promote renewal of the midgut epithelium during metamorphosis. However, many mitotic cells were detected in the midgut

epithelium of both pink-eyed and black-eyed pupae in the present study. This may be due differences in the procedures used because Martins et al. (2006) used a pulse of bromodeoxyuridine during the larval stage, whereas we detected mitotic cells using a specific antibody against mitotic phosphorylated histone H3. We suggest that the reestablishment of new regenerative cell nests occurs in black-eyed pupae to support midgut renewal throughout the adult lifespan of *M. quadrifasciata*.

Apoptosis plays an important role in the development, maintenance, and senescence of organisms and is one of the mechanisms used to remove damaged cells during the natural process of tissue aging (Ward et al. 2008). Thus, apoptosis is expected to occur in the midgut of *M. quadrifasciata anthidioides* adults. However, this process was not observed. Cell death can be classified into three types according to morphological features: apoptosis, autophagy, and necrosis (Ferreira and Malaspina 2009). During the pupal stages of *Drosophila melanogaster* (Diptera) (Denton et al. 2009) and *Bombyx mori* (Franzetti et al. 2012), autophagy is the primary process of cell death. In Protura, midgut degeneration begins with autophagy, followed by apoptosis (Rost-Roszkowska et al. 2010). Our ultrastructural findings suggest that both autophagy and apoptosis occur during the metamorphosis of *M. quadrifasciata anthidioides*.

Apoptosis and autophagy are types of programmed cell death, while necrosis is a pathological and genetically non-programmed cell death (Ward et al. 2008; Ferreira and Malaspina 2009). However, recent studies have shown physiological regulation of necrotic cells. Although necrosis occurs in pathological conditions, it is also a component of some physiological processes (Ferreira and Malaspina 2009). Therefore, we assume that necrosis is responsible for bee senescence in *M. quadrifasciata anthidioides* adults. Perhaps, this type of cell death does not allow the apoptotic molecule markers to enter the cells. Another hypothesis is that midgut cell senescence is absent and the cell death rate is lower than the rates during metamorphosis because workers bees have a shorter life span than the queens. Thus, the death of midgut cells in *M. quadrifasciata anthidioides* may be the result of concomitant apoptosis and autophagy and perhaps minor necrosis during metamorphosis.

The increased number of midgut digestive cells and reestablishment of regenerative cell nests in the midgut of *M. quadrifasciata anthidioides* during the final phase of metamorphosis occurs as a consequence of mitosis and the absence of apoptosis in black-eyed pupae.

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Prolifération et mort cellulaire dans l'intestin moyen de l'abeille sans aiguillon *Melipona quadrifasciata anthidioides* (Apidae, Meliponini) durant la métamorphose

Intestin moyen / cellule régénératrice / mort cellulaire / apoptose / métamorphose / Hymenoptera

Zellteilung und Zelltod im Mitteldarm der Stachellosen Biene *Melipona quadrifasciata anthidioides* (Apidae, Meliponini) im Verlauf der Metamorphose

Mitteldarm / regenerative Zellen / Zelltod / Metamorphose / Hymenoptera

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