

## SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Ultrastructural Modifications in the Venom Glands of Workers of *Apis mellifera* L. (Hymenoptera: Apidae) Promoted by Topical Application of Juvenile Hormone

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Modificações Ultra-Estruturais nas Glândulas de Veneno de Operárias de *Apis mellifera* L. (Hymenoptera: Apidae) Promovidas pela Aplicação Tópica de Hormônio Juvenil

RESUMO - O presente estudo analisou, através de estudos ultra-estruturais a influência do tratamento com hormônio juvenil sobre as glândulas de veneno de operárias de *Apis mellifera* L. Para tanto, operárias recém-emergidas receberam aplicação tópica de 1µl de hormônio juvenil, na concentração de 2 µg/µl, sendo usado o hexano como veículo. Foram feitos dois controles, um que não recebeu nenhum tipo de tratamento (grupo C1) e o outro que recebeu aplicação tópica de 1 µl de hexano (grupo C2). O aspecto das células glandulares, em operárias recém-emergidas, mostra que estas não estão ainda secretando ativamente. Observa-se que alterações celulares ocorrem de acordo com a idade da operária e da região glandular considerada no controle C1. Assim, a fase de secreção mais ativa da glândula ocorre entre a emergência e os 14 dias de idade; aos 25 dias as células já perderam sua característica secretora, sendo a região distal a primeira a sofrer degeneração. Os tratamentos com hormônio juvenil e com hexano alteram a cronologia do ciclo glandular, antecipando o início da secreção e da degeneração da glândula.

PALAVRAS-CHAVE: Ciclo secretor, degeneração glandular, hexano, abelha

ABSTRACT - The present study analyzed, the influence of the treatment with juvenile hormone on the ultrastructure of *Apis mellifera* L. workers' venom glands. Newly emerged workers received topical application of 1 µl of juvenile hormone diluted in hexane, in the concentration of 2 µg/µl. Two controls were used; one control received no treatment (group C1) and other received topical application of 1 µl of hexane (group C2). The aspect of the glandular cells, in not treated newly emerged workers, showed that they are not yet secreting actively. Cellular modifications happened according to the worker age and to the glandular area considered. The most active phase of the gland happened from the emergence to the 14th day. At the 25th day the cells had already lost their secretory characteristic, being the distal area the first to suffer degeneration. The treatment with juvenile hormone and hexane altered the temporal sequence of the glandular cycle, forwarding the secretory cycle and degeneration of the venom gland.

KEY WORDS: Secretory cycle, glandular degeneration, hexane, bee

The venom glands of workers of *Apis mellifera* L. presents a single secretory cycle characterized by an intense synthetic stage in young workers of up to 16 days after emergence. The venom produced is then stored inside the reservoir and the venom gland begins to degenerate (Cruz-Landim & Kitajima 1966, Cruz-Landim *et al.* 1967, Owen & Bridges 1976, Abreu *et al.* 2000). Thus, in foraging workers, the glandular cells present evident signs of degeneration, lacking most distinguishable cellular

structures, with the nuclei and the microvilli surrounding the canaliculi as the only discernable structures. Vesicles with irregular sizes and shapes are found in the remaining cytoplasm. Structures in the form of concentric lamellae frequently appear in degenerating cells (Cruz-Landim & Kitajima 1966).

Studies developed by Cruz-Landim *et al.* (1967) and Abreu (2000) in *A. mellifera* showed that, in addition to the differential glandular activity observed through the life of

the worker, there is also a differential activity among the cells of the distal and proximal regions of the glandular filament, with the cells of the distal region being more active in the synthesis and secretion of the proteic components of the venom.

Modifications in the secretory cycle of insect's glands during their life translate into modification in the genetic program for their functioning. The genetic program also changes during glandular degeneration, in which the genes that lead to the process of programmed cell death are activated (Amabis *et al.* 1981).

The control over the changes that occur on the genetic programming in insect's organs is carried out by the hormones, particularly the juvenile hormone and ecdisone. In the adult, the juvenile hormone has a somatotrophic effect, which in bees controls the development of specific organs, specially the fat body, ovaries, and hypopharyngeal glands. The hormone also controls age polyethism, which characterizes the division of labor in the worker caste (Jaycox 1976). The level of juvenile hormone in the haemolymph of workers rises when they progress from performing tasks inside the colony to tasks outside the colony (Akamatsu *et al.* 1975, Rembold 1976, Robinson 1987). Adult workers of *A. mellifera* present low titers of juvenile hormone in the haemolymph during the first two weeks of adult life, when they perform tasks related to brood care (Rutz *et al.* 1976, Fluri *et al.* 1982), and higher titers when they become foragers from the third week of life (Robinson *et al.* 1989; Huang *et al.* 1991, 1994; Robinson 1992). The rise in the titer of juvenile hormone induces a precocious foraging behavior (Robinson 1985, Sasagawa *et al.* 1989, Sullivan *et al.* 2000), thus suggesting that the mechanism through which this control is attained is through the effect of the juvenile hormone over the glandular development of the workers.

Therefore, these observations justify studies that aimed at identifying the role of the juvenile hormone on the development of the glands of the exocrine system (Bonetti *et al.* 1994, Abdalla *et al.* 2001, Paes de Oliveira & Cruz-Landim 2001), with the intent of understanding its effect on either the immature stages or on the adult individual. In this work we take into consideration that these glands do not present innervations, thus being under hormonal control, and presenting different developmental degrees among workers performing tasks inside or outside the nest. Therefore, it is possible that the juvenile hormone controls the development of the exocrine glands rendering the worker apt to perform the different tasks according to age.

The present study had the objective of verifying the effect of the treatment with juvenile hormone on the ultrastructure of the venom glands of workers of *A. mellifera*.

## Materials and Methods

For the study of the venom glands, workers of *Apis mellifera*, were collected at the Apiary of the Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), in Rio Claro, SP, Brazil.

About 150 newly emerged workers were divided into

three experimental groups by marking them with non-toxic paint of different colors on the thorax. The first group did not receive any kind of treatment, thus constituting the control group (group C1). The second group received the topical application of 1 µl of 2 mg/µl juvenile hormone (JH-III, Sigma) diluted in hexane (group JH) on the dorsal portion of the abdomen, and the third group received the topical application of 1µl of pure hexane (group C2). After marking and treatment, the bees were returned to the colony and 10 individuals of each group were collected after reaching 14 and 25 days of age in order to study their venom glands. Ten newly emerged workers were also collected.

**Transmission electron microscopy.** The venom glands of all individuals collected were removed in insect saline (NaCl 7.5g/l, Na<sub>2</sub>HPO<sub>4</sub> 2.38g/l e KH<sub>2</sub>PO<sub>4</sub> 2.72g/l). The glands were divided into proximal and distal portions. These portions were fixed separately in modified Karnovsky (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2h at 4°C. Once fixed, the glands were washed in the same buffer for 30 min. Next, the material was post-fixed in 1% osmium tetroxide in the same buffer for 2h at room temperature. Afterwards, the glands were washed again in the same buffer for additional 30 min. The material thus post-fixed were contrasted for 2h in a solution of 2% uranyl acetate in 10% acetone and then was subjected to dehydration in a standard acetone series. The material was included in Epon-Araldite resin and ultrathin sections were obtained in an ultra-microtome. The sections were contrasted with lead citrate for 15 min. and examined under the TEM.

## Results

The venom gland of workers of *A. mellifera* presents a layer of flat epithelial cells known as the intima, which is internally coated by a thick cuticle. These cells present flat nuclei and the cytoplasm may present or not rough endoplasmic reticulum and free ribosomes (Fig. 1A) depending on the age of the worker and/or on the glandular stage. Externally to the intima there is another layer of epithelial cells, the secretory cells (Fig. 1A). The intracellular collecting canaliculi (terminal apparatus) are found dispersed throughout the cytoplasm. These canaliculi are cuticular structures formed by a discontinuous epicuticle and a porous procuticle. Around these canaliculi the plasma membrane form long microvilli of uniform extension that are sustained by microfilaments that project radially towards the wall of the canaliculi (Figs. 1A, C). At the base of the microvilli there are numerous vacuoles with different electron densities and at their apex we observed pinocytosis vesicles (Fig. 1C). The nucleus of the secretory cell appears dislocated towards the basal region and contains dispersed accumulations of chromatin. The cytoplasm is rich in rough endoplasmic reticulum, polymorphic mitochondria and Golgi complexes (Fig. 1A). The basal plasma membrane may infold to form a peripheral labyrinth (Fig. 1B). The collecting intracellular canaliculi are linked to an extracellular duct that opens into the glandular lumen, perforating the intima (Fig. 1A).

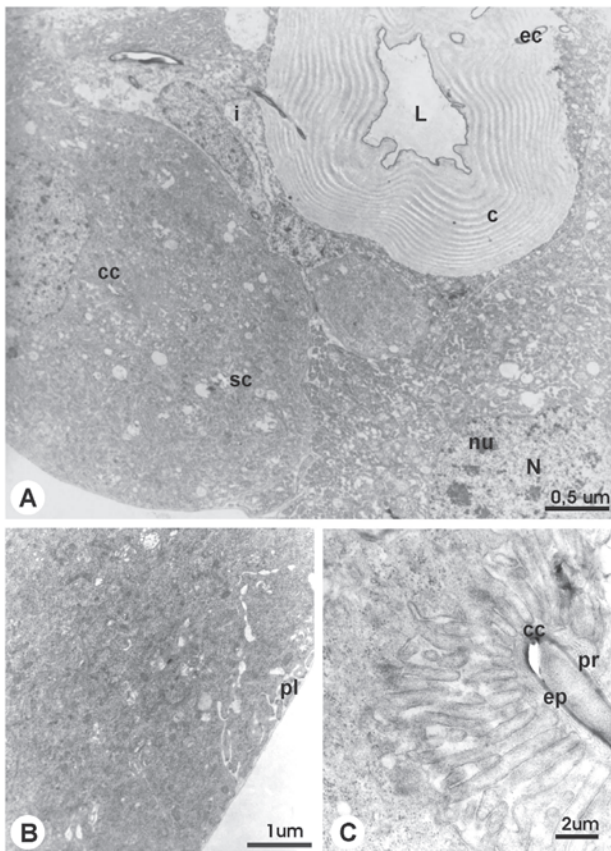


Figure 1. TEM micrographs showing the venom gland general organization of newly-emerged worker of *A. mellifera*. A. General aspect of intima (i) cells covered by cuticle (c) around the lumen (L) and the excretory canaliculi (ec). Notice the secretory cells (sc) with collector canalicules (cc); N = nuclei; nu = nucleoli. B. Distal region with peripheral labyrinth (pl). C. Detail of collector canaliculi (cc) showing the epicuticle (ep) and the pro-cuticle (pr).

At all life stages of the workers, the glands present this same general constitution. However, according to age and glandular region a few variations may be found mainly with regards to the secretory cells. These aspects characterize their current state of the functional cycle.

Newly emerged workers present a gland with cylindrical secretory cells that appear intact in all regions studied (Figs. 1A-C). The secretory space is enclosed by long microvilli (Figs. 1A, C). At 14 days of age, in the untreated workers (Group C1), the secretory cells appear slightly altered. The cytoplasm appears vacuolated, with disarrayed reticulate and presence of concentric lamellar structures (Figs. 2A, B). The nuclei present an irregular shape and condensed chromatin (Fig. 2A). The lumen is filled by an homogeneous and electron dense secretion (Figs. 2A, B). These characteristics are observed at all glandular regions, but are more pronounced at the distal region (Fig. 2B). The characteristics of cellular degeneration are still more evident at all glandular regions of untreated workers with 25 days of age. These workers present an evidently altered

epithelium, with secretory cells of irregular shape (Fig. 3A). In addition to the concentric lamellar structures associated or not to electron dense granules that are dispersed throughout the cytoplasm (Fig. 3C), structures in a paracrystalline arrangement are also observed associated or not to membranous structures (Fig. 3B). The nucleus acquires a completely irregular shape showing a highly condensed chromatin and granular materials are found in the glandular lumen (Fig. 3A).

The treatment with juvenile hormone alters the sequence of this glandular cycle. Therefore, workers subjected to this treatment presented at 14 days of age a glandular epithelium with more alterations than in individuals of group C1 at this

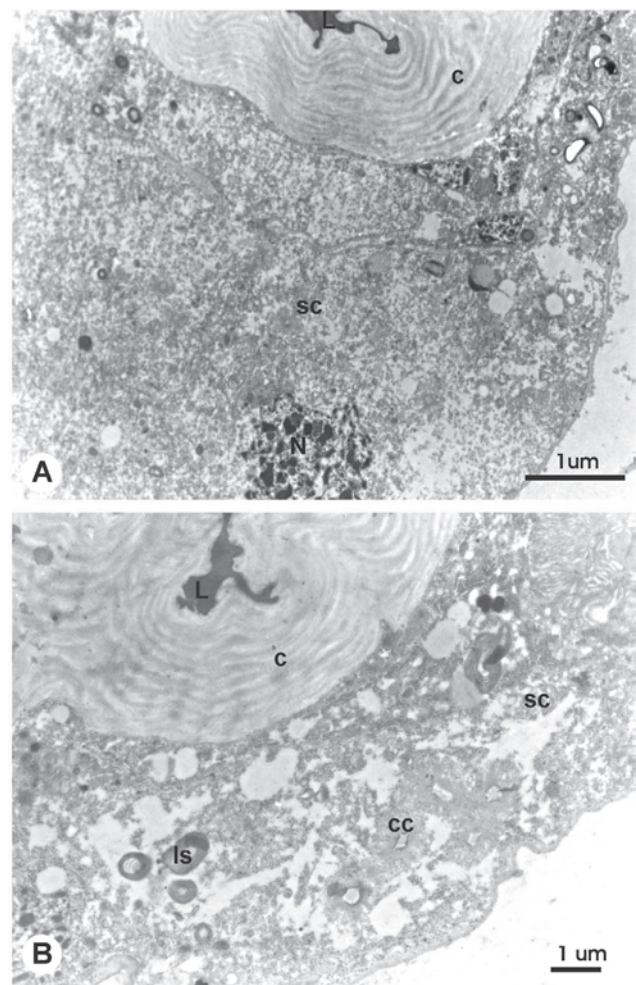


Figure 2. TEM micrographs of the gland venom of 14 day-old worker of *A. mellifera* from the control without treatment (C1 group). A. Proximal region of the gland, showing the secretory cell (sc) and the nucleus (N) with modified aspect. Note the presence of secretion in the glandular lumen (L); c = cuticle. B. Distal region general view, showing the vacuolated cytoplasm of the secretory cell (sc) and the presence of lamellar structures (ls). Note the secretion in the lumen of the glandular tube (L); cc = collector canaliculi; c = cuticle.

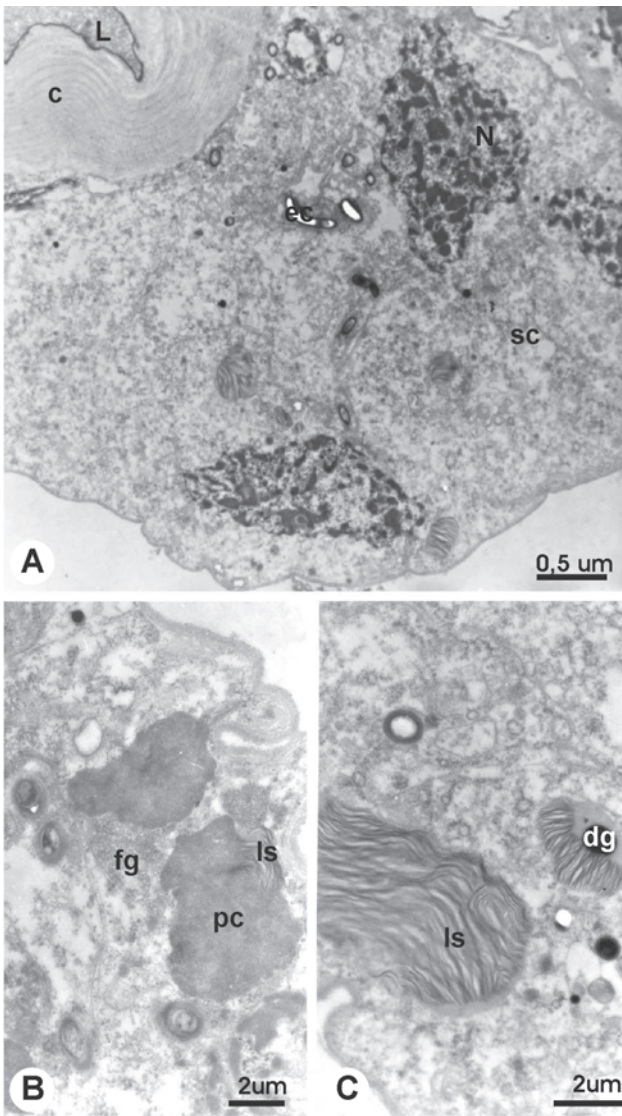


Figure 3. TEM micrographs of the venom gland of 25 day-old workers of *A. mellifera* from the control without treatment (C1 group); A. Distal region general view, showing the secretory cell (sc) with nucleous (N) with irregular shape and condensed chromatin; L = lumen; c = cuticle. B. Magnification showing structures with para-crystallin (pc) associated to membranous lamellas (ls) and regions showing thin granulation (fg) which can be precursory of the crystal. C. Lamellar structures (ls) with dense granules (dg).

age. The cellular structures with characteristics of cellular degeneration are more evident (Figs. 4A-D). The cytoplasm appears completely vacuolated (Figs. 4A, C), with numerous concentric lamellar structures usually located around the terminal apparatus (Fig. 4B). At this age, in the treated group, we noted the presence of structures that were only observed in the C1 control group at 25 days of age, such as the presence of concentric lamellar structures associated to electron dense

granules (Fig. 4D) and structures with a paracrystalline arrangement at the distal region (Fig. 4C).

Workers treated with the solvent of the juvenile hormone, hexane (Group C2), presented at 14 days of age a proximal region with a fairly intact glandular epithelium (Fig. 5A), even more preserved than in group C1. At this region, the intracellular spaces are dilated, the rough endoplasmic reticulum is well developed, and several regions of Golgi complexes can be observed in the cytoplasm (Fig. 5A). Nevertheless, the secretory cells of the distal region appear more altered, showing a great loss of cytoplasm (Fig. 5B).

At 25 days, the differences between the group treated with juvenile hormone and the groups C1 and C2 are more discreet, since at this age, independently of the treatment

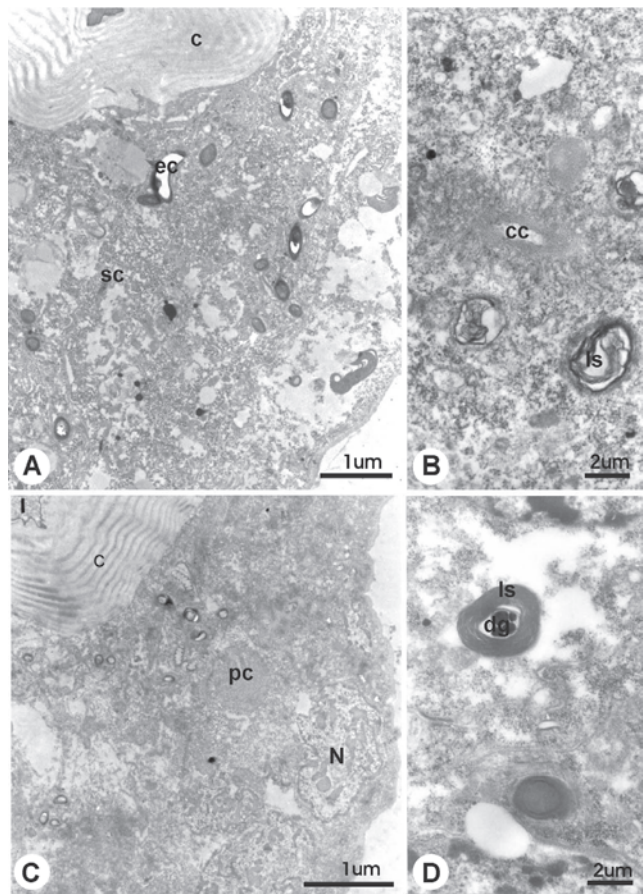


Figure 4. TEM micrographs of the venom gland of workers of 14 days of *A. mellifera* of the group treated with juvenile hormone (HJ group); A. Proximal region general view of the gland, showing the modified secretory cells (sc); c = cuticle; ec = excretory canaliculi. B. Detail of the proximal region, showing the collector canaliculi (cc) with lamellar structures (ls) around showing the secretion release. Note the cytoplasm altered. C. Distal region general view. Note the presence of structures with para-crystalline arrangement (pc) and nuclei (N) with irregular shape and chromatin condensed; l = lumen; c = cuticle. D. Detail of the distal region with lamellar structures (ls) and electron dense granules (dg).

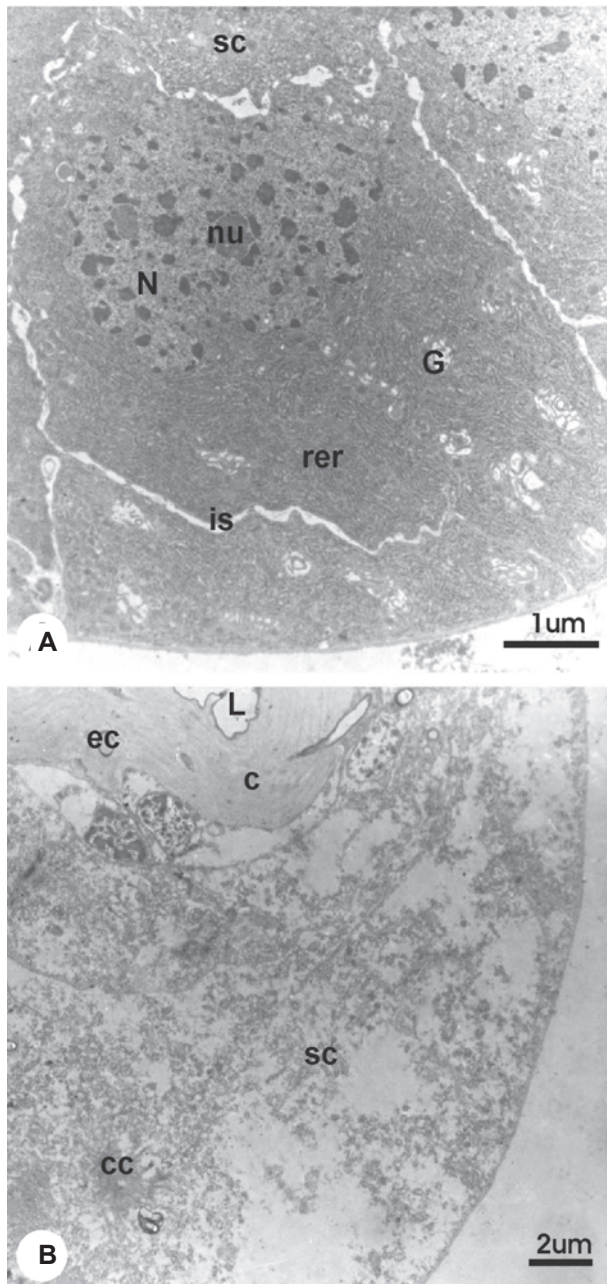


Figure 5. TEM micrographs of the venom gland of 14 day-old workers of *A. mellifera* from the control with the hexane treatment (C2 group). A. General aspect and details of the proximal region. Note secretory cells (sc) preserved, opened intercellular spaces (is), rough endoplasmic reticulum (rer) and many Golgi regions (G). The nuclei (N) is with irregular shape, chromatin condensed (cr), and nucleolus (nu); B. Distal region general view, showing altered structure; l = lumen; c = cuticle; ec = excretory canaliculi; cc = collector canaliculi; sc = secretory cell.

and region under consideration, the glandular epithelium appears completely degenerated. Figs. 6A and 6B show the characteristics observed in the group treated with juvenile

hormone and Figs. 6C through 6E show the characteristics observed in group C2, treated with hexane. In Fig. 6C it is possible to observe the presence of an homogeneous mass that represents the condensation of a secretory cells, probably in process of cellular death.

## Discussion

Accompanying the ultrastructural modifications of the glandular cells of the untreated control group, it is possible to observe that cellular alterations occur according to the age of the worker and glandular region under consideration. The stage of greater development of the secretory apparatus of the cell, and therefore the stage of active secretion of the gland, must occur between emergence and 14 days of age, since at this age we found some alterations at the distal region of the gland, even though the proximal region showed intact cells. The fact that there was secretion inside the lumen shows that this secretion is still being produced or that it is still being transported into the reservoir. At 25 days, the cells have already lost their secretory characteristics, such as the presence of collecting canaliculi in the cytoplasm, presence of rough endoplasmic reticulum and nuclei with disperse chromatin and regular shape.

The venom gland presents a single secretory cycle, which begins at the end of pupation and reaches its maximum around the 16th day of the worker's adult life. From this point, the venom is stored inside the reservoir and the gland enters the degeneration process, which is completed around 30 days after emergence (Cruz-Landim & Kitajima 1966, Cruz-Landim *et al.* 1967). The present study confirms this result and shows an asynchrony in the cellular function between the glandular regions studied, since at 14 days the signs of cellular degeneration are more frequent in the distal region than in the other regions of the gland studied.

The venom is used by the workers for defense and, thus, must be available at the time when the task they execute exposes them to the greater dangers, that is, in the workers performing the tasks of guardians, which precedes the foraging task. Therefore, the secretion of the venom must precede the onset of this activity. On the other hand, the worker that stings loses its sting and the posterior part of the abdomen, leading to its death. Thus, there are no reasons that justify another secretory cycle under natural conditions. Nevertheless, it is interesting to point out that if the venom reservoir is emptied artificially without harming the sting, it could fill up again, thus indicating that the venom gland is able to secrete again (Abreu 1996). Therefore, in this case, as in others reported previously, the gland is capable of responding to external stimuli, altering its secretory cycle.

The treatment with juvenile hormone acts on the venom gland maintaining the pattern of cellular asynchrony, with the distal region of the gland of individuals of the treated group presenting at 14 days characteristics that were only observed at 25 days in the untreated C1 group. Abreu (2000), through histological and histochemical studies of the venom gland of workers, also observed that the glandular filament presents asynchrony. However, according to this author the

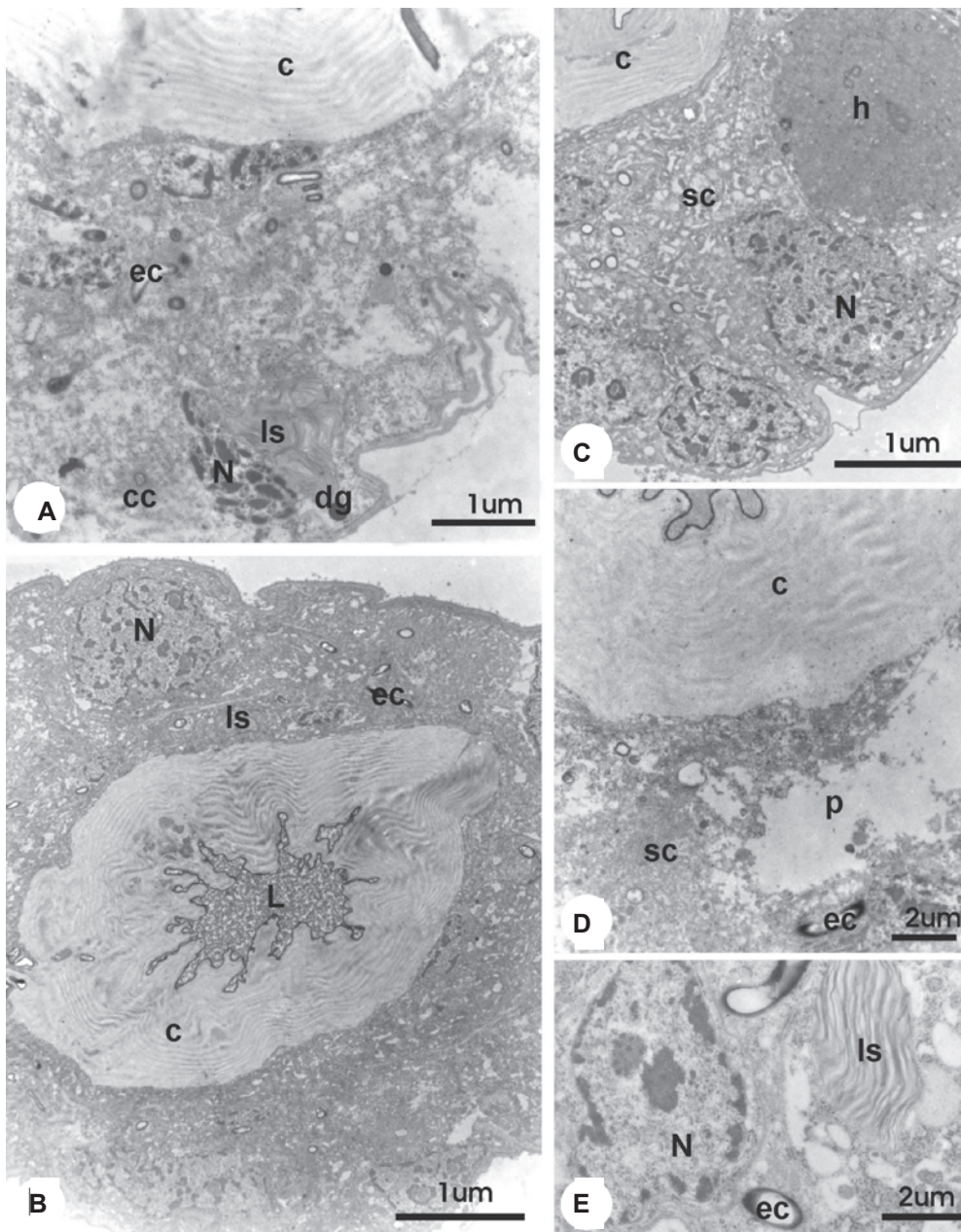


Figure 6. TEM micrographs of the venom gland of 25 day-old workers of *A. mellifera* from the group treated with juvenile hormone (HJ group) and the control with the hexane treatment (C2 group). A e B. Proximal region general view of the gland of workers treated with juvenile hormone, showing the presence of lamellar structure (ls) and nuclei (N) with irregular shape and condensed chromatin; c = cuticle; ec = excretory canaliculi; cc = collector canaliculi; dg = dense granules; L = lumen. C. Distal region general view of the gland of workers treated with hexane. Note the secretory cell (sc) with an homogeneous mass (h) and nuclei (N) with irregular shape and condensed chromatin; c = cuticle. D. Proximal region general view of the gland of workers treated with hexane, showing the secretory cell (sc) with cytoplasmic loss (p); c = cuticle; ec = excretory canaliculi. E. Aspect of cytoplasmic desorganization of the gland of workers treated with hexane, showing concentric lamellar structures (ls) and condensation of the nuclei (N); ec = excretory canaliculi.

proximal region degenerates first, which contradicts the results presented here but agrees with observations in other glands (Cruz-Landim & Mello 1981, Silva & Silva de Moraes 1999), in which the glandular degeneration begins

at the distal end.

The juvenile hormone III is the only form found in the workers of *A. mellifera* (Fluri et al. 1982, Robinson & Ratnieks 1987, Robinson et al. 1989) and the titer of this

hormone increases with the age of the worker, being that immediately after emergence, at the first day of adult life, these bees present approximately 5 pmol of this hormone for every 100 ml of haemolymph, but at 21 days of age they present about 20 pmol per 100 ml of haemolymph (Fluri *et al.* 1982, Robinson & Ratnieks 1987). Thus, the variations in the levels of juvenile hormone during adult life, mainly during the transition from performing activities inside the colony to foraging, affect the secretory cycle of the venom gland, as was shown through the ultrastructural analysis. When the workers present low levels of juvenile hormone, the cells of the venom gland present characteristics of active secretory cells. These characteristics were observed in newly emerged workers and in the untreated control group C1 at 14 days of age, although a few signs of degeneration were detected in the individuals of this group. Nevertheless, when the titer of juvenile hormone is high, the venom gland presents evident signs of degeneration, similar to the ones observed in workers of the untreated group C1 but at 25 days of age. These facts show that the anticipation of the secretory cycle and the degeneration of the venom gland promoted by the treatment with juvenile hormone is reflected on the glandular ultrastructure as a disarray of the secretory apparatus of the cells.

A similar effect was observed in other glands that are related to intra-colonial tasks. In nurse workers, the hypopharyngeal glands secrete substances that serve as food for the larvae. The treatment with juvenile hormone (Jaycox *et al.* 1974, Rutz *et al.* 1976) on newly emerged workers causes the premature degeneration of these glands, a process that would normally occur when the workers cease to perform tasks inside the colony. This result shows, once more, that the hormonal determinism of behavior is expressed through the maturation of the organs involved in the functions to be performed during each life stage.

In the present work, the treatment with hexane (group C2) produced at 14 days of age workers with secretory cells more preserved than group C1. In addition, hexane presented an effect that was similar to the one induced by the treatment with juvenile hormone, but with less intensity. A similar effect for the treatments for the hormone and its solvent hexane was verified by Nocelli *et al.* (2000) through morphological analyses of the venom gland of *A. mellifera* and by Roat *et al.* (2004) through biochemical analysis by means of polyacrylamide gel electrophoresis (SDS-PAGE) on the band pattern of the glandular extracts.

We conclude that the treatment with juvenile hormone causes a precocious maturation or aging in the venom gland of *A. mellifera*; thus, in this case, its effect is contrary to the one exerted during the larval stage when its topical application reverts the condition of the gland into a previous stage.

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