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# Regenerative Cells in the Midgut of the Honey Bee *Apis mellifera* (Apidae: Apini) Queens with Different Ages

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**HIGHLIGHTS**

- Honey bee queens have the long lifespan in the colony.
- Queen midgut undergoes epithelial cell death with age.
- The midgut epithelium renewal decreases with queen age.
- Midgut stem cells differentiate without proliferation.

**Abstract:** The honey bee *Apis mellifera* is a plant-pollinator that produces commercial products. It has female castes with workers performing tasks in the colonies and a queen, it has the longest lifespan, in charge of reproduction. This bee undergoes a population decline worldwide. Therefore, it is important to understand how aging affects the digestive tract of this insect. In the midgut, regenerative cells are organized in nests replacing the dead cells. This study verified the hypothesis that the number of regenerative cells decreases as *A. mellifera* queen ages. The midgut was evaluated in queens at the age of four days and eight, 11 and 25 months. The midgut of 11- and 25-month-old queens presented signs of epithelial disorganization in comparison with younger queens. The number of regenerative cell nests in the midgut decreases according to the age of the queen, but the number of cells per nest is similar. The decreased number of regenerative cell nests reveals a potential loss in the amount of these cells available for the renewal of the midgut epithelium. The absence of variations in the number of regenerative cells per nest according to the queen age indicates that these cells do not undergo proliferation before the differentiation.

**Keywords:** Aging; digestive tract; epithelium renewal; Hymenoptera; stem cells.

## INTRODUCTION

Bees have economic and environmental importance due to the production of honey, wax, propolis, royal jelly and venom [1], besides plant pollination. Among bees, *Apis mellifera* Linnaeus (Hymenoptera: Apidae)

is a pollinator of native and crop plants in an ecosystem service estimated at 153 billion euros [2].

Adapted to the Brazilian environmental conditions, the Africanized *A. mellifera* is a hybrid between the European *Apis mellifera mellifera* Linnaeus 1758 and South African *Apis mellifera scutellata* Lepeletier 1836 [1] that spread in America [3].

*Apis mellifera* is eusocial, with caste organization and labor division. Workers are partially sterile and perform several tasks, including feeding larvae and queens, colony defense, and foraging for resources. The queen is the reproductive caste, which produces the eggs. The social organization is kept mainly with pheromones produced by the queen, which inhibit worker ovarian activation [4]. The female castes are dimorphic, with trophogenic determination. The queen larvae receive food in higher quantity and quality than the larvae of workers [5]. In the early developmental stage, queen larvae receive royal jelly, produced by the hypopharyngeal glands of workers, which has high sugar content, protein royalactin [6,7] and compounds from the mandibular gland [8]. These compounds also increase the longevity of the queen, which can survive for years, whereas workers live for ca. 2 months [5].

In recent years, stressor agents have increased bee mortality and caused population decline [3]. Among the stressors, pathogens [9] and pesticides can damage the midgut cells of the bees [10-14] and induce colony decline [1]. Although queens are important for colony maintenance, they can die due to the shortage of workers in the colony population [15].

Food digestion occurs mainly in the midgut, which is longer in *A. mellifera* queens than in workers [16]. This organ has a single-layered epithelium with columnar digestive cells that absorb substances and produce digestive enzymes and peritrophic matrix components [16,17]; regenerative cells that replace dead ones [18,19]; and some endocrine cells producing hormone peptides [20-22].

Regenerative cells are organized in nests scattered at the base of the midgut epithelium [23]. These cells have undifferentiated features, including cytoplasm poor in organelles [24,25].

During post-embryonic development, regenerative cell nests increase in number until metamorphosis is complete [26]. However, in adults, digestive cells do not originate from the proliferation of regenerative cells, which indicates that during midgut metamorphosis, larval regenerative cells differentiate into digestive cells [18,27]. Thus, it is plausible to suggest that the amount of regenerative cells decreases with aging, as a consequence of cell renewal in adult insects.

In the mosquito *Culex quinquefasciatus* Say 1823 (Diptera) adults, the number of regenerative cells decreases after each blood meal, since digestive processes result in the death of digestive cells, which are replaced by the differentiation of regenerative cells [28]. In worker bees, has been claimed that increased digestive cell renewal occurs in the midgut region with more number of regenerative cell nests [29].

Data on the regenerative cells in the digestive tract of bees are mainly related to workers, while few studies have focused on queens. This work evaluated the hypothesis that the number of regenerative cell nests and the number of cells per nest in the midgut decrease as *A. mellifera* queen ages.

## MATERIAL AND METHODS

### Bees

Africanized *A. mellifera* queens were obtained from colonies kept in the apiary of the Regional Laboratory for Research in Apicultural Health (LASA; 22º 57' S, 45º 27' W, 560 m a.s.l.), Instituto Biológico (IB), Agência Paulista de Tecnologia dos Agronegócios (APTA), Secretaria de Agricultura e Abastecimento (SAA), Pindamonhangaba, State of São Paulo, Brazil. The queens were virgins < 4 days old (n = 3), physogastric, at the ages of 8 (n = 4), 11 (n = 3) and 25 months (n = 3).

### Light microscopy

The queens were transferred to 4% paraformaldehyde and kept at 6-10 °C. Subsequently, they were dissected in a saline solution for insects (0.1 M NaCl + 0.2 M $KH_2PO_4$ + 0.2 M $Na_2HPO_4$), pH = 7.2, and the midguts were dehydrated in a graded ethanol series (70%, 80%, 90%, and 95%), for 10 minutes each concentration. The samples were embedded in glycol methacrylate historesin (Leica), cut into 3 µm thick sections in a rotatory microtome (Leica), stained with hematoxylin (12 min) and eosin (30 s) and analyzed under an Olympus BX60 microscope coupled with an Olympus Q-Color 3 camera.

### Quantification of regenerative cells

In order to quantify the regenerative cells, 15 midgut sections of each queen were randomly selected using a 20X objective lens, numerical aperture 0.20, and the numbers of nests and regenerative cells per

nest were counted.

The data obtained were analyzed using descriptive statistics with arithmetic mean, median, standard deviation and standard error of the mean.

### Transmission electron microscopy

The queens were transferred to 2.5% glutaraldehyde in 0.15 M sodium cadodylate buffer pH 7.2 for 12 h. Then the queens were dissected and small fragments of the median subregion of the midguts were post-fixed in 1% osmium tetroxide in the same buffer for 2h following dehydration in a graded ethanol series (70%, 80%, 90%, 95%, and 98%). Subsequently, the samples were embedded in LR-White medium grade resin (Sigma-Aldrich) and ultra-thin (70-90 nm) sections obtained in ultramicrotome (RMC), stained in 1% aqueous uranyl acetate and lead citrate, following analysis in a LIBRA 120 Zeiss transmission electron microscope.

## RESULTS

The midgut of A. *mellifera* queens had a folded single-layered epithelium with columnar digestive cells presenting an enlarged nucleus rich in decondensed chromatin and a well-developed apical brush border. In addition, regenerative cells were organized in nests placed in the basal region of the epithelium, without reaching the organ lumen, which had an evident peritrophic matrix (Figures 1A, 1B). Externally, the midgut epithelium was on a thin basement membrane followed by muscle layers (Figure 1C). The ultrastructural analyses reveal that these cells had long and organized apical microvilli and cytoplasm rich in mitochondria and rough endoplasmic reticulum (Figure 2A).

The digestive cells of the virgin queens (4 days-old) presented a homogeneously acidophilic cytoplasm (Figure 1A). In eight and 11-month-old queens, enlarged apical protrusions were observed, as well as the release of cell fragments, some containing the cell nucleus, into the lumen (Figures 1B, 1C). In 25-month-old queens, there was disorganization of the epithelium architecture characterized by irregular apical surface (Figure 1D), increased release of cell fragments into the lumen and cytoplasm vacuolization of the digestive cells (Figure 3). The apical protrusions in the digestive cells found in eight, 11, and 25-month queens were rich in mitochondria and large vacuoles with electron-lucent content (Figure 2B). The cell fragments that were released from the protrusion to the midgut lumen were rich in a flocculent content without cytoplasm (Figure 2B).

In all queens age analyzed, the regenerative cells were similar and organized in the shape of an onion bulb in nests, with those at the periphery narrowed and straightened to reach the midgut lumen, whereas the central cells were almost spherical, with well-developed nucleus (Figures 4A, 4B). The central regenerative cells had the nucleus rich in decondensed chromatin and cytoplasm with many mitochondria (Figure 5) some rough endoplasmic reticulum profile. The regenerative cells in the nest periphery that undergo differentiation, characterized by the straightening, presented the nucleus with decondensed chromatin and cytoplasm rich in mitochondria (Figure 5).

As queens age, the number of regenerative cell nests in the midgut decreases. Four-day-old and eight-month-old queens presented a mean of 14 regenerative cell nests per midgut section, with a median of 16 and 13 nests, respectively (Table 1). The mean and median decreased to nine regenerative cell nests in the 11-month-old queen (Table 1). The 25-month-old queen presented a mean of five regenerative cell nests per section, with a median of four nests (Table 1). All queens analyzed presented two central regenerative cells per nest (Table 1).

**Table 1.** Number of regenerative cells and cells per nests obtained from 15 midgut sections of *Apis mellifera* (Hymenoptera) queens with different ages.

| Queen age | Regenerative cell nest | | | Cell per nest | |
|---|---|---|---|---|---|
| | Mean ± sd | SE | Median | Mean ± sd | SE |
| 4 days | 14.75 ± 4.55 | 0.67 | 16 | 2.22 ± 0.75 | 0.029 |
| 8 months | 14.36 ± 5.87 | 1.07 | 13 | 2.32 ± 0.56 | 0.027 |
| 11 months | 9.63 ± 5.03 | 0.91 | 9 | 2.16 ± 0.47 | 0.027 |
| 25 months | 5.33 ± 4.32 | 0.91 | 4 | 2.15 ± 0.59 | 0.037 |

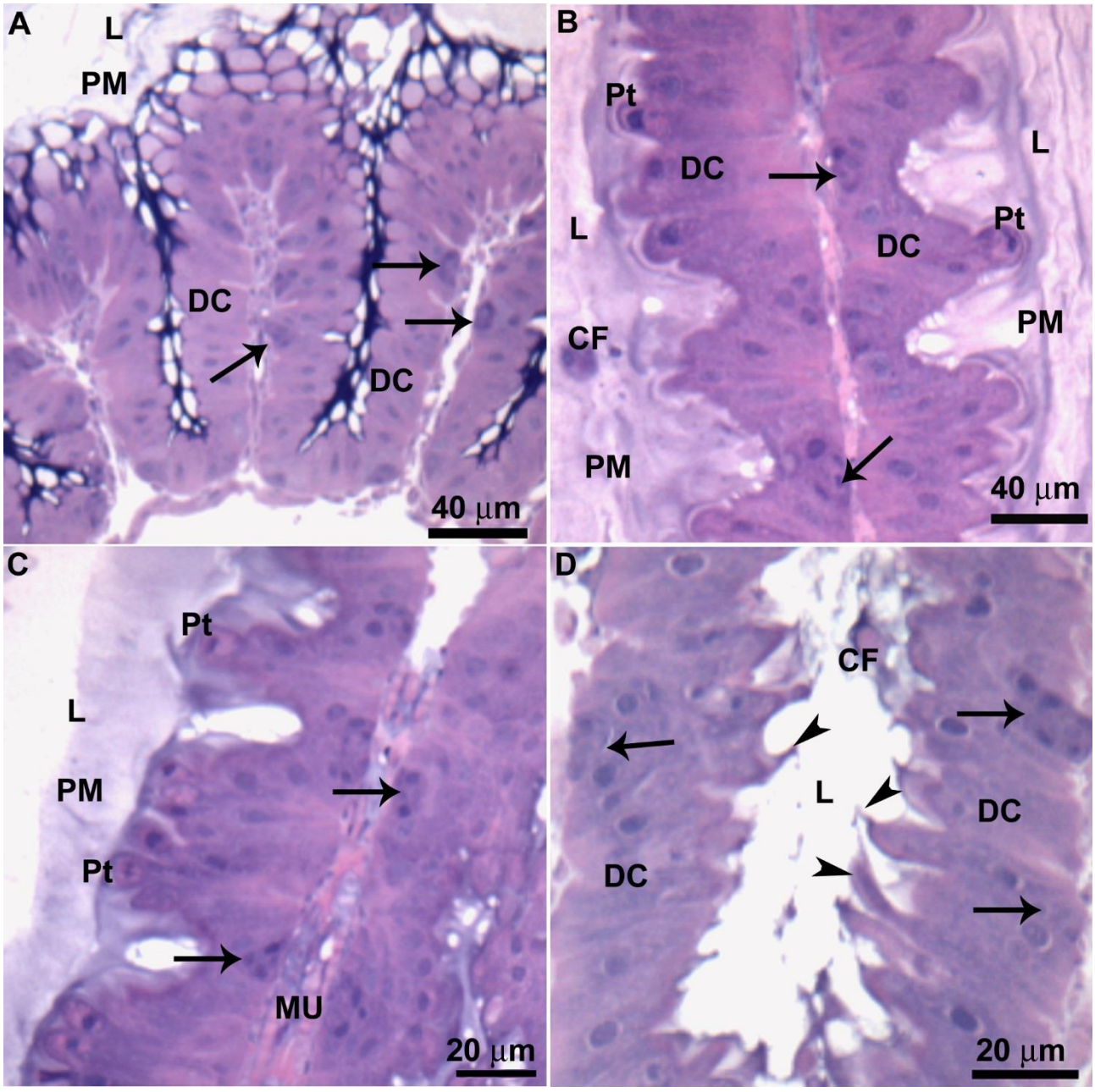


**Figure 1.** Light micrographs of the midgut of *Apis mellifera* queens. **A)** Folded epithelium of four-days-old virgin queen showing digestive cells (DC) and regenerative cell nests (arrows). **B)** Eight-month-old queen showing epithelium with apical protrusions (Pt) some released as cell fragments (CF) to the lumen (L) and the peritrophic matrix (PM). **C)** 11-month-old queen showing epithelium with apical protrusions (Pt), regenerative cell nests (arrows) and circular muscle (MU). **D)** 25-month-old queen showing epithelium with irregular apical surface (arrowheads), cell fragment (CF), and nests of regenerative cells (arrows).

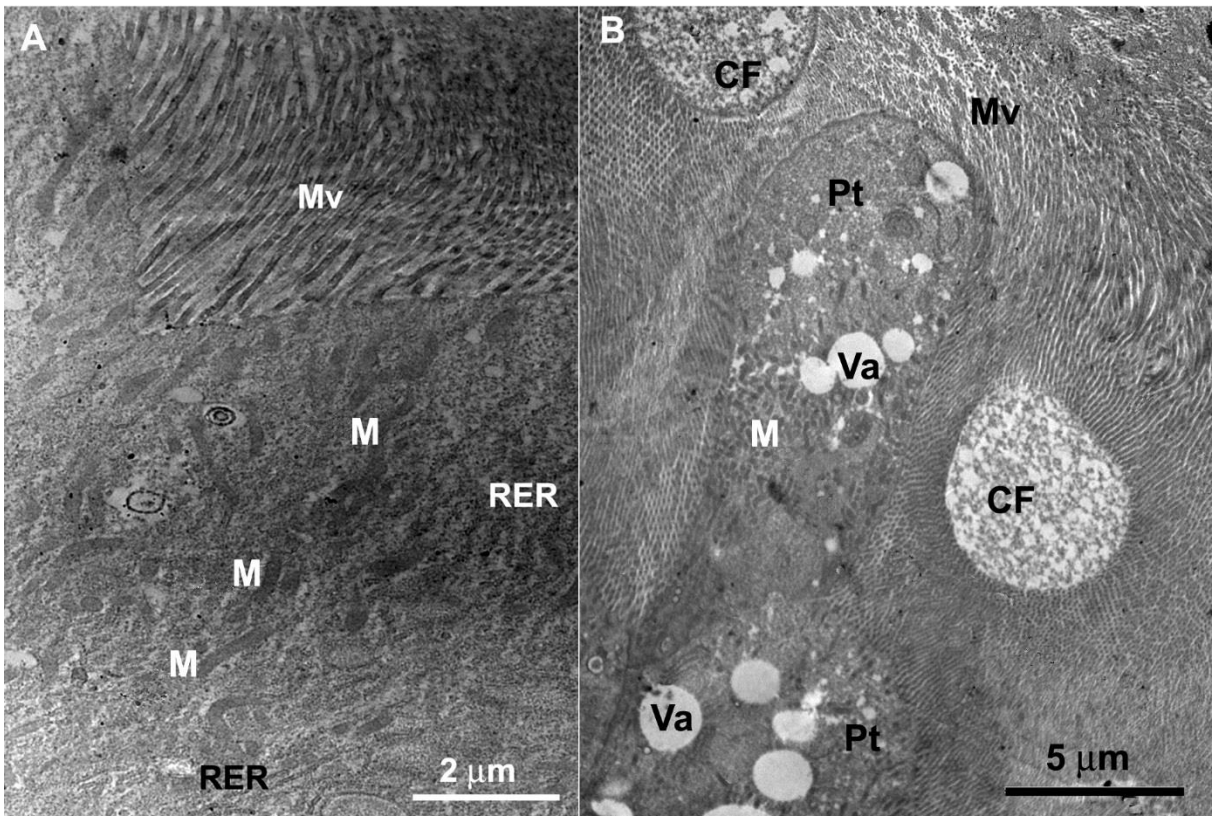


**Figure 2.** Transmission electron micrographs of the midgut digestive cells in *Apis mellifera* queens. **A**) Four-days old queen showing cell with well-developed apical microvilli (Mv) and cytoplasm rich in mitochondria (M) and some rough endoplasmic reticulum (RER). **B)** 11-month-old queen showing cell with apical protrusions (Pt) rich in vacuoles with electron-lucent content (Va) and some mitochondria (M). Note cell fragments (CF) with flocculent content released into the midgut lumen.

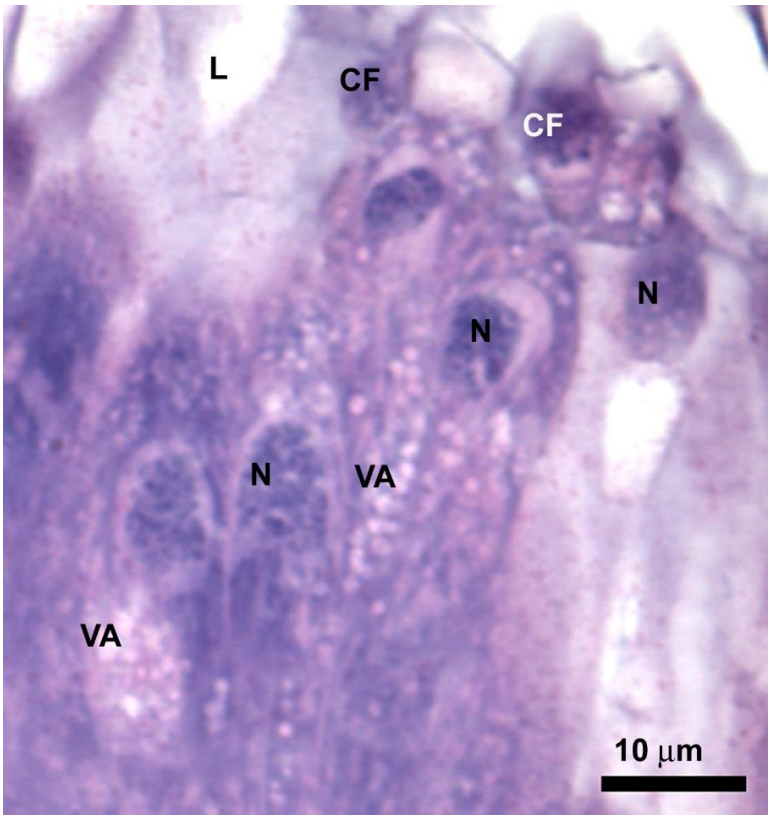


**Figure 3.** Light micrographs of the midgut of 25-month-old *Apis mellifera* queen showing digestive cells with cytoplasm rich in vacuoles (VA) and cell fragments (CF) release to the lumen (L), some with the cell nucleus (N).

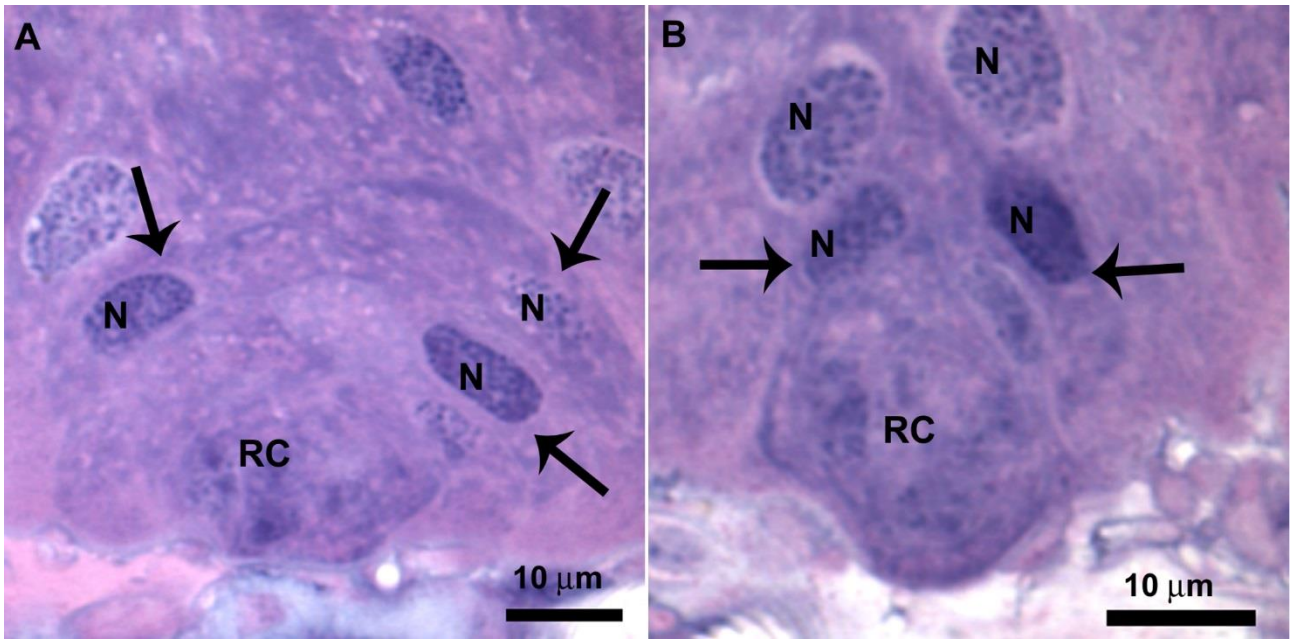


**Figure 4.** Light micrographs of the midgut of *Apis mellifera* queen showing regenerative cell nest. Note central regenerative cell (RC) and peripheral ones narrowed and straightening (arrows) with enlarged nucleus (N). **A)** 11-month-old queen. **B)** 25-month-old queen.

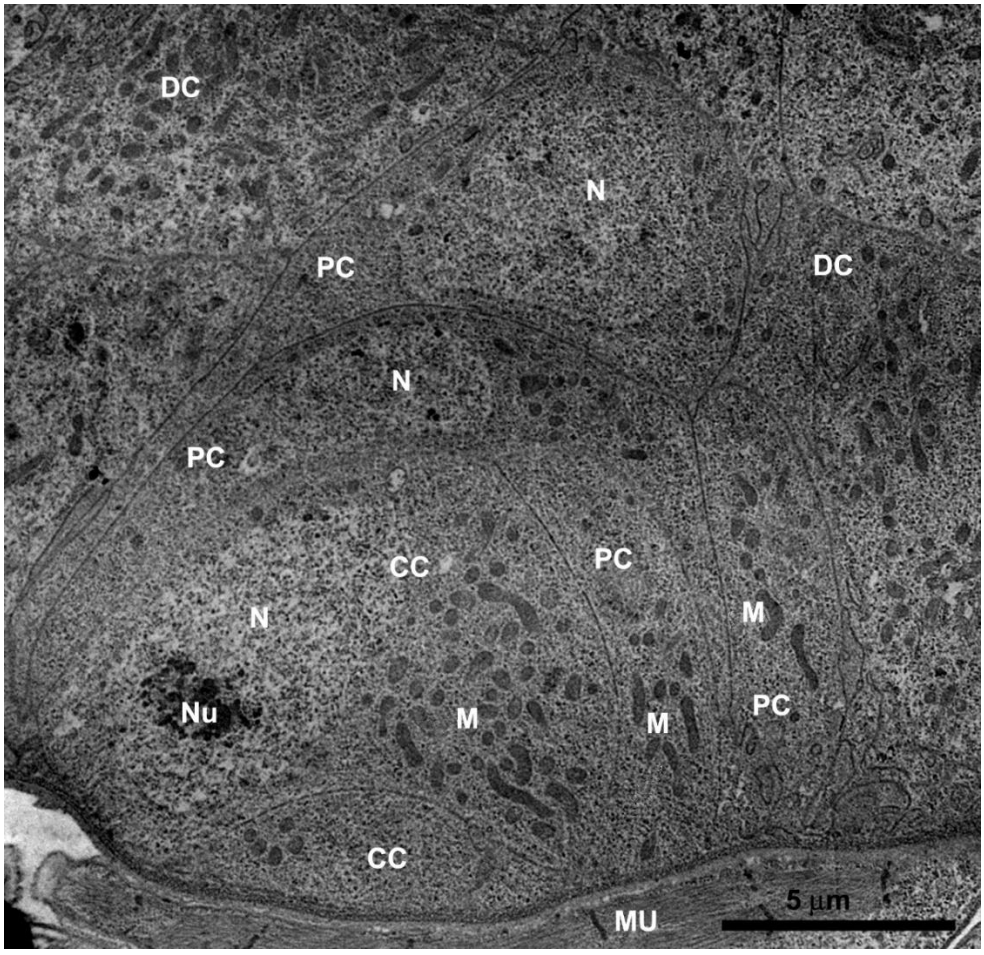


**Figure 5.** Transmission electron micrograph of the regenerative cell nest in the mdigut of eight-month-old *Apis mellifera* queen, showing central regenerative cells (CC) with nucleus rich in decondensed chromatin (N) and evident nucleolus (Nu) and cytoplasm with mitochondria (M). Note peripherial regenerative cells (PC) straightening with nucleus (N) with predominance of decondensed chromatin and cytoplasm with mictochondria (M). DC – Digestive cell

## DISCUSSION

The midgut wall of the Africanized *A. mellifera* queen, with a folded simple epithelium with digestive cells, apical brush border and nests of regenerative cells, is similar to that found in workers of this bee [23].

The digestive cells of the midgut epithelium of *A. mellifera* queens, with well-developed nuclei rich in decondensed chromatin and evident brush border, indicate that these cells may be multifunctional, similar to worker bees, with participation in the synthesis of digestive enzymes [30], nutrient absorption [31] and the production of peritrophic matrix components (17,32].

The aging of *A. mellifera* queens affects the digestive cells, as revealed by the occurrence of apical protrusions and the release of cell fragments, which are characteristics of cell degeneration, commonly found in the midgut epithelium of bees exposed to stressors, including pesticides [10-13,33]. Cell fragments result from the secretory activity of digestive cells and may contain cytoplasmic inclusions, which indicates osmotic regulation and detoxification [34-37]. Furthermore, these protrusions may be apocrine secretion that results in the loss of organelles, including the nucleus [38], as observed here. On the other hand, the release of digestive cell fragments may indicate apoptotic bodies and cell death [39,40].

*Apis mellifera* queen at the age of 25 months reveals the disorganization of the midgut epithelium architecture and cytoplasm vacuolization, which may be due to autophagy and characterize the turnover of cell constituents, a role of autophagy reported in the digestive cells of workers [12,26].

The digestive cell changes aforementioned indicate the degeneration of these cells in *A. mellifera* queens, which need to be replaced by regenerative ones. In *A. mellifera* queens, the regenerative cells in the nest periphery are narrowed and tall, whereas the central cells are globular, similar to those described in other bees [41,42], ants [43] and Hemiptera [27]. In adult insects, it has been claimed that the regenerative cells do not divide, and when they onset to differentiate push the digestive cells above them, which are released into the midgut lumen [44]. When these differentiating cells reach the midgut of the lumen, they are columnar-shaped and develop the brush border [18,44-46].

Our findings indicate that the midgut epithelium renewal of *A. mellifera* queens occurs by the differentiation of regenerative cells, but without the occurrence of cell proliferation, since aging decreases the number of regenerative cell nests in the digestive tract. The queen has the longer lifespan in the honey bee colony, which suggests the need for several cell renewals in the midgut epithelium. In fact, queens have more regenerative cell nests than workers, since the latter has shorter lifespan [42]. The decreased number of regenerative cell nests may disrupt the midgut epithelium and affect digestibility, which has been reported in the stingless bee *Scaptotrigona postica* (Latreille 1807) [29].

The results obtained here reveal a decreased number of regenerative cell nests in the midgut of older (25-month-old) Africanized A*. mellifera* queens, which are believed to survive for up to five years [47,48]. Therefore, it is plausible to suggest that the decreased number of regenerative cell nests affects nutrient digestion and absorption, due to the lower renewal of digestive cells, which compromises queen physiology. Queens of Africanized *A. mellifera* over 12 months old drop egg production and pheromones [47], consequently reducing honey production and jeopardizing colony productivity and survivor [47]. Thus, it is recommended that beekeepers replace the queens annually [47,48]. However, that is a complex operation, since young queens may be rejected by the workers, and larvae can be injured during handling and transfer to the artificial queen brood cells [47].

Although the number of regenerative cell nests decreases as *A. mellifera* queen ages, the number of central (undifferentiated) cells per nest remains constant. Therefore, aging does not seem to affect the number of cells available in each nest, but leads to the absence of regenerative cell proliferation [17,45], since the number of cells per nest does not increase. The midgut epithelial homeostasis in adult *Drosophila melanogaster* Meigen 1830 (Diptera) has been claimed to involve proliferation of the regenerative cells, before differentiation in digestive ones [49,50]. Thus the absence of proliferating regenerative cells in the midgut of the honey bee queens here evaluated indicates the need for further studies.

## CONCLUSION

Our results demonstrate that the number of regenerative cell nests decreases in the midgut of Africanized *A. mellifera* queens from eight months of age onwards, which may compromise the renewal of digestive cells in this insect.

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