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Electron microscope studies on meroistic ovarioles of *Spilostethus pandurus* (Hemiptera: Lygaeidae) and *Bactrocera zonata* (Diptera: Tephritidae)

Heba A S Elelimy
Department of Entomology, Faculty of Science, Cairo University, Giza, Egypt

Abstract

The ovarioles, which are cylindrical or tapering units, compose the majority of insect ovaries. Moreover, each ovariole comprises the terminal filament, the egg tube (germarium and vitellarium), and the pedicel. Additionally, a mature egg tube is divided into two parts: i) Germarium, which includes germ cells that are actively dividing, and ii) Vitellarium, the location where egg cells develop and reach their full size. Following the presence or lack of nutritive cells in the egg tube, the egg tube may be classified as panoistic or meroistic. Furthermore, the meroistic ovariole type is divided into the telotrophic and polytrophic types. Consequently, this study aims to observe differentiation between telotrophic and polytrophic types according to the position of nutritive cells in egg tubes using transmission electron microscopy.

Keywords: ovarioles; meroistic; telotrophic; polytrophic; transmission electron microscope

Introduction

During ovarian differentiation, viable insect oocytes are produced by close contact between the oocyte and several cell types. The roles of these auxiliary cells include organelle formation, transport of cytoplasmic materials, yolk precursors, control of oocyte development, and creation of egg envelopes. Meroistic ovaries are one of the insects' most complicated ways of contact (Gross, 1901; Telfer, 1975) [12, 42]. Gross (1903) [13] classified meroistic ovaries as either polytrophic or telotrophic (also known as acrotrophic) types. Thus, a thorough picture of the anatomy of meroistic ovaries is beneficial for comprehending insect reproduction, evolution and for many other fields focused on fundamental cellular and biochemical principles (Garel, 1982; Huebner, 1984) [14].

Each type of meroistic ovarioles was studied separately, and this study examined the ultrastructural arrangement of telotrophic and polytrophic ovarioles to show differences between them in their internal cell structure by using transmission electron microscopy.

Materials and Methods

Transmission Electron Microscope (TEM) Preparations

Detailed examination of female ovaries ultrastructure was carried out on specimens taken from the 5-day old adult female for *Hemiptera* insect and 25-day old adult female for *Diptera* insect.

The specimens of mature female *Hemiptera* insects were pinned to a Petri plate with paraffin via the pronotum. On both sides of the insect's body, a longitudinal incision was performed to remove the abdominal tergites, followed by removing the ovaries. The adult female *Diptera* was anesthetized with ether and dissected microscopically. Pins were inserted into the female adult's thorax, and a tiny forceps was used to remove the ovaries.

Adult female ovaries of *Hemiptera* and *Diptera* were dissected in a drop of prefixation solution (2.5 percent glutaraldehyde in 0.1 M phosphate buffer pH 7.3) and stored at 4°C for at least two hours before being washed overnight in 0.1 M phosphate buffer. Then, postfixed for 30 minutes at 4 degrees Celsius in phosphate-buffered, 1% osmium tetroxide, pH 7.3. After that, washing for one hour in a phosphate buffer with a pH of 7.3, followed by dehydration in a graded series of alcohol (50-90% for 15 min and 100% for 1 h) and pure acetone for one min. Following that, we performed infiltration in 2:1, 1:1, and 1:2 acetone:epon mixture for 2 h, overnight, and for 3 h, respectively, then in pure resin overnight to guarantee appropriate epon penetration. Then, following 20 hours of polymerization at 70 degrees Celsius in epon. Thin sections (60-90 nm) were cut with a glass knife using RMC, Inc. microtomy, collected on colloid grids. Ultra-thin sections were subjected to 15 minutes of uranyl acetate staining, followed by 20 minutes of lead citrate counterstaining (Reynolds, 1963) [34]. Using a Joel JEM-1200 EX II transmission electron microscope, sections were analyzed. Toluidine blue (1 percent) was used to stain semithin sections, which were then viewed using a Cambridge light microscope.

Results

Ultrastructure of telotrophic-meroistic type ovariole of *Spilostethus pandurus*

1. The tropharium region

The tropharium region is divided into two regions, the anterior region containing syncytial lobes with multiple trophocyte nuclei embedded in a common cytoplasm (Fig. 1a), and the posterior region containing early previtellogenic (arrested) oocytes, oocyte nucleus, small pre-follicular cells, and nutritive cord (Figs. 1b and 1c). As illustrated in Figure (1c), the trophic core and nutritive cord are devoid of cells. The trophic core extends peripherally into cytoplasmic lobes and connects proximally to arrested and maturing oocytes (Fig. 1b). The trophocyte nuclei have scattered chromatin and nucleoli with an uneven shape (Fig. 1a).

The posterior part of the tropharium is populated with small pre-follicular cells and arrested oocytes (Fig. 1a). Large and spherical oocyte nuclei (germinal vesicles) contain decondensed chromatin (Fig. 1b). In the posterior part of the tropharium, cytoplasmic bridges link the previtellogenic oocytes (Fig. 1d).

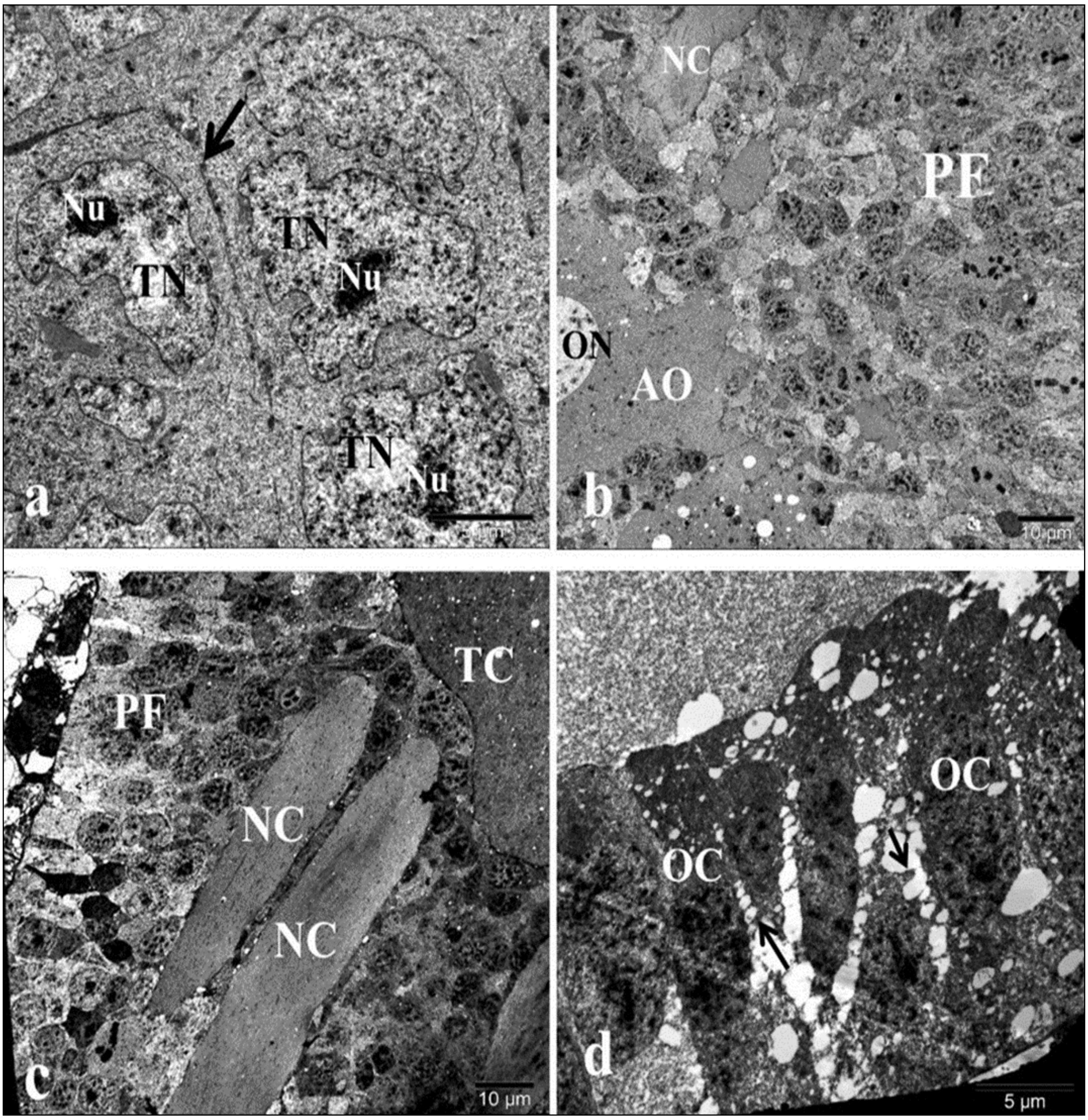

Fig 1: (a-d) Transmission electron micrographs of the telotrophic ovariole of *Spilostethus pandurus*. a) TEM micrograph in the anterior region of tropharium showing multiple trophocyte nuclei (TN) in a common cytoplasm, trophocyte nucleolus (Nu), and membrane of syncytial lobes (arrow). b) TEM micrograph showing nutritive cord (NC) is continuous with arrested oocyte (AO), oocyte nucleus (ON) also observed, and pre-follicular cells (PF). c) TEM micrograph showing trophic core (TC), nutritive cord (NC), and pre-follicular cells (PF). d) TEM micrograph showing previtellogenic oocytes (OC) connected by cytoplasmic bridges (arrows).

2. The vitellarium region

The vitellarium area contains oocytes at various phases of development, including previtellogenesis, vitellogenesis, and choriogenesis, which are grouped linearly. The synthesis of RNA reserve substances (during vitellogenesis) and egg envelopes (during choriogenesis) takes place in the vitellarium.

During previtellogenesis, developing oocytes are surrounded by a monolayer of columnar and mononucleated follicular cells (Fig. 2a). As seen in Figure (2b), follicular cells become binucleated at the onset of vitellogenesis. Each oocyte's oolemma begins to produce microvilli at the beginning of vitellogenesis (Fig. 2c). Each developing oocyte is separated from the next by an interfollicular plug (Fig. 2d), which most likely comprises trapped pre-follicular cells. The follicular cell in the vitellogenesis stage shows vacuoles and lysosomes, as shown in Figure (2c). The ovariole is surrounded by an epithelial sheath, scattered lumen cells with irregular shape nuclei, and a tunica propria, as shown in Figures. (2a and 2d).

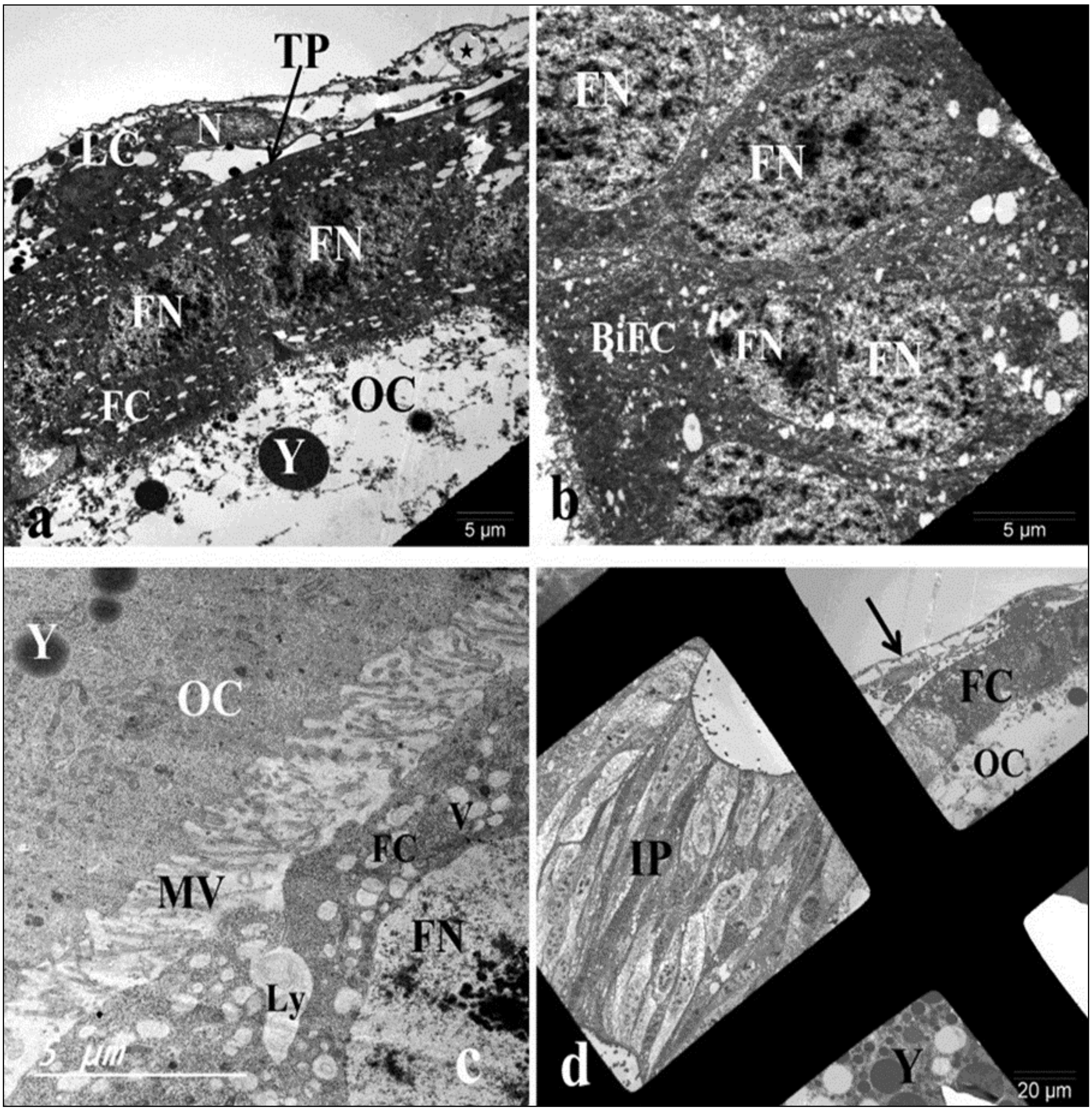

Fig 2: (a-d) Transmission electron micrographs of the telotrophic ovariole of *Spilostethus pandurus* showing developing oocytes in the vitellarium region. a) TEM micrograph of a fragment of the oocyte in early vitellogenesis showing yolk granule (Y), tunica propria (TP), and lumen cell (LC) with irregular shape nucleus (N) of the outer ovariole sheath and columnar follicular cells (FC) with one nucleus (FN). b) TEM micrograph of a fragment of the oocyte in the vitellogenesis stage showing binucleate follicular cell (BiFC) with two nuclei (FN). c) TEM micrograph of the developing oocyte in the vitellogenesis stage showing oocyte microvilli (MV), yolk granule (Y), and follicular cell (FC) contain nucleus (FN), vacuoles (V), and lysosomes (Ly). d) TEM micrograph showing the developing oocyte (OC) is separated from the next by an interfollicular plug (IP), which probably contains trapped pre-follicular cells, and the oocyte (OC) surrounded by one layer of follicular cells (FC), yolk granules (Y), and outer ovariole sheath (arrow).

Ultrastructure of polytrophic-meroistic type ovariole of *Bactrocera zonata*

The ovary of *Bactrocera zonata* consists of polytrophic ovarioles, each ovariole composed of egg chambers in a different stage of development, as shown in Figures (3b and 3c). Each egg chamber contains one oocyte with its nurse cells, and the egg chamber is surrounded by follicular cells epithelium (Figs. 3b and 3c). The ovariole consists of a distal germarium and a string vitellarium of ovarian chambers. In the germarium region, the follicular epithelial cells cannot be differentiated from oögonia, as shown in Figure (3a). The oögonia are very small, poor in the cytoplasm, and have spheroidal nuclei. The nucleus seems filled with a coarse granular substance (Fig. 3a). The cell boundaries are very tortuous and almost appear in contact, as shown in Figure (3a).

The oöcyte and the nurse cells in the vitellarium region differ in size according to the stage of development, as shown in Figures (3b and 3c). The egg chamber contains either a mature ovum in which the nurse cells completely disappeared or a nearly mature ovum in which the nurse cells are still attached to the developing egg's anterior portion (Figs. 3b, 3c, and 3f). These two kinds of egg chambers indicated the continuous process of vitellogenesis. The entire egg chamber is surrounded by follicular cell epithelium with a circular nucleus, as shown in Figures (3b, 3c, 3d, and 3e). The mature oocyte is surrounded by follicular cell epithelium with oval shape nuclei, as shown in Figure (3f). The nurse cell nucleus appears spherical, as shown in Figures (3b, 3c, 3d, and 3e).

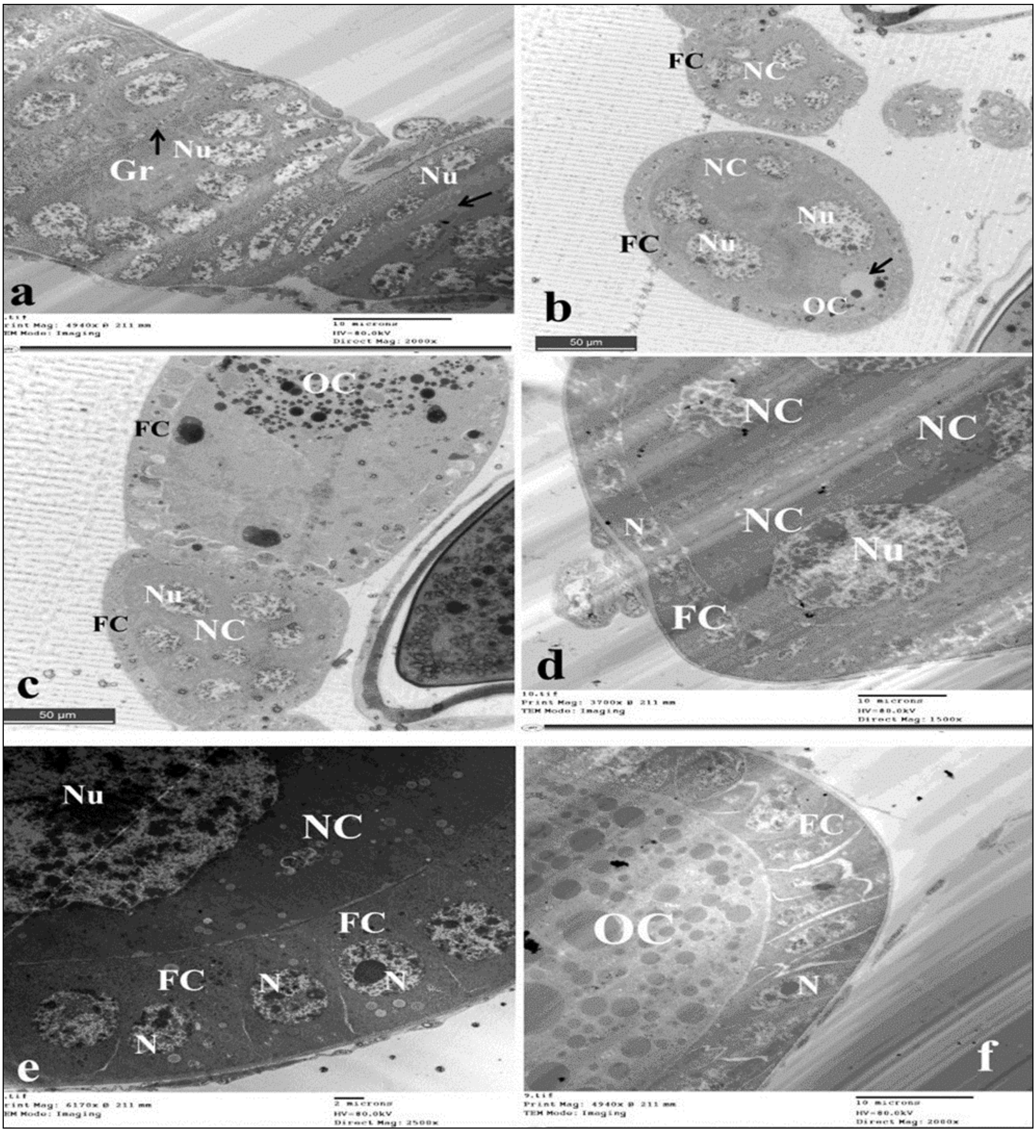

Fig 3: (a-f) Transmission electron micrographs of the polytrophic ovariole of *Bactrocera zonata*. a) TEM micrograph in the germarium region showing the cell boundaries (arrows), the small oögonia, poor in the cytoplasm, and spheroidal nuclei (Nu). b) and c) Semi-thin photograph of a well-developed egg chamber

showing oocyte (OC) and its nucleus (arrow), nurse cells (NC), nurse cell's nucleus (Nu), follicular cells epithelium (FC) surrounding the entire egg chamber. d) and e) TEM micrograph showing nurse cells (NC) and their nucleus (Nu) surrounded by follicular cell epithelium (FC) with circular nucleus (N). f) TEM micrograph of the mature oocyte (OC) surrounded by follicular cell epithelium (FC) with oval nucleus (N).

Discussion

Telotrophic-meroistic type ovariole of *Spilostethus pandurus*

The ovary of the *Spilostethus pandurus* is of telotrophic-meroistic type. The front portion of the ovariole is known as the germarium, whereas the remaining portion is known as the vitellarium. During the vitellogenesis phases, three to four oocytes are detected in the vitellarium area of *S. pandurus*. During the vitellogenesis phases, however, just one oocyte has been documented for *O. urticae*, *D. coccus*, and *E. grisea* (Vogelgesang & Szklarzewicz, 2001; Ramírez-Cruz *et al.,* 2008) [44, 33].

Extensive ultrastructure analyses of the ovaries of aphids (*Aphidinea*), scale insects (*Coccinea*), whiteflies (*Aleyrodinea*), psyllids (*Psyllinea*), cicadas and leafhoppers (*Cicadomorpha*), and true bugs (*Heteroptera*) have shown that the tropharia of these organs vary in structure (Büning 1985, 1994; Szklarzewicz *et al*., 2000) [5, 6]. Numerous investigations on the ovaries of hemipterans have shown that their tropharium structures vary (Szklarzewicz *et al*., 2007) [41]. In the tropharium, oocytes are generated by consecutive mitotic divisions of germinating cells and are surrounded by one layer of follicular epithelial cells (Bonhag, 1958; Büning, 1979) [3, 4].

In the present study, transmission electron micrographs of *S. pandurus* showed clearly that the structure of the cells of the tropharium is similar to other hemipterans. In *S. pandurus*, the tropharium is lined by a single layer of cells covered with the tunica propria. Also, tracheoles form part of this sheath. They do not penetrate it, and all the oxygen consumed by the ovariole uses diffuses to the ovariole from them (King and Aggarwal, 1965) [20].

The tropharia in most hemipteran insects are syncytial. This is characteristic of females of advanced *Heteroptera* (*Reduviidae*, *Aradidae*, *Coreidae*, *Pyrrhocoridae*, *Lygaeidae*, *Pentatomidae*) (Simiczyjew *et al*., 1998) [39]. Although the construction of the trophic chamber of hemipterans has been extensively studied (Huebner and Anderson, 1972a; Choi and Nagl, 1976; Lutz and Huebner, 1980, 1981) [16, 8, 24, 25] the origin of syncytial lobes remains obscure. Choi and Nagl (1976) [8] hypothesize that syncytialization of tropharia arises from the amitotic division of trophocyte nuclei, but Lutz and Huebner (1981) [25] assert that the fusing of trophocyte membranes forms cytoplasmic lobes. In the tropharium region of *S. pandurus* ovariole, multiple trophocyte nuclei are embedded in a common cytoplasm. All trophocytes are surrounded completely by a plasma membrane and are joined to a centrally located trophic core. The trophic core is connected to trophocytes, and the nutritive core is connected to oocytes (Teresa *et al*., 2000) [43]. The ovarioles of *S. pandurus* are covered by an epithelial sheath, scattered lumen cells, and a tunica propria. These three elements of the ovariole coating were also observed in many other insects (Miya *et al*., 1970b; Cruickshank, 1973) [29, 9].

The presence of arrested oocytes in the tropharium region of *S. pandurus* was observed as described for telotrophic ovaries in other insects (Büning, 1994; Michalik *et al.,* 2013) [6, 28]. In teleotrophic ovaries, this was never reported that the previtellogenic oocytes are connected by cytoplasmic bridges. However, Papacek and Soldan (2008) [31] showed the presence of cytoplasmic bridges in previtellogenic oocytes of *Aphelocheirus aestivalis* only in a transmission electron micrograph, without any comments. In *S. pandurus* tropharium region, cytoplasmic bridges in previtellogenic oocytes were observed.

Following the tropharium in the ovarioles is the vitellarium, where oocytes collect yolk via vitellogenesis, increasing in size until they are ready to be fertilized. In this area, oocyte and follicular cells participate in the synthesis (endogenous generation) and deposition (exogenous incorporation) of yolk in oocytes. In the oocytes of *S. pandurus*, lipid droplets and protein granules contained by membranes were detected. Protein transport into oocytes is selected by intercellular spaces of the follicular epithelium, indicating that these cells are involved in the uptake of proteins from the hemolymph (Anderson & Telfer, 1970) [1]. Caperucci and Camarago-Mathias (2006) reported the presence of viscous yolk due to the lysis of yolk granules.

During vitellogenesis, the developing oocytes of fulgoromorphans are surrounded by a single layer of binucleate follicular cells (Kaulenas, 1992; Büning, 1994). There have been no reports of binucleate follicular cells in non-heteropteran hemipterans, although they are prevalent in heteropterans (Huebner and Anderson, 1972b; Simiczyjew, 1999) [17, 38]. It is hypothesized that the existence of binucleate follicular cells is associated with creating egg envelope precursors. In most insects, follicular cells have a single polyploid nucleus enclosed by a folded nuclear envelope.

During vitellogenesis, the follicular cells surrounding the expanding oocytes in the telotrophic ovarioles of *Rhodnius prolixus* play a crucial role as they actively synthesize and integrate macromolecules into the oocyte (Huebner & Anderson, 1972; Raikhel & Dhadialla, 1992) [15, 32].

The dense appearance of microvilli suggests an active involvement of these cells during vitellogenesis. The synthesized proteins are transported via the microvilli. Also, pinocytosis at the oocyte surface suggests the transfer of macromolecules from follicle cells and hemolymph into oocytes. Pinocytosis in insect oocytes was previously reported by several authors (Kamal *et al.*, 2005) [18].

Like hemipteran insects, oocytes grow in the vitellaria close to follicular cells. During oogenesis and vitellogenesis, mitosis of pre-follicular cells experienced a series of morphological and physiological modifications, which resulted in the formation of follicular cells (Caperucci and Camarago-Mathias, 2006) [7].

Natalie *et al.* (2006) [30] reported the presence of an interfollicular plug that separates the oocytes. The plug may contain trapped pre-follicular cells. Ultrastructural examination of developing oocytes of *S. pandurus* ovariole showed the presence of an interfollicular plug.

Polytrophic-Meroistic Type Ovariole of *Bactrocera zonata*

Like all other *Diptera*, the ovariole of female *Bactrocera zonata* is of polytrophic-meroistic type (Gross, 1903) [3]. Germarium refers to the front portion of the ovariole, which contains germline stem cells. One germ cell becomes an oocyte, while the remaining 15 become nurse cells. The stem cells of follicle cells give birth to follicle cells (Margolis, 1995) [26]. The follicle cells create an egg chamber by forming a basic epithelium surrounding the cyst (Roth *et al.*, 1995; Ruohola *et al.*, 1991) [35, 36]. The freshly created egg chamber is distinguished from the germarium by differentiating 5-8 stalk cells. Due to the ingestion of yolk protein generated in the follicle cells and fat, the oocyte develops fundamentally (Koch & King, 1966; King *et al.*, 1968). The majority of follicular cells transform into columnar epithelium around the oocyte. The follicle cells cover the oocyte with the vitelline membrane and eggshell. When oocyte maturation is complete, nurse and follicle cells undergo apoptosis (Cooley *et al.*, 1992) [10].

In *Drosophila melanogaster*, the follicular epithelium is generated in the germarium's posteriormost zone, where pre-follicular cells encircle eggs and associated nurse cells (Mariusz *et al.*, 2008) [27]. The resultant egg chambers move from the germarium to the vitellarium. The homogeneous follicular epithelium is then formed shortly afterward by cuboidal cells. Two groupings of up to five cells, known as polar cells, are designated at the poles of the ovarian follicle at the beginning of this procedure (Besse and Pret, 2003) [2]. During the last stages of oogenesis, the subpopulations of follicular cells are responsible for eggshell production (Wu *et al.*, 2008) [4].

Each nurse cell nucleus of *Bactrocera zonata* contains chromatin material dissociated and randomly fills the nuclear volume, a sign indicating the completion of the endomitotic process (Lorz, 1947; Shehata *et al.*, 2011) [23, 37]. Shehata *et al.* (2011) [37] observed that the nurse-cell nucleus located in the oöplasm is spherical, and its size differs according to the developmental stage of the egg chamber. The internal structure of the *Bactrocera zonata* ovary is similar to that of the other species in this genus (Shehata *et al.*, 2011) [37].

Conclusion

From our results, it can be concluded that the ultrastructure of telotrophic-meroistic type ovariole differs from polytrophic-meroistic type ovariole. The telotrophic-meroistic ovariole type consists of two main regions, the tropharium, and vitellarium. The tropharium region is divided into the anterior and posterior regions. The anterior region is occupied by trophocytes or nurse cells, and the posterior region is occupied by small pre-follicular cells and arrested oocytes. The vitellarium region of the telotrophic type houses linearly arranged oocytes at different stages of development.

The polytrophic-meroistic ovariole type consists of two main regions: germarium and vitellarium. The germarium region houses the oogonia and the follicular epithelial cells. The vitellarium region is occupied by the oocyte and nurse cells. Each oocyte possesses its nurse cells to form an egg chamber surrounded by follicular cell epithelium.

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