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Note

Ultrastructural changes in the oocytes and hepatocytes associated with the maturation of gonads in the protogynous spinycheek grouper *Epinephelus diacanthus* (Valenciennes)

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ABSTRACT

Ultrastructural changes in the oocytes and hepatocytes in the female *Epinephelus diacanthus* were studied with the progress of maturation. Transmission electron microscopic (TEM) observations revealed cytological changes associated with ovarian development. Nucleolus number increased in the perinucleolus stage, which is an indirect indication of increase in protein synthesis with the onset of oogenesis. Zonation of yolk sphere and presence of microvilli in the zona radiata were observed in mature oocytes. In comparison to the immature phase, mature/ripe satge hepatocytes showed greater development of both endoplasmic reticulum and increased density of mitochondria in the cytoplasm which is an evidence of progress in vitellogenin synthesis.

Keywords : Gonad maturation, Hepatocyte, Nucleolus, Oocyte, Ultrastructure, Zonation of yolk

Reproduction in most animals undergoes cyclic rhythms and the patterns of these changes in the gonads are characteristic of each species. One of the convenient methods of elucidating the reproductive cycle including the spawning period of a fish is to study the seasonal developmental stages of the gonads through macroscopic and microscopic observations. Macroscopic examination alone has its limitations, which may be useful for gonochoristic fishes for identification of gonadal stages, where as in case of hermaphroditic fishes, microscopic observation is the only alternative. In macroscopic observation, actual developmental stages of growing oocyte may not be discernible. Though histological studies give details of changes associated with maturation of the ovary, this will not give clear picture about intercellular and intracellular changes. The cellular and dynamic aspects of vitellogenesis and oocyte growth can only be known by ultrastructural examination. Ultrastructural studies of ovary and liver by transmission electron microscopy (TEM) will give a better description of cytological and nuclear processes such as yolk accumulation and formation of yolk nucleus, egg membranes, lipid droplets as well as cortical alveoli.

In understanding breeding related morpho-functional changes of the fish, liver is the first organ to be considered for study. Hepatocytes play a major role in the production of both yolk precursors and egg shell components, namely the vitellogenin and the zona radiata proteins (Arukwe and Goksoyr, 2003). The histomorphology of liver in teleosts varies considerably with sex and sexual activity (Ishii and Yamamoto, 1970, Aida *et al.,* 1973, Welsch and Storch, 1973, Yamamoto and Egami, 1974, Varghese, 1976, Vander Gaag *et al.,* 1977, Peute *et al.,* 1978, Olivereau and Olivereau, 1979, Van Bohemen *et al.,* 1981, Nunomura *et al.,* 1984, Eurell and Haensly, 1982, Avila, 1986, Leatherland and Sonstegard, 1988, Ribeiro *et al*., 2006).

In India the major limitation with development of grouper aquaculture is the lack of seed availability. To develop hatchery technology, generation of information on biological and physiological changes associated with the maturation of gonads is very much essential. In view of this, ultrastructural examination of oocytes and hepatocytes were carried out in order to understand the changes associated with the maturation of gonads in the spinycheek grouper, *Epinephelus diacanthus*.

Live specimens of *Epinephelus diacanthus* were collected onboard Fishery Survey of India (FSI) vessel during the cruises off Quilon region (lat. 8º 55'N; long. 76º 30' E) and off Ratnagiri region (lat. 15º 42' N; long. 73º 16' E) at 50 m depth. The maturity stages were assigned after histological observations following the method described by Moe (1969). The tissue processing and sectioning were done as per Dawes (1988) with slight modifications.

Ovary and liver tissues were subjected to ultrastructural studies by transmission electron microscopy (TEM). For TEM analysis, ovary and liver tissues were collected from live fishes at different stages of maturaty. Fishes were anaesthetised onboard the vessel, tissues (ovary and liver) were excised and cut into one mm cubes size and immediately fixed in 3% buffered gluteraldehyde solution for 12 h at 4 °C. Subsequently the tissue samples were washed with buffer (0.1M sodium cacodylate; pH 7.3) and then transferred to fresh cacodylate buffer and kept until the vessel landed. The fixed tissues were brought to the laboratory, washed three times (30 min each) in 0.1M sodium cacodylate buffer and kept overnight. Tissues were

then post-fixed in 1% osmium tetroxide, dehydrated in acetone series, infiltrated in Spurr's resin (Spurr, 1969) and blocks were prepared. From the polymerised blocks, ultrathin sections (60 – 90 nm) were cut, double stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshally, 1965), mounted on grids and the images observed and photographed in a Hitachi-H-600 Transmission Electron Microscope.

In the primary stage oogonia, nucleus is large and oval in shape (Fig. 1). Nucleoplasm appears electron dense, containing small clumps of chromatin, which was found more near the nuclear envelope. Oogonia have nucleus with a distinct envelope and cytoplasm has polar distribution of cell organelles. In *E. diacanthus,* primary stage oogonia showed presence of mitochondria associated with cement and nuages. High nucleus to cell ratio was observed. The oogonial cytoplasm contains mitochondria, free ribosomes and scant endoplasmic reticulum. Golgi complex was not distinctly seen. Few granulocytes observed in the periphery of oogonial cytoplasm. The electron dense nuages were observed scattered in the cytoplasm.

In chromatin nucleolus stage (stage I – Immature) (Fig. 2) roughly spherical, large and eccentrically located nucleus is well developed and occupies greater part of the cell. The nuclear envelope is highly wavy or undulating in nature. Ooplasm occupies major part of the oocyte after nucleus. This is strongly basophilic and electron dense. Ribosomes are numerous and densely packed in the cytoplasm. Mitochondrial aggregations are conspicuously arranged near the nuclear envelope. Few concentric profiles of endoplasmic reticulum observed near the mitochondria and nuages are scarce.

In peri-nucleolus stage (Fig. 3), the nucleus increases in size and the nucleoli increase in number. The nuclear envelope is some what irregular in outline and runs rather smoothly, occasionally ruptured by nuclear pores. The cytoplasm is increasingly dense and still basophilic and homogenous in appearance.

In maturing ovary (Fig. 4) , the oocytes have dense aggregation of mitochondria near the zona radiata. The cytoplasmic organelles observed are smooth endoplasmic reticulum and free ribosomes spreading in cytoplasm. Thin zona radiata is present. Few granulose cells are observed near the zona radiata. Basal lamina and thecal cells are also observed

In vitellogenic oocytes (Mature) (Fig. 5), mitochondrial aggregation in the cytoplasm is observed. In the early vitellogenic stage, oocytes with lipid droplets were observed. Dense rough endoplasmic reticulum and enlarged mitochondria with tubular cristae are noticed in the cytoplasm (Fig. 6). Microvilli are seen in the thick zona radiata (Fig. 7). Basal lamina well developed and occupied the middle of granulosa layer and thecal layer (Fig. 8). Yolk globules have occupied the most part of the cytoplasm (Fig. 9). Yolk globules have showed zonation of electron dense inner layer and lighter outer layer (Fig. 9).

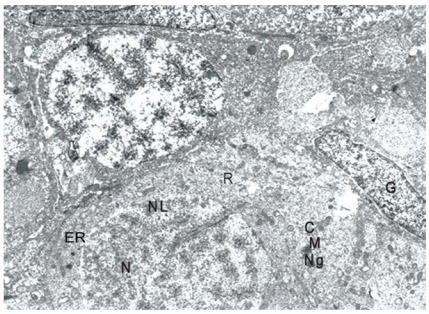

Fig. 1. Electron micrograph of oogonia showing nucleus (N) with distance envelope, nucleolus (NL), cytoplasm with mitochondria (M), cement (C), nuage (Ng), granulocyts (G), ribosomes (R) and endoplasmic reticulum (ER) (X5000)

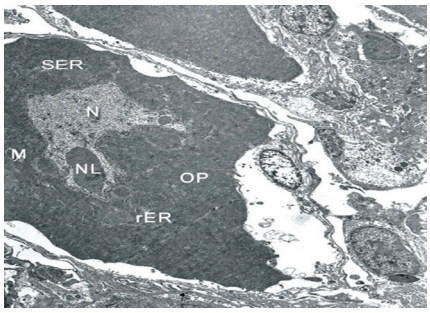

Fig. 2. Electron micrograph of chromatin nucleolar oocyte OP : Electron dense ooplasm, N: Nucleus, NL: nucleolus, M: mitochondria, rER: rough endoplasmic reticulum (rER), SER: smooth endoplasmic reticulum (X3500)

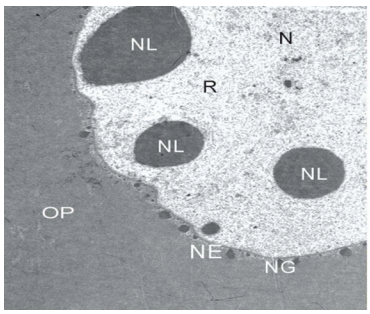

Fig. 3. Electron micrograph of perinucleolar oocyte with electron dense ooplasm (OP), nucleus (N), nucleolus (NL), ribosomes (R), nuages (NG) and nuclear envelope (NE) (X3500)

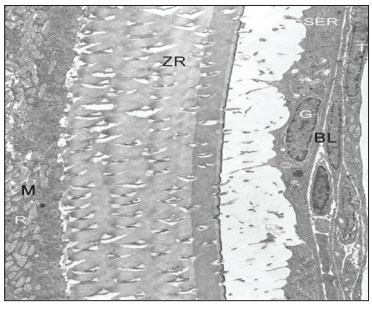

Fig. 4. Electron micrograph of maturing ovary with developing thin zona radiata (ZR), mitochondria (M), basal lamina (BL), granulocyte (G), thecal cell (T), ribosomes (R), smooth endoplasmic reticulum (SER) (5000X)

Ultrastructure of hepatocytes in immature fishes (Fig. 10 and 11) revealed presence of large, round, centrally situated nucleus with a prominent nucleolus. Scattered rough endoplasmic reticulum is observed around the nucleus. Oval shaped mitochondria are present. Dense lipid droplets occupied most of the cytoplasmic area. Dispersed glycogen granules were also observed.

In fishes with maturing ovary, electron dense cytoplasm is seen in the hepatocytes. Lipid droplets are scarce in the cytoplasm compared to immature female hepatocytes. Glycogen granules are scattered in the cytoplasm (Fig.12).

In ripe females hepatocytes are having dense rough endoplasmic reticulum with flat cisternae. Cytoplasm contains dense electron regions with few smooth endoplasmic reticulum. In the ripe female hepatocytes enlarged mitochondria are observed (Fig. 13, 14, and.15).

Oogenesis is the preparation for embryogenesis and it is characterised by the progressive accumulation of reserve materials used later in embryonic development. The storage of ribosomes during oogenesis is sufficient to ensure organogenesis and even cell differentiation. It is also clear that early germ cells have been studied almost exclusively in freshwater teleosts mostly by light microscopy and only few accounts are available on marine species (Brusle and Brusle, 1978). Brusle and co-workers have established the cytological criteria for the identification of early germ cells by electron microscopic studies in *Mugil auratus*

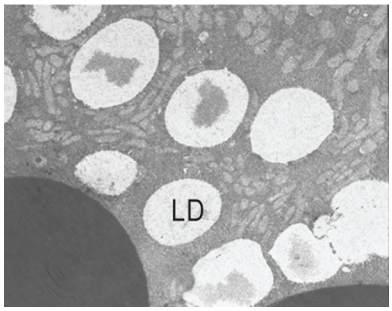

Fig. 5. Electron micrograph of early vitellogenic oocytes with lipid droplets (LD) (X5000)

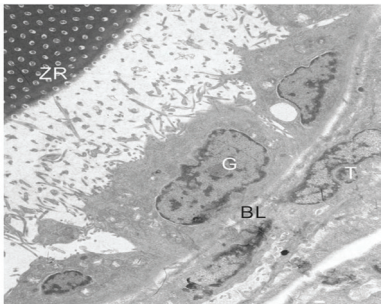

Fig. 8. Electron micrograph of vitellogenic oocytes with well developed basal lamina. ZR : zona radiata, BL : basal lamina, G : granulocyte, T : thecal cell (X4000)

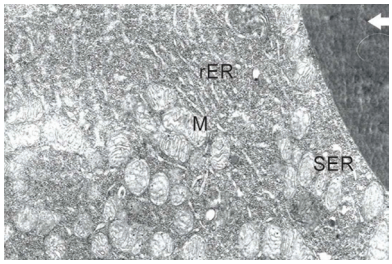

Fig. 6. Electron micrograph of late vitellogenic oocytes with dense and enlarged mitochondria (M), dense rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (SER) (X12000)

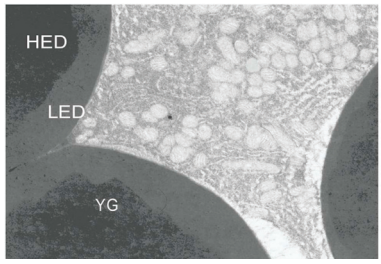

Fig. 9. Ultrastructure of protein yolk globule (YG) showing transitional yolk spheres. Note the central high electron dense (HED) layer surrounded by low electron dense (LED) fluid layer (X6000)

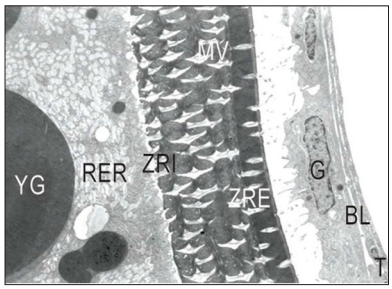

Fig. 7. Electron micrograph of vitellogenic oocytes with fully differentiated zona radiata (ZR), zona radiata interna (ZRI), zona radiata externa (ZRE), microvilli (MV), yolk globules (YG), granulocytes (G), thecal cell (T), basel lamina (BL) (3500X)

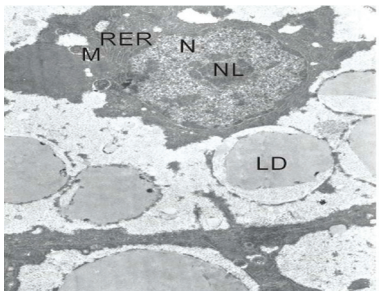

Fig. 10. Hepatocyte of immature female *E. diacanthus*, N : nucleus (N), NL: nucleolus, rER : rough endoplasmic reticulum, M: mitochondria (X8000)

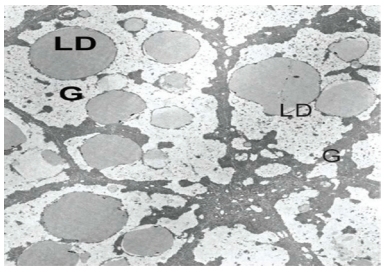

Fig. 11. Hepatocyte of immature female *E. diacanthus* with lipid droplets (LD) and dispersed glycogen granules (G) (X4000)

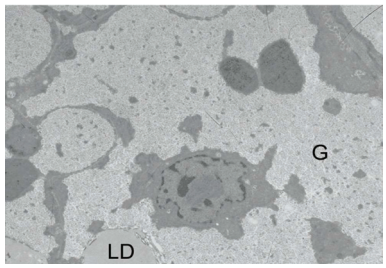

Fig. 12. Hepatocyte of maturing female *E. diacanthus* with electron dense cytoplasm and scattered glycogen granules (X5000)

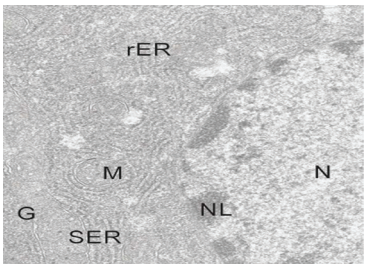

Fig. 13. Hepatocyte in mature/ripe stage. Rough endoplasmic reticulum (rER) with parallel cistrane, smooth endoplasmic reticulum (sER) glycogen granules (G) (X30000)

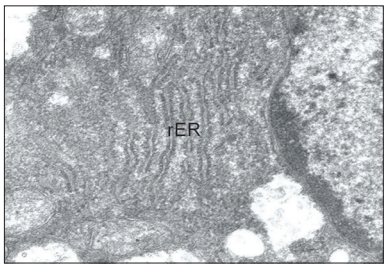

Fig. 14. Electron micrograph of ripe female hepatocyte with dense rough endoplasmic reticulum (rER) with flat cistermae (X35000)

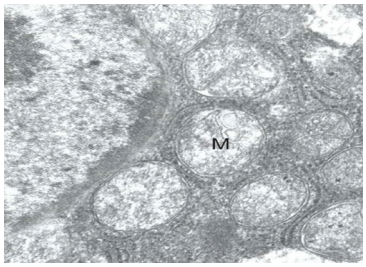

Fig. 15. Electron micrograph of ripe female *E. diacanthus* hepatocyte with dense and enlarged mitochondria (M) (X35000)

(Brusle, 1980), *Epinephelus microdon* (Brusle-Sicard *et al*., 1992), *Serranus hepatus* (Brusle, 1983) and *Amphiprion frenatus* (Brusle-Sicard *et al*., 1994). They have reported cytological criteria of high nucleus to cell ratio, abundant free ribosomes, a few mitochondria often forming association with nuages in the oocytes of immature, maturing and ripe female ovaries.

In the present study in female *E. diacanthus,* with the progress of oogenesis from oogonia to perinucleolus stage, small nucleoli increased in number. Similar observations were also reported in *Barbus barbus* by Thiry and Poncin (2006). They have concluded that these small nucleoli could originate from the activation of some amplified rRNA genes. This indicates the activation of protein synthesis with the progress of oogenesis. In the present study, maturing oocytes showed thin zona radiata in the process of oogonial development. Gopalakrishnan (1991) also made similar observations in *M. cephalus*. In ripe female *E. diacanthus* vitellogenic oocytes showed increase in dense rough endoplasmic reticulum, movement of germinal vesicle towards the periphery, yolk globules formation, development of thick zona radiata, zonation in yolk globules and mitochondrial aggregation. Goplakrishnan (1991) has also observed increased intensity in rough endoplasmic reticulum, yolk globule presence in the cytoplasm and thick zona radiata in the vitellogenic oocytes of *M. cephalus*. Lal (1991) also made similar observations in the vitellogenic oocytes of *Lates calcarifer*.

Yolk globules in vitellogenic oocytes of *E. diacanthus* showed zonation of electron dense inner region and electron lighter outer region. It indicates transition of yolk spheres in the penultimate stage of vitellogenesis. Lal (1991) also observed similar zonation of yolk globule in *L. calcarifer*. However in *M. cephalus*, yolk globules did not show zonation (Gopalakrishnan, 1991).

Liver produces vitellogenin after receiving estradiol stimulation from the ovary and it also plays a role in the synthesis of hormones. Hepatosomatic index (HSI), energy storage capacity of the hepatocytes and cytochemical characters of the hepatocytes depend on the physiological condition of the fish, feeding habits and nutrient availability (Svedong and Wickstorm, 1997). In the immature stage, female hepatocytes of *E. diacanthus* showed centrally located nucleus and scattered rough endoplasmic reticulum around the nucleus. Gopalakrishnan (1991) has also observed scattered endoplasmic reticulum in the immature female hepatocytes of *M. cephalus*. Similar cytological characters of hepatocytes in immature female fishes were also reported in the earlier works by Peute *et al*. (1978) in *Brachydanio rerio*; Bohemen *et al*. (1981) in *Salmo gairdneri* and Ribeiro *et al*. (2006) in *Steindachnerina insculpta*.

The maturing and ripe stage female hepatocytes of *E. diacanthus* have shown rapid proliferation of rough endoplasmic reticulum with flat cisternae and scant smooth endoplasmic reticulum. Enlarged mitochondria have also been observed in the ripe female hepatocytes of *E. diacanthus*. The observations in the present study are in agreement with the earlier works reported in *S. gairdneri* (Bohemen *et al*., 1981), in *Clupea harengus pullari* (Gillis *et al*., 1990), in *M. cephalus* (Gopalakrishnan, 1991) and in *S. insculpta* (Ribeiro *et al*., 2006).

Rapid proliferation of rough endoplasmic reticulum in mature female hepatocytes could be attributed to increasing vitellogenin (glycolipoprotein) production. Similar view has also been expressed by Peute *et al*. (1978) and Ribeiro *et al*. (2006). However, Bohemen *et al*. (1981) detected no vitellogenin in the liver, suggesting that this protein is released into the blood stream immediately after its synthesis by the liver. Therefore increase in liver weight which resulted in increase of HSI with gonadal maturation may be related to the non-proteic substances and or development and proliferation of organelles in the cytoplasm of the hepatocytes during vitellogenesis (Bohemen *et al*., 1981). According to Leatherland and Sonstegard (1988) presence of smooth endoplasmic reticulum could be related to the role played by the liver in metabolising and converting sex hormones. Increase in density of cytoplasmic organelles in the mature female hepatocytes of *E. diacanthus* could be attributed to increasing energy demand and metabolic rate related to the maturation of gonads.

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