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PROGRAMMED CELL DEATH DURING OVARIAN DIFFERENTIATION IN QUEENS OF *Apis mellifera* LINNÉ, 1758 (HYMENOPTERA, APINI)

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ABSTRACT

In this study, we examined various aspects of ovarian development in adult honey bees queens (*Apis mellifera*). Caged honey bee queens showed an initial programmed germ cell differentiation that was independent of any external or environmental stimulus. In young queens, division of the stem germ cells resulted in cysts of clone cells (cystocytes) that were connected through intercellular bridges and appeared as rosettes. The cystocytes started differentiate shortly before the queens reached sexual maturity (about 5 days old). The oocyte subsequently appeared as a large, stained cell connected to parallel, double rows of smaller cells, or nurse cells. If the queens did not mate, germ cell differentiation was "switched off", and development of the ovary was interrupted in an intermediate developmental stage, without follicular cell organization. Therefore, in such queens, there were no previtellogenic follicles. This finding could explain why virgin queens rarely lay eggs and why after fecundation they require several days to start laying. The absence of previtellogenic follicles may also indicate that some stimulus is required for continuation of the vitellogenesis and ovary development.

Key words: Carbon dioxide, cell death, nurse cells, oocytes, ovarian follicles

INTRODUCTION

Insect ovaries are classified as either: panoistic or meroistic. Meroistic ovaries are subdivided according to the localization of the nurse cells or trophocytes: telotrophic ovaries are those in which all of the nurse cells remain in the germarium, and polytrophic ovaries are those in which a group of nurse cells is closely associated with each oocyte and is enclosed within a follicle in the vitellarium [3]. Meroistic politrophic ovaries occur in bees. During development of the bee ovary, the oocyte and respective nurse cells differentiate and are kept separate in two interconnected follicular chambers (the oocyte and the nurse chambers) [3]. The nurse cells and oocyte originate from the same cystoblast or second oogonium and therefore have the same genome. The nurse cells retain cytoplasmic connections with the oocyte through cellular bridges that persist because of incomplete cell division [11]. The main function of the nurse cells is to support the oocyte in the synthesis of the euplasm (egg's cytoplasm without yolk) [8]. The deutoplasm (cytoplasm with yolk) appears only after vitellogenesis.

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In vertebrates, the oocytes have special nuclear regions, known as lumpbrush chromosomes, in which the DNA is amplified during the stationary phase of the meiotic prophase I. In this phase, the lumpbrush chromosomes produce all of the RNA needed for the first steps of the developmental and embryological regulation of the zygote. This RNA is arranged in very heavily stained micronucleoli at the nucleus periphery and, during oogenesis, will pass through the nuclear pores to accumulate in the cytoplasm. In insects, especially in those with meroistic ovaries, this nuclear activity is very low, with the nuclear or germinative vesicle being very small and weakly stained for RNA. This germinative vesicle releases many small vesicles that contain RNA and DNA. However the main oocyte growth and RNA production involves the nurse cells, which function like the lumpbrush chromosomes of vertebrates and transfer all the necessary substances to the oocyte during its development [5].

The ovaries of the honey bee *Apis mellifera* are of the meroistic polytrophic type, and are much more developed in queens than in workers [12] since the queen is the reproductive caste in these eusocial insects [7]. In *A. mellifera* queens the ovaries are paired structures composed of 180-200 ovarioles [12]. Each ovary gives off a lateral oviduct that converges with its counterpart to form a common

oviduct. The ovarioles are long, cylindrical units that normally converge at the anterior end of the corresponding lateral oviduct and consist of a terminal filament, a germarium, and in mature ovaries, a vitellarium [2,12].

A fully-developed ovary with ovarioles containing an extensive vitellarium is only seen in mated queens [10]. Virgin queens usually do not lay eggs and if they do not mate there is a marked degeneration of the ovarian tissue after the reproductive period (when they are around 5 days old) that culminates in total regression of the organ [10]. Fecundation of the queens stimulates development of the ovaries by a mechanism that is still unknown and leads to vitellogenesis and maturation of the ovarian follicles [10].

In *Drosophila melanogaster*, sex peptides target the female neuroendocrine axis to stimulate juvenile hormone biosynthesis by the *corpora allata* [6,9], which then controls the oogenic cycle in the ovaries [13,14]. Although the existence of such sex peptides in *A. mellifera* has been investigated, the results have been inconclusive. Colonello and Hartfelder [4] isolated three main peptides from the mucus gland of mature adult drones of *A. mellifera,* which may be candidates for controlling female vitellogenesis activation after mating.

As part of an investigation into the mechanisms involved in the initial activation of the ovary during the life cycle of *A. mellifera* queens, we investigated the morphological changes associated with germ cell differentiation in the ovaries of caged virgin queens of various ages, and compared this development with that of mated queens.

MATERIAL AND METHODS

Queens of Africanized honey bees *A. mellifera* were produced artificially by the larval transfer method into queen cups containing royal jelly. The colonies were maintained in the apiary of the Department of Biology (IB, UNESP, Rio Claro, Brazil) using standard beekeeping procedures. The newly-emerged queens were caged individually with 4-6 workers in small wooden cages at room temperature. The workers were fed with a mixture of sugar and honey. Virgin queens 3, 5, 10, and 15 days old and fecundated with 10 and 15 days old were studied. The artificially inseminated queens were paint-marked and reintroduced into the colony for subsequent collection at specific ages.

The ovaries were dissected in insect saline solution (7.5 g of NaCl, 2.38 g of Na_2HPO_4 and 2.72 g of KH_2PO_4 in 1000 mL of distilled water). For light microscopy, the ovaries were fixed with 4% paraformaldehyde for 24 h and dehydrated in increasing concentrations of ethanol (70% to 95%), prior to infiltration and embedding in historesin (Leica). Sections 7 µm thick were cut with a Leica RM 2145 microtome and mounted on glass slides for staining with hematoxylin and eosin, 1% toluidine blue, and the Feulgen reaction. The sections were examined with a Zeiss photomicroscope.

For fluorescence microscopy the ovaries were dissected in insect saline solution, and incubated with Hoechst dye (10 mg/ml Bisbenzimide 33342, Sigma) for 5 min in the dark. Subsequently, material was incubated with propidium iodide (960 µl of 3% sodium citrate, 10 µl of formalin 1:80, 20 µl of FDA 460 µg/ml, 10 µl of propidium iodide 125 µg/ml) for a further 5 min. The material was analyzed immediately after staining.

RESULTS

In three-day-old virgin queens, the ovarioles consisted of a long terminal filament and a long germary with a large number of cystocytic rosettes (Fig. 1A), that originated from the incomplete mitotic division of a germ cell, known as a cytoblast or secondary oogonium.

At approximately 5 days of age, when the queens were able to mate, the cystocytic rosettes disappeared as a result of differentiation. In this phase, the ovarioles had a shorter terminal filament and a larger, longer germary (Fig. 1B). A single cystic cell differentiated into an oocyte while the remaining cells differentiated into nurse cells that were connected to the oocyte by two lateral rows of smaller cells (Fig. 1B). This pattern of ovarian organization was maintained if the queens did not mate (Fig. 1D). There was no follicle formation and no previtellogenic follicles were seen. The somatic cells that normally form the pre-follicular cells remained undifferentiated and were randomly scattered among the differentiating germ cells.

After the mating phase, the ovarioles of 10 day-old unmated caged queens had an increased number of pycnotic nuclei with highly condensed chromatin, as shown by the Feulgen reaction (Fig.1C). At 15 days of age, certain regions of the ovaries of virgin queens showed tissue disorganization and an increasing number of cells with pycnotic nuclei (Fig. 1D). This high level of cellular death was confirmed by the staining with Hoescht dye and propidium iodide (Fig. 1E). With the former dye, living cells were stained blue, whereas with propidium iodide the nuclei of dead cells with a damaged nuclear membrane were stained in red (Fig. 1E).

After mating the ovaries of fecundated queens were “switched on”, the germ cells continuing their differentiation to complete follicles and undergo vitellogenesis. The ovarioles consisted of a long vitellarium containing the oocyte and well defined

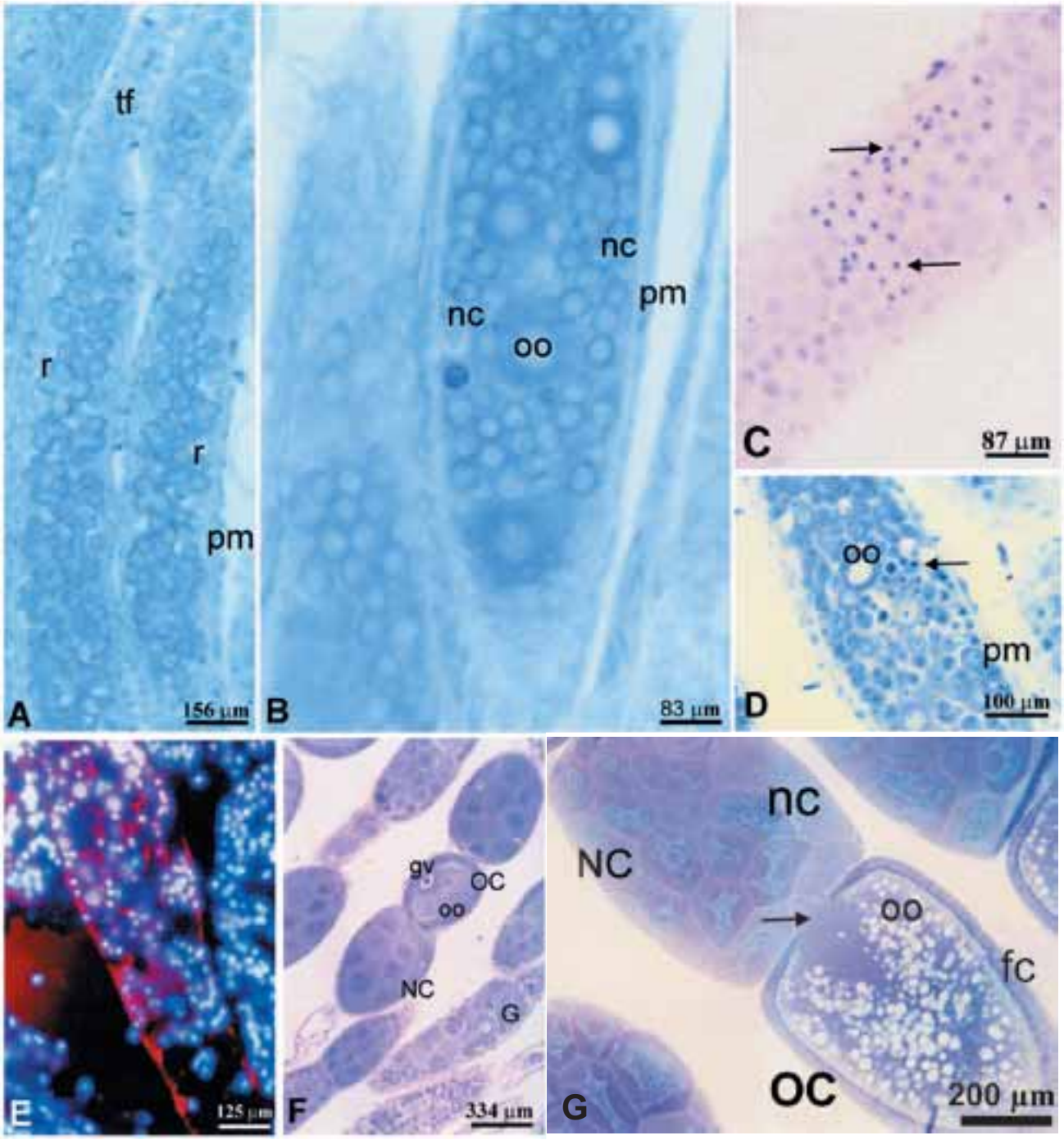

Figure 1. Morphological aspects of ovaries from caged virgin and fecundated *A. mellifera* queens. **A**. Ovarioles of a 3-day-old virgin queen showing a long terminal filament (**tf**) and the cystocyte rosettes (**r**) of a germarium. **pm** = peritoneal membrane. Toiludine blue staining. **B**. Ovariole of a 5-day-old queen showing oocytes (**oo**) connected to parallel rows of differentiating nurse cells (**nc**). **pm** = peritoneal membrane. Toluidine blue staining. **C**. Feulgen reaction showing cells with pycnotic nuclei (**arrows**) in an ovariole of a 10-day-old virgin queen. **D**. Detail of an ovariole in a 15-day-old virgin queen showing tissue disorganization and some cells with pycnotic nuclei (**arrow**). **oo** = oocyte, **pm** = peritoneal membrane. Toluidine blue staining. **E**. Fluorescence technique showing many dead cells with red stained nuclei (propidium iodide) and healthy cells with blue stained nuclei (Hoechst dye). **F**. Ovarian follicle of a 15-day-old fecundated queen showing a developed vitellarium in which nurse (**NC**) and oocyte (**OC**) chambers are clearly visible. A germinative vesicle (**gv**) is present in the oocyte (**oo**). **G** = germarium. HE staining. **G**. Detail of a follicle from a 15-day-old fecundated queen showing the oocyte chamber (**OC**) connected (**arrow**) to nurse chamber (**NC**). **fc** = follicular cells, **oo** = oocyte, **nc** = nurse cells. Toluidine blue stainig.

nurse chambers surrounded by follicular cells (Fig. 1F,G). After mating, the appearance of the ovaries was totally different from that of the ovaries from unmated caged queens of the same age (Fig. 1D,F).

DISCUSSION

In agreement with a previous report for honey bee queens under normal conditions [4,10], our results showed that there was also a clear sequence of programmed germ cell differentiation in caged queens of Africanized honey bees. In young queens, the germ cells produced by the mitotic division of an oogonium, give rise to groups of cystoblastic clone cells [3]. Each cytoblast enters mitosis to produce the cystocytes that remain connected through intercellular bridges to eventually form cystocyte rosettes or cysts [3]. Shortly before the queens reach sexual maturation (at about 5 days of age), the cystocytes start to differentiate, with each cyst producing one oocyte that is connected to parallel, double rows of smaller nurse cells [3]. As shown here, if the queen did not mate, then germ cell differentiation was "switched off", and ovarian development was arrested in a differentiation intermediate developmental stage, i.e., in the early stage of oocyte and nurse cells differentiation, and there was no previtellogenic follicle and no vitellogenesis.

The chronological sequence of initial germ cell differentiation in *Apis mellifera* queens seen here differed from that described by Patrício and Cruz-Landim [9] and Tanaka and Hartfelder [14]. According to the former authors, cystocytes appear in the ovarioles of virgin queens from four days of age onwards. However, as shown here, previtellogenic follicles were not observed in virgin queens, even in those more than 10 days old (an age at which the previtellogenic oocyte and differentiating nurse cells had already divided into oocyte and nurse chambers and had evolved into follicular cells). According to Zacaro (Zacaro, Master's dissertation, UNESP, Rio Claro, SP), cystocytic rosettes are already present in newly emerged queens and persist in 4-day old virgin queens.

As shown by Patrício and Cruz-Landim [10] and Tanaka and Hartfelder [14], the initial stages of oogenesis are not dependent on a stimulus, whereas the continuation of oogenesis is stimulus-dependent since, after the mating period, the ovaries start to degenerate as also demonstrated here by fluorescence microscopy. The nature of the stimulus is unknown but is probably multifactorial and may include the levels of mating hormones and social interactions between the castes.

Interruption of the initial stages of oogenesis may explain the finding on that virgin queens rarely lay eggs and, after being fecundated, they require several days (on average 7.5 days) to start laying [1]. This pattern of ovarian development may be an additional adaptive strategy to guarantee the reproductive dominance of the fecundated queen. Since if the ovaries of virgin queens could develop without mating or any other stimulus, then they might compete with the fecundated queen in producing males.

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