MATING INFLUENCE IN THE OVARY DIFFERENTIATION IN ADULT QUEENS OF *Apis mellifera* L. (HYMENOPTERA, APIDAE)

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Received May 31, 2001 – Accepted September 11, 2001 – Distributed November 30, 2002

(With 4 figures)

ABSTRACT

The present results show that in the ovarioles of a newly emerged (0 day) queen of *A. mellifera* only two regions may be distinguished: a proximal, short germarium and a very long distal, terminal filament. As the queen matures and gets ready for the nupcial flight, the germarium increases in lenght, advancing towered the distal end, as the terminal filament shortens. The ovarioles of queens ready to mate (6 to 8 days old) have, already one or two ovarian follicles, i.e. a very short proximal vitellarium, but a real vitellogenesis only starts after the fecundation. If the queen does not mate the ovarioles structure is disrupted (12-16 days old). In mated queen eggs the ovarioles present three differentiated regions, from the apice to the basis: a short terminal filament, a medium size germarium, and a very long basal vitellarium. As the eggs are laid, the emptied follicle collapses, degenerates and produces a *corpus luteum*.

Key words: mating, germarium, vitellarium, oocyte.

RESUMO

Influência do acasalamento na diferenciação do ovário em rainha adulta de *Apis mellifera* L. (Hymenoptera, Apidae)

Os resultados do presente trabalho demonstram que nos ovaríolos da rainha de *Apis mellifera* recém-emergida (0 dia) apenas duas regiões podem ser distinguidas: um curto germário proximal e um longo filamento terminal distal. À medida que a rainha amadurece e fica pronta para o vôo nupcial, o comprimento do germário aumenta e avança em direção à região distal e o filamento terminal diminui. Os ovaríolos da rainha pronta para acasalar (6 a 8 dias) têm 1 ou 2 folículos ovarianos, isto é, um vitelário proximal muito pequeno, mas a vitelogênese propriamente dita somente se inicia após a fecundação. Nas rainhas não acasaladas os ovaríolos se desestruturam (12-16 dias). Nas rainhas acasaladas os ovários apresentam os ovaríolos divididos em três regiões, do ápice para a base: um filamento terminal pequeno, um germário médio e um vitelário muito longo. Quando os ovos são postos, os folículos esvaziados colapsam, produzem um corpo lúteo e finalmente degeneram.

Palavras-chave: acasalamento, germário, vitelário, ovócito.

INTRODUCTION

The rudiments of the ovaries, in the newly hatched larva consist of two elongated masses of cells, situated lateral to the dorsal body surface, near the midline of the mid-gut. In honey bees, as in other insects the ovaries are derived from the genital ridge, formed by the visceral wall of the mesodermal tubes (Nelson, 1915). The mesoderm provides the epithelial gonadal contour and the small cells which lie between the large germ cells (King, 1970). But the germ cells originate elsewhere in the embryo and later migrate to the gonadal rudiment (Counce, 1961; King, 1970; Büning, 1994).

The general pattern of embryological development of the ovaries is very similar in the holometabolous insects (King, 1970; Lello, 1979; Büning, 1994) and most of the ovary differentiation into its constituent ovarioles takes place during late larval phase and pupation.

The adult bee's ovary consists of a bunch of parallel ovarioles arising from the anterior ends of the lateral oviducts. A fully developed ovariole, in this meroistic polytrophic ovary, consists of a terminal filament containing only somatic cells, a germarium that accommodates oogonia and somatic cells and the vitellarium, which looks like a string of beads increasing in size toward the oviduct. The bead-like swellings contain the oocyte and nurse cells, enclosed by a sheath of epithelial follicular cells, in successive stages of development.

Zacaro (1993) verified that the queens of *Apis mellifera* emerge with the ovarioles in a state in which a vitellarium cannot be distinguished, that is, the whole ovariolar filament is occupied by the germarium, except for the apical terminal filament and the basal pedicel. The author distinguishes three areas in this "germarium". The distal area is a proliferative area in spite of mitoses having been observed there, only a few times. The medium area is characterized by the presence of large cells interlinked by cytoplasmatic bridges. In the proximal region, oocytic and nurse cells are already differentiated. The ovary remains like this until the fourth day after the emergence of the queen (Zacaro, 1993).

Having in mind that Zacaro's observations were limited to the first four days of the queen's adult life and were destined to subsidize oogenesis studies, the purpose of the present work was to study the effect of fecundation over the imaginal differentiation of the queen's ovary.

MATERIAL AND METHODS

Queens of *Apis mellifera* were produced artificially in colonies maintained in the apiary of the Biosciences Institute of UNESP, Rio Claro, SP, by the larval transfer method into queen cups containing royal jelly, using the usual beekeeping procedures. These queens were studied 0, 2, 4, 6, 8, 12, and 15 days after emergence. Newly fecundated and laying queens were also studied, these being captured from the apiary colonies.

The previously anesthetized queens had their ovaries dissected in insect saline solution. For light microscopy (LM) the ovaries were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.0 during 24 hours; washed in the buffer and dehydrated through a series of increasing ethanol concentrations (70% to 95%); infiltrated with histo-resin and imbedded in the same resin with catalist. The 6 μm-thick sections were put onto histological slides and routinely stainded with Hematoxilin and aqueous Eosin.

For Scanning Electron Microscopy (SEM), the ovaries were dissected in insect saline solution and fixed in Karnovisk (2% paraformaldehyde, 2.5% glutaraldehyde in Sörensen buffer 0.1 M) during 24 hours; washed in the buffer and dehydrated through a series of increasing ethanol concentrations (from 70% to 95%); dried in the "Critical Point Drying"; placed in aluminum stubs with the aid of double faced adhesive tape and covered with gold. Some ovaries were frozen in liquid nitrogen and fraturated before preparation to SEM examination. Analysis of the ovaries was made with a Scanning Electronic Microscope JEOL JSM-P15.

RESULTS

The ovary of the queen of *Apis mellifera* is constituted by a great number of ovarioles (up to more than a hundred per ovary). The analysis with light microscopy (LM) enables to follow the aspects of the differentiation of the cellular types that compose the ovarioles. These results will be presented according to the age of the queens after emergence.

Newly emerged queens (zero days old)

In a newly emerged queen these ovarioles are seen at SEM examination, as long and thin cylinders that maintain the same caliber along the entire lenght, only becoming thinner in the terminal filament region. The ovarioles are closely united by a vast tracheolar net. The ovarioles in the distal extremity roll up giving the ovary a round shape (Fig. 1A).

Histologically almost the whole ovariole is constituted by undifferentiated cells, having square to rectangular sections, with spherical central nuclei, forming a line inside the peritoneal sheath of the ovariole, the terminal filament. The cytoplasm and the nuclei of these cells were poorly stained by hematoxilin and eosin. Sections of the numerous tracheoles (Figs. 1B and C) are seen among the ovarioles.

In the proximal portion of this ovary, two regions are distinguished one basal constituted of polyedric cells forming piles, the ovariole pedicel (Fig. 1D), and over that, the germarium contains round large cells, interpreted as germinative cells. Among the germinative cells are seen small, round cells interpreted as somatic cells. The cytoplasm of the poliedric cells is clear and their nuclei present one to three nucleoli. The cytoplasm of the big round cells also stains poorly by hematoxilin, but their nucleus is large, with disperse chromatin and only one evident nucleolus (Fig. 1D). The small round cells have little cytoplasm and almost only the nucleus is visible. The ovarioles are widened only very close to the pedicels that link them to the eggs' cup, when the lateral oviduct begins.

Two day-old queens

In longitudinal ovary sections of queens of this age the ovarioles present the same organization already described (Fig. 1E). They are composed in most of their extension by undifferentiated cells with rectangular sections wrapped up by the peritoneal membrane. However, the basal zone with differentiated large round cells is longer having has already moved a little ahead toward the distal end of the ovariole. The sub mediam zone of the ovaries already show thicker ovarioles having a larger number of cells in their interior (Fig. 1E), and the presence of sporadic large cells surrounded by many small ones (Fig. 1F). The differentiation of all the ovarioles is not synchronous. Tracheoles are still present in large amounts among them.

Four day-old queens

The ovarioles of queens of this age still have a similar aspect to the one observed in ovaries of queens with 0 and 2 days of adult life. However, in the proximal zone of the ovarioles, the number of large cells surrounded by small ones, increased and present more regular arrangement (Fig. 2A). The terminal filament suffers a retraction to the distal end compared to the ovarioles of 0 and 2-day-old queens, due to moving forward the portion of the ovariole containing germinative cells (the germarium).

Six day-old queens

In nature *A. mellifera* queens start to mate after the fifth day of adulthood (Chaud-Neto, personnal information). The examination of histological slides of virgin queen ovaries shows thicker ovarioles than the previous phases. The condition, previously mentioned, of a big cell surrounded by several small cells is more frequent than in the previous ages and this aspect is now restrict to the most proximal portions of the ovarioles, and not randomly arranged in all proximal region as seen in zero and two day old queens. Therefore, in these ovarioles, three zones may be distinguished: a proximal zone with big cells surrounded by small ones, a middle-zone with large and small cells but lacking this arrangement and an distal zone formed by the terminal filament. In the more basal portion of the proximal zone, cysts are already present, which means that the large cell has already undergone the mitotic divisions that originate the cystocytes (Fig. 2B).

Eight day-old queens

The ovarioles of virgin queens with 8 days of adult life are very similar to those of six day old queens, except the observation of transverse strings of cells formation in the medium portion of the ovarioles. A larger cell occupies a central position and the other cells linked to it become slowly smaller. In the most basal portion of the ovariole the difference between nurse cells and oocyte is already noticeable, i.e., the vitellarium starts to appear.

Twelve and fifteen day-old queens

Although Chaud Netto informs that even virgin queens as old as 30 days may still be mated, and lay eggs normally, the natural mating occurs between 5 and 8 days after emergence. Virgin queens with 12 and 15 day have therefore surpassed the optimal age to mate.

In virgin queen ovaries of these ages signs of cell reabsorption may be observed as cell vacuolization (Fig. 2C) and cyst desorganization (Figs. 2D and E). The desorganization is characterized by the cyst collapse and degeneration with cell nuclei condensation and picnosis (Figs. 2D and E).

Newly mated queens

In these queens the ovaries already present the ovarioles clearly divided into a vitellarium (proximal), germarium and a terminal filament (distal). The germarium occupies a small terminal portion and most of the ovariole become constituted by the vitellarium, where oocytes in all phases of development are found (Figs. 3A, B and C).

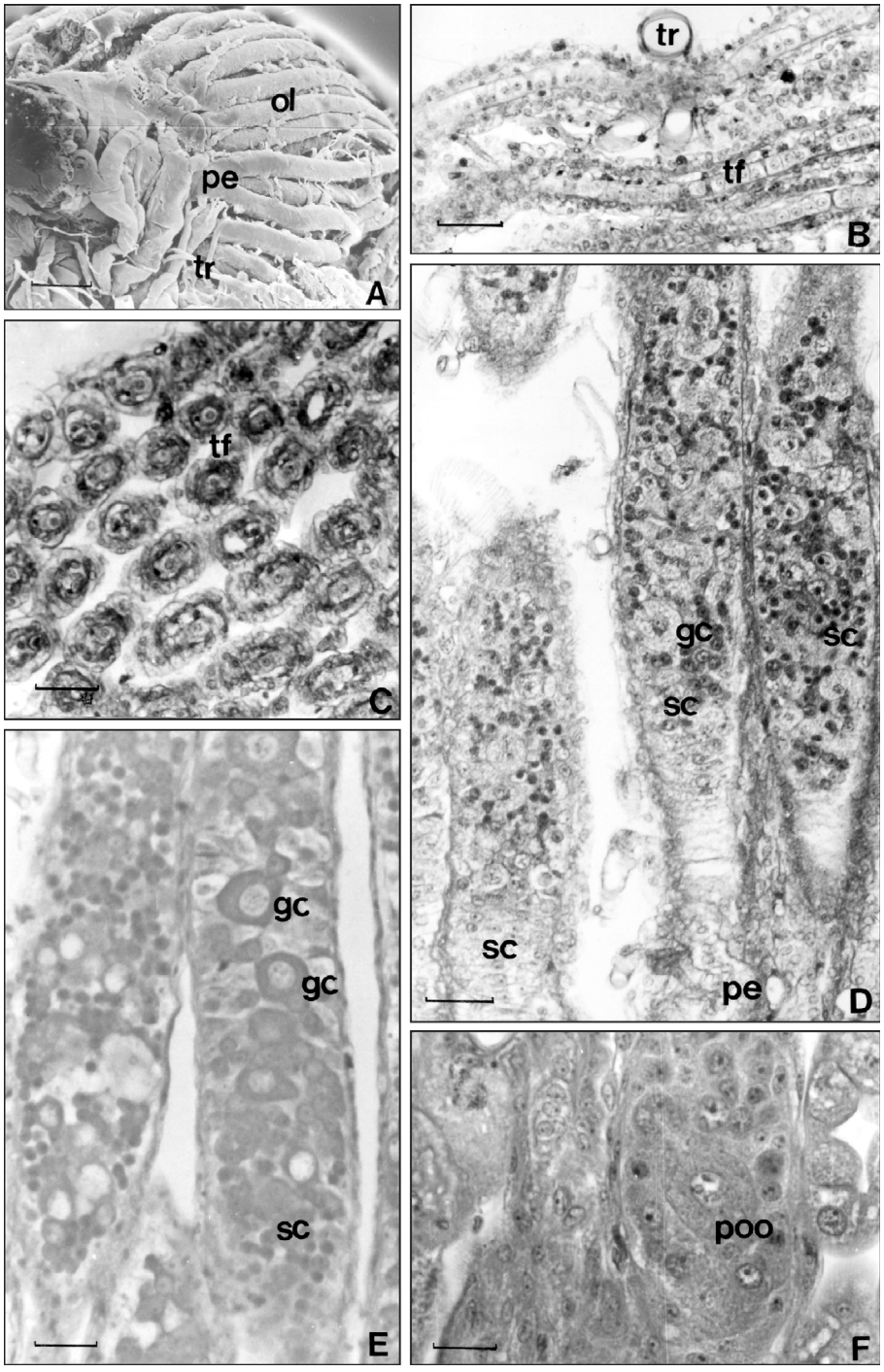

Fig. 1 — A – Scanning electron microscope micrograph showing the ovariole (ol) of a newly emerged queen. **B** and **C** – Longitudinal (B) and cross (C) sections of terminal filament (tf) of virgin queen ovaries. **D** – Basal zone of two days old queen ovarioles (ol). Notice the ovariole pedicel, still closed, formed by piles of prismatic somatic cells. **E** – Basal to median zone of two days old queen ovarioles (ol) showing a germinative large cell surrounded several somatic mall cells. **F** – Sections of ovarioles (ol) of two days old virgem queen, showing a primary oogonia cell (poo). tr = tracheoles; pe = pedicel; cy = cyst of cells; sc = somatic cells; gc = germinative cells. Bars of Figs. A = 330 µm, B = 20 µm, C = 40 µm, D = 30 µm, E = 25 µm, F = 25 µm.

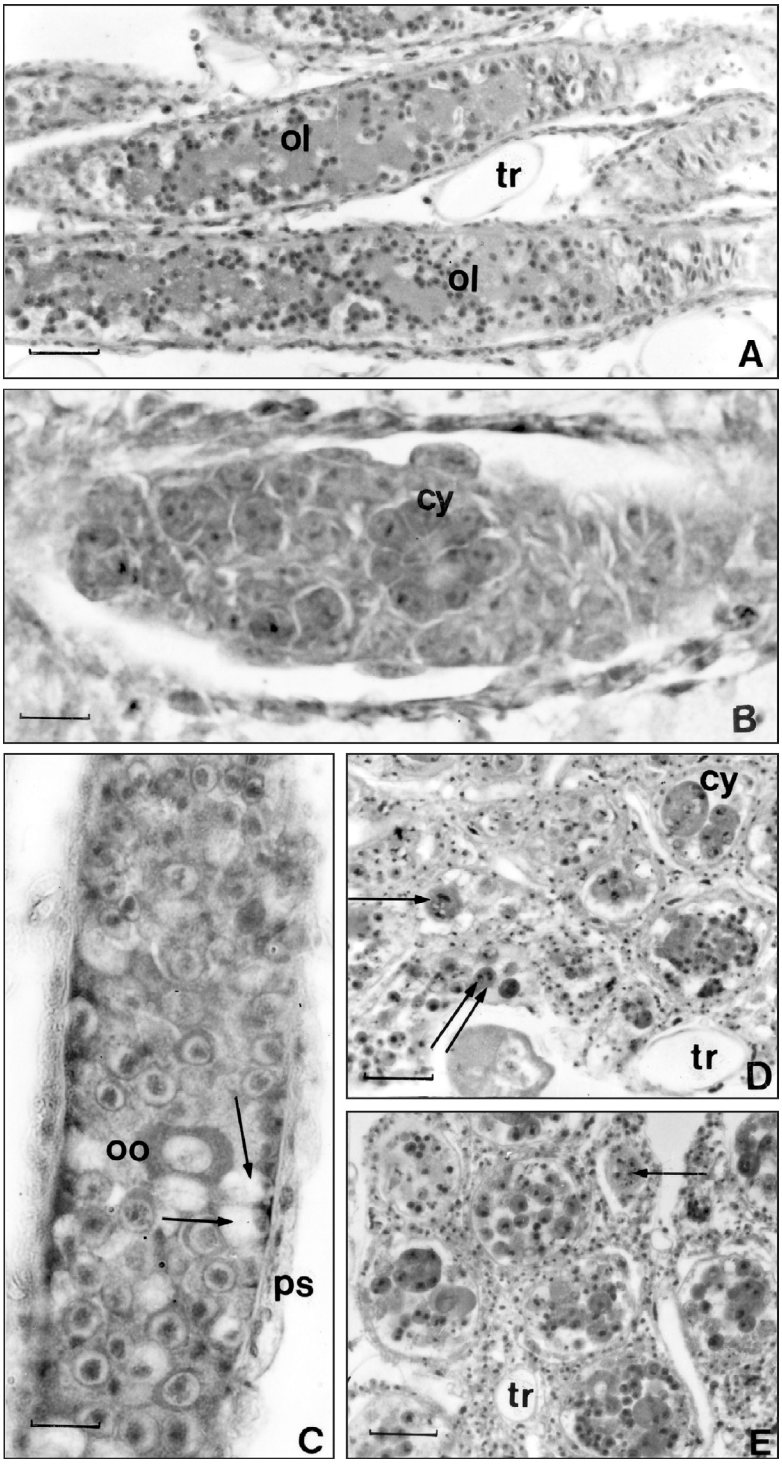

Fig. 2 — A – Light microscopy micrograph of ovary of a 4 days virgin queen, showing disorganized of ovarioles (rol). **B** – Sections of ovarioles (ol) of 6 days old virgin queen showing in the more basal portion cysts (cyc). **C** – Longitudinal section of ovarioles (ol) of 15 days old virgin queen showing signs of cell reabsorption may be observed as cell vacuolization (arrows). **D** – Cross sections of 15 days old virgin queen showing cyst (cy) reabsorption and also somatic cell reabsorption (arrows). ps = peritoneal sheath; tr = tracheoles. Bars of Figs. A and B = 40 µm, C = 10 µm, D = 20 µm.

About three days elapse between the queen's insemination and the beginning of posture, and in this period, great amounts of oocytes mature, that is, undergo vitellogenesis. In this short time the ovary acquires the characteristics and dimensions of an adult ovary. Therefore, during posture the cysts are continually formed from the oogonia present in the germarium. The somatic cells originate the cells that surround the cysts in the germarium and follicular cells that surround the follicle in the vitellarium.

Laying queens

Young queens in the beginning of posture (2 days after the beginning of posture) and old queens that were being substituted were studied. In both the aspect of the ovary is very similar. The vitellarium is the largest portion of the ovary and oocytes in all phases of development can be found, the younger ones placed in the more distal zones. The difference between old and young queens is that in old queens the germarium has the aspect of being in exhaustion phase. The exhaustion of the germarium is characterized by the shortening of this zone and the vacuolization of its content. Once again, cellular reabsorption may be observed, characterized by cytoplasm vacuolation and nuclear picnosis (Fig. 3D).

In the ovaries of laying queens, the ovarioles show an anatomical differentiation along their length, being thicker proximally, and becoming thinner in the distally. In this type of ovary, the germarium appear as a sinuous cylinder of uniform caliber. The beginning of the vitelarium is marked by an increasing caliber and the appearance of thickenings (the follicles), separated by narrowings giving the ovariolar filament the aspect of a string of beads. In the vitellarium the follicles are constituted of two chambers; the oocytic and the nurse chambers. In queen ovarioles of in this phase, it can be observed that the proximal portion of the ovarioles presents some of its follicles empty, indicating the occurrence of posture (Fig. 4A). It can be verified that a good number of the ovarioles from each ovary discharged oocytes at same time. The discharge promotes the collapse of the follicle walls, which become a narrow and wrinkled tube. Numerous tracheal branches surround these structures, resulting from the follicular cell collapse, denominated *corpus luteum* by analogy to vertebrates (Fig. 4A).

The preparations of these ovaries after criofracture and examination with SEM, enabled to visualize internal aspects of the nurse and oocytic chambers (Fig. 4B). In cross fractures at the beginning of the vitellarium; it is noticed that yolk deposition begins in the oocyte's periphery and that around each granule there is a space, probably formed by contraction of the granule inside the membrane that separates it from the cytoplasm of the oocyte (Fig. 4C). The yolk is accumulated until the oocyte becomes filled with yolk granules, which are compact and without the cavity around them in their majority (Fig. 4D). The cytoplasm that fills the space among the yolk globules presents small cavities.

DISCUSSION

The present data show that the *A. mellifera* queen emerges with an undifferentiated ovary concerning the oogenesis process. The germinative cells are restricted to the very proximal region of the ovariole and are identified as few large round cells immersed in surrounding small cells. It is difficult and very rare to observe mitosis into the ovarioles, but with the passing of time the number of large cells increase and the length of the segment recognised as germarium elongates. Zacaro (1993) describes the ovarioles of newly emerged queens as constituted almost only by the germarium, but contrary to our observations, show that they are formed almost by the terminal filament, being the germarium restricted to a small proximal segment. As the virgin queen grows old, the germarium progressively encreases. In the ovarioles of 4-6 days old adult queens ovariolar cists are already present.

The large cells seen in 2 and 4 days old queens seem to be a primary oogonium and the small somatic cells that will probably originate the cells that will surround the cysts and the follicular cells (Fig. 1E) that will surround the follicle. In 8 day-old queen the oocyte are already differentiated from the future nurse cells, configurating the formation of a follicle, or the beginning of a vitellarium. However a real vitellogenesis or the yolk depositions do not occur in virgin queens.

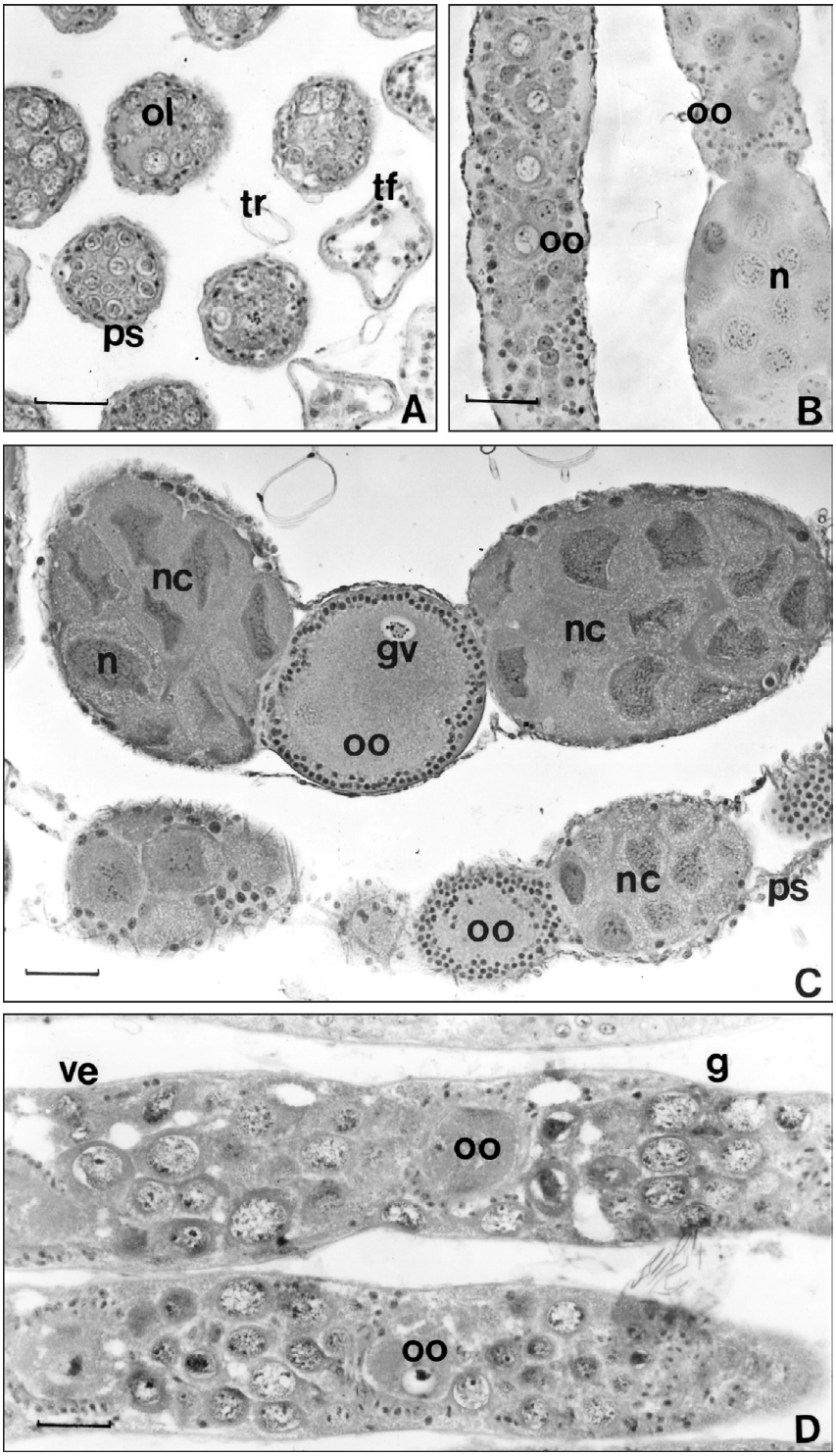

Fig. 3 — Ovarioles (ol) of egg laying queen showing the germarium (**A**), the begin of vitellarium (**B**) and a well differentiated vitellarium (**C**). Notice the oocyte (oo) and nurse cells (nc) differentiation. **D** – Transition between germarium (g) and vitellarium (ve) in na old mated queen. Notice cell reabsorption (arrows). ps = peritoneal sheath; gv = germinal vesicle; fc = follicular cells; tf = terminal filament; cy = cyst. Bars of Figs. A = 47 µm, B = 40 µm, C = 47 µm, D = 40 µm.

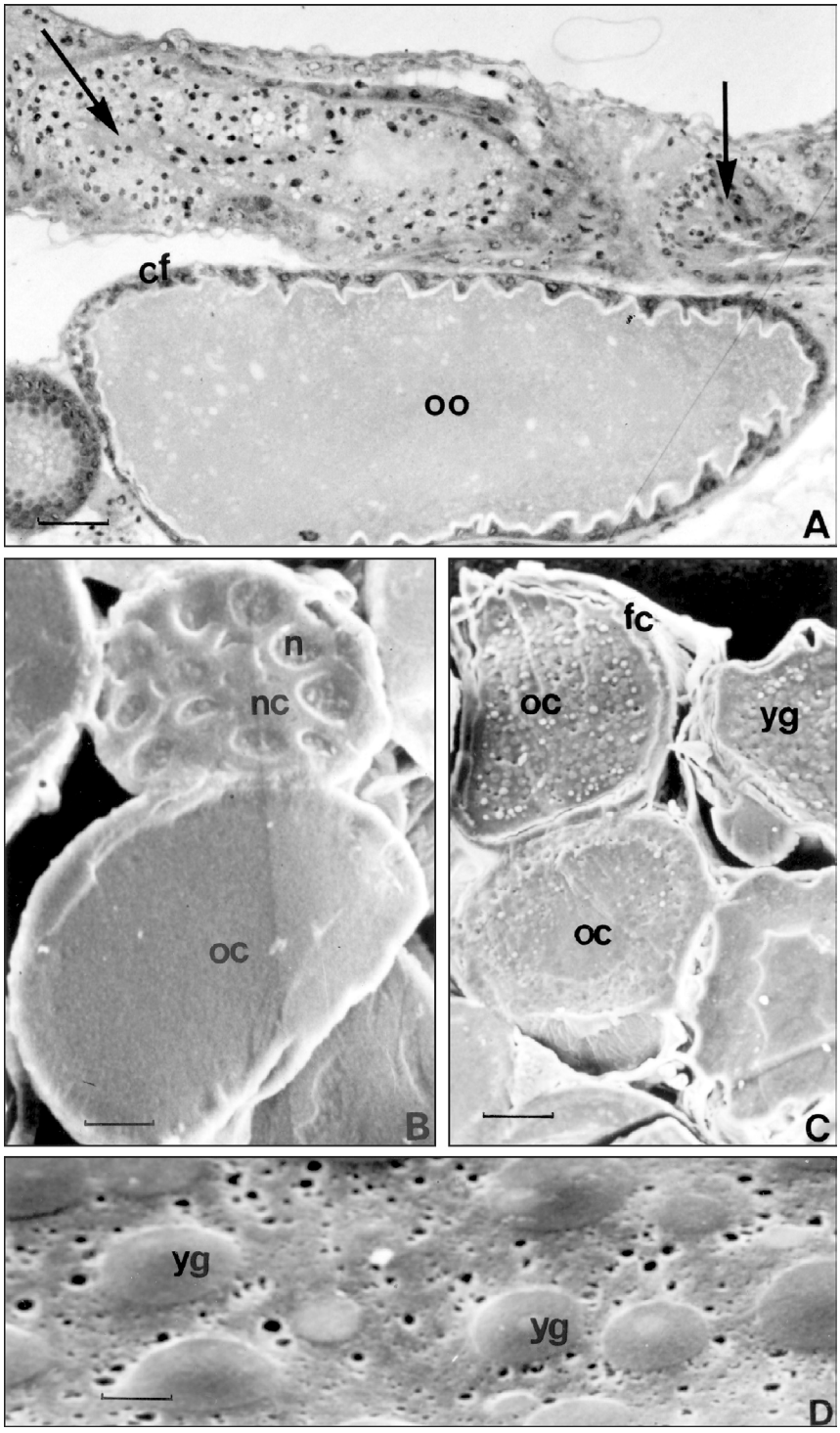

Fig. 4 — A – Light microscopy micrograph of ovarioles (ol) of egg laying queen showing in the proximal portion follicles empty, indicating the occurence of posture (arrows). **B**, **C**, and **D** – Scanning electron microscope micrograph of ovarioles of egg laying queen after criofracture. Internal aspect of the nurse (nc) and oocyte chambers (oc) (B and C). **D** – Yolk is accumulated until the oocyte becames filled of yolk granules (yg). fc = follicular cells, n = nuclei. Bars of Figs. A = 47 μm, B = 10 μm, C = 3 μm, D = 15 μm.

The *A. mellifera* queen generally leaves for the nuptial flight between 5 to 8 days after emergence as adult. Queens of this age have the ovariolar germarium occupying half of the ovariole lenght and, as can be said, a incipient proximal vitellarium, constituted by early pre-vitellogenis follicles. As soon as the mating occurs the follicle differentiates in an anterior nurse and a posterior oocytic chamber. The transferring of material from the nurse cells to the oocyte, starts as well and later begins the yolk deposition. In one or two days the ovarioles present a predominant length of the vitellarium becoming the germarium restrict to the distal end of the ovariole, with radical shortening of the terminal filament, and the queen begins to lay eggs.

The posture determines the appearance of a new structure in the ovariole, resulting from the emptying of the follicle from where an oocyte leaves. The follicular cells collapse, their cytoplasm fills up with lipidic droplets, and a yellow plug is formed in the proximal end of the ovariole, that delivers one oocyte, called *corpus luteum*. This structure is soon reabsorbed and the way out for a new oocyte, becomes open again.

Therefore in virgin queens, only two regions may be distinguished in the ovarioles (the terminal filament and the germarium) although the germarium growth and iniciates differentiation, with prodution of germarial cysts and early follicles, yolk is not deposited in oocyte. Soon after mating the queenthe yolk deposition begins and the ovarioles show three differentiated regions (terminal filament, germarium and vitellarium). This condition remains during the queen's entire fertile life, with only a shortenning of the germarium as the queen grows old.

If the queen is prevented mating the early vitellarium do not continue differentiation, the vitellogenesis does not occur, and the cysts inside the ovarioles starts to desorganize, and some cells show signs of dying. Therefore the mating is a stimulating factor to start vitellogenesis and oocyte maturation. Several results obtained mainly studying *Drosophila* (Schmidt *et al*., 1993; Coleman *et al*., 1995; Wolfner, 1997; Tram & Wolfner, 1998) demonstrate that the mating affect the ovarian development, the egg laying rates, and even the female longevity. These effects are attributed to substances introduced into the genital tract of the female with the sperm, produced by the accessory glands of the male reproductive apparatus.

The *A. mellifera* queen mates with several males and stores their semen in the spermatheca during all her fertile life. The present results confirm the effect of fecundation in the ovary development, but the kind of effect and the causing substances, demand further investigation.

Acknowledgments — The authors acknowledge the financial support of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (95/0256-6) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (351016194-5).

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