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Morphological Aspects of Cluster Formation in the Germarium of the Sugarcane Borer *Diatraea saccharalis* Fabricius (Lepidoptera: Pyralidae)

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Aspectos Morfológicos da Formação do Cisto no Germário da Broca da Cana *Diatraea saccharalis* Fabricius (Lepidoptera: Pyralidae)

RESUMO - *Diatraea saccharalis* Fabricius é uma das maiores pragas da cultura da cana-de-açúcar. Este trabalho visou caracterizar a região do germário da broca da cana pela microscopia de luz e eletrônica de transmissão, enfatizando as etapas morfológicas da formação do cisto ovariano. No germário desse inseto, quatro zonas puderam ser identificadas morfologicamente durante a formação do cisto. Na extremidade apical de cada ovaríolo – Zona I – as células germinativas primordiais sofrem divisão mitótica completa, originando os cistoblastos. Na Zona II, cada cistoblasto produz um grupo de oito células, os cistócitos, os quais são interconectados por canais circulares. Grupos contendo cistócitos na meiose caracterizam a Zona III. Células germinativas com características ultraestruturais de apoptose são também detectadas nesta zona. Na Zona IV os cistócitos se diferenciam, morfologicamente, em um oócito e sete células nutridoras. As células somáticas intersticiais e células pré-foliculares exibem em seus citoplasmas vacúolos heterogêneos contendo fragmentos celulares degenerados, caracterizados como corpos apoptóticos. Os resultados apontam evidências morfológicas relacionadas com importantes mecanismos de controle para a produção de novos cistos/folículos e para o rearranjo celular no germário, resultante da morte celular programada. Assim, acredita-se que a caracterização morfológica da formação dos cistos ovarianos em *D. saccharalis* forneceu informações valiosas para o entendimento das etapas iniciais da oogênese e contribuiu para o conhecimento dos mecanismos celulares relacionados com a produção de oócitos e com a reprodução nos insetos.

PALAVRAS-CHAVE: Ovaríolo, cistoblasto, cistócito, ultra-estrutura, inseto

ABSTRACT - *Diatraea saccharalis* F. is one of the greatest pests of the sugar cane culture. This report aimed to characterize the germarium region of the sugarcane borer by light and transmission electron microscopy, emphasizing the morphological steps of the ovarian cluster formation. In the germarium of this insect, four zones could be morphologically identified during the cluster formation. In the most apical end of each ovariole – Zone I – the germ line stem cells undergo complete mitotic division, originating the cystoblasts. In the Zone II, each cystoblast produces a group of eight cells, the cystocytes, which are interconnected by the ring canals. Clusters containing all the cystocytes in the meiosis, characterizes the Zone III. Germ cells with ultrastructural features of apoptosis are also detected in this Zone. In the Zone IV the cystocytes differentiate, morphologically, into one oocyte and seven nurse cells. Interstitial somatic cells and pre-follicle cells exhibit, in their cytoplasm, heterogeneous vacuoles containing degenerated cellular fragments, characterized as apoptotic bodies. Our results pointed out to the morphological evidences related with important control mechanisms for new clusters/follicles production and for the cellular arrangement into the germarium, resulting from the programmed cell death. We believe that the morphological characterization of ovarian cluster formation in *D. saccharalis* provided valuable information for the understanding of the initial steps of oogenesis and contributed for the knowledge of the cellular mechanisms related with the oocyte production and with reproduction in insects.

KEY WORDS: Ovariole, cystoblast, cystocyte, ultrastructure, insect

The paired ovaries of the lepidopteran species are each composed of four polytrophic meroistic ovarioles, which contains a linear array of follicles in progressive stages of development. Each ovarian follicle is made up of an oocyte and seven nurse cells surrounded by a single layer of follicular epithelium (Telfer 1975, King & Büning 1985, Büning 1994). During Lepidoptera oogenesis, clusters are formed following the division of germ line stem cells (King & Aggarwal 1965, Mandelbaum 1980, Zimowska *et al*. 1991). These stem cells are located at the anterior tip of the germarium and divides asymmetrically to produce a daughter stem cell and a differentiated daughter cell called cystoblast. The cystoblast then undergoes three rounds of asymmetric and incomplete mitosis to produce a germ line cluster consisting of eight cells, the cystocytes, interconnected by cytoplasmic bridges called ring canals (King & Aggarwal 1965, Miya *et al*. 1970, Mandelbaum 1980, Büning 1994). The cystocytes of a cluster get arranged like the elements of a rosette, while the interconnected ring canals are centralized by an axial structure called fusome (Mandelbaum 1980, Marec *et al*. 1993, de Cuevas & Spradling 1998). Later on, a determination process starts in each cluster, leading one cystocyte to develop as oocyte, whereas the other cystocytes become nurse cells that provide an assortment of RNA and proteins to be transported into the future oocyte (Büning 1994).

The insect germarium has been morphologically divided into a variable number of regions, depending on the insect species, to facilitate the understanding of the events concerning the germ cell cluster formation (Brown & King 1964, Koch & King 1966, Miya *et al*. 1970, Lin & Spradling 1993). Although there are many morphological studies on the Lepidoptera oogenesis (King & Aggarwal 1965, Mandelbaum 1980, Yamauchi & Yoshitake 1984), the topological characterization of the different steps from the germ cell to the cluster formation into the germarium is only described for *Bombyx mori* (L.) (Miya *et al*. 1970).

Diatraea saccharalis (Fabricius) is one of the greatest pests for the sugar cane culture in many countries (Graça 1976); however, almost nothing is known about the reproductive system of this insect, except few works related to the morphological aspects of their ovariole (Santos & Gregório 2002). The knowledge of the sugar cane borer´s ovaries morphology is very important to found the basic researches related to the insect reproduction and to found the applied studies concerning the development of new mechanisms of biological control of this pest. This work aimed to characterize the germarium region of this insect by light and transmission electron microscopy, emphasizing the morphological steps of the ovarian cluster formation.

Material and Methods

The *D. saccharalis* larvae were reared on artificial diet (Hensley & Hammond 1968), and the pupae were kept in a recipient without food, until the emergence as adults. Both larvae and pupae were maintained in laboratory under controlled temperature (25-27°C) and humidity (70%). Ovaries removed from larvae (last larval instar - 30 days) were fixed for 24h in 2% glutaraldehyde - 4% paraformaldehyde solution buffered in 0.1M buffer phosphate (pH 7.3). For the light microscopy (LM) observations, the ovaries were dehydrated through a graded series of alcohol and embedded in historesin (JB4 – Polysciences); 5-µm sections were stained with hematoxylin-eosin and examined under a Zeiss Axiophot photomicroscope. For the transmission electron microscopy (TEM) observation, the ovaries were post-fixed in 1% osmium tetroxide for 2h, dehydrated through a graded series of acetone and embedded in Araldite; ultra-thin sections were double stained with uranyl acetate and lead citrate and examined under a Philips CM 100 transmission electron microscope.

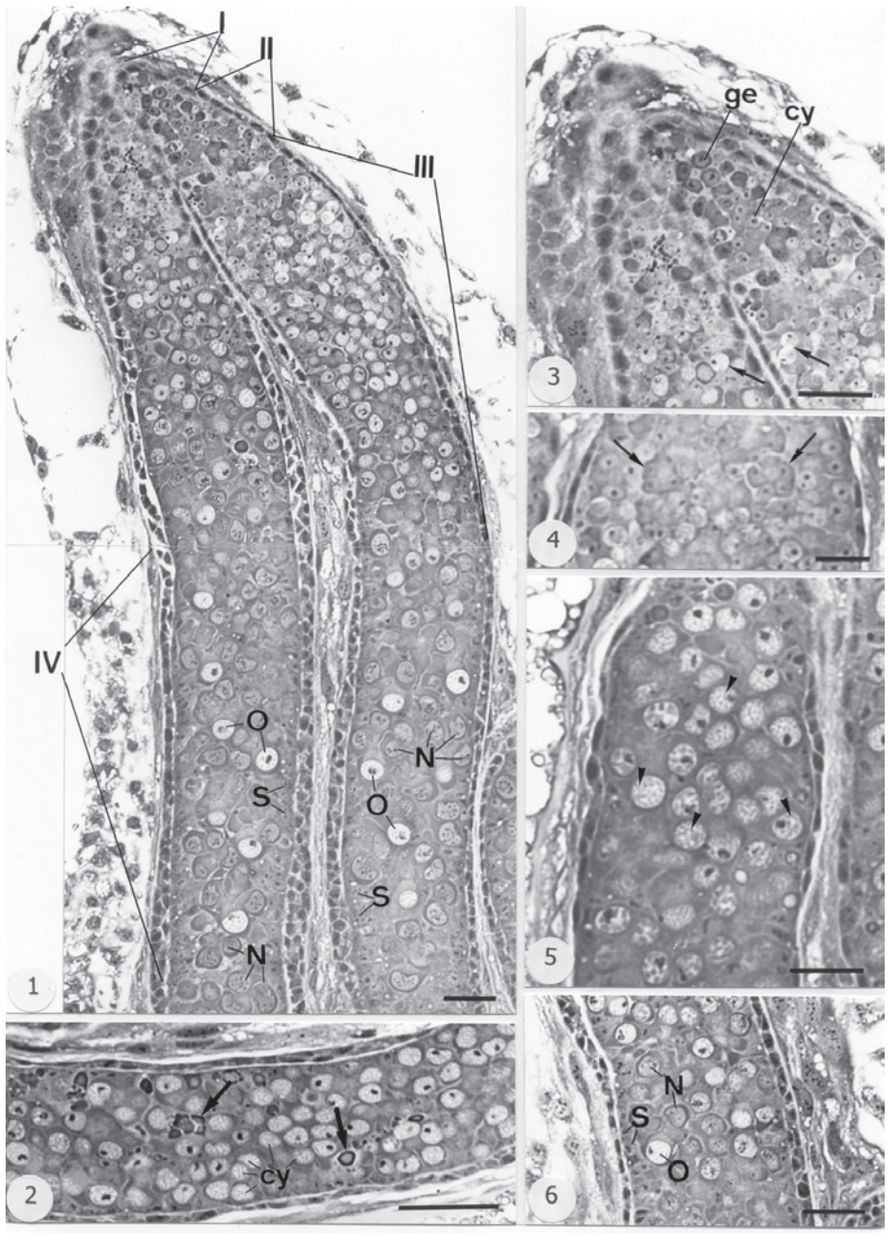
Results

In the *D. saccharalis* ovariole, the germ line cluster formation occurs in the germarium, where four regions are identified: Zones I – IV (Figs. 1, 25).

Zone I. It is the most apical end of the ovariole, where the germ line stem cells (oogonia) differentiate into cystoblasts (Fig. 25), but there is no clear morphological differentiation possible between them. They exhibit round and large nuclei, dense and reduced cytoplasm (Figs. 1, 3, 7, 9); the cytoplasm exhibits scattered mitochondria and discreet membranous cisterns (Fig. 9); we could not observe the spectrosome into these germ cells. A large and flat apical cell encloses the anterior tip of each ovariole (Figs. 7, 8, 9, 10), presenting abundant smooth endoplasmic reticulum, dense mitochondria, small and scattered Golgi complexes, and a great amount of microtubules (Figs. 7, 8, 10). The apical cell is in direct contact to the germ line stem cells (Fig. 9) and interstitial somatic cells are visualized among the germ cells exhibiting irregular nuclei and reduced cytoplasm with some heterogeneous vacuoles (Figs. 7, 8, 10).

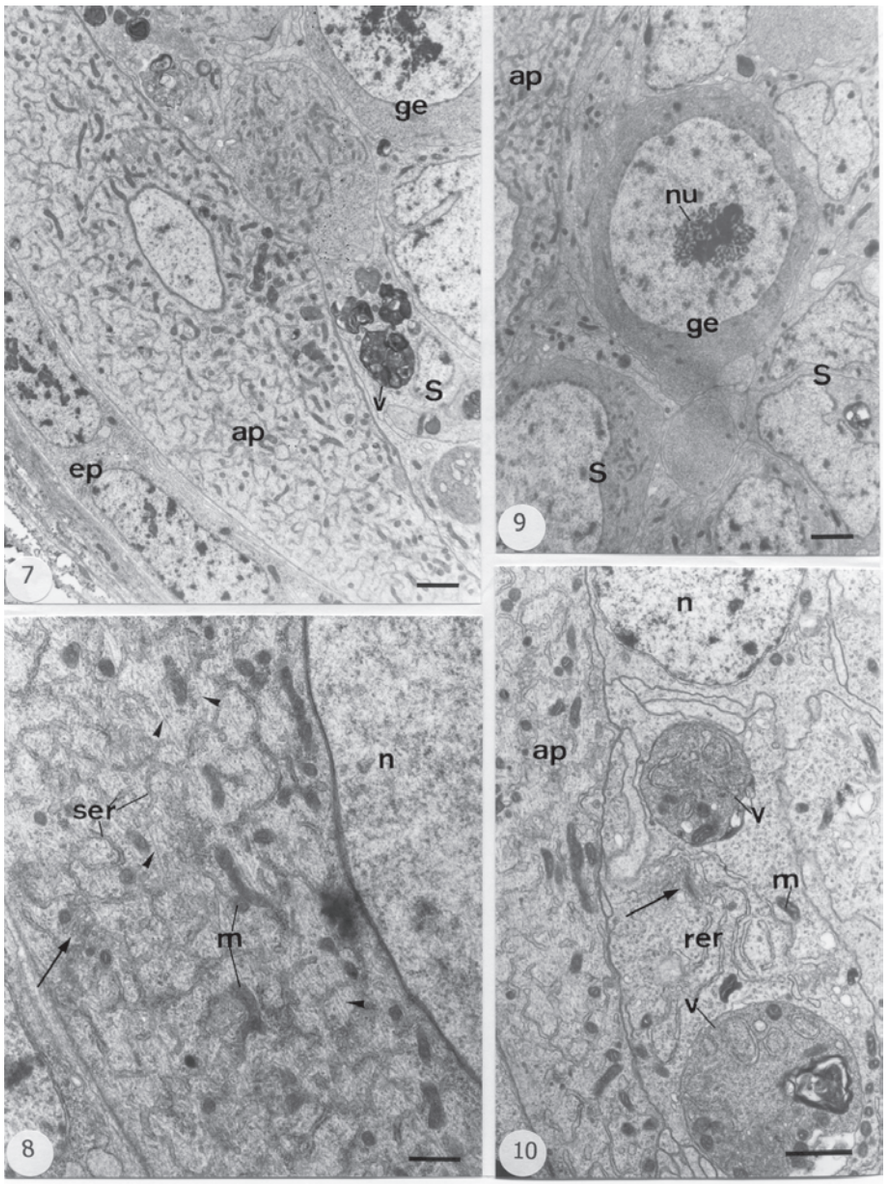
Zone II. In this region, each cystoblast produces a cluster of interconnected cystocytes by mitotic division (Figs. 1, 3, 4, 11, 13, 25). The cystocytes are always smaller than the cystoblasts (Figs. 1, 3, 25); their cytoplasm shows scarce organelles and the mitochondria are concentrated in the middle of the cluster, where the ring canals are located, next to the fusome material (Fig. 11). The newly formed ring canals exhibit mitotic spindle residues with a dense midbody structure (Fig. 12). In established ring canals the microtubules of mitotic spindle residues are replaced by fusome material, an intracellular branched membranous structure with high levels of cytoskeleton proteins, lined up by dense mitochondria and stretched through all intercellular bridges (Fig. 14). The canal rims shows a discreet layer of densely staining material at the cytoplasmic surface (Figs. 12, 13, 14). The interstitial somatic cells are detected among the germ ones and exhibit the same features observed in the Zone I.

Zone III. Now, the cluster genesis is complete; each one is composed by eight cystocytes (Fig. 25). It is the region



Figs. 1-6. Light microscopy of *D. saccharalis* ovarioles. Fig. 1. General aspect of two adjacent ovarioles (tangential section), showing the different Zones: I (I), II (II), III (III) and IV (IV). Oocyte (O); nurse cell (N); somatic cell (S). Bar = 100 µm. Fig. 2. Zone III showing meiotic cystocytes (cy) and dense bodies (arrows) among the cystocytes. Bar = 100 µm. Fig. 3. Apical region of the germarium: Zone I with germ cells (ge); Zone II with small cystocytes (cy); Zone III with meiotic cystocytes (arrows). Bar = 100 µm. Fig. 4. Zone II showing clusters (arrows) of cystocytes. Bar = 50 µm. Fig. 5. Zone III with meiotic cystocytes with synaptonemal complexes (arrow heads). Bar = 100 µm. Fig. 6. Zone IV exhibiting oocyte (O), nurse cells (N) and somatic ones (S). Bar = 100 µm.

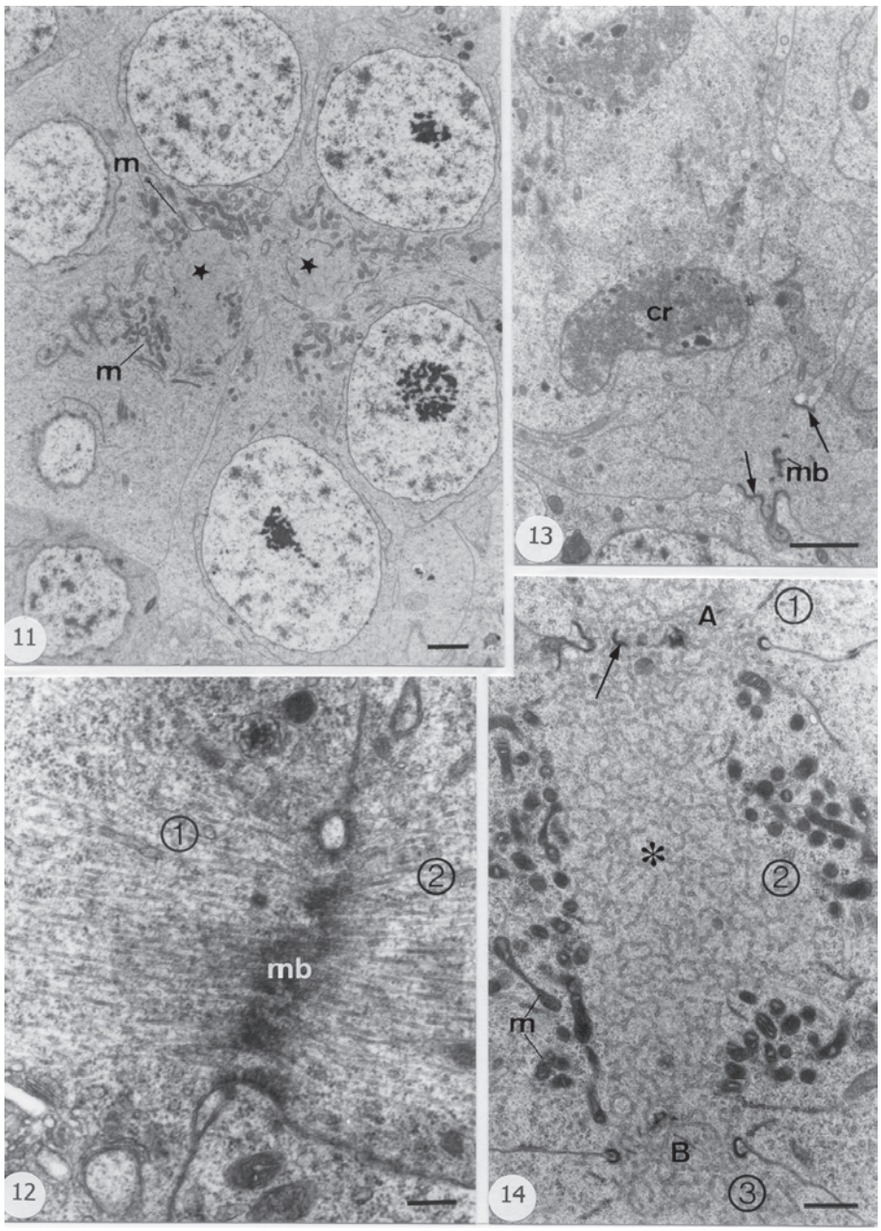
where the cystocytes are found in the prophase of meiosis. They show large and round nucleus with dense filamentous structures, recognized as synaptonemal complexes, and evident nucleolus; the cytoplasm is quite reduced (Figs.1, 2, 5, 15, 16, 17). Germ cells with morphological signs of death are observed in this zone; some of them show reduced and dense cytoplasm, and nucleus with flocculated and compacted chromatin (Fig. 16), whereas others are visualized as masses of apoptotic bodies (Fig. 18) among interstitial somatic cells. Round and irregular bodies are also detected in this zone by LM (Fig. 2); the TEM shows that they represent large cytoplasmic vacuoles with heterogeneous material and cellular fragments into the somatic cells cytoplasm in different degrees of



Figs. 7-10. Transmission electron microscopy of the Zone I in *D. saccharalis* germarium. Fig. 7. Apical cell (ap) with abundant smooth endoplasmic reticulum and dense mitochondria. Germ cell (ge), and interstitial somatic cell (S) with dense vacuoles (v). Epithelial sheath of ovariole (ep). Bar = 1 µm. Fig. 8. Detail of the apical cell with many microtubules (arrow heads), small and dense mitochondria (m), smooth endoplasmic reticulum (ser) and Golgi complex (arrow); nucleus (n). Bar = 0.5 µm. Fig. 9. Germ (ge) adjacent to the apical cell (ap). Germ cell nucleus with evident nucleolus (nu). Interstitial somatic cells (S). Bar = 1 µm. Fig. 10. Detail of the interstitial somatic cell with vacuoles (v) containing cellular fragments, mitochondria (m), rough endoplamic reticulum (rer), Golgi complex (arrow) and free ribosomes. Apical cell (ap); nucleus (n). Bar = 1 µm.

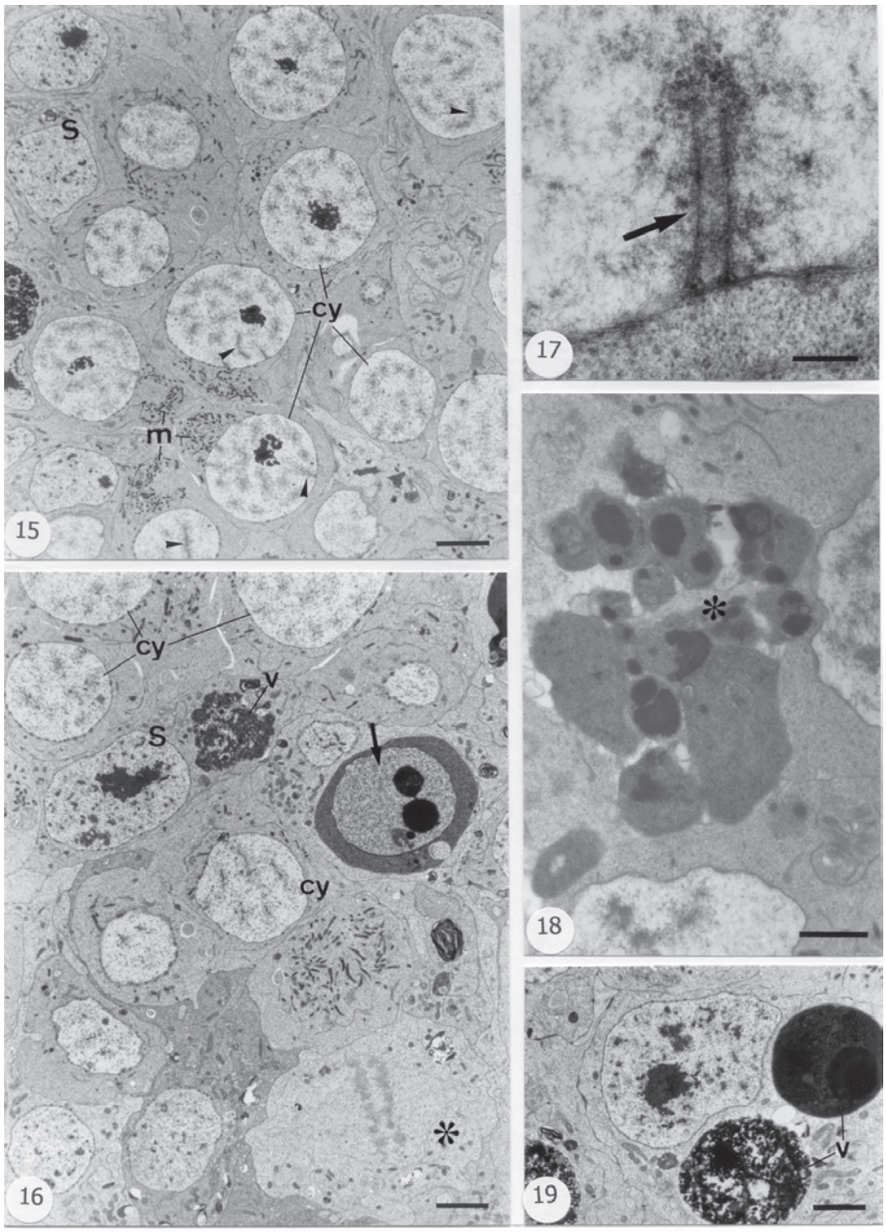
degeneration (Figs. 16, 19). Interstitial somatic cells are visualized in mitosis (Fig. 16).

Zone IV. The cystocytes differentiated in oocyte and nurse cells (Fig. 25). The oocyte is distinguished from the nurse cells by its nuclear morphology: the chromatin in oocyte nucleus is more uncondensed than in the nurse cell nucleus (Figs. 1, 6, 20); besides, small fragments of modified synaptonemal complexes and an evident nucleolus are only recognized in the oocyte nuclei (Figs. 20, 22). In both cell types the number of elongated mitochondria increases, still concentrated in the middle of the cluster, near to the ring canals (Figs. 20, 21). Into the intercellular bridges, the membranous elements of fusome are observed among a great amount of microtubules and dense granules of variable sizes running through the ring canals (Fig. 21). The ring canals expand in diameter and exhibit morphological changes in their rims, which become more convoluted and exhibit a



Figs. 11-14. Transmission electron microscopy of the Zone II in *D. saccharalis* germarium. Fig. 11. Section through an eight-cell cluster in which one we can see six cystocytes interconnected by ring canal filled by the fusome material (★). Mitochondria (m) concentrated in the center of the cyst, around the fusome. Bar = 1 µm. Fig. 12. Detail of newly formed ring canal connecting two cystocytes (1, 2) filled by microtubules and exhibiting the midbody (mb). Bar = 0.25 µm. Fig. 13. Telophasic stage of cystocyte division: chromosome (cr) partially surrounded by a double membrane structure. Ring canal with dense rim (arrow) and midbody (mb). Bar = 1 µm. Fig. 14. Part of a fusome between two ring canals (A and B), connecting three cystocytes (1, 2 and 3). The fusome is formed by smooth membranous structures (*) lined up by dense mitochondria (m); midbody fragments (arrow). Bar = 0.5 µm.

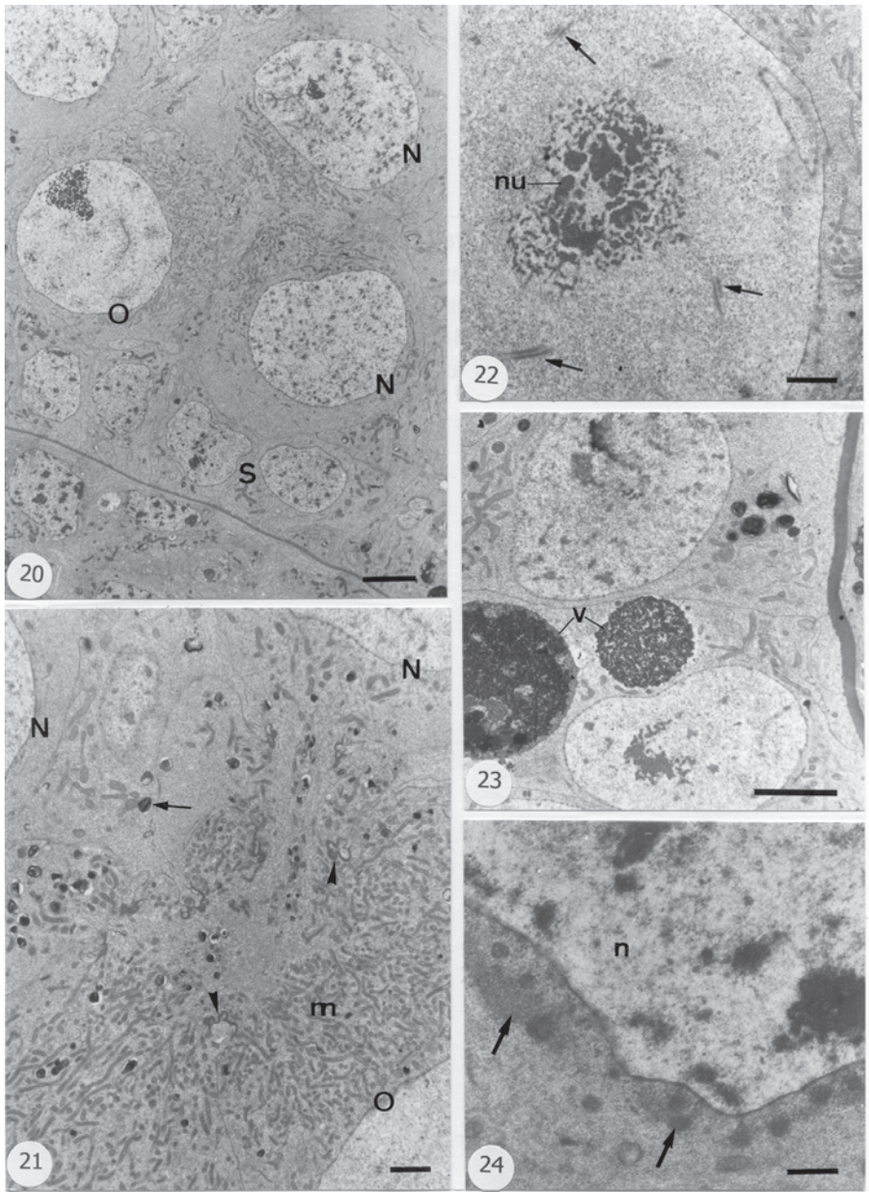
discreet increase of densely staining material at the cytoplasmic surface (Fig. 21). The nurse cells show cytoplasmic electron dense materials not surrounded by membrane concentrated around the nuclear envelop (Fig. 24), known as nuages. Some columnar somatic cells located at the ovariole periphery, termed pre-follicle cells "sitting" upon a proeminent basement membrane, the tunica propria (Figs. 20, 23), and seems to enclose each cluster located near the ovariole periphery (Fig. 20). These pre-follicle cells show small and oval nuclei, and the cytoplasm exhibits scattered mitochondria and large vacuoles with different size and content, interpreted as apoptotic bodies (Fig. 23). Few interstitial somatic cells are also observed among the clusters, which exhibit the same morphological features described in anterior zones.



Figs. 15-19. Transmission electron microscopy of the Zone III in *D. saccharalis* germarium. Fig. 15. General aspect of the meiotic cystocytes (cy) with lucent nucleus presenting synaptonemal complexes (arrow heads) and mitochondria (m) concentrated in one of the cell pole. Interstitial somatic cells (S). Bar = 3 µm. Fig. 16. Some of the cystocytes are visualized with reduced and dense cytoplasm, and nucleus with flocculated and compacted chromatin (arrow); normal cystocytes (cy). The interstitial somatic cells (S) show dense vacuoles (v) with heterogeneous material. Interstitial somatic cell in anaphase (*). Bar = 2 µm. Fig. 17. Detail of the cystocyte nucleus showing fragments of synaptonemal complex attached to the nuclear envelope (arrow). Bar = 0.4 µm. Fig. 18. Detail of mass of electron dense cellular fragments (*), interpreted as apoptotic bodies observed among the cystocytes. Bar = 1 µm. Fig. 19. Interstitial somatic cell containing cellular fragments into the heterogeneous vacuoles (v). Bar = 2 µm.

Discussion

The germarium of the *D. saccharalis* can be subdivided in four Zones, based on the morphological features of the cells during the ovarian cluster formation. There are few works establishing different regions in insect germarium, but there is no agreement neither in the number of the regions nor in their characteristics, even in the same insect. For instance, Brown & King (1964) characterize five distinct zones for *Drosophila melanogaster* (Meigen), while Koch & King (1966) describe three regions for the same insect. The morphological subdivision of the *D. saccharalis* germarium adopted by us was partially based on the results described by Miya *et al*. (1970) for *B. mori*.



Figs. 20-24. Transmission electron microscopy of the Zone IV in *D. saccharalis* germarium. Fig. 20. Pre-follicle cells (S) mainly located at the ovariole periphery. Oocyte (O) and nurse cells (N). Bar = 3 µm. Fig. 21. Detail of the central region of a cluster showing the ring canals interconnecting the oocyte (O) and the nurse cells (N). Mitochondria (m) and dense granules (arrow) are concentrated near and into the ring canal. Convoluted ring canals rims (arrow heads). Bar = 1 µm. Fig. 22. Detail of the oocyte nucleus showing modified fragments of synaptonemal complex (arrows); nucleolus (nu). Bar = 1 µm. Fig. 23. Pre-follicle cells at the ovariole periphery with vacuoles (v) containing degenerated cellular fragments. Bar = 0.25 µm. Fig. 24. Detail of nurse cell exhibiting perinuclear nuages (arrows); nucleus (n). Bar = 0.5 µm.

In *D. saccharalis*, clusters arise from germ line stem cells located at the anterior tip of each ovariole. These stem cells divide asymmetrically to produce a new stem cell and a cystoblast (Zone I), which then goes through synchronized rounds of mitosis to form a cluster of cystocytes (Zone II).

A large and flat apical cell encloses the anterior tip of each ovariole, the apical cell. This somatic cell was first described by Verson (1889) apud Buning (1994) in Lepidoptera testes. The role played by this somatic cell in the ovariole development is still controversial. The great amount of microtubules observed in the apical cell of the *D. saccharalis* germarium point out a sustentation function for the adjacent cells, as suggested by some authors (Miya *et al*. 1970, Büning 1994). According to Büning (1994), the apical cell is thought to serve as "docking cell" for the germarial stem cells, since the contact of the apical cell–stem cell is an important event for cystoblast determination. In *D. saccharalis* ovarioles we adopt the term cystoblast

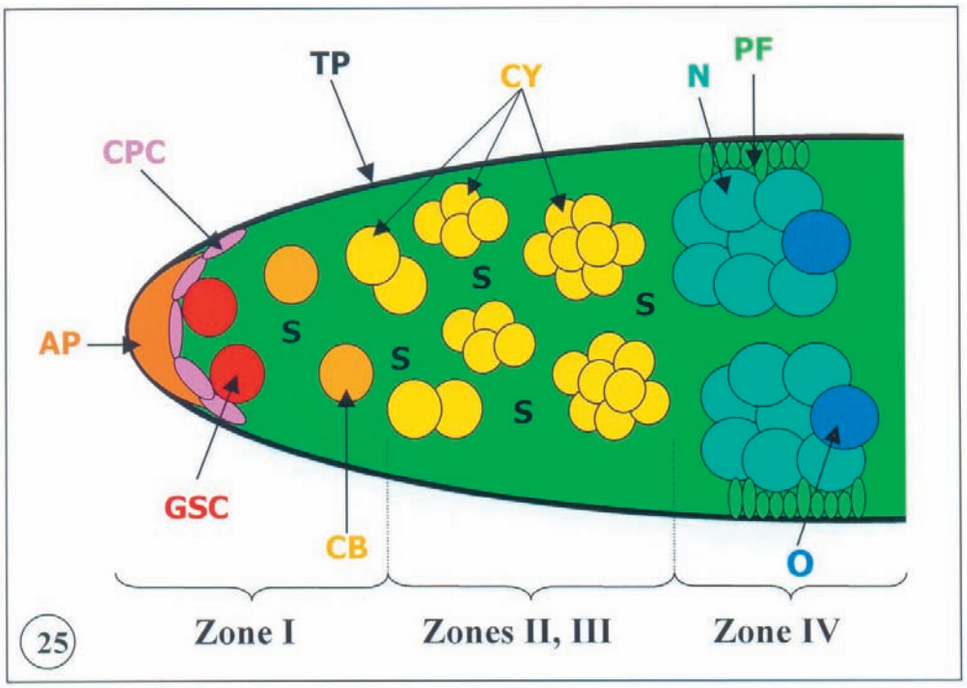


Fig. 25. Model of the *D. saccharalis* germarium in cross section indicating Zones I, II, III, IV and germline stem cells (GSC), apical cell (AP), cap cells (CPC), cystoblast (CB), cystocytes (CY), interstitial somatic cells (S), pre-follicle cells (PF), nurse cells (N), oocyte (O) and tunica propria (TP).

for germ cells which are clearly not in contact to the apical cell. Recently, it has been proposed that the somatic cells at the anterior tip of the *Drosophila* ovariole (termed cap cells) are organized into a niche that maintains and controls the germ line stem cells behavior (Xie & Spradling 2000).

Stem cell behavior is thought to be controlled by neighboring stromal cells that create special microenvironments known as stem cell "niches" (Xie & Spradling 2000). Stem cells in many different tissues and organisms may be regulated in a similar manner. In the *Drosophila* testis, five to seven stem cells are anchored on terminally differentiated somatic hub cells, suggesting that both the ovary and the testis could use similar strategies to regulate their stem cells (Fuller 1993). In *Caenorhabditis elegans* (Maupas), distal tip cells have been directly implicated in the maintenance of the germ line stem cell population (Kimble & Simpson 1997). In *D. saccharalis* ovariole, the germ line stem cells are enveloped by the apical cell. We believe that these cell act as *Drosophila* cap cells (Xie & Spradling 2000), maintaining and controlling the germ line stem cells behavior.

The cluster formation starts in the Zone II of the *D. saccharalis* germarium, as a result of the incomplete mitotic divisions of the cystoblasts. Clusters with variable number of interconnected sibling cells, the cystocytes are detected in this Zone. All the cystocytes of a cluster exhibit similar morphologic features, and are smaller than the cystoblast. The morphological characteristics of the *D. saccharalis* cystocytes are similar to the ones described for the other insects with polytrophic meroistic ovarioles (reviewed by Büning 1993, 1994, 1996, 1998), exhibiting mitochondria concentrated in the middle of the cluster, near the ring canals.

The ring canals are found between all the cells that divide and develop synchronically, being extensive cytoplasmic communications formed by incomplete cytokinesis; this structure has been studied extensively in insects cystocytes by TEM (King & Mills 1962, Carcupino *et al*. 1992, Robinson *et al*. 1994), and recently also by immunolabeling of their components (de Cuevas *et al*. 1996, Cooley 1998, Hudson & Cooley 2002).

In *D. saccharalis* the newly-formed ring canals present great amount of microtubules, remnants of the mitotic spindle, and a plate of densely staining material, the midbody, that has also been observed in other insects (Telfer 1975, Mandelbaum 1980, Büning 1994). In established ring canals of the sugarcane borer clusters, the microtubules and the midbody are replaced by the fusome material, a prominent cytoplasmic structure containing membranous elements and scarce amount of ribosomes, excluding further cytoplasmic organelles. The role played by the fusome in the cluster formation is discussed in several papers (Lin *et al*. 1994, McGrail & Hays 1997, Lilly *et al*. 2000), been postulated that the fusome helps to form ring canals and determine the pattern of nurse cell-oocyte differentiation (Lin *et al*. 1994).

According to Rasmussen (1976, 1977), in a cluster of polytrophic meroistic ovarioles, all the cyst cells enter simultaneously in meiotic prophase and progress as far as pachytene, when synaptonemal complexes are observed into their nucleus; this happens in the Zone III of *D. saccharalis* germarium, which all cystocytes enter meiotic prophase and

remain in this phase for a long time. Only the cell that is destined to be the oocyte progresses to the diplotene stage, while the other cells in the cluster lose their meiotic characteristics and become the nurse cells (Zone IV). This Lepidoptera result is different from the one observed by Carpenter (1975, 1979), which showed clearly, that in *Drosophila* wild-type only the two pro-oocytes of the 16 cell cluster remain in the prophase of meiosis for a prolonged time. According to de Cuevas *et al*. (1997) the maintenance of the meiotic cycle might represent the switch that signals a cystocyte to differentiate as an oocyte. In *D. saccharalis* the oocyte and the nurse cells are initially differentiated by the nuclear characteristics, while the cytoplasm features remain similar.

The nuages detected in the perinuclear region of *D. saccharalis* nurse cell were also described in *D. melanogaster* (Koch *et al*. 1967). This structure is abundant during animal oogenesis and spermatogenesis (Russel & Frank 1978, Gruzova & Batalova 1993, Quagio-Grasiotto & Lello 1995), being composed of RNA and proteins (Fuge 1976). The presence of nuages in the nurse cell cytoplasm at this initial point of germ cell differentiation emphasizes the role played by the nurse cells contributing euplasm to the oocyte.

The interstitial somatic cells are easily distinguished from the germ cells along the *D. saccharalis* germarium. They exhibit similar nuclear and cytoplasmic morphology throughout the four germarium zones presenting large and heterogeneously vacuoles with cellular fragments and/or digested materials. The detection of cellular fragments in the somatic cells in *Dermatobia hominis* (Linnaeus Jr.) germarium was interpreted as degenerated germ cells (Secco *et al*. 1992). Büning (1994) reports germ cell death in ovarioles of different insect species; the author suggested that during the time of insect reproduction, the stem cells undergo a limited number of mitotic divisions and the number of prospective oocytes becomes fixed. This number of prospective oocytes is much larger than the capacity of the species to raise all these cells to eggs. In our material, the observations of cells with morphological signs of programmed cell death, mainly in Zone III, may represent masses of apoptotic bodies resulting of the germ cell death, probably cystocytes; until now we could not determine in which step of the cluster formation the control by death occurs.

Finally, the presence of pre-follicle cells in the ovariole periphery, enclosing each cluster in the Zone IV, suggests that an important process occurs in this region of the *D. saccharalis* germarium: the ovarian follicle formation.

During the ovarian cluster formation in *D. saccharalis*, our results pointed out to the morphological evidences related with important control mechanisms for the new clusters/ follicles production (cap cell maintaining and possible controlling the germ line stem cells behavior) and for the cellular arrangement into the germarium, resulting from the programmed cell death (apoptosis) detected. We believe that the morphological characterization of ovarian cluster formation in *D. saccharalis* provided valuable information for the understanding of the initial steps of oogenesis in the sugarcane borer, besides the contribution for the knowledge of the cellular mechanisms related with the oocyte production and with the reproduction in insects.

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