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Histological and ultrastructural studies on oogenesis of the freshwater bivalve *Caelatura* (*Horusia*) *parreyssi* (Philippi, 1848)

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ABSTRACT

The present study was carried out to describe the ovary and oogenesis in the freshwater bivalve *Caelatura parreyssi* from histological and ultrastructural points of view. Ten adult healthy clams collected from the River Nile were used in this study. The specimens were dissected and gonads were obtained and processed for histological and ultrastructural examination. Histologically, the ovary of *C. parreyssi* is composed of numerous of follicles which are connected together by connective tissue. Each follicle contains different stages of developing oocytes; oogonia, previtellogenic oocytes, vitellogenic oocytes and postvitellogenic oocytes (mature oocytes). At ultrastructural level, stages of female gametogenesis were examined; the process of oocytes formation and yolk deposition were described in successive stages of oogenesis. The structure and function of auxiliary cells and follicle cells in ovarian follicles during vitellogenesis were discussed. In conclusion, this study showed that the follicle cells play an important role during oogenesis but probably are not the major source of yolk precursors. The vesicular connective tissue is probably the main source of nutrients for vitellogenesis. The auxiliary cells appear to play an integrate role in the development of the oocytes as their functions can permit a transfer of precursors necessary to vitelogenesis.

INTRODUCTION

Caelatura parreyssi is a common freshwater mussel of Egypt that belongs to family Unionidae. It is widely distributed in the bottom of the River Nile (Ibrahim *et al*., 1999; Bogan, 2004; Lopes-Lima *et al.* 2017). It forms a vital source of feed for various animals and the nacreous shell is used for different industrial use (Yeemin, 1997). These mussels are major filter feeders and serve to extract large amounts of fine particulate organic materials, heavy metals and large organic molecules, thus having a direct impact on water quality (Soto and Mena, 1999). These animals are eaten in Asia as a supplemental protein source, but apparently not in Egypt.

Most of the researchers studied the reproduction and other aspects of the biology on marine species of bivalves, especially those of commercial values such as *Ruditapes philippinarum* (Drummond *et al*., 2006), *Ostrea edulis* (da silva *et al.*, 2009); *Ensis magnus* (Hernandez- Otero *et al.*, 2014). On the other hand, limited studies of ovarian morphology and oogenesis had been conducted on freshwater bivalves.

The most detailed description of the reproductive biology of the freshwater bivalves, histologyical descriptions of the ovary and oogenesis were conducted only at the light microscopic level (Grand *et al.*, 2001; Chatchavalvanich *et al.*, 2006; Hulya *et al*., 2009; Lima *et al*., 2012; Hinzmann *et al.*, 2013; Rybalkina *et al.*,2013; Wu *et al*., 2017).

Comprehensive ultrastructural studies of oogenesis had been reported for few species of marine bivalves, such as *Atrina pectinata* (Fang and Qt, 1988); *Pecten maximus* (Dorange and Le Pennec, 1989); *Pinna nobilis* (Gaulejac *et al.*, 1995) and *Crassostrea virginica* (Eckelbarger and Davis, 1996).

In the same context, few previous ultrastructural studies had been documented on the freshwater bivalves or unionids found in Egypt. Therefore, the present investigation was designed to study the histological and fine structural characteristics of different stages of oogenesis in *C. parreyssi* to provide valuable basic information in the development of the germ cells.

MATERIAL AND METHODS

Sexually mature freshwater bivalves *C. parreyssi* were collected from Ayatt canal, Giza governorate in March, 2017. They were preserved in a container with some water from the canal, then transported to the laboratory at Biological Department, Faculty of Education, Ain Shams University.

For light microscopy, specimens were dissected and pieces of the ovary were rapidly fixed in aqueous Bouin's solution for 24 hours. After that, the fixed tissues were dehydrated, cleared, embedded in paraffin and sectioned at 5µm (Bancroft and Gamble, 2002). Then, the sections were stained with hematoxylin-eosin, microscopically examined and photomicrographs were made as required.

For transmission electron microscopic preparation, very small pieces of the ovary ($1mm^3$) were immediately fixed in cold mixture of 4% formalin and 1% gluteraldehyde and then washed in phosphate buffer (pH 7.3) for 24 h. The fixed tissues were transferred into phosphate buffer; two changes for one hour, and post-fixed in 1% osmium tetroxide for 2-4 hours and then washed in the same buffer. The specimens were dehydrated in ascending grades of ethanol, cleared in propylene oxide and embedded in resin (Dykstra *et al*., 2002). Ultrathin sections (80 nm) were cut and mounted on copper grids. The sections were stained with uranyl acetate and lead citrate, examined and photographed with a JEOL-JEM-1400 EX- electron microscope at Regional center for Mycology and Biotechnology (RCMB), AL Azhar University.

RESULTS

The ovary of *C. parreyssi* consists of a creamy soft tissue of irregular shape located within the visceral mass and occupies two-third of the visceral area. The ovarian tissue lies adjacent to the dark brown digestive gland surrounding the coiled digestive tract.

Histological study

Light microscopic examination of the ovary showed that, it composed of a large number of ovarian follicles which connected together by a connective tissue (Fig. 1). Each follicle contains different stages of oogenesis; oogonia, previtellogenic oocytes, vitellogenic oocytes and postvitellogenic oocytes (mature oocytes). The oogonia embedded in the follicle wall and they were found in groups. They appeared rounded to oval in shape with spherical nuclei having a prominent nucleolus and their cytoplasm are stained light bule with hematoxylin-eosin (Fig. 2). The volume of the nucleus exceeds the volume of the cytoplasm as clearly observed in Fig. (2).

The previtellogenic oocytes appeared rounded, cuboidal, clyndrical or hemispherical in shape. They bulge from the follicle wall, and the largest ones being attached to the follicle wall by short, broad stalk. The nuclei had spherical shape and being relatively large occupying a central position. It had dispersed chromatin with one prominent nucleolus. Both cytoplasm and nucleus stained deep blue with hematoxylin-eosin (Figs. 2, 3). The vitellogemic oocytes were elongated and protruded into the center of the follicles and connected to the follicle wall by stalk. The stalk became thinner and the oocytes increased in size due to yolk accumulation. Bundles of fibrillar material were seen in the stalk and in the cytoplasm close to the stalk (Fig. 2). The spherical nucleus appeared and migrated to the distal or free end of the cell. Then, nucleus enlarged and stained less heavily as the chromatin dispersed. The nucleoli continued to grow and in some oocytes were seen to be eosinophilic or basophilic. The cytoplasm showed acidophilic yolk vesicles at the periphery of the nucleus. They were surrounded by a vitelline layer which was connected to the stalk at the base of the cell (Fig. 2). The postvitellogenic oocytes (mature oocytes) lost contact with the follicle wall and became free in their lumens (Figs. 2, 3). They were rounded and more regular in outline than the attached oocytes. The nucleus appeared spherical and prominent.

Ultrastructural study

Electron microscopical examination of the ovary of *C. parreyssi* revealed that the germ cells are distributed along the inner wall of each acinus in all stages of differentiation, ranging from oogonia to late-stage vitellogenic oocytes. Few follicle cells were observed in association with oocytes at all stages. Early oocytes were completely or partially surrounded by follicle cells (Fig. 4). These cells appeared with variable sizes but tend to be squamous with prominent, oblong nuclei containing scattered clumps of heterochromatin as clearly observed in Fig. (4). Four stages of oogenesis were observed in every ovarian acinus including oogonia, previtellogenic oocytes, vitellogenic oocytes and postvitellogenic oocytes (mature oocytes). The oogonia appeared in layers, being one or two cells thick along the edge of the follicle in contact or separated from the edge by pseudopodia-like projections of auxiliary cells. They observed in close contact by means of desmosomes. They have large nucleus with a prominent nucleolus. Their cytoplasm characteristically contained parallel stacks of rough endoplasmic reticulum, scattered mitochondria, Golgi complexes and glycogen-like granules (Figs. 4, 5).

The previtellogenic oocytes were distinguished by ooplasm initially devoided of organelles, except a few lipid droplets. The late stage of previtellogenic oocyte growed longer and appeared nuclear and cytoplasmic modifications. The nucleus migrated to an apical position with its double-membranes, nuclear envelope possessed numerous pores and its nucleolus displayed an acentric position. The mitochondria appeared numerous and scattered in the cytoplasm (Fig. 6). In the vitellogenic oocytes, the nucleus lost their round shape and their nuclear envelope became

undulating besides its nucleolus formed a nuclear ring that appeared in close contact with the nuclear envelope (Fig. 7). Moreover, dense accumulations were seen near the inner membrane of the nuclear envelope. Tiny microvilli were clearly seen (Figs. 7, 9). The cytoplasm displayed curls of the rough endoplasmic reticulum, abundant mitochondria and lipid globules (Figs. 8, 9). Two types of yolk granules dominate the ooplasm of vitellogenic oocytes were obviously observed including spherical electron- dense, membrane-bound granules and slightly larger-spherical lipid droplets (Figs. 8, 9). In the postvitellogenic oocytes (mature oocyte), the nuclear envelope appeared twisted and the nucleolus occupied a marginal position and in close contact with the nuclear envelope which has abundant pores. The two types of yolk granules and lipid globules were abundantly seen in the cytoplasm. The microvilli observed along the oolemma (Fig. 10). A fibrous intermicrovillar matrix arises simultaneously with the appearance of the microvilli, forming a uniform egg envelope.

The Auxiliary cells were present throughout oogenesis. They were observed united by desmosomes. These cells had an irregular shape and anchored to the walls of the follicles by numerous digitate processes of the cell and developed pseudopodia-like projections between germ cells (Fig. 11). No junctions between auxiliary cells and oogonia were observed. During the vitellogenic stage, the oocyte appeared completaly surrounded by several layers of auxiliary cells. On formation of the vitelline coat around the oocyte, the auxiliary cells being restricted to the stalk region and contacts maintained by desmosomes. During the postvitellogenic oocyte, the oocyte appeared free in the lumen of the follicles without contact with auxiliary cells. The nucleus of the auxiliary cell was often marginal or eccentric in position. Annulate lamellae were present along the nuclear envelope (Fig. 11). The cytoplasm possessed numerous ovoid mitochondria, cisternae of rough endoplasmic reticulum, myelinic figures and voluminous autophagic vacuoles (Fig.11).

The ovarian follicles were supported and surrounded by a matrix of large plemorphic somatic cells termed "vesicular connective tissue". These cells appeared bag-like, with an irregularly shaped nucleus containing a single nucleolus and cytoplasm filled with glycogen, few mitochondria and peripherally positioned lipid droplets. The outer wall of the follicles was defined by a discontinuous layer of thin squamous myoepithelial cells that formed a partial barrier between developing oocytes and the hemocoel (Fig. 4)

DISCUSSION

Fershwater bivalves in Egypt represent a neglected animal group and little is known about them, perhaps due to the fact that have no apparent economic or medical importance. But, on the other hand, they represent an important items to the bottom-feeder fishes in the ecosystem, and an important trophic level for environmental stability. The present study showed that the ovary of *C. parreyssi* formed of many follicles; each of them contained all the different stages of oocyte development throughout the year, suggesting that oocytes are continually produced with no reproductive seasons. The present results are in parallel with the previous microscopic observations on different species of freshwater bivalves including *Dreissena polymorpha* (Juhel *et al*., 2003); *Hyriopsis bialatus* (Chatchavalvanich *et al*., 2006); *Corbicula japonica* (Rybalkina *et al*., 2013) and *Sinohyriopisis schlegelii* (Wu *et al*., 2017).

The current investigation showed that, the oogenesis of *C. parreyssi* had four stages represented as oogonia, previtellogenic oocytes, vitellogenic oocytes and

postvitellogenic oocytes (mature oocytes). The general process of oogenesis is similar to that of other bivalves such as *Anodonta gabillotia pseudodopsis* (Sereflisan *et al*., 2009), *Anodonta cygnea* (Lima *et al*., 2012) and *Sinohyriopisis schlegelii* (Wu *et al*., 2017).

The present study indicated that, the oogonia are distributed along the inner wall of female follicles, previtellogenic oocytes attached by a stalk to the follicular wall, the stalk became thinner and longer in vitellogenic oocytes. Eventually, mature oocytes became free in the lumen of ovarian follicles. These results agree with the results reported by Grand *et al*. (2001), Henley (2002), Juhel *et al*. (2003) and da Silva *et al*. (2009) in their studies of freshwater and marine bivalves.

On the other hand, the ultrastructural features for the general process of oogenesis in *C. parreyssi* showed that, oogonia are maintained in close contact by desmosomes, which permits synchronous development. This result concides with the views of Gaulejac *et al*. (1995) and Eckelbarger & Davis (1996). The previtellogenic stage is characterized by nuclear and cytoplasmic modifications suggesting major synthetic activity. The high density of pore complexes in the nuclear envelope indicated an intensive transport activity. These observations accord with Dorange and Le Pennec (1989). The growth of cytoplasm during the vitellogenic stage is large due to the accumulation of organelles and inclusions. Various cytoplasmic constituents have been proposed as being involved in the formation of yolk granules as well as lipid droplets of various sizes. These were documented by Pal and Hodgson (2002), Amor *et al*. (2004) and Roy *et al.* (2016).

In the present study, two main types of yolk granules were morphologically distinguished. The same results were observed by Jong-Brink, *et al.* (1976); Gaulejac *et al*. (1995), Eckelbarger & Davis (1996) and Roy *et al.* (2016) in other molluscs. The endogenous yolk formation predominates during the vitellogenic stage with proliferation of ooplasmic organelles. Worles of stacks of rough endoplasmic reticulum were observed in the cytoplasm of the vitellogenic oocytes being surrounding the yolk granules. Similar investigations have been reported by Popham (1975), Pipe (1987) and Gaulejac *et al*. (1995) in other bivalves like *Bankia australis*, *Mytilus edulis* and *Pinna nobilis*. The present observations revealed that parts of the cytoplasm are often encircled by curls of rough endoplasmic reticulum. This result run paralled with those obtained by Reverberi (1966) who suggested that the substances are used for the construction of yolk granules filter gradually through the different order of lamellar rings and thereafter concentrate in the central area. The mitochondria became abundant in the vitellogenic oocytes of *C. parreyssi* where the mitochondria are produced essentially during the previtellogenic stage. This is due to that the respiratory activity is higher in early oocytes than in the later stages as described for *Biomphalaria glabrata*(Jong-Brink *et al*., 1976) and *Pinna nobilis* (Gaulejac *et al*., 1995). The multiplication of mitochondria is a common phenomenon in the oocytes of the bivalves.

Microvilli appeared along the membrane of vitellogenic oocytes of *C. parreyssi*, the microvilli were unbranched and their tipe project slightly above the egg envelope surface into the follicular cavity. This is also in accordance with the results obtained by Beams and Sekhon (1966), Jong-Brink *et al*. (1976), Gaulejac *et al*. (1995) and Eckelbarger & Davis (1996) in other moluscs, *Anodonta*; *Biomphalaria glabrata*; *Pinna nobilis* and *Crassostrea virginica* consequntly.

At the ultrastructural level, the results of the present study showed, the presence of several layers of auxiliary cells surrounding the oocyte only during the early stages of development. On formation of microvilli around the oocyte, the auxiliary cells are

restricted to the stalk region and desmosome-like gap junctions were present; interdigitations between oocytes and auxiliary cells were observed, as in *Lymnaea stagnalis* (Rigby, 1979) and *Pinna nobilis* (Gaulejac *et al*., 1995). This associations suggest that the auxiliary cells play an integral role in the development of the oocyte. Desmosomes between auxiliary cells were observed in *C. parreyssi* as in *Trachydermon cinereus* (Durfort, 1980). On the other hand, morphologically these cells were similar to the vesiculous cells which described by Medhioub and Lubet (1988) at the beginning of gametogenesis in *Ruditapes philippinarum* which stock both glycogen and lipids. These observations incline us to propose different functions for the auxiliary cells during oogenesis. It might play an important role in oocyte nutrition and partially vitelline envelope formation due to the presence of extensive rough endoplasmic reticulum cisternae. The same functions were suggested by Griffond and Gomot (1979), Gaulejac *et al*. (1995), Eckelbarger & Davis (1996) and Rybalkina *et al.* (2013) in different species of mollusks, such as *Viviparus viviparous, Pinna nobilis, Crassostrea virginica* and *Corbicula japonica.*

The vesicular connective cells of *C. parreyssi* resemble those in *Mytilus edulis* (Eckelbarger and Davis, 1996).

The present investigation revealed the presence of follicle cells surround previtellgenic and early vitellogenic of *C. parreyssi*. These cells were described in *Crassostrea virginica* by Eckelbarger and Davis, (1996). The follicle cells appear nearly universally in invertebrate gonads and they are playing some role in oocyte nutrition (Wourms, 1987; Eckelbarger and Davis, 1996).

In conclusion, As far as we know, the present study probably is the first to describe the ultrastructural features of vesicular connective cells in freshwater bivalves in Egypt. The present observations indicated that, the auxiliary cells play an important role in oocyte nutrition and vitelline envelope formation, the vesicular connective cells serve as nutrient storage function and the follicle cells play some important role during oogenesis.

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Explanation of Figures

Figs. 1-3: Photomicrographs of ovary sections of *C. parreyssi* stained with Hx & E.

Fig. 1: Showing the ovarian follicles (OF) connected together by a vesicular connective tissue (VCT), Digestive tubules (DT) are clearly seen.

Fig. 2: Showing the ovarian follicles contain deferent stages of oogenesis; oogonia (Og) previtellogenic oocy (PVO) vitellogenic and late stage of vitellogenic oocyte (VO), Fibrilles (F) and mature oocyte (MO).

Fig. 3: Showing early stage of previtellogenic oocyte (PVO) and mature oocyte (MO).

Figs. 4-11: Electron micrographs of the ovary of *C. parreyssi*.

Fig. 4: Showing myoepithelial cells (My) form a partial barrier between developing oocyte, early oocytc (EO), follicle cell (FC).

Fig. 5: Showing an oogonium possessing spherical nucleus, mitochondria (M) and rough endoplasmic reticulum (ERE), as well as nucleus (N) having a nuclear envelope (NE), nucleolus (Nu), heterochromatin (Hc) and euchromatin (Ec).

Fig. 6: Showing late-stage previtellogenic oocyte, illustrating the nucleus (N) which is polylobed in apical position, scattered mitochondria (M) and voluminous lipid droplets (LD).

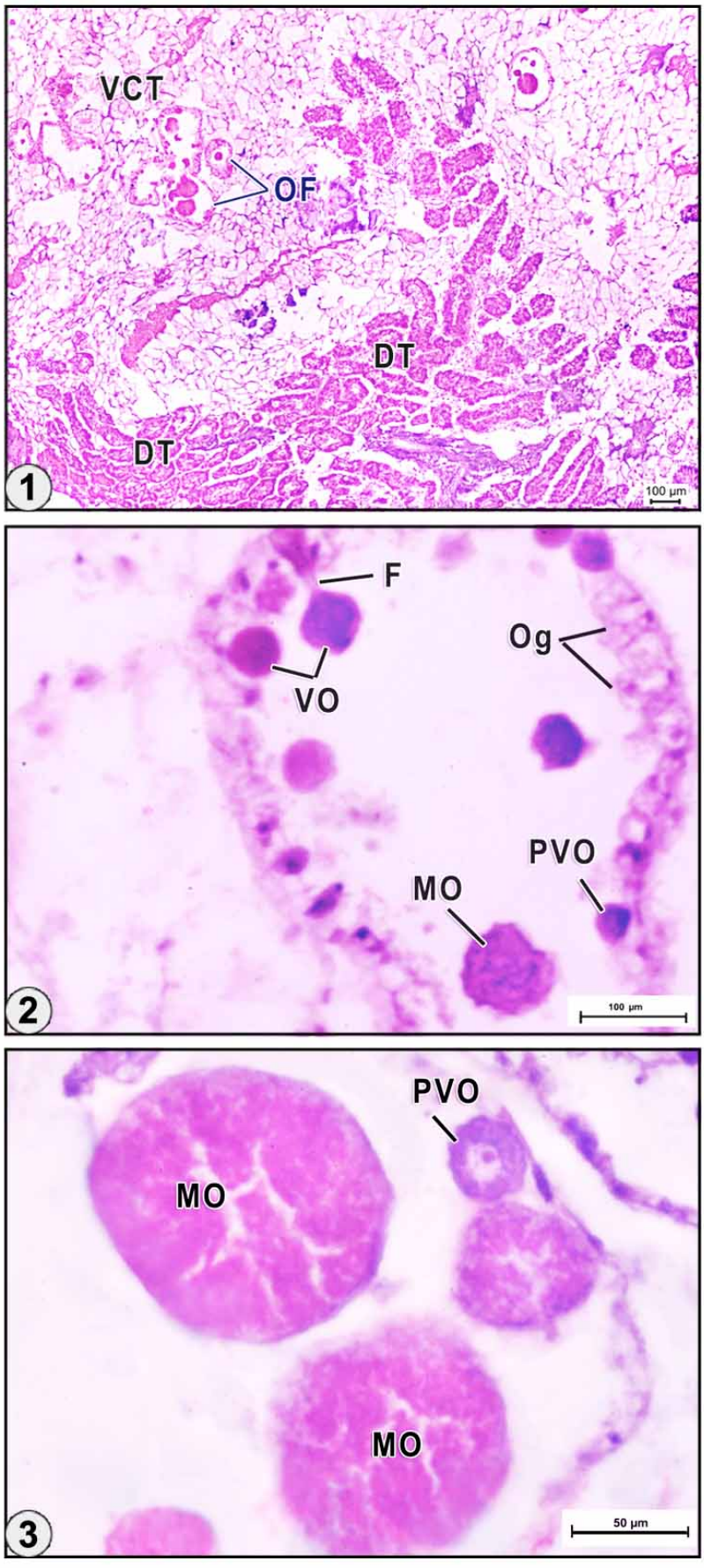
Fig. 7: Showing vitellogenic oocyte possessing a nucleus (N) with irregular nuclear envelope (NE), dense heterochromation (Hc) accumulated on the inner membrane of nuclear envelope and spherical nucleolus (Nu) appear in contact with the nuclear envelope. Tiny microvilli (Mv) is obviously seen.

Fig. 8: Showing vitellogenic oocyte having a ring of rough endoplasmic reticulum (RER) surrounding a yolk granule (YG) which can distinguished into two main types; proteinaceous yolk granules (PYG) and lipid proteinaceous granules (LPG).

Fig. 9: Showing higher magnification of late stage of vitellogenic oocyte revaling apical microvilli (Mv) and ooplasm filled with both lipid droplets (LD) and electron dense yolk granules (YG).

Fig. 10: Showing mature oocyte showing acpical microvilli (Mv), lipid droplets (LD) and two types of yolk granules (PYG & LPG).

Fig. 11: Showing the nucleus (N) of the auxiliary cells (AC) which is marginal and polylobed. Annulate lamellae are present along the nuclear envelope ( ).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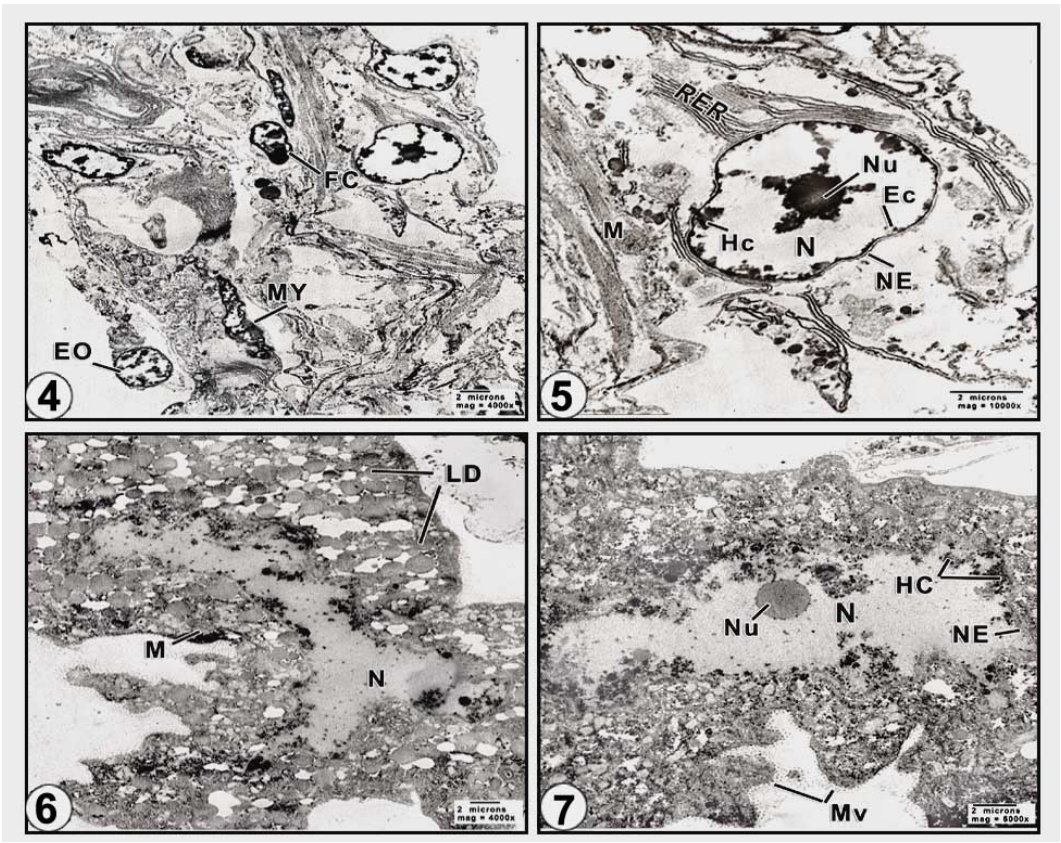
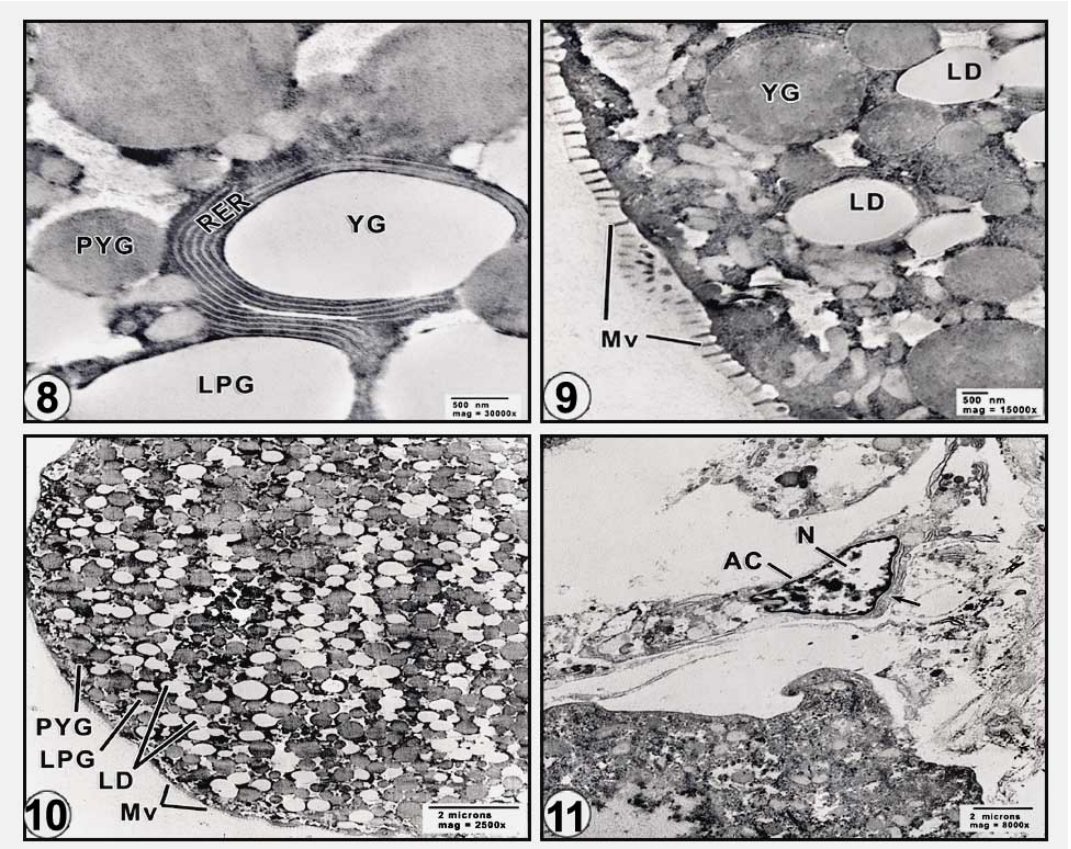
3
50 µm


FC
MY
EO
4
RER
Nu
Ec
M
Hc
N
NE
5
LD
M
N
6
HC
Nu
N
NE
Mv
7


PYG
RER
YG
LPG
8
YG
LD
LD
Mv
9
PYG
LPG
LD
Mv
10
N
AC
11

ARABIC SUMMARY

دراسات هستولوجية وتركيبية دقيقة على مراحل تكوين البويضات فى محار الماء العذب

Caelatura (*Horusia*) *parreyssi* (Philippi, 1848)

إلهام رافت عواد و أميمة محمد مصطفى
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تهدف الدراسة الحالية لوصف التركيب الهستولوجى والتركيب الدقيق للمبيض ومراحل تكوين البويضات فى محار الماء العذب *Caelatura parreyssi*. تم جمع 10 عينات من نهر النيل وتشريحها للحصول على المبيض وتم فحصها من الناحية الهستولوجية والتركيبية الدقيقة. يتكون المبيض هستولوجيا من حويصلات عديدة مرتبطة معاً بنسيج ضام . وجد أن كل حويصلة تحتوى على المراحل المختلفة من الخلايا البيضية النامية : أمهات البيض وبويضات قبل المرحلة المحية، والبويضات المحية، والبويضات بعد المحية (البويضات الناضجة). واشتملت الدراسة الحالية على وصف التركيب الدقيق لهذه المراحل وتم وصف عملية تكوين الخلايا البيضية وتكوين المح فى المراحل المتتالية. ونوقشت بنية ووظيفة الخلايا المساعدة والخلايا الحويصلية. واظهرت الدراسة أن الخلايا الحويصلية لها دور مهم فى عملية تكوين البويضات ولكن ربما ليست المصدر الرئيسى لمخازن المح. والنسيج الضام الحويصلى ربما هو المسئول ع ن المغذيات أثناء مراحل تكوين المح. وتقوم الخلايا المساعدة بدور مكمل فى المراحل التكوينية لنمو البويضات حيث أنها تنقل المخزنات الضرورية اللازمة لتكوين المح.