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Original Article

OVARY DEVELOPMENT IN HONEYBEE (*APIS MELLIFERA* L.) WORKERS UNDER CO_2 NARCOSIS, CAGED OUTSIDE OF THE COLONY

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Abstract

Exposure of *A. mellifera* workers to CO_2 has been reported to decrease life span, induce behavioral changes, and inhibit the development of some glands and the ovaries. However, the use of CO_2 is currently practiced among beekeepers and how the development of workers' ovaries are affected is unknown. The present work aimed to evaluate the effect of CO_2 on the ovaries of *A. mellifera* workers, using a morphological approach. Newly emerged, and 3, 5, and 10-day-old workers were exposed to saturated CO_2 for 30 seconds, more than once. The ovaries were examined under light (LM) and transmission electron microscopy (TEM). The ovaries of narcotized, 5-day-old workers exhibited long ovarioles with well-developed germarium, although oogenesis showed little development. While in the control group, the ovaries already exhibited cysts with cystocytes forming rosettes. At 10-days-old, the ovaries feature was variable; some of the ovaries showed ovarioles with many rosette cysts while others showed a high intensity of disorganization due to the beginning of cell death. The ovarioles of the ovaries of 15-day-old treated workers were morphologically varied as oogenesis showed little or no advance. In others cases, a complete disorganization with swollen cells and cell death features were observed. Germ cells of the ovaries of CO_2-treated workers exhibited well-structured organelles, such as the Golgi complex, and larger amounts of mitochondria in the cytoplasm than the control group, but there was disorganization of the endoplasmic reticulum membranes. Our findings revealed that the exposure of workers to CO_2 promotes impairment of oogenesis and ovarian degradation.

Keywords: anesthesia, cell death, eusocial insects, light microscopy, oogenesis, transmission electron microscopy.

INTRODUCTION

In apiculture practice, CO_2 is currently used as a tool in bee handling or as a way of accelerating the beginning of queen laying. Many studies have been done on the effect of CO_2 treatment in *Apis mellifera* (Mackensen, 1947; Berger et al., 2005; Cobey, 2007), however, most of the research concerned queen ovary functioning and little is known about the ovaries of the workers.

The exposition of *A. mellifera* newly emerged workers to a saturated CO_2 atmosphere promotes several changes: physiological, as inhibition of the ovaries, hypopharyngeal, and wax glands development (Fyg, 1950; Skowronek and Jaycox, 1974; Harris and Harbo, 1990) and behavioral, as a precocious acquirement of the forager activities. Moreover, there is a decrease in the lifespan, pollen collection, and consumption (Skowroneck and Jaycox, 1974; Ebadi et al., 1980; Berger and Abdalla, 2004). The effect on the ovaries was attributed to a decreased synthesis of vitellogenin (Vg), which is dependent on the intake of pollen (Bitondi and Simões, 1996). The underdevelopment of the glands is due to the same reason (Cruz-Landim and Akahira, 1966). Therefore, the CO_2 treatment affects a worker's organism by decreasing nutrition. Consequently, the development of the worker is slowed down, since the ingestion of proteins is essential for organ development at the beginning of adulthood. The way CO_2

acts is not completely understood. It seems that for workers but not queens, feeding, as shown above, is influenced by the effects of CO_2. Since the Vg is a precursor of the yolk vitellin, there is a possibility that the final action of CO_2 on Vg synthesis occurs. In fact, studies made by Koywiwattrakul et al. (2005) show that the CO_2 treatment reduces the expression of Vg and the transferrin (TRF) genes, suggesting that these genes could be involved in the sterility of treated workers. In an attempt to explain the other effects of the CO_2 narcosis on the worker's organism, Koywiwattrakul et al. (2005) stated that the Vg is a conditioning factor regulating the worker's sequence of activities or labor division. This is in accordance with the current point of view of Vg as a multifunctional molecule (Guidugli et al., 2005).

The developmental stage of the worker's ovaries varies with age, and depends on the colony conditions. The queen's presence and colonial constraints regulate the colony homeostasis, and consequently, the possibility of worker reproduction (male production). As colony fumigation is a current practice in beekeeping, the present work aimed to separate the colony effect from the CO_2 narcosis by evaluating the gas effect on the workers' ovarian functioning, in workers caged outside of the colony. The alterations on ovary morphology were studied under light and electron microscopy levels.

MATERIAL AND METHODS

In a queenright colony, the *A. mellifera* workers start their duties outside of the nest when they are about 25-days-old. Before that age, they work in the nest and eventually lay eggs that might originate drones. Therefore, the effect of the narcosis in the ovaries was studied in the period that workers were within the colony. A virgin queen was caged with the workers in order to simulate a colony condition, while at the same time avoiding the mated queen pheromonal which has control over a worker's ovary development. The queens were not the object of this study.

Obtaining and maintaining the workers

Newly emerged workers were collected in colonies from the apiary of the Departamento de Biologia, Instituto de Biociências, UNESP, Rio Claro, SP, Brazil. During the experiment, the 50 workers were kept caged in 10 wooden boxes that were 15x15x7 cm with a virgin queen, and candy and water *ad libitum*. The temperature was kept at a constant 32°C degrees and the relative humidity was 70%. The control workers had not been exposed to the gas, and were kept under the same conditions. All analysed workers were selected randomly from wooden boxes.

CO_2 exposition

The workers were exposed to a CO_2 saturated atmosphere for 30 seconds. The analysis of the ovaries' morphology is shown in Table 1. The exposure time was based on the studies of Ebadi et al. (1980) and Berger and Abdalla (2004) where different times of CO_2 exposure and longevity were tested. The workers analysed at 5 days-old were exposed twice: when newly emerged and at 3 days-old (and analysed at 5 days-old, two days after the second exposure). Workers analysed at 10 days-old were exposed three times: when newly emerged, at 3 days-old, and at 5 days-old (and analysed 5 days after the last exposure).

Table 1.

Workers of *Apis mellifera* were treated under narcosis when newly emerged and at 3, 5, and 10-days old. The morphology of the ovary was analysed at 5, 10, and 15-days old

Narcosis treatment				Morphological analysis		
Newly emerged	3 days-old	5 days-old	10 days-old	5 days-old	10 days-old	15 days-old
X	X			X		
X	X	X			X	
X	X	X	X			X

Workers analysed at 15 days-old were exposed four times: when newly emerged, at 3 days-old, at 5 days-old, and at 10 days-old (and analysed 5 days after the last exposure).

Light microscopy (LM)

The ovaries of 10 workers of each age (5, 10, and 15 days-old) from the experimental and control groups were dissected and fixed in 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer with a pH of 7.4. After being dehydrated in a graded ethanol series (70-95%), the ovaries were embedded in Leica historesin. Sections that were 5 µm thick were obtained, put in histological slides and

stained with 1% toluidine blue with a pH of 7.0 (TB) and hematoxylin-eosin (HE). The sections were observed and photographed in a Zeiss photomicroscope.

Transmission electron microscopy (TEM)

The ovaries of 10 workers of each group and age (5, 10, and 15 days-old) were fixed for 2 hours in Karnovsky's solution, and post-fixed in 0.5% osmium tetroxide containing 0.8% potassium ferricyanide dissolved in 0.1 M sodium cacodylate buffer with a pH of 7.4, for 1 hour in a refrigerator. After this, the ovaries were washed 2 times. Each bath lasted 15 minutes each in the same buffer. Then the washed ovaries were incubated for 3 hours in an aqueous solution of 0.15% tannic acid, left for 2 hours in 1% uranyl acetate, and then washed with 10% ethanol. The ovaries were dehydrated in an acetone series (70-100%) and embedded in Epon-Araldite resin. The obtained ultra-thin sections were collected on copper grids and contrasted with uranyl acetate and lead citrate before being examined and photographed in a Philips TEM.

RESULTS

The control group: Ovary development in caged workers

The ovaries are formed by ovarioles whose numbers vary from 2 to 12 in *A. mellifera* workers. Each ovariole is formed by terminal filament, germarium, and vitelarium. The ovaries of workers present the same organisation as found in queens but the ovarioles are shorter and fewer in number. In newly emerged workers, the ovarioles are extremely thin and involve a net of tracheoles. Under stereoscope examination, no differentiation can be noted along the length. However, the histological preparations showed the presence of a very short basal germarium and a longer terminal filament at the apical region.

The germarium initiates the oogenesis process until oocyte differentiation among the cyst cystocytes and vitellogenesis start. In some of the newly emerged workers, some advance in oogenesis could already be noticed, that is, the oocyte differentiated from the remaining cystocytes that appear in two

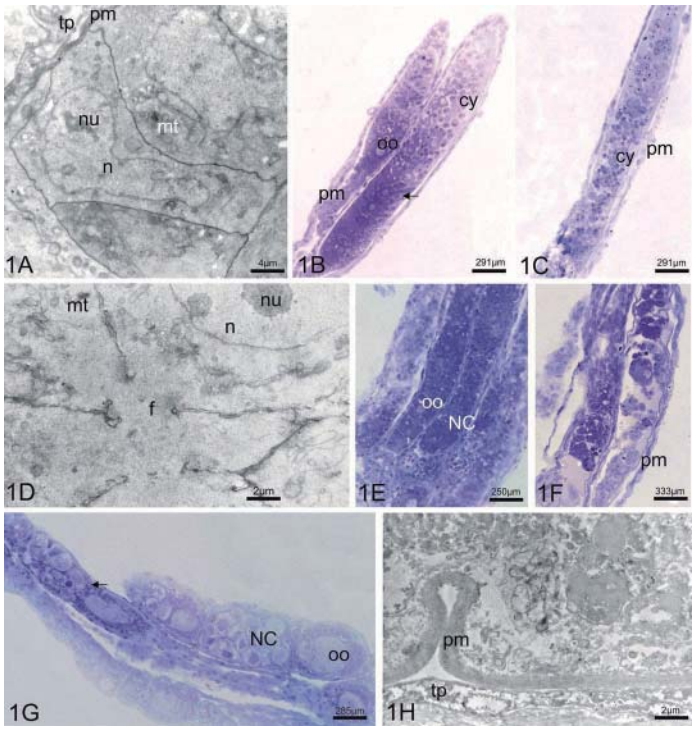

Fig. 1. A (TEM), C and D (LM, TB) of 5-day-olds, and B (LM, TB) of newly emerged worker. A. Terminal filament showing the arrangement of cells stacked to form a single row. Ovaries demonstrating different levels of ovarioles differentiation in B. Undifferentiated cells are observed in C. The fusoma (f) central region and a few mitochondria (mt) concentrated in a small region of the cytoplasm (D) in 5-day-old workers' ovariole. Ovarioles showing different degrees of degeneration, as observed in E (10-day-olds) the well preserved ovarioles while in F (10-day-olds) they are disorganised. G (LM) and H (TEM) of 15-day-olds. G showing differentiation between the nurse cell (NC) and oocyte (oo) chambers and the canal between them, and H showing completely disorganised ovariole. arrow = tissue degeneration; cy = cystocytes; mt = mitochondria; n-nucleus; nu-nucleolus; pm = peritoneal membrane; oo = oocyte; tp = tunica propria ; LM = light microscopy; TB = staining with toluidine blue; TEM = transmission electron microscopy.

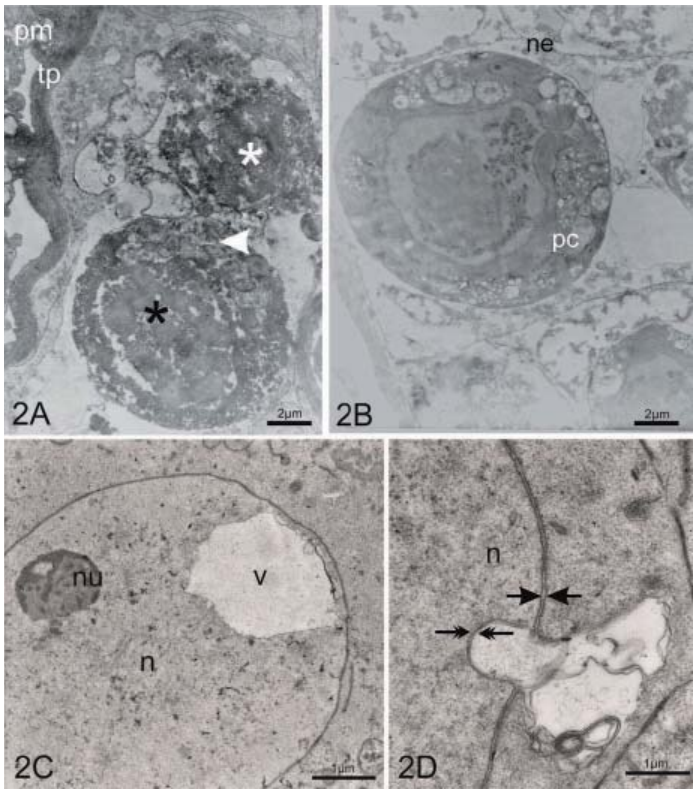

Fig. 2. A, B, C and D (Transmission Electron Microscopy) Micrographs of 15-day-olds, control group II, newly emerged workers showing different aspects of cell death. In A, chromatin condensation (asterisk) and disorganization of the cytoplasm (arrow head) can be observed.
B. Condensed cell being phagocytosed by neighboring cell. C. Vacuole (v) inside the nucleus (n). D. Formation of loops by the swollen perinuclear space by separation of inner and outer membranes of the nucleus (arrows).
ne = neighbour cell; pc = phagocytized cell; pm = peritoneal membrane; tp = tunica propria

rows diverging from the oocyte sides (Fig. 1B). Nevertheless, most of the workers develop cysts in the ovaries where the oocyte is not yet distinct (Fig. 1C). The terminal filament presents the usual morphology of rectangular cells arranged in a single line and surrounded by the peritoneal membrane. Between the terminal filament and the germarium, the single line becomes cells with a wide outer side, opposed to the peritoneal membrane, and the inner side is funneled in such way that a kind of lumen is open in the base of the terminal filament. The cells of the terminal filament have a large central nucleus, poorly developed endoplasmic reticulum and many small mitochondria (Fig. 1A).

Five-day-old workers had a longer germarium in which the number of cysts was augmented and the oogenesis advanced. Cysts in the rosette stage are frequent as well as cysts with differentiated oocyte. The oocyte can be recognised by its larger size, central nucleus with disperse chromatin, and few cytoplasm organelles (Fig. 1D).

The aspect of the ovaries of the 10-day-old workers was variable. Some of them had ovarioles with many rosette cysts (Fig. 1E) while others showed a high intensity of disorganisation due to cell death (Fig. 1F).

The ovaries of the 15-day-old workers were in an advanced stage of degeneration, showing a higher number of cells with pyknotic nuclei. In some cases the degeneration is so advanced that only the peritoneal membrane remained (Fig. 1H). Nevertheless, the degeneration does not attain all the ovary ovarioles or its total length. So, in non-degenerated regions, it was possible to observe oogenesis advancement with the beginnings of follicle formation, that is, separation of the oocyte chamber from the nurse chamber containing the remaining cystocytes, now nurse cells (Fig. 1G).

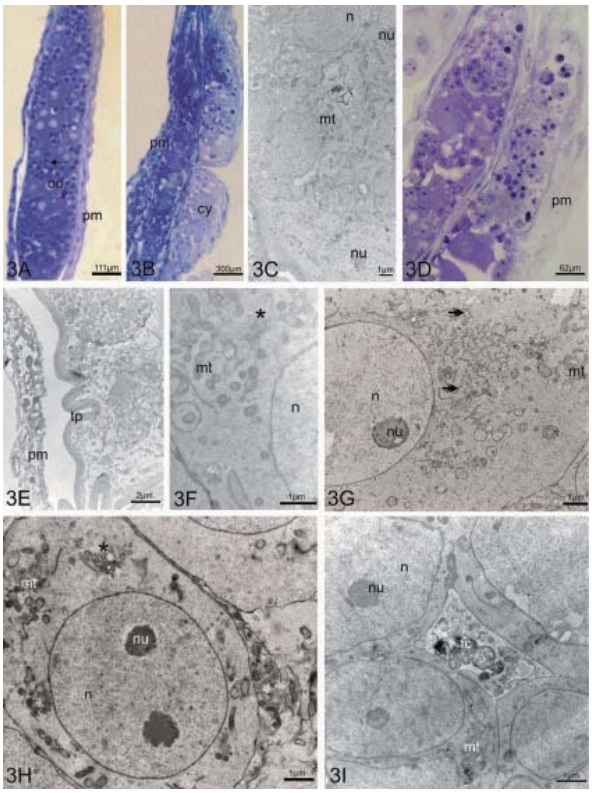

Fig. 3. A. LM micrograph of ovariole of a 5-day-old worker treated with CO_2. Note that the cells present inside are less differentiated than in untreated workers of the same age. B, C, D and E. Micrographs of ovarioles of 15-day-old workers treated with CO_2. B. LM of an intact ovariole with no signs of tissue degeneration. C. TEM showing ovariole with few signs of tissue damage. D and E. Micrographs showing disorganisation of the ovariolar tissue. F and H. TEM showing an increase in the number of mitochondria (mt) in the cytoplasm as well as better visualisation of the Golgi complex (asterisk). G. Disorganisation of endoplasmic reticulum membranes (arrows). I.- Death of follicular cells (fc).
oo = oocyte; tp = tunica propria; cy = cystocytes; pm = peritoneal membrane; mt = mitochondria; n = nucleus; nu = nucleolus ; LM = light microscopy; TEM = transmission electron microscopy

At fifteen-days-old, the ovariole's dying cells present a morphology of the apoptotic cells. The cytoplasm appears condensed and is showing signals of reabsorption (Fig. 2 A, B). The nucleus presents compacted chromatin or vacuoles in the nucleoplasm and irregular outlines (Fig. 2A, C), enlargement of the perinuclear space, and bleb formation (Fig. 2D).

Treatment group: Ovary development in caged workers treated with CO_2

The treated 5-day-old workers presented ovarioles with long germaria, but with oogenesis less advanced than the control. The cysts in rosettes observed in the control, were not present (Fig. 3A).

The ovaries of 10 and 15-day-old workers presented ovarioles in different stages of development. In some cases, the ovarioles are well-preserved although with a poor or absent advance in oogenesis (Fig. 3B, C) or are completely disorganised, showing empty regions, and a great amount of dying cells with swelled cytoplasm, making it difficult to identify the cell type (Fig. 3D, E). The CO_2 narcosis seems to affect the mitochondria, increasing their amount in the germ cells (Fig. 3F). The Golgi apparatus appear more conspicuous (Fig. 3F, H), while the endoplasmic reticulum appear disorganised (Fig. 3G). The ovariole somatic cells also attained cell death (Fig. 3I).

DISCUSSION

The ovaries of *A. mellifera* workers are functional. Under favorable conditions the workers produce mature eggs, although underdeveloped in comparison with the queen's eggs. Nevertheless, in most of the workers from queenright colonies, the vitellogenesis (oocytes maturation) is partially inhibited by queen pheromones. In the present experiment, the workers were kept caged outside of the colony with a virgin queen, soon after the emergence of the workers. In this way, these workers did not suffer the colonial

and mated queen constraints. The CO_2 has an aging effect on bees (Berger and Cruz-Landim, 2009) and Skowronek (1979) verified that treated workers become physiologically 'foragers" early; at the age in which the ovaries develop.

During the whole studied period, anatomical aspects did not change in either the control or with the CO_2 treatment. However, histological and ultrastructural changes were observed. Some of the remaining workers in the colony develop ovaries and may lay eggs. However, the period during which a worker's ovaries remain functional is short, since near the age at which a worker becomes a forager, the ovary has already degenerated. This explains why, even in the control group, the developmental state of the ovaries in 10-day-old individuals is variable, and in 15 days almost completely reabsorbed. This condition explains why the effect of narcosis is best observed in ovaries of 5-day-old workers and less evident in 10 and 15-day-old workers.

The present results differ from those of Patricio and Cruz-Landim (2003) for caged workers. Two factors might have contributed to the difference:

1) The presence of a virgin queen caged with the workers

It is know that some workers in orphaned colonies start vitellogenesis and might even do oviposition, acquiring the status of a false queen, according to Butler and Paton (1964). Even as a virgin when becoming ready for the nuptial flight (around 5 days-old), the queen starts to produce the queen substance pheromone that inhibits the worker's ovarian development. Therefore, after the age mentioned above, the presence of a virgin queen arrested ovary development of the control workers, exerting an effect similar to CO_2.

2) Poor nutrition due to lack of proteins

The workers kept in orphaned colonies (Patricio and Cruz-Landim, 2003) have better developed ovaries. This is because the absence of the queen meant the workers could feed on pollen stored in the colony; which is an important requisite for Vg synthesis (Bitondi and Simões, 1996), and thus, for vitellogenesis.

In queens, CO_2 positively affects the ovary function, promoting vitellogenesis even without fertilisation, and CO_2 also accelerates the start of egg laying after the nuptial flight (Mackensen, 1947; Berger et al., 2005) as a result of the aging effect. Nevertheless, Pain et al. (1967) and Skowronek (1979) verified that although treated queens present precocious ovary development they eventually lose ovary efficiency in comparison with non-treated queens of the same age.

The effects of CO_2 narcosis on the various systems of the workers and queens show that the gas promotes an advance in individual maturity or aging. Concerning workers, one of the effects is the advancement of the beginning of the forager phase in which the worker does not eat pollen, but nectar (Machado and Camargo, 1972; Serrão and Cruz-Landim, 1995) and the ovaries are in a regressive phase.

Koywiwattrakul et al. (2005) stated that those *A. mellifera* workers submitted to CO_2 narcosis and examined 4 hours later, showed the same development of the ovary as the control, but the situation is reversed 48 and 96 hours later: the treated workers have less developed ovaries than the control. According to Engels et al. (1976) the genes regulating the synthesis of Vg and TRF are responsible for ovary activation. Koywiwattrakul et al. (2005) verified that Vg and TRF are under-expressed in the bees treated with CO_2.

The correlation between nutrition and Vg synthesis, and also between nutrition, Vg synthesis, and ovary activation seems clear, but the effect of nutrition is indirectly exerted. It is known, that the hemolymph juvenile hormone (JH) titers rise in the transition from hive to field workers (Jaycox, 1976; Huang and Robinson, 1996; Amdam and Omholt, 2003). According to Ebadi et al. (1980) the treatment with CO_2 causes the same behavioral changes as the JH. Therefore, it seems that the gas effect in the workers mimics the rising of the JH titers (Engels et al., 1976; Koywiwattrakul et al., 2005).

CONCLUSIONS

The present results show that even when away and free from the colony, the ovaries of the control workers were not activated or were only very slightly activated in 5 and 10-day-old individuals. The presence of a queen among the workers, even if the queen is a virgin, might have inhibited the development of the ovaries, but most probably the main cause was protein deficiency. In comparison, CO_2 had a delayed effect on the ovaries of the treated 5-day-old workers. But the delayed effect was not visible in the following studied ages of the workers. Fifteen-day-old workers seemed to have less ovariolar disorganisation than the control.

The narcosis can promote the same effects observed in queenright colonies, inhibiting ovary development, although other causes that control ovary activation in *A. mellifera* workers cannot be neglected. In addition, CO_2 promotes impairment of oogenesis and ovarian degradation.

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