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CELL DEATH AND OVARIAN DEVELOPMENT IN HIGHLY EUSOCIAL BEES (HYMENOPTERA, APIDAE): CASTE DIFFERENTIATION AND WORKER EGG LAYING

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ABSTRACT

The development and functioning of the ovary in highly eusocial bees is one of the most prominent differences between the castes in these insects, with queens having very large ovaries and a high capacity to produce eggs while the workers have small, sub-functional ovaries. The differences in ovary size and function are established during larval and pupal development and are hormonally controlled. Differential cell death has a prominent role in modulating the ovarian differences during development and adulthood. In this review, we discuss the forms of cell death, the types of cells affected and the timing of death in relation to the function of the female castes in the colony.

Key words: *Apis mellifera*, cell death, eusocial bees, *Melipona quadrifasciata*, ovariole, queens, *Scaptotrigona postica*, ultrastructure, workers

INTRODUCTION

Cell death is an important mechanism for development, maintenance of homeostasis and defense against disease. Consequently, this phenomenon is often temporally and spatially programmed throughout the life-time of an organism. In eusocial bees, reproduction by females is almost completely restricted to the queen, with egg production by workers being inhibited by pheromones or aggression. Nevertheless, in most eusocial bees, the workers have potentially functional ovaries and may lay eggs under specific conditions that vary according to the peculiarities of the social organization in each species.

Sex determination in bees occurs through a haploid-diploid system in which the females develop from diploid fertilized eggs, while males develop from haploid unfertilized eggs through arrenothocous parthenogenesis. The late separation of females into two castes, one fertile (the queens) and the other sterile (workers), is, with rare exceptions, attributable to the nutritional profile of the larvae during development. Differences in the quantity and quality of the larval food in *Apis mellifera* and in the quantity in Meliponini (except for *Melipona* species) result in divergences in the development of the reproductive apparatus, with atrophied ovaries and genital ducts leading to impaired mating in workers [67]. Hence, atrophy of the genital ducts is the main physiological (and morphological) cause of worker sterility.

Although nutritional variations appear to be responsible for queen-worker differences, the physiological regulation of the development of the reproductive tract is hormonal. Consequently, variations in the levels of juvenile and ecdysteroid hormones account for the morphogenetic and reproductive differences between castes during development and adulthood [31-33,61,62]. These hormones exert their effects mainly on egg production because this activity requires a high investment that cannot be wasted. In addition to the physiological condition of individual workers, several environmental factors may also influence ovarian activity in order to minimize the loss of valuable resources invested in egg production.

Bees have a meroistic polytrophic ovary. The sequence of oogenesis in this type of ovary is well-known in insects [12,21,57,71], including bees. In this article, we will discuss the circumstances leading to cell death in ovaries, based on morphological studies using light and transmission electron microscopy. The physiological significance of cell death

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as a tool for controlling reproduction within and between the female castes in highly eusocial bees will also be considered.

Cell death has a key role in establishing differences between the female castes in several organs, including the ovary. Queens always have large ovaries and produce many more eggs than workers. *Apis mellifera* and Meliponini species differ in the stage of development in which queens and workers start to diverge and in the regulation of egg production. In the Meliponini, there are also differences in the role of nutrition in queen-worker differentiation. In *Melipona* species, food does not interfere with or has only a minor role in caste differentiation [39,40,42]. In the following discussion, we will compare the role of cell death in modulating ovarian development and function in *Apis mellifera, Scaptotrigona postica* and *Melipona quadrifasciata*.

Cell death in immature bees and caste differentiation

Apis mellifera: In this species, the castes are determined trophically, with the larvae of each caste being fed food that differs qualitatively and quantitatively. The nutritional differences between larvae that will become queens and workers start 2-3 days after eclosion and are controlled daily by the nurse workers that progressively supply increasingly different food. Hence, caste determination in this species is a sequential process, as demonstrated by the effect of the larval age grafted to produce queens artificially [4,26]. As a consequence, ovarian dimorphism between the castes also begins early and is progressive.

Cell death is common during organ development and its occurrence in the ovaries of three-day-old worker larvae (Fig. 1A) indicates that this phenomenon is not entirely attributable to nutritional differences. Moreover, as the larvae grow older, the rate of cell death increases, resulting in some re-absorption of the ovarioles (Fig. 2B). Cell death starts with germ line cells but also affects somatic cells, with disruption of the ovariole being a later event resulting from the death of these cells. The entire process results in a decrease in the number of ovarioles in worker ovaries, although the latter effect is only observed sometime after cell death has occurred.

Different types of cell death affect the germ line and somatic cells. Reginato and Cruz-Landim [64] found three morphologically distinct types of cell death in the ovary of *A. mellifera*, namely, (a) death of germ line cells that was morphologically similar to apoptosis, with the dead, very condensed cells appearing in the cytoplasm of somatic cells (Fig. 2A), (b) death of somatic cells via an authophagic process in which the cell cytoplasm became filled with autophagic vacuoles (Fig. 2B) after the phagocytosis of dying germ line cells, and (c) death characterized by cell disruption.

In contrast to cells dying by apoptosis, which show no acid phosphatase activity, the cells affected by an autophagic process of death show a positive reaction for acid phosphatase in the autophagic vacuoles present in the cytoplasm and in the condensed nuclear chromatin [11,63,64]. Although not all ovariole cells die, the death of primordial germ line cells and of some somatic cells causes disintegration of the ovariole. The remaining ovariolar cells are added to the stromata that separate one ovariole from another. These stromatic cells, as well as ovarian capsular cells, contain large amounts of glycogen (Fig. 1B) and store nutrients in a manner similar to fat body cells. The presence of glycogen in young larval ovarioles is one of the factors that distinguishes somatic cells from germ cells. During pupation, the capsular and stromatic cells disintegrate, thereby discharging their contents into the hemolymph. This event is typical of death by cell disruption and is also seen in the trophocytes of the prepupal fat body.

The germ line cells are located at the distal end of the ovariole anlage because the anlage base consists of a penduncule formed by radially arranged flat cells that connect the ovariole to the oviduct [67]. Hence, since cell death initially affects the germ line cells to cause disappearance of the ovariole, the distal end of the ovariole is the first to be affected. Cell death during the development of worker larvae causes a drastic decrease in the number of ovarioles present in the ovaries of this caste. Thus, whereas queens have almost 200 ovarioles per ovary, the workers have only 2-12.

Cell death occurring during worker larval development is induced by a deficient production of juvenile hormone by the corpora allata because of the type of food fed to the larvae by nurse workers [21,34,61,62,65]. This finding is corroborated by the observation that supplying juvenile hormone to developing worker larvae partially mimicks the effect of food by inhibiting cell death and consequently preventing the ovarioles (Fig. 4A, B) from

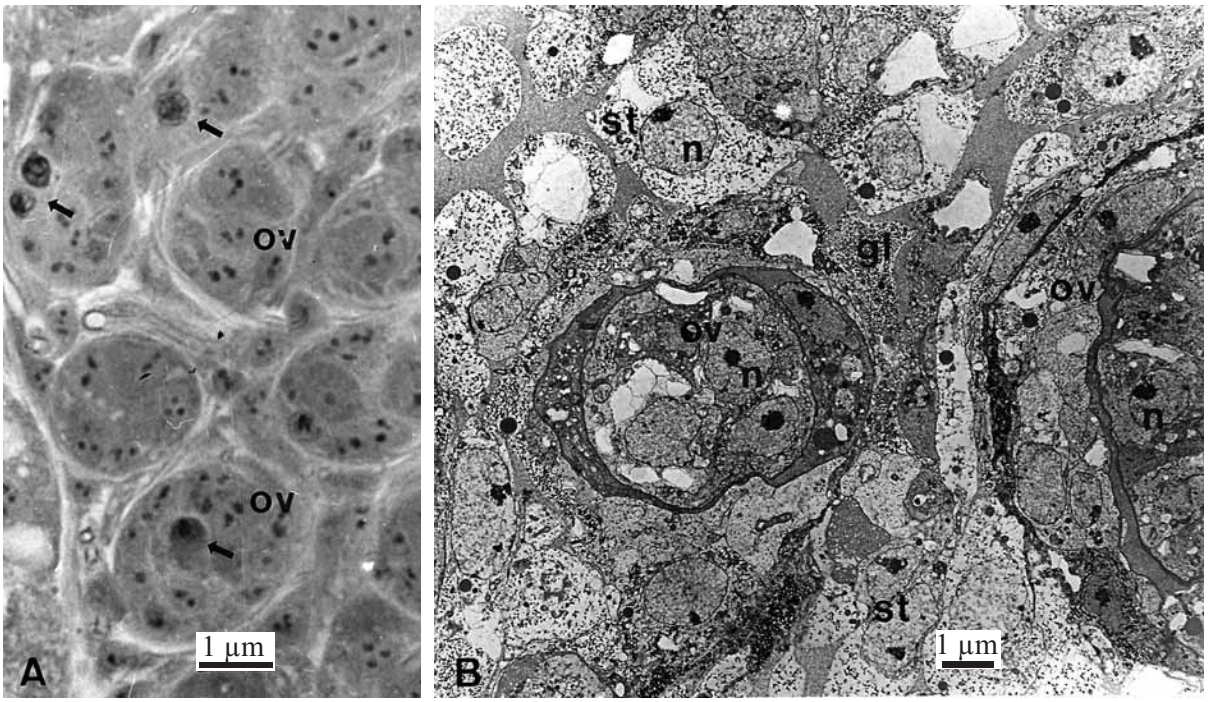


Figure 1. Cell death during larval development of the ovary in an *Apis mellifera* worker. (**A**) Light micrograph of ovarioles (**ov**) in a three-day-old worker showing cell death (**arrows**). (**B**) Transmission electron microscopy (TEM) micrograph of an ovariole (**ov**) during resorption in a four-day-old worker larval. **st -** stromatic cells, **n -** nuclei, **gl -** glycogen.

degradation [Antonialli Jr. WF, PhD thesis, Paulista State University (UNESP), Rio Claro, Brazil]. When three-day-old worker larvae were treated with 1 μL of juvenile hormone dissolved in hexane (1 μg/μL), the decay of the ovarioles seen on the 5th day was lower [2] than that in non-treated larvae. Double staining with Hoescht dye and propidium iodide showed that the ovarioles of treated larvae were healthy (blue staining) whereas those of non-treated larvae were red, indicating dying cells (Fig. 3A, B). (Since the Hoechst dye is a supravital fluorochrome, it stains only healthy cells, whereas cells with damaged cytoplasmic and nuclear compartments, including condensed nuclear chromatin, are stained by propidium iodide).

Cell death in the worker ovary is therefore triggered by environmental conditions that produce a form of nutritional castration. Since this cell death is mediated by programmed mechanisms designed to eliminate unnecessary or non-viable cells, this phenomenon is referred to as programmed cell death. Since the egg and the newly ecloded larva can equally give rise to a queen or a worker, there has been evolutionary selection in this eusocial species to drastically reduce the worker's capacity to produce eggs. Hence, the function of cell death in this case is to economize on the resources invested in the development of ovaries in reproductively unfit individuals that will not produce offspring. In primitive eusocial species, this mechanism does not exist and most of the females have well-developed ovaries; in these cases, the queen controls the reproductive activity of the workers by her aggressive behavior [53].

Scaptotrigona postica: This species also shows trophic determination of the castes. However, the amount of food provided to the brood is not increased progressively, as in *A. mellifera*, but is given all at once in a large quantity. The cells in which the eggs will be laid by the queen are first provisioned by the nurse workers with all of the food that the larvae will need and, after the egg is laid, the cell is then sealed to prevent the workers from having further contact with it. For rearing queens, the workers build larger cells that hold more food such that the nutritional differences between queen and worker larvae are only related to the amount of food provided. Consequently, nutritional differences begin to exert an affect only at the end of the larval feeding stage, i.e., when the larvae in worker cells have exhausted

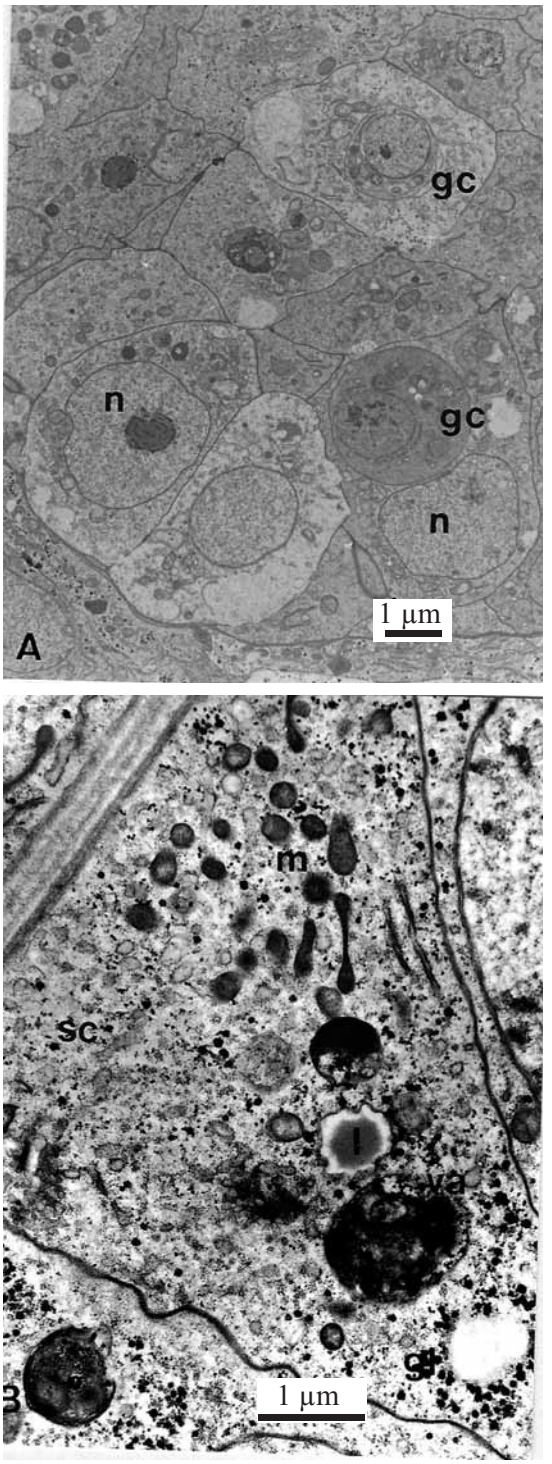


Figure 2. TEM micrographs of different types of cell death in the ovarioles of worker larvae. (**A**) Apoptosis-like cell death in germ line cells (**gc**). Note that the dying cell is located in the cytoplasm of another cell. (**B**) Autophagic death of ovarian somatic cells (**sc**). **n -** nuclei, **av -** autophagic vacuole, **l -** lipid, **m -** mitochondria, **gl** - glycogen.

their quota, the larvae in queen cells still have a considerable amount of food to eat. Therefore, although the ultimate factor that determines to which caste the larvae will belong continues to be hormonal [30,33], this determination will occur only at the end of the larval phase and is not progressive as in *A. mellifera.* This conclusion is confirmed by the observation that the supplying of juvenile hormone to worker larvae of this species at the end of the last instar can transform them into queens [14,15], by mimicking the extra amount of food that the queen larvae usually receive. In *A. mellifera*, this same procedure produces only inter-castes that become close to the queen caste the earlier the hormone is supplied [4,31]. In addition, the provision of extra food to larvae developing in worker brood-cells increases the emergence of queens [13,46].

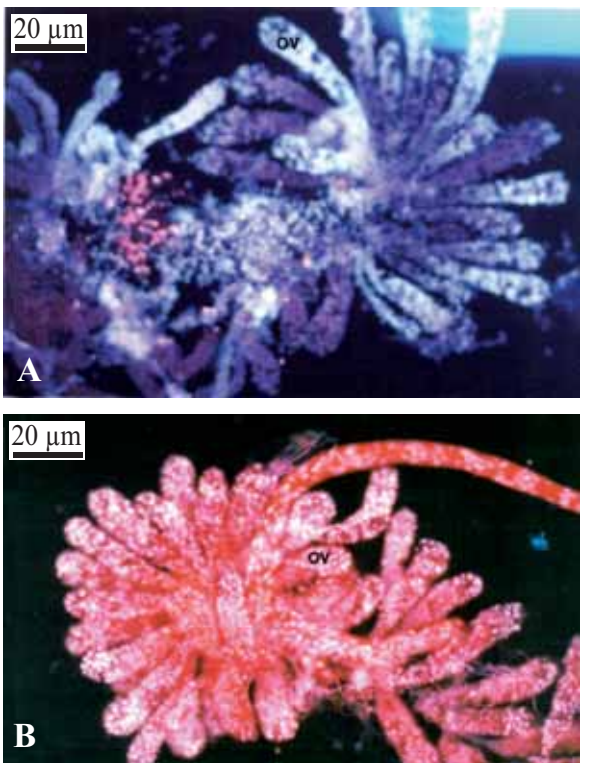


Figure 3. Fluorescence microscopy of ovaries from five-day-old *Apis mellifera* worker larvae double stained with Hoechst dye and propidium iodide. (**A**) Ovary of a larval worker treated with a topic application of juvenile hormone on the 3rd day after eclosion. Note the ovarioles (**ov**) stained blue with Hoechst dye. (**B**) Ovary from a worker of the same age but without exposure to juvenile hormone. Note the red ovarioles (**ov**) after staining with propidium iodide.

Workers and queens of this species have the same number of ovarioles in their ovaries. However, the ovarioles are much longer in the queens than in the workers [21,51]. Some cell death occurs in the ovaries of worker larvae and queens, but no consistently divergent rates of cell death between the castes have been observed in this phase. Indeed, the ovaries of both castes show similar rates of cell death up to the beginning of the last larval instar.

After the worker larvae have defecated and become upright in the brood cell during the prepupal stage, extensive cell mitosis occurs in the developing queen (Fig. 4A). This mitosis occurs preferentially in the distal end of the ovariole to greatly increase its length, particularly in the terminal filament (Fig. 4B). In contrast, there are fewer mitoses in the worker, with most occurring in the proximal region of the ovary, probably in the somatic cells of the gonadal

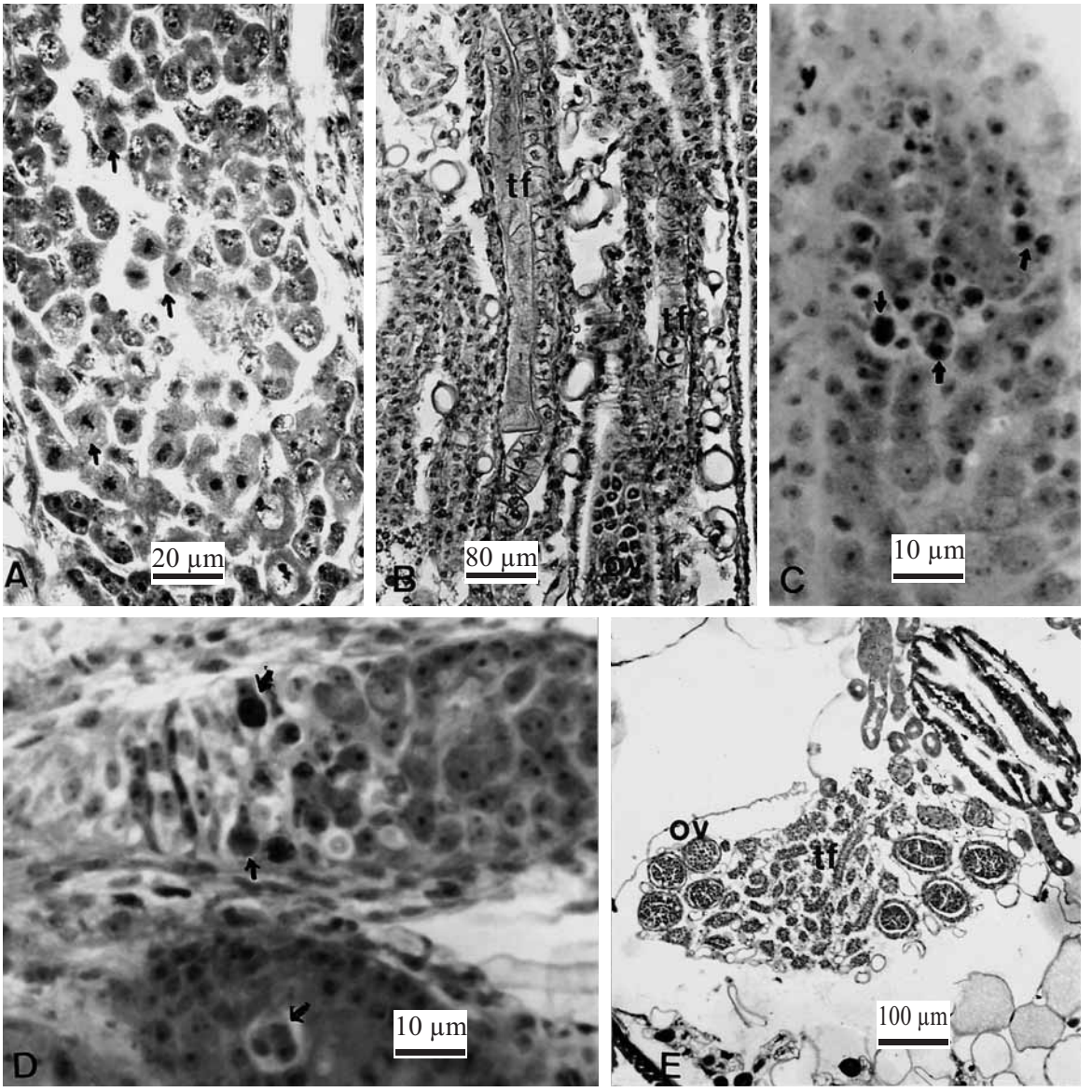


Figure 4. Light micrographs showing differentiation along an ovary of *Scaptotrigona postica*. (**A**) Cell divisions (**arrows**) in the ovariole of a larval queen. (**B**) Terminal filament (**tf**) of queen ovarioles (**ov**). (**C**) Cell death (**arrows**) in the apex of a worker ovariole. (**D**) Cell death (**arrows**) at the base of a worker ovary. (**E**) A view of the four ovarioles (**ov**) of the queen ovaries in cross-section. Note the long terminal filament (**tf**).

ducts. Whereas cell death is rarely observed in the queen, it occurs at the apical end of the ovarioles in workers (Fig. 4C), which suggests that the affected cells are probably the germ line cells. Cell death is also observed in the basal region of the ovary (Fig. 4D) where the ducts differentiate into those of a queen or a worker. This cell death probably accounts for the inability of the workers to mate.

Although the timing and mechanisms of the ovarian changes in *S. postica* are different from those in *A. mellifera,* the final result is similar, i.e., the worker has a lower capacity to produce eggs than the queen. In this case, the queen's fertility does not derive from a larger number of ovarioles, but from having much longer ovarioles (Fig. 4E).

The cell death in the worker ovary at the beginning of the pupal phase may be so extensive in some species of Meliponini that no functional ovary will be present in the adult. This is the case of some *Friesomelitta*, in which the ovary is almost completely reabsorbed during pupation [9,20,72,73].

Atrophy of the worker ovary serves to control egg production by this caste. Moreover, in Meliponini species, the presence of the queen generally does not impede vitellogenesis, i.e., oocyte maturation in the worker ovary, as in *A. mellifera*. On the contrary, in most species, the workers frequently lay eggs, most of which are given to the queen as food. In *Friesomelitta* species, the workers never lay eggs. This condition in which the worker caste is completely sterile was described by Zucchi [78] as hypersocial.

In some species of Meliponini with trophic determination of the castes, the large amount of food eaten by the larvae leads to an increased number of ovarioles in the ovary, as demonstrated by Lisboa *et al.* [46] in *Trigona spinipes.* In this species, the worker larvae eat about 36 μL of food, while the queens consume 360 μL. Although there is some correlation between the amount of food ingested and the number of ovarioles per ovary, the process is self-limiting. With a food intake of up to 72 μL, there is a rise in the percentage of ovaries with an increased number of ovarioles, whereas for a food intake of ≥108 μL, all of the worker larvae are transformed into queens. The number of ovarioles does not increase anymore with a further rise in food intake, indicating that the proces is self-limiting.

The queens always have longer ovarioles with a very long terminal filament, and the workers have short ovarioles with a very short terminal filament [23,46,51]. The terminal filaments are generally considered to connect the distal extremities of the ovary to the dorsal tegument, but the differences in their sizes according to the castes suggest a more complex function. One possibility is that the primordial stem germ line cells are lodged there, or that the cells in the filament are actually primordial germ line cells [20,21,75]. Presumably, these cells may represent undifferentiated stem cells that can give rise to germ cells as required.

Melipona quadrifasciata: Caste determination is not trophic in the genus *Melipona*. The size of the brood cells is the same for queens, workers and males. Kerr [39,40] proposed a genetic determination of the castes based on the fact that queens constantly emerge from each queen posture in such numbers as to remind one of the Mendelian laws for heterozygous individuals. Nutrition appears to play a role since larvae weighing less than 72 μg do not generate morphological queens [39,40,43], but rather generate genetic queens as worker phenocopies [43].

In this genus, supplying juvenile hormone to larvae at the end of the last instar turns them into queens, regardless of the genotype [41]. Bonetti [10] obtained 100% queens from larvae treated topically with a juvenile hormone analog. Hence, even if the caste is determined by a specific genetic constitution, its nature is evident throughout the differential development of the respective corpora allata [66] and, consequently, throughout the different titers of juvenile hormone [41].

In *Melipona* species, the adult queens emerge with a very small abdomen, which means that the ovaries occupy little space within this region. In these species, as in *S. postica*, there are four ovarioles in both castes, with the difference between them being their length [51]. Apparently, the increase in ovariole length occurs late, during pupation (although this requires confirmation), and continues into adulthood. Cell death is sporadic and does not differ between castes, but cell proliferation is greater in queen than in worker ovaries.

In the three species discussed above (*A. mellifera, M. quadriafasciata* and *S. posticas*), the queen ovarioles are much longer than those of workers

such that the differences in ovary size in *Meliponini* castes reflects differential cell division rather than differential cell death. Nevertheless, in all of these cases, cell death provides an interesting means of reducing worker fecundity.

Differences in cell death in the ovaries of adult workers and queens

In adult bees, cell death influences ovary functioning by regulating the relationship between the normal progression of oogenesis and the environmental conditions, e.g., the conditions for posture. Cell death generally affects the follicular and oocyte nurse cells. In addition, pathological or environmental factors may cause varying degrees of germ line cell death and, when germ line cells die, the related follicular or nurse cells will also die.

The function of cell death in the adult ovary is therefore different from that in immature ovary. In the latter, cell death serve to model the organ whereas in adults, this death is related to the reproductive function of the individual. Thus, in eusocial bees, cell death in the adult ovary depends on the caste and on the type of queen-worker interaction during the reproductive process.

Cell death in queen ovaries

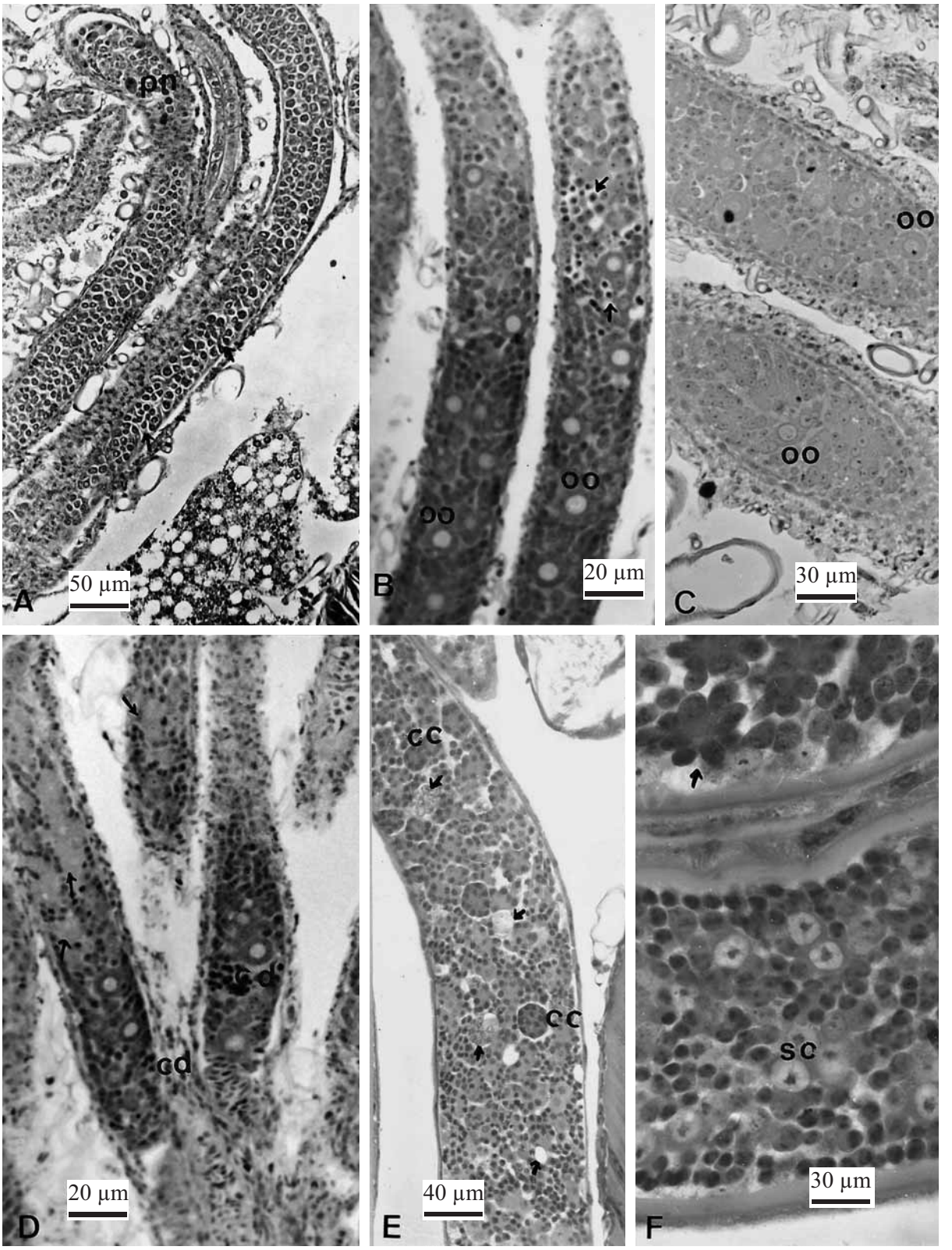
Virgin queens generally do not start vitellogenesis before mating. Consequently, the long ovarioles of virgin queens at emergence consist of only the germarium and the terminal filament (Fig. 5A). Nevertheless, during the pre-mating period, the early stages of oogenesis occur in the ovarioles, with this process possibly extending as far as the cystocytes, rosette stages or beyond, to the stage of already differentiated oocytes (Fig. 5A-C), but without follicular formation. Vitellogenesis starts only after mating. If mating does not occur in due time, cell death initiates in the ovarioles.

Eight-day-old virgin queens of *A. mellifera* show many signs of cell death in the ovary, including cells with pycnotic nuclei (containing higly condensed chromatin) (Fig. 5B,D) and structural disorganization of the ovariolar structure [7,58,59]. Similar features are seen in *S. postica* in which, if the queen does not mate, the clusters of cystocytes collapse into condensed cell aggregates that are eventually reabsorbed, leaving empty spaces in the ovariolar structure (Fig. 5E). In four-day-old virgin queens of *M. quadrifasciata*, the ovarioles contain many cells with a very clear cytoplasm and tiny condensed nuclei. In addition, the ovarioles also have empty spaces, suggesting that cell death is implemented by cell swelling and disintegration (Fig. 5F), in a manner similar to necrosis. In all of these cases, a delay in mating impedes the progress of oogenesis and increases the incidence of cell death in the ovarioles.

Although cell death is more frequent at the proximal end of the ovariole, where oogenesis is more advanced, with the presence of clusters of cystocytes, rosettes and even differentiated oocytes, the exact timing and mechanism of this event is unknown. Nevertheless, the occurrence of cell death in most advanced stages of oogenesis, after the germ line cells have left the stage of latency and have entered the proliferation phase, appears to be a conserved mechanism since it has also been observed in vertebrates [1]. Although sometimes only some cells of the cluster die, in most cases the entire cyst undergoes degeneration. In this case, transmission electron microscopy (TEM) shows collapse of the entire cyst, with the cystocytes having apoptotic features (Fig. 6). The cystocytes collapse around the fusome, a feature that agrees with the view of Schmidt-Capella and Hartfelder [68,69] in which cytoskeletal disruption is the cause of this cell death. However, using specific antibodies coupled to FITC, [Patrício K, 2000, MSc dissertation, Paulista State University (UNESP), Rio Claro, Brazil] reported a normal distribution of actin and tubulin in the germarium of ovaries from virgin and fecundated queens of *A. mellifera* and *S. postica* (Figs. 7A, B).

During mating, compounds that affect female fertility and are produced in the male genital tract, mainly in the accessory glands, are transferred to the female with the spermatozoa [18,28,70,76,77]. In bees, the function of the accessory gland and the presence in semen of substances that can control female reproduction are not well-understood. Nevertheless, Koeniger [45] speculated that *A. mellifera* queens display a sign of mating after copulation, and Collonelo and Hartfelfer [19] found that a peptide present in mucus gland secretion was a potential stimulator of oogenesis in mated queens. However, the Meliponine reproductive tract does not have accessory glands [22,25,27] mating influences the start of vitellogenesis in the ovary [52].

After mating, a vitellarium differentiates in the proximal end of the ovarioles as a result of the oocytes entering the maturation phase in which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yolk is deposited in the ooplasm. In the meroistic polytrophic ovary of bees, the vitellarium consists of a linear series of follicles containing oocytes in a progressive sequence of maturation extending from the apex to the base. Each follicle consists of two connected chambers: the oocyte chamber and, on top of this, the nurse cell chamber, both of which are enclosed by the follicular epithelium [21,57,71]. In the fecundated laying queen, cell death in the ovaries always occurs as a physiological consequence of oocyte maturation and ovulation.

In the pre-vitellogenic follicles located at the distal end of the vitellarium, the nurse chamber is larger than the oocyte chamber (Fig. 8A) and is increased by polyploidization of the cells [Patrício K, 2005, PhD thesis, Paulista State University (UNESP), Rio Claro, Brasil]. Moreover, the product synthesized by these cells is transferred to the oocyte through the bridge (trophic stalk) between the two follicular chambers (Fig. 8B) and, at the end of vitellogenesis, all of the cytoplasmic content of the cells is dumped into the oocyte (Fig. 8C). The nurse cells are clones of the oocyte and synthesize RNA and other morphogenes of maternal origin, subsequently transferring them to the oocyte (the nucleus in these oocytes is almost totally inactive). Thus, the nurse cells are programmed to die at the end of oocyte maturation. These cells die via a progressive loss of cytoplasm (Fig. 8C) such that, in the end, only debris from condensed chromatin is left in the nurse chamber (Fig. 8D). The cytoskeleton is reorganized during cell death [38,54,55]. In the fruit fly *Dacus oleae*, this reorganization prevents the nuclei of nurse cells from obstructing the canal of communication with the oocyte. The reorganization of cytoskeletal actin during the accumulation of nurse cell material in the oocyte is a developmentally regulated physiological mechanism that is phylogenetically conserved in higher Diptera [55], but has, until now, not been demonstrated in bees.

Although TUNEL-labeling gives a positive reaction in these dying cells, and even though Cavaliere *et al.* [16] refer to the process of nurse cell death as apoptosis, this type of cell death, although programmed, does not have the morphological and physiological characteristics of apoptosis. Indeed, a positive reaction to TUNEL is not exclusive to apoptotic cells since DNA fragmentation, with its production of free reactive radicals, also may occur in other types of cell death.

Another type of ovarian cell programmed to die as a consequence of oocyte maturation is the follicular cell. These cells have many roles in oocyte development and are involved in the uptake of vitellogenin, the yolk protein precursor, from hemo-

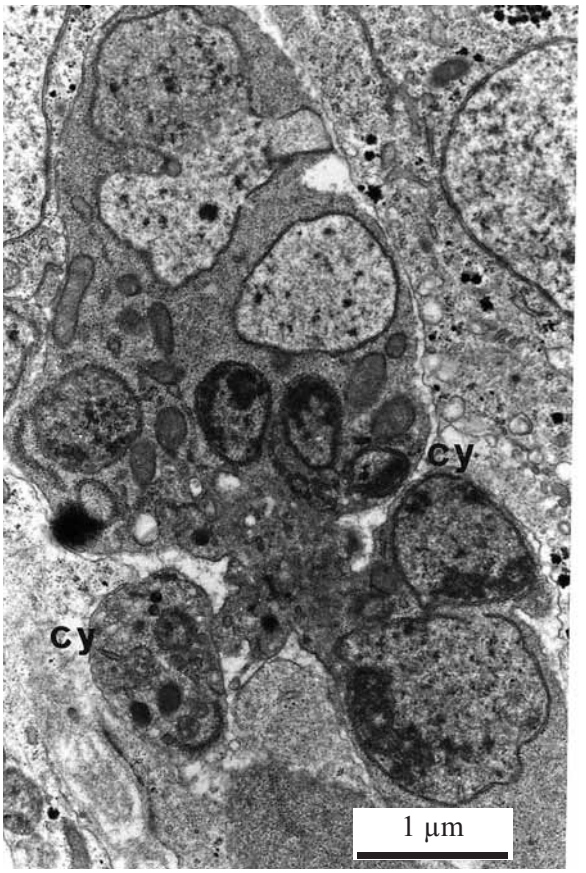

Figure 6. TEM micrograph of a cyst affected by cell death in a 10-day-old virgin queen of *Apis mellifera*. Note the apoptosis-like death of the cystocytes (**cy**).

Figure 5. Light micrographs of ovarioles in virgin queen ovaries. (**A**) Ovarioles of a virgin queen of *Scaptotrigona postica* showing only the germarium with numerous cysts in the rosette phase (**arrows**). Note the cell death represented by pycnotic nuclei (**pn**). (**B**) Ovarioles of a six-day-old virgin queen of *Apis mellifera* showing the germarium cysts with differentiated oocytes (**oo**), but no follicular organization. Note the cell death (**arrows**). (**C**) A two-day-old virgin queen of *Melipona quadrifasciata* showing cysts with differentiated oocytes (**oo**) in the ovarioles. (**D**) An *Apis mellifera* ovary showing ovarioles with no zones of cells (**arrows**) and extensive cell death (**cd**). (**E**) Ovariole of a virgin queen of *Scaptotrigona postica* showing condensed cysts (**cc**) and the empty space resulting from cyst degeneration (**arrows**). (**F**) Swollen cells (**sc**) in in a four-day-old virgin queen of *Melipona quadrifasciata*. Note the presence of some rosettes (**arrow**).

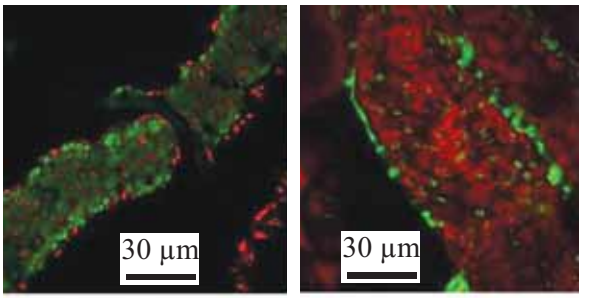

Figure 7. Confocal microscopy of the actin distribution in the intercellular bridges of cystocytes in the germarium of virgin queens. (**A**) *Scaptotrigona postica*. (**B**) *Apis mellifera*.

lymph, as well as in egg shell (chorion) deposition and the formation of special features of this chorion structure.

Distributed among the nurse cells in the nurse chamber are border cells (Fig. 8B) that originate from the pre-follicular cells and represent a type of follicular cell that gives rise to the chorion micropyle through which the spermatozoa enter to fertilize the egg. When the nurse cells complete the transfer of their contents to the oocyte (the so-called "dumping" process) and die, the border cells collapse into the communicating bridge between the two chambers, become filliform, and occupy the bridge inter-space that protrudes into the perioocyte space. Around each border cell there is a deposition of chorion material that surrounds the space occupied by the border cells to provide for the micropyle openings. After formation of the chorion, the border cells die (Fig. 9A) leaving many perforations that form the egg micropyle [24,49]. In *Drosophila,* the first signs of death in follicular cells occur at the anterior pole of the egg chamber and are characterized by a loss of the microvilli of the apical cell membrane, in addition to cytoplasmic and nuclear alterations [56]. In bees, the follicular cells of the posterior pole of the oocyte chamber detach from the oocyte and die after the first layer of the chorion has been deposited (Fig. 9B); this results in the appearance of the egg smooth chorion at this pole [21,74].

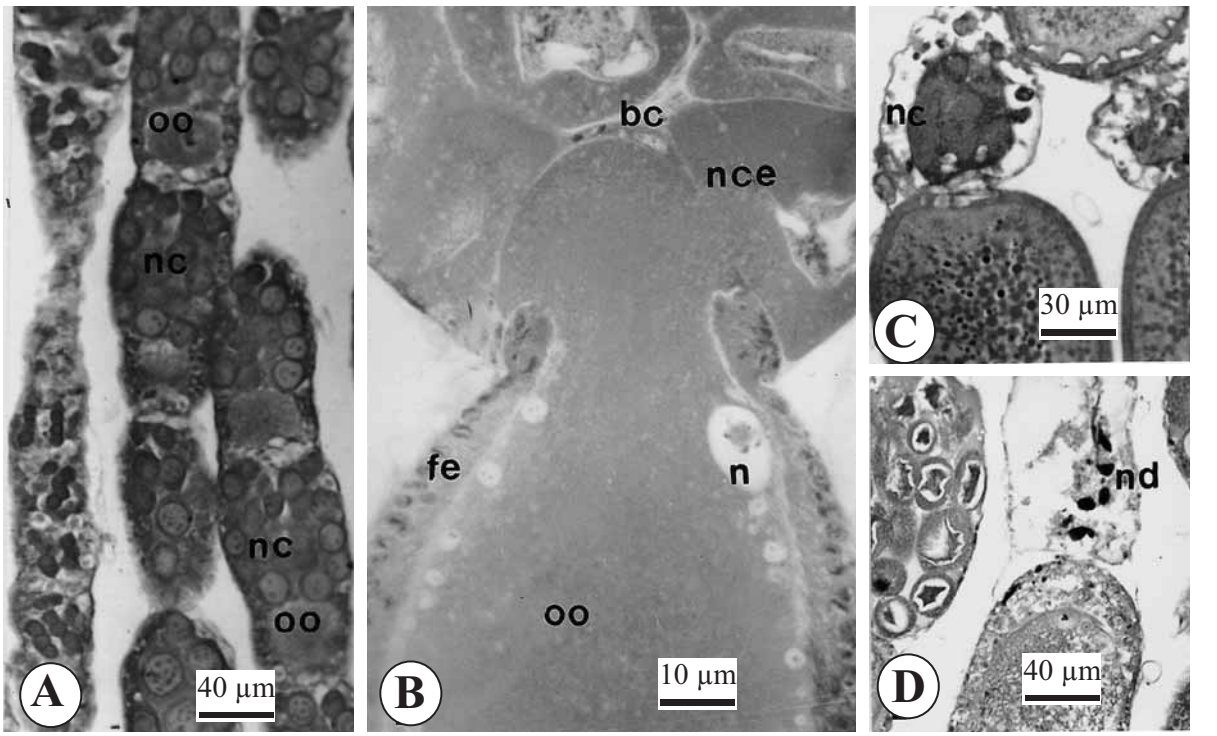

Figure 8. Light micrographs showing the development and death of nurse cells. (**A**) Pre-vitellogenic follicles of *Apis mellifera* showing that the nurse cell chamber (**nc**) is larger than the oocyte (**oo**) chamber. (**B**) Material from a nurse cell (**nce**) passing into the vitellogenic oocyte (**oo**). (**C**) Empty nurse cell chamber (**nc**) after death of the nurse cells through massive and complete loss of cytoplasm. (**D**) Nuclear debris (**nd**) from nurse cells in the mature follicle. **bc -** border cells, **fe -** follicular epithelium. (**A)** and (**D**) Bar = 40 µm, (**B)** Bar = 10 µm, (**C**) Bar = 30 µm.

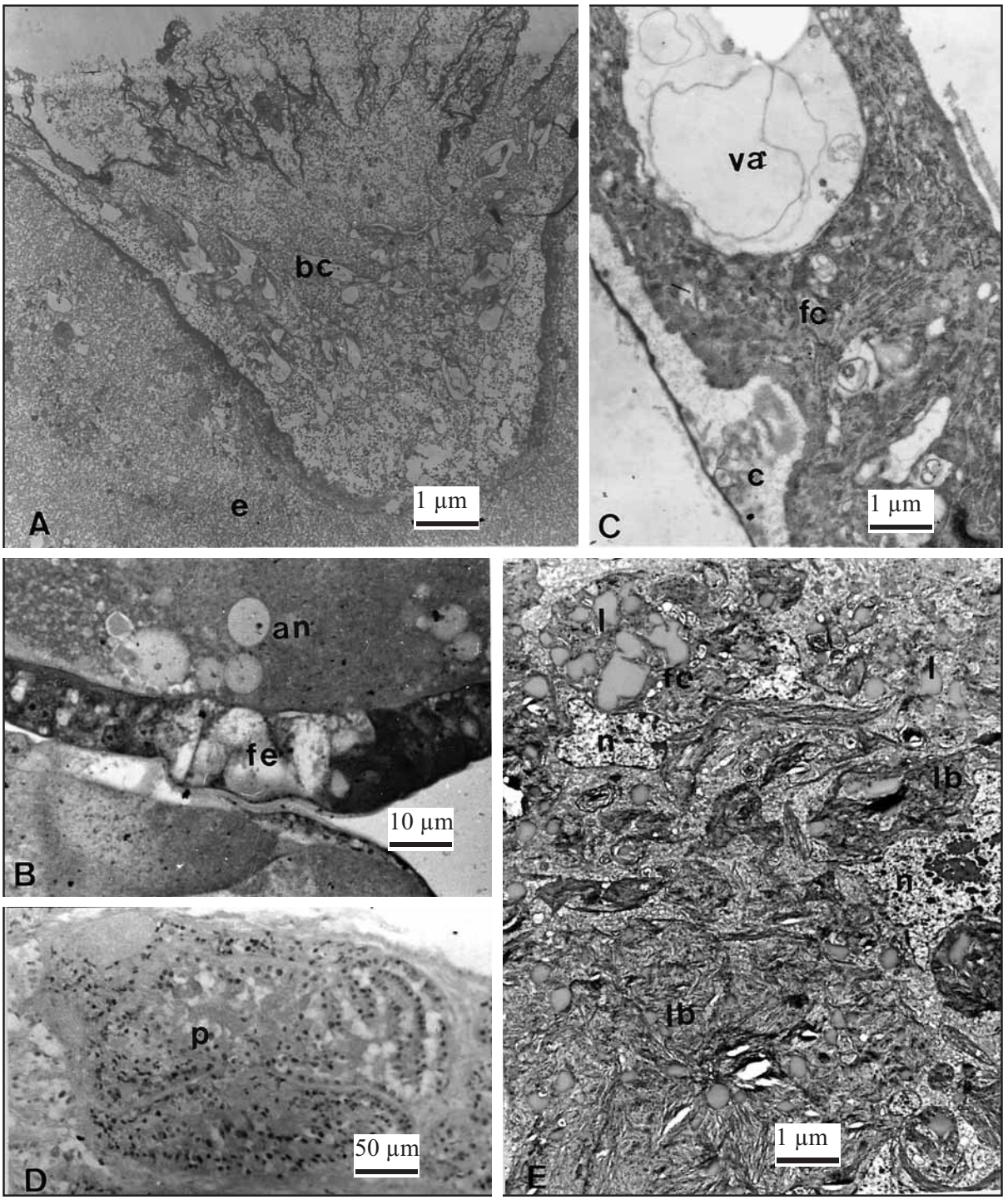

Figure 9. Micrographs showing death of the follicular cell layer. (**A**) TEM of dead border cells (**bc**) during formation of the micropyle of an *Melipona quadrifasciata* egg (**e**). (**B**) Light micrograph of dying cells from the follicular epithelium (**fe**) in the posterior pole of a *Melipona quadrifasciata* oocyte. Note the extensive "vacuolation" of the cells. (**C**) TEM showing "vacuolation" (**va**) of the follicular cell layer (**fc**) after deposition of the chorion (**c**). (**D**) Light micrograph of the plug (**p**) of follicular cells formed after ovulation in the distal end of the ovariole. (**E**) TEM showing resorption of the plug by autophagic death of the follicular epithelial cells (**fc**). **lb -** lamellar bodies, **l -** lipids, **n -** nuclei, **an** - accessory nuclei. (**A),** (**C**) and (**F**) Bar = 1 µm, (**B)** Bar = 10 µm, (**D**) Bar = 50 µm.

When deposition of the chorion has been completed, the follicular cells around all of the eggs become empty and very flat (Fig. 9C). Ovulation makes these cells collapse into the ovariole lumen to form a temporary plug that, during a certain time, closes the passage for other eggs (Fig. 9D). The presence of a plug is evidence of ovulation or egg laying and is usually considered to be analogous to the corpus luteus because of the yellowish color conferred by lipids; this plug is also analogous to the structure seen in the vertebrate ovary after ovulation. After a variable, species-dependent period of time, the follicular cells die and the plug is reabsorbed. In this process of death, the cellular organelles are completely disorganized and the cytoplasm becomes filled with bodies consisting of concentrically arranged membranous lamellae and lipid droplets (Fig. 9E). This type of cell death was usually referred to as fatty tissue degeneration**.** The cytoplasm of follicular cells in this type of death has high acid phosphatase activity and the membranous lamellae are sugestive of a form of autophagic death.

The types of cell death described in the present review are common to *A. mellifera* and Meliponini species and are physiologically and genetically determined to occur during egg production. However, incorrect oogenesis and environmental factors may trigger other types of cell death in the germarium or vitellarium of queen ovaries. Cell death caused by errors in oogenesis may occur at any developmental stage and the morphology seen in the germarium is similar to that observed in virgin queens. Cell death in the vitellarium generally starts with the death of the oocyte, which is soon followed by the death of the whole follicle. The death of a follicle during development is seen as an amorphous mass of tissue that interrupts the sequence of maturating follicles in the ovariole. The death of follicles containing non-viable oocytes is frequent and occurs at a given physiological rate since errors may occur in the course of cell division during oogenesis or on other occasions. This type of death is considered pathological when, for genetic or other (i.e. nutritional) reasons, there are detrimental factors so powerful and extensive that they produce a significant reduction in fertility.

When cell death is caused by environmental factors, it initially affects oocytes that are ready to be ovulated into the proximal end of the ovariole or are already in the ovariole peduncle. If the queen is caged and prevented from laying, the mature eggs wrinkle into the chorion (Fig. 10A) and the follicular epithelium collapses around this structure. In this case, the exit of the ovariole is also closed by a plug during a certain period of time but, unlike the post-ovulation plug, this plug contains oocyte debris (Fig. 10B). The follicular cells are responsible for resorption of the oocyte debris, and are themselves also resorbed. The structure formed by resorption of the follicle is sometimes referred to as corpus atresicus because of the physiological resemblance to its vertebrate analog. This pattern of cell death is frequent in insects when the environmental conditions for egg development are unfavorable [6,35,36 and Patrício K, 2000, MSc dissertation, Paulista State University (UNESP), Rio Claro , Brazil]. Such cell death also prevents the waste of nutrients deposited in the oocyte.

Cell death in the adult worker ovary

Although workers have smaller ovarioles than those of queens and their genital tract does not allow mating, most retain their capability to produce and lay eggs. In *A. mellifera*, egg laying by workers is restrained by queen pheromones, but even in queenright colonies vitellogenesis and even posture may occur [3,59] in some workers. Nevertheless, most of the workers spend their lives inside the colony with their ovaries in a previtellogenic stage and, when they leave their intranidal activities to become foragers, the ovaries degenerate. Juvenile hormone, which is important in promoting ovarian development in queens, has an opposite function in workers since in this caste the ovaries degenerate when the titers of this hormone rise [37]. Thus, workers can produce oocytes and potentially lay eggs while they remain in the colony, but lose this potential when they leave the colony and no longer participate in intra colony-related tasks. This switch in activities provides a means of economizing energy that would otherwise be directed to the maintenance of a functional ovary.

In most Meliponini species, the queen does not inhibit vitellogenesis in worker ovaries, but rather appears to stimulate egg production [20] and laying; indeed, in some cases, the queen eats some of the worker eggs. The workers also lay functional eggs that will develop parthenogenetically to produce males [5,44]. The maintenance of egg production by workers is an interesting strategy used by

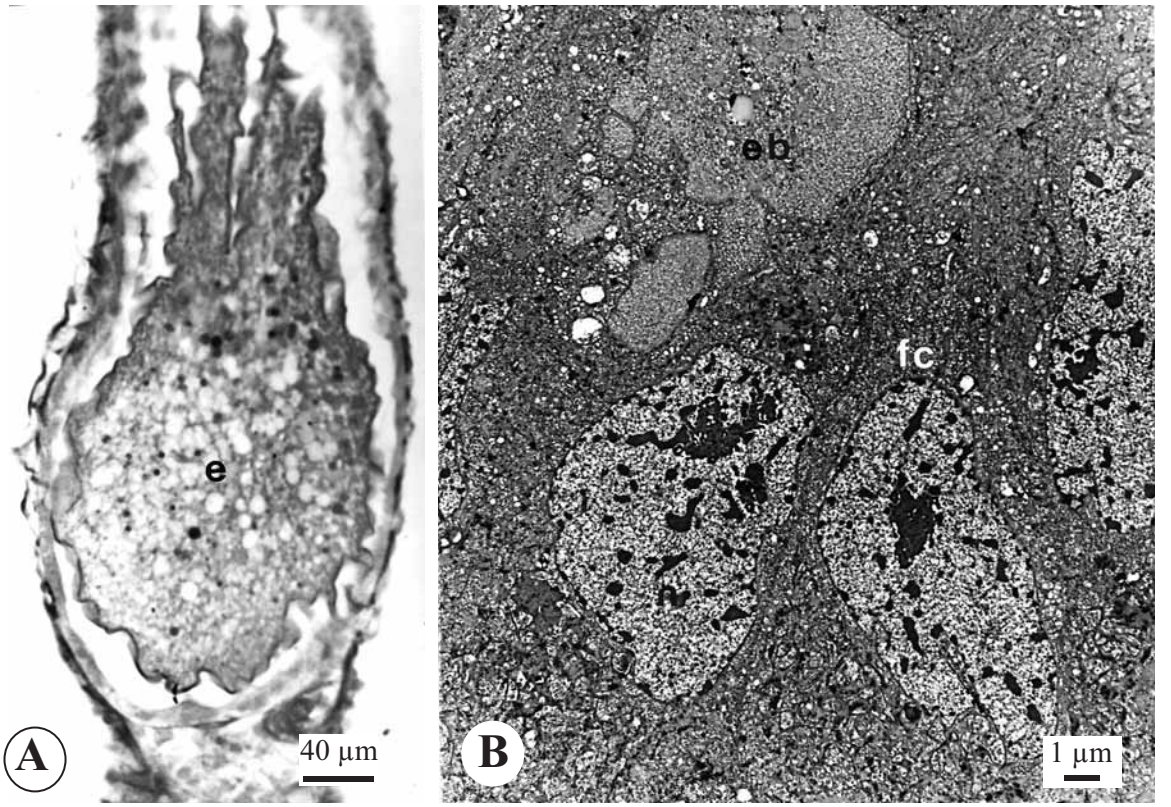

Figure 10. Resorption of mature eggs that did not ovulate. (**A**) Light micrograph of a mature egg (**e**) being reabsorbed. (**B**) TEM of the resorption body. **ed -** egg debris, **fc** - follicle cells being reabsorbed, **n** - nucleus.

queens to obtain food from workers, even if this may result in competition for reproduction. Thus, with rare exceptions, e.g., *Leurotrigona* species in which vitellogenesis is inhibited by the queen's presence as in *A. mellifera* [78], the ovaries of Meliponini workers are functional during the period corresponding to brood provisioning, which is when trophic or functional eggs may be laid. The absence of cell death in the ovaries of Meliponini workers working inside the colony supports their role in a special form of food production to feed the queen. Cell death in the ovaries of foragers is related to an inability to lay eggs. As in *A. mellifera*, the ovaries of workers in most species of Meliponini also become non-viable when the workers leave their intranidal activities. However, in contrast to *A. mellifera* in which there are generally no vitellogenic follicles, these structures are frequent in Meliponini foragers. The inability to lay mature eggs results in the appearance of atresicus bodies in these degenerative ovaries (Fig. 10A,B), in a manner similar to that seen in caged queens.

FINAL COMMENTS

Programmed cell death is the most common fate of female germ cells in most animals. In *Drosophila*, 15 nurse cells die for every oocyte that is produced [50]. In bees, this number is also high and may involve other types of cell death. Programmed cell death in bee ovaries is genetically determined when castes are also genetically determined, as occurs in *Melipona*, or is mediated by environmental factors, as in species in which caste determination is trophic. However, in highly eusocial bees, programmed cell death may also be environmentally triggered by the quantity and type of food the larvae receive during post-embryological development; this nutritional profile regulates the rate of juvenile hormone production. This last type of induced programmed cell death produces females with the same genotype but different reproductive capabilities, perhaps by promoting differential gene expression in the ovaries of each caste.

In conclusion, all cell death can be considered as programmed, but may not always be classified as

apoptosis. Apoptosis has well-defined morphological and biochemical characteristics [17] that are only partially observed in insects. Other types of programmed cell death previously described in insects are also present in bee ovaries [47,48], and may result from autophagy, as in pre-follicular and follicular cells, or from cytoplasmic disintegration, as in stromatic and nurse cells.

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