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ULTRASTRUCTURAL COMPOSITION OF STEROID PRODUCING CELLS IN OOCYTE WALL, AND MICROPYLAR APPARATUS AS A SPERM GUIDANCE SYSTEM IN *OREOCHROMIS NILOTICUS* REARED IN FRESHWATER ENVIRONMENT

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Key words: Oocyte ultrastructure, steroid producing cells, Oreochromis niloticus, spermatozoa, micropylar apparatus.

ABSTRACT

The oocyte wall includes several steroid producing cells such as thecal and granulosa cells. On the prosecution of these cells, it was found that their ultrastrucutral composition permit them to produce steroids which are important in the continuity of oogenesis. The wall of the perinucleolar stage shows one layer of follicular epithelial layer with oval nuclei separated from the cytoplasm by a basal lamina. The epithelial follicle of vacuolized oocyte increased in thickness and differentiated into an outer thecal layer with clear basal lamina and an inner granulosa layer. In yolk deposition ocyte a considerably high nucleoplasmic ratio is displayed in the thecal cells. The granulosa layer has an amoeboid large nucleus displaying a granular electron dense chromatin arranged on its periphery with a clearly detected uniformly electron dense nucleoli located near the nuclear membrane. The granulosa's cytoplasm is characterized by the existence of plentiful number of mitochondria and a network of smooth endoplasmic reticulum. The thecal cells of tertiary yolk deposition oocyte show a considerable amount of secreted oil or lipid droplets, larger sized oval mitochondria and clearly detected golgi bodies. Ripe ova wall shows a tremendous number of mitochondria in the granulosa cells in which they are scattered filling the whole cytoplasmic area.

The spermatozoa of *Oreochromis niloticus* have long flagellar tail reaching 18 in length and the head's diameter ranges between 1.5-1.3 along its long and short axes. The ripe oocyte wall of the studied fish has a non-adhesive wall with floor tiles- like pores and a special micropylar apparatus including cristae corresponding to similar irregularities in the sperm head ,such pattern could act as a sperm guidance system.

INTRODUCTION

Tilapia is the common name of over 300 species of perch like fishes of family Cichlidae native to fresh waters of tropical Africa (Trewavas, 1983; Stiassny 1991). Tilapias are the major protein source in developing countries. Their fast growth rate and high disease resisting character give them the ability to survive in crowded media with low oxygen tension. In addition, their acceptability in markets recommends them as the most potential group of fishes for aquaculture as they are considered as the prime domesticated teleosts.

Family Cichlidae includes the mouth brooding genera *Sarotheroden* and *Oreochromis*, also the substrate spawner Tilapia. Nile tilapia belongs to *Oreochromis* genera named as *Oreochromis niloticus* (Trewavas 1983).*Oreochromis niloticus* is

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characterized by prolonged spawning season lasts in several months; also, the average eggs count in one patch of mature oocyte ready for fertilization is about 2000 eggs. Spawning occurs in shallow water up to 8 meters on muddy sandy substrates.

The study of fish reproduction usually requires information about the stages of gonadal development in an individual fish. Such information is often based on visual inspection of external appearance of gonads. A more precise and accurate detailed analysis required the use of histological methods. Identification of cellular changes, however, may be difficult by light microscopy alone. Ultrastructural studies using scanning and transmission electron microscopy can resolve some of the difficulties, as well as, providing additional valuable information to fish biologists (Selman and Wallace, 1986). For the purpose of taxonomic identification, many authors were encouraged to apply ultrastructural technique to identify species of teleosts. As the ultrastructural features of micropyle and egg, surface composition can be used as a criterion to identify species (Riehl andGotting, 1974 and Riehl, 1993).Moreover, in the natural environment the micropylar apparatus acts as a sperm guidance system to ensure proper fertilization.

In 1993 Riehl and Kokoscha were interested in the study of unique surface pattern and the micropylar apparatus in the eggs of *Luciocephalus* sp. In addition, they discussed the role of surface pattern in fertilization.

Yao *et al.* (1995) illustrated the ultrastructure of eggs of the ocean pout *Macrozoarces americanus* that is internally fertilized marine teleosts. Eggs were examined by scanning and transmission electron microscopy.

Vorobyeva and Markov (1998) studied the morphology of egg membranes in seven genera of Acipenseridae using electron microscope. Also they studied the specific structural features of the external membranes of the egg beside the micropylar apparatus.

Chen *et al.* (1999) identified four species of family Sparidae using micropylar ultrastructural using scanning electron microscope. They compared the microstructural characters such as the diameter of micropyle, number and arrangement of accessory opening and the reinforcement direction of micropylar canal.

Andrade *et al.* (2001) studied the gametogenesis in neotropical fresh water teleosts *Bryconops affinis*. They explained the oocyte maturation using ultrastuctural analysis.

Rizzo *et al.* (2003) described the ultrastructure of the oocytes of *Prochilodus marggraii* during their work on the short-term storage of these teleost eggs. In addition, a fully detailed idea was given about the oocyte surface concerning zona radiata and micropylar region.

MATERIALS & METHODS

The females and males of the studied fish species were reared in the reproduction laboratory of the National Institute of Oceanography and Fisheries in large fiberglass aerated aquaria in fresh water media till breeding season. Spawning was managed by photoperiod manipulation as reproductive inducing method (18L: 6D). The temperature was maintained at 26 ±2ؚc.

Samples preparation for Transmission electron microscopy:

i. Fixation and Post Fixation:

After dissection of fish, female gonads were cut into several parts then fixed immediately in 4% glutraldehyde in 0.1 M cacodylate buffer pH 7.4 at 4ºc for 2-3 hours. The specimens were washed in 5% sucrose in 0.05 M cacodylate buffer pH 7.2 – 7.4 three times, each for 15 min. Specimens could be left in the fourth change overnight at 4ºC. Specimens were fixed in 1% osmium tetra oxide in 0.2 M cacodylate buffer pH 7.2 -7.4 ,

for 2 hours at 4ºc, then rinsed in 0.1 M cacodylate buffer pH 7.2-7.4 for two hours at 4ºC.

ii. Dehydration and embedding: The tissues were dehydrated in an ascending series of ethanol and embedded in epoxy resin. The process of dehydration, infiltration and embedding were done as indicated in schedule of "D0166 Durcupan ACM Set".

iii. Sectioning and Staining:.

Ultra thin sections also were cut on a JEOLJUM.7 ultra microtome with glass knives, and placed on 200 – mesh copper grids. The sections were then stained with uranyl acetate (Saturated in 70% ethyl alcohol) followed by lead citrate (Reynolds 1963) and examined with a JEM = 10cx transmission electron microscope at 80 K.v.

Preparation of eggs, and sperms for scanning electron microscopy:

1. Unfertilized eggs:

Unfertilized eggs were collected by stripping of ripe females , then cleaned first using fish saline buffer, prefixed in 4% glutraldehyde, washed with phosphate buffer pH 7.4 and post fixed in 1% osmium tetraoxide. The samples were then washed with buffer and dehydrated in a graded series of ethanol. Samples were transferred into amylacetate then dried in a critical point dryer with liquid carbon dioxide. The dried samples were mounted on stubs then gold coated. Then they were studied by scanning electron microscope.

2. Spermatozoa preparation:

After stripping of ripe males, milt was immediately fixed in 4% glutraldehyde buffered to pH 7.2 with sodium cacodylate then filtered on 0.22 µm Millipore filter. Sperms dehydrated in a graded series of Ethyl alcohol, critical point dried using CO2 and gold coated then examined.

RESULTS

In the present study, different developmental stages of oocytes walls were examined using transmission electron microscope. The most important reason of this investigation is to know the fine structure and the role of the steroid producing cells such as thecal and granulosa cells during the oogenesis of *Oreochromis niloticus.*

In early stage of oocyte maturation, the epithelial follicle shows differentiated layers. Using transmission electron, the wall of the perinucleolar stage shows one layer of follicular epithelium with oval nuclei separated from the cytoplasm by a basal lamina as shown in Figure (1). The nuclei are highly granulated electron dense showing an elongated pattern, including clumps of condensed chromatin that is irregularly distributed and some times associated with the nuclear envelope. They range between 1.3µm and 1.6µm in diameter. A rough endoplasmic reticular network surrounds these nuclei. The basal lamina which separates this layer from the cytoplasm reaches 0.6µm in thickness. The total thickness of the epithelial follicle reaches 2.5µm. The cytoplasm of this developmental stage shows scarcely dispersed mitochondria in different shapes including oval and rounded ones.

Concerning the vacuolized oocyte wall, it was found that the thickness of the epithelial follicle showed an increase reaching about 2.8µm. In this stage, the epithelial follicle starts to show a clear differentiated outer thecal layer with clear basal lamina ranging up to o.6µm in thickness and an inner granulosa layer as shown in Figure (2). On investigating the granulosa layer, the most obvious characteristic feature is the appearance of oval or spherical mitochondria with lamellar cristae. The nuclei of these cells are oval with granular election dense chromatin material arranged on its periphery. As to the cytoplasmic inclusions which are dispersed in the cytoplasm of the oocyte, one can notice an increase in the number and size of the mitochondria. The Balbiani body is clearly detected showing a spherical structure with two distinguished zones. Also, some oil droplets can be seen above the Balbiani body.

Later on, in yolk deposition oocyte represented in Figure (3), a considerably high nucleoplasmic ratio is displayed in the thecal cells. The nucleus is granularly electron dense showing some darker points. A good variety of cytoplasmic inclusions is dispersed in the cytoplasm of the thecal cells. Some mitochondria of different sizes appear with lamellar cristae, and golgi bodies also are well detected accompanied by vesicles. A long lipid droplet can be detected in the thecal cells showing an electron dense oval body. The basal lamina increases in thickness to reach 0.8µm.

On the other hand, the granulosa layer has an amoeboid large nucleus displaying a granular electron dense chromatin arranged on its periphery with a clearly detected uniformly electron dense nucleoli located near the nuclear membrane. A very clear characteristic feature was shown in the granulosa cytoplasm, which is the existence of plentiful number of mitochondria and a network of smooth endoplasmic reticulum. The rough endoplasmic reticulum appears to be ill developed, nevertheless is often present. The granulosa cells develop a certain microvillar processes arising from the oocyte surface and projecting deeply into the extra cellular spaces of the overlying granulosa cells forming pore canals.

In the subsequent stage, which is known as tertiary yolk deposition Figure (4), thecal cells start to show a considerable amount of secreted oil or lipid droplets, larger sized oval mitochondria and clearly detected golgi bodies. A fusiform granular electron dense nucleus was shown in the same Figure. The basal lamina of the thecal layer increases in thickness to reach 1µm.

Figure (5) shows that the steroid producing granulosa cells embodying different size nuclei with granular electron dense chromatin material. Well-developed networks of smooth and rough endoplasmic reticulum are well marked in granulosa cells, as well as plentiful mitochondria exhibiting (oval and rod-like type). The microvillar process of zona radiata increased in thickness to reach about 1µm showing an electron dense zones of the pore canals (Figure 6).As regards the cytoplasmic inclusions of the oocyte represents large vacuole accompanied by a long rod shaped crystalloid body as demonstrated in the same figure.

On further development of the oocyte, ripe ova wall shows a tremendous number of mitochondria in the granulosa cells in which they are scattered filling the whole cytoplasmic area as shown in Figure (7). The oblong nuclei of the granulosa cells are arranged in a specific manner and having a well marked nucleoli either on the periphery or in the middle of the nucleoplasm. Oil droplets increase gradually to fill in between the granulosa cells as shown in the same figure. They are secreted by granulosa cells as main source of steroids. It is indicated in Figure (8), that microvillar processes of zona radiata are occluded by an electron dense material which gives the zona radiata a solid appearance when cut transversely. A great number of small electron dense bead-like structures fill the cytoplasm of both granulosa and oocyte as evident in Figure (8), these are known as lysosomes.

Spermatozoa of most teleosts are monoflagellates, in the present study the scanning electron microscope investigation revealed that *Oreochromis niloticus* spermatozoa have long flagellar tails reaching 18 µm in length. The head and collar region length is about 2.5 µm as shown in Figures (9) and (10). The mitochondrial region only reaches 1µm in length, while the head of spermatozoon measures between 1.3 µm and 1.5 µm in diameter along its short and long axes. Figure (9) confirms that the head of spermatozoon lacks an acrosome.

Fertilization process is such a complicated phenomenon that occurs in natural environment, to ensure that the proper sperm should fertilize the proper egg, oocyte surface and micropylar apparatus must be adapted to suit the specific structure of the spermatozoon of such species.

According to this fact, the micropylar structure must constituted to suit the sperm. The following ultrastructural investigations will explain how both micropylar structure and sperm structure harmonize to ensure fertilization.

It is clear in Figure (11) that the oocyte of *Oreochromis niloticus* has a non-adhesive surface lacking filaments.The surface of the oocyte shows a compact vitelline envelope surrounding the egg with a single micropyle located in the animal pole as shown in Figures (11), (12) and (13). The envelope in the vegetative pole shows a polyhedral surface that resembles the floor tiles. The distance between pores of the envelope is approximately 0.8 µm before fertilization as shown in Figure (12). Over the equatorial region, these tiles change their shape and elongate to form isolated crests. Near the animal pole, these crests fuse together and become furrow like structure converge directly into the micropyle pit. In the center of this pit, the micropylar canal is located with a diameter reaching 11 µm .The sperm head consists of a corresponding crest as shown in Figure (14), as they fit in the micropylar canal like a key and its lock.

DISCUSSION

Teleostian ovaries generally are known to secrete steroid hormones (Fostier *et al*., 1983), and according to Nagahama (1983), at least five different cellular sources have been considered to implicate in the process of steroid production in ovaries of such fish.

The outer most layer of the follicle, that is known as thecal layer, was known to synthesize testosterone (Kagawa *et al*., 1982). Formation of testosterone is subsequently aromatized by cytochrome P_{450} aromata (CYP_{19}) to eastradiol by the granulosa cells (Kagawa *et al*., 1982; Nagahama, 2000). While the inner layer of the follicle possessed a well-developed granulosa layer as reported in the present study confirming the previously mentioned postulation of Fostier *et al.* (1983) and Nagahama (1983) in steroidogenesis. The cells of this layer have manifested marked alterations, being reflected in the form of an obvious increase in both number and size. These features were taken as an indication of the obvious involvement of those cells in the process of steroidogenesis accompanying the development of the ovaries and formation of ripe oocyte. Those observations conform those presented by Rosenblum *et al*. (1987) in *Ictalurus nebulosus*, Matsuyana *et al.* (1991) in *Pagrus major*, concerning such successive changes. Furthermore, the present data were supported by the observations reported by Mousa (1994) in *Mugil cephalus*, inhabit in El-bardaweill lagoon who presented a positive correlation between oocytes dimensions and their estradiol -17β hormones content.

Underneath granulosa layer, zona radiata appeared in ultrastructural examinations. It was well developed reaching a maximum thickness in ripe stage having a specific striated appearance. This layer is a complex structure showing pore canals containing microvilli or follicular cell processes (Guraya, 1996). The microvilli get in contact in different ways allowing the oocyte to grow up by transferring nutrients and different types of junctions, gaps and tights (Selman and Wallace, 1986), should link growing factors. Normally, we could not detect the kind of junction in the present that join the microvilli and follicular cell surface. On the other hand,this complex structure started to be clear in some sections taken in the yolk deposition stages reaching its maximum thickness in ripe stage.

In other teleosts, zona radiata was called zona pellucida, it begins to be formed in the previtellogenic oocyte. Its outer layer begins to be formed through electron dense material deposited between microvilli of the oocyte (Rizzo and Bazzoli, 1991 and Cruz-Hofling and Cruz-landin, 1993). The origin of zona radiata of teleosts is still controversial issue, yet it is postulated that the oocyte follicular cells and hepatic cells may play an important

role in the formation of this structure and that specializations associated to it may originate from follicular cells (Guraya, 1996).Coward and Bromage (1998) described similar feature in *Tilapia zillii*. This Some studies reported that the interstitial cells which may be involved in steroidogenesis, originate from atretic previtellogenic follicles (Guraya, 1973; 1976 and Saidapur, 1978). Few studies reported the presence of postouulatory follicles in Tilapia (Dadzie and Wangila, 1980 and Coward and Bromage, 1998).

The morphology of spermatozoa highlights the potential application of phylogenetic relationship between fish species and serves as taxonomic criterion (Jamieson, 1991; Mattei, 1991; Gwo *et at.,* 2004).Spematozoa of teleost fishes exhibit a great variety in shapes and structures reflecting, to a great extent, the reproductive patterns.

The present study revealed that, the studied fish has anacrosomal sperm that has been related to the presence of a micropyle in the egg. Those of Mattei (1970) and Riehl (1993) supported this result. To guarantee the efficient fertilization, special structures should be present in the sperm as a guiding criterion to certain micropyle (Hart, 1990).

The present study revealed a special micropylar apparatus including crests corresponding to an equivalent irregularity in the sperm head. The structure of the micropylar apparatus of the studied fish was supported by the opinion of Ainanze & Iyengar (1990) confirming that such a pattern could function as a sperm guidance system. Various eggs surface pattern emerged from scanning microscopical- analyses declared that, teleost eggs have variable unique surface patterns.*Oreochromis nitoticus* eggs had a non adhesive surface with a floor tiles like pores.Rizzo *et al*., (2003) studied the adhesiveness and surface patterns of neotropical fresh water eggs of several families. They recorded several adhesive egg surfaces in family Characidae, Erythrinudae and Doradidae.

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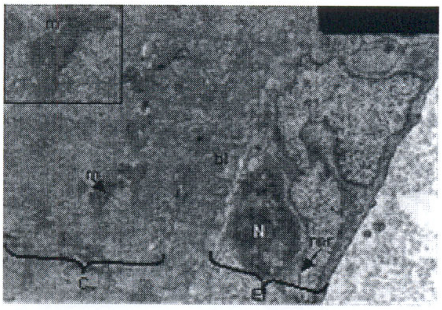
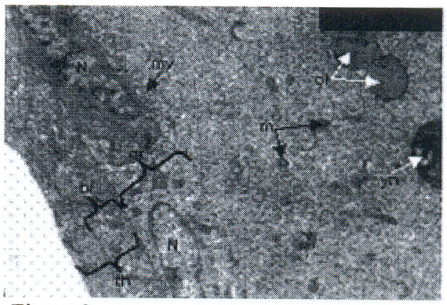


Figure 1



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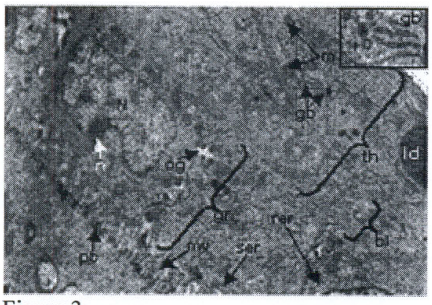

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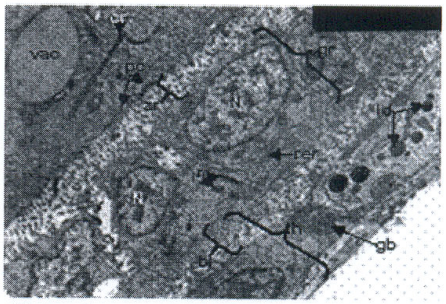

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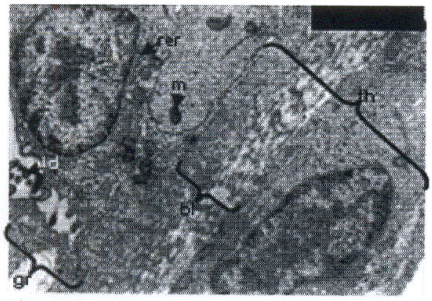


Figure 5

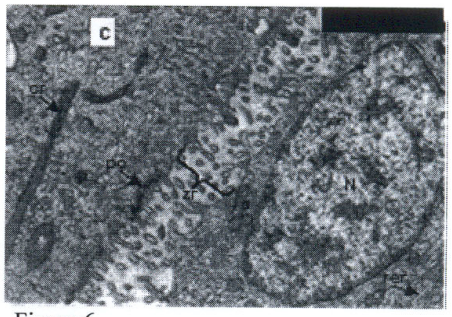
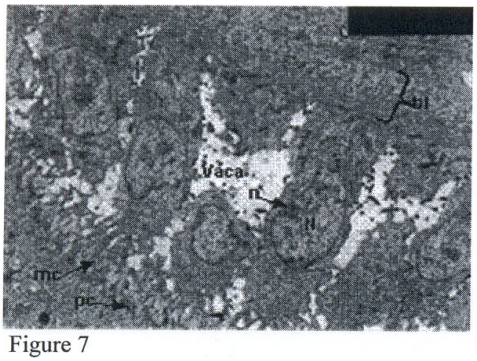


Figure 6



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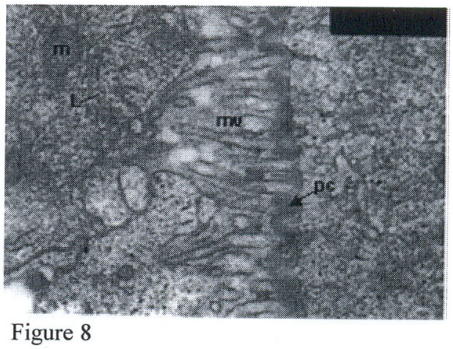

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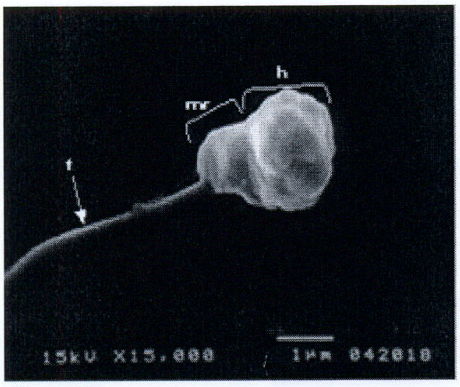

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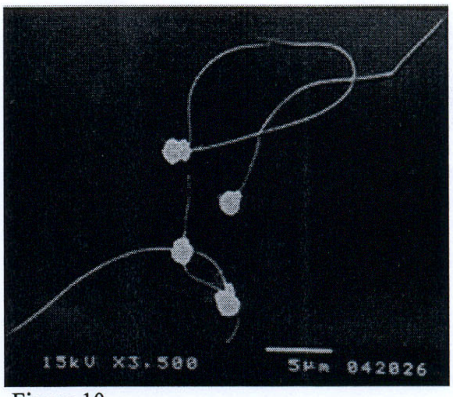

Figure 10

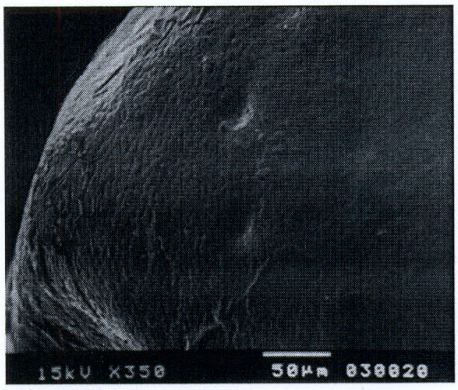

Figure 11

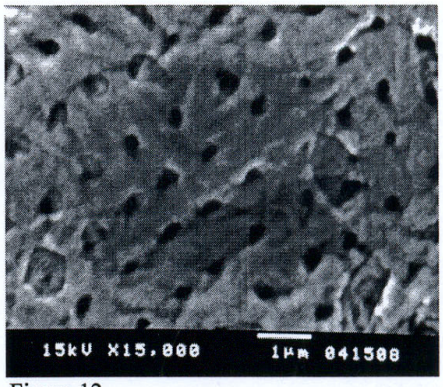

Figure 12

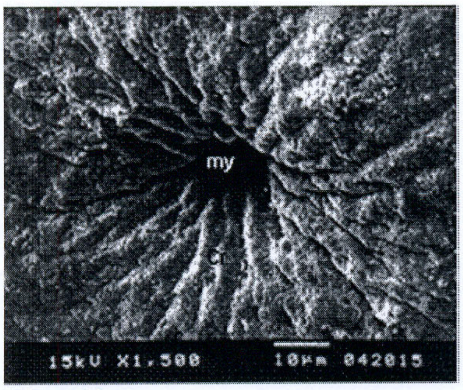

Figure 13

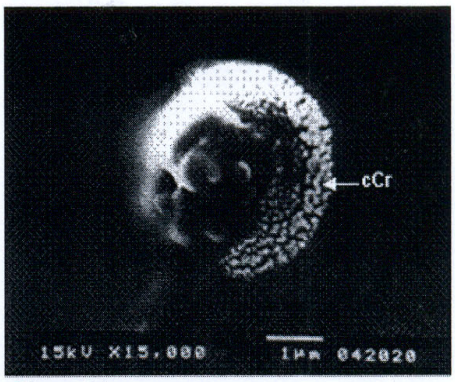

Figure 14

FIGURE'S DEFINITION

Figure (1): An electron micrograph in perinucleolus oocyte wall of *Oreochromis niloticus* showing the epithelial follicle (Ef), basal lamina (bl), cytoplasm of the oocyte (C) with few mitochondria (m) and rough endoplasmic reticulum (rer). Uranyl acetate- lead citrate stain X 7500.

Figure (2): An electron micrograph in oocyte wall of vacuolized stage in *Oreochromis niloticus* showing epithelial follicle which is differentiated into an outer thecal layer (th) with clear basal lamina (bl) and an inner granulosa layer (gr) exhibiting oval nuclei (N), mitochondria (m) & yolk nucleus (Yn). Uranyl acetate-lead citrate stain X 7500

Figure (3): An electron micrograph in the wall of primary yolk deposition stage of *Oreochromis niloticus* a differentiated outer thecal layer (th) and an inner granulosa layer (gr), some cytoplasmic inclusions such as large lipid droplet (ld), golgi bodies (gb), mitochondria (m) ,large nucleus of granulosa layer (N) containing an obvious nucleolus (n), oil globules (og). Smooth and rough endoplasmic reticulum (ser) & (rer) and zona radiata microvilli (mv) with distinct pore canals (pc). Uranyl acetate-lead citrate stain X 7500

Figure (4): An electron micrograph in the wall of tertiary yolk deposition oocyte of *Oreochromis niloticus* showing lipid droplets (ld) in the thecal layer, basal lamina (bL), granulosa layer (gr) with large oval nuclei (N), mitoochondria (m), golgi bodies (gb),vacuole (vac) and crystalloid (cr). Uranyl acetate- lead citrate stain X 5000.

Figure (5): An enlarged part of the previous figure showing the thecal layer (th) and it's basal lamina (bL), granulosa nucleus (N), mitochondria (m), rough endoplasmic reticulum (rer) and lipid droplet (Ld). Uranyl acetate-lead citrate stain X 10000.

Figure (6): An enlarged part of figure 25 showing granulosa layer (gr), zona radiata (Zr), pore canals (pc), active cytoplasm of the oocyte (C) and crystalloids (cr). Uranyl acetate-lead citrate stain X 10000.

Figure (7): An electron micrograph in a ripe oocyte wall of *Oreochromis niloticus* showing large vacuolated areas in the granulosa layer. Uranyl acetate-lead citrate stain X 4000.

Figure (8): An electron micrograph in zona radiata layer of ripe oocyte showing the microvilli (mv) with electron dense pore canals (pc) and lysosomes (L). Uranyl acetate-lead citrate stain X 20000.

Figure (9): A scanning electron micrograph showing the external shape of *Oreochromis niloticus* spermatozoon with it's distinctive regions head (h), mitochondrial region (mr) and tail (t). Gold coating technique X 1500.

Figure (10): A scanning electron micrograph showing a group of sperms of *Oreochromis niloticus.* Gold coating technique X 3500.

Figure (11): A scanning electron micrograph showing the surface pattern of *Oreochromis niloticus* ripe oocyte. Gold coating technique X 3500.

Figure (12): A scanning electron micrograph of *Oreochromis niloticus* oocyte showing the floor tiles like pores. Gold coating technique X 1500

Figure (13): A scanning electron micrograph of ripe oocyte of *Oreochromis niloticus* showing the aperture of micropylar canal (my) and the convergent crests (Cr).Gold coating technique X 1500.

Figure (14): A scanning electron micrograph of sperms head of *Oreochromis niloticus* showing the corresponding crests which fit in the micropyle of the egg (cCr). Gold coating technique X 1500.