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Observation on the Ultrastructural Alternations in Ova of Leiognathus equulus. (Perciformes) from AL-Shabab Lagoon, in Jeddah Province

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Abstract: *The common ponyfish Leiognathus equulus was caught from the Red Sea and Al-Shabab lagoon. This study was carried out to investigate the ultrastructure alternations induced by the Al-Shabab lagoon pollutants in Leiognathus equulus ova chorion. The fine structure of the chorion of non-polluted and polluted mature unfertilized egg was examined respectively. The chorion of fish ova caught from Red Sea consists of three layers: theca, granulosa and zona radiata which separated into two layers external and internal zona radiata consisting of 6 lamellae. The outer surface of the egg was granulated. The micropyle is simple. While, fish caught from Al-shabab lagoon shows variable deformations in egg including deterioration and degeneration of zona radiata, hypertrophy of both theca and granulosa layers with deformed and shrinkage nuclei and increased number of mitochondria.*

Key words: *Common ponyfish., ultrastructure., Red Sea., Al-Shabab lagoon., deformations., chorion.*

I. Introduction:

Leiognathids, commonly known as ponyfishes, are characterized by a strongly protrusible mouth. The family is distributed in coastal and estuarine waters of the Indo-West Pacific (Nelson, 2006). The family is commercially important in Asian wild fisheries and aquaculture as a fresh or processed product (Woodland, et al., 2001).

Water pollution affects the ecological conditions which usually affect the biological and physiological conditions of the fish, especially reproduction, which is reported to be affected seriously (Santiago, et al., 1985).

Pollution of the aquatic environment is a serious and growing problem. Increasing number and amount of industrial, agricultural and commercial chemicals discharged into the aquatic environment having led various deleterious effects on the aquatic organisms (McGlashan and Hughies, 2001). Aquatic organisms, including fish, accumulate pollutants directly from contaminated water and indirectly via the food chain (Sasaki, et al., 1997).

Identification of cellular changes however, may be difficult by light microscopy alone. Ultrastructural studies using scanning and transmission electron microscopy can resolve some of these difficulties, as well as provide additional valuable information to fish biologists (Selman and Wallace, 1986).

Ultrastructural changes in cells, tissues or organs can afford good biomarkers of pollutant stress. Using of ultrastructural changes as a biomarker has the benefit of permitting researchers to examine specific target organs and cells as they are affected by exposure to environmental chemicals (Salamat and Zarie, 2012).

Wourms (1976) and Wourms & Sheldon (1976) described by means of electron microscopy the formation of primary and secondary egg envelope in the Cyprinodontiformes Cynolebias melanotaenia and C. ladigesi. In the oviparous teleost Blenniuspholis, two types of follicle cells with different functions in the formation of zona radiata were identified (Shackley & King, 1977). While, Schmehl & Graham (1987) studied the comparative ultrastructure of the zona radiata of six species of salmonid eggs. In the cod, Gadus morhua, the egg chorion grows to a tripartite structure, an outer thin porous layer, an intermediate homogeneous layer and an inner thick helicoidal layer (Kjesbu & Kryvi, 1989). The chorion surface was also analysed by scanning electron microscopy by Johnson & Werner (1986), who described the external morphology of the chorion of five species of freshwater fish and concluded that scanning electron microscopy was a powerful tool for identifying fish eggs. Riehl (1993) pointed out the importance of egg surface and micropyle morphology for taxonomic purposes and stated that the structure of the egg surface, the diameter and distance of the pores, and the micropyle morphology are important and necessary criteria for fish egg characterization. More recently Li et al. (2000) underlined the importance of micropyle ultra structural features for egg identification in three genera of Perciformes. **Ultrastructural aspects of the ovarian follicle.pdf 2000**

Saad and Fahmy (1994) recorded four main pollution sources at Jeddah coast: the untreated domestic sewage wastes, oil pollution from oil refinery of factory Petromin, fish wastes from the big fish market of Bankalah region and probably desalination plant effluents.

This study aimed to evaluate the occurrence of ultrastructure alternations in egg (not fertilized) of L. equulus. It is expected that this study will contribute to our knowledge useful information for providing a basis on which future monitoring of the pollutants or assessment of the fate of environment in this area can based.

II. Materials and Methods:

Fish samples:

Fish samples in the present work were obtained from the Red Sea waters in front of Jeddah (178 fish) and from Al-Shabab lagoon in Jeddah (226 fish). The fish were transported to the laboratory in ice aquarium.

The fish were weighted to the nearest gram. Directly, the fish were dissected to determine sex and maturity stage. The ovaries were removed, weighed and thoroughly examined.

Electron microscopy technique:

Immediately after dissection of the fish, the ovaries were cut several parts. Those assigned for the ultra structural studies were fixed in 2% gloter aldehyde in 0.2 M sodium cacodylate buffer pH 7.2.

1. Scanning Electron microscopy (SEM):

Samples were fixed in Trump's fixative from half to one hour, after which samples were rinsed three times in distilled water (10 min each), fixed in Osmium Tetra oxide ($OsO4$) (1 hour) , rinsed twice in distilled water (1min each), 50 % Ethanol 1 min , 100 % Ethanol (10 min), ethanol : acetone (1:1) for 10 minute, pure acetone three changes (10 minutes each), Acetone: Hexamethyldisilazane (1:1) for one hour, pure Hexamethyldisilazane also one hour and remove the Hexamethyldisilazane and was put the sample under vacuum (10 min (H.P) or Overnight (L.P) then critical –point dried and gold coated. Preparation were viewed in a QUANTA FEG 450 at Faculty of Science Biology Department King Abdul-Aziz University (Electron Microscope laboratory).

2. Transmission electron microscopy (TEM):

2.1 Dehydration and embedding:

The tissues were dehydrated in ascending series of ethanol and embedded in epoxy resins.

2.2 Sectioning and Staining:

For light microscopy, semithin sections (0.5-1 um) were cut on a JEOL JUM-7 ultra microtome with glass knifes and stained with 1% toluidine blue in 1% sodium borate. Ultrathin sections (70 - 90 nm) were placed on 200- mesh copper grids. The sections were stained with uranyl acetate (saturated in 70% ethyl alcohol) followed by lead citrate and examined with PHILIPS CM 100 transmission electron microscope at 60 kv at King Fahd Medical Research Center (Electron Microscope Unit).

III. Results:

Ultra structure of non-polluted ova:

The unfertilized eggs of non polluted L. equulus at ripe stage were spherical in shape and covered with granulated egg shell (Fig. 1). SEM observations of crossed-fractured egg showed that envelope consisting of three distinct layers. The eggs were enveloped by relatively thick zona radiata (1.598 µm ± 0.246). The surface of the zona radiata externa maintains a compact homogenous structure with a uniform distribution of almost round pores (Fig. 2). The two most external layers, the outer and the middle layer, appeared compact. The third inner layer, which is the thickest one consisted of 6 lamellae (Fig.3).

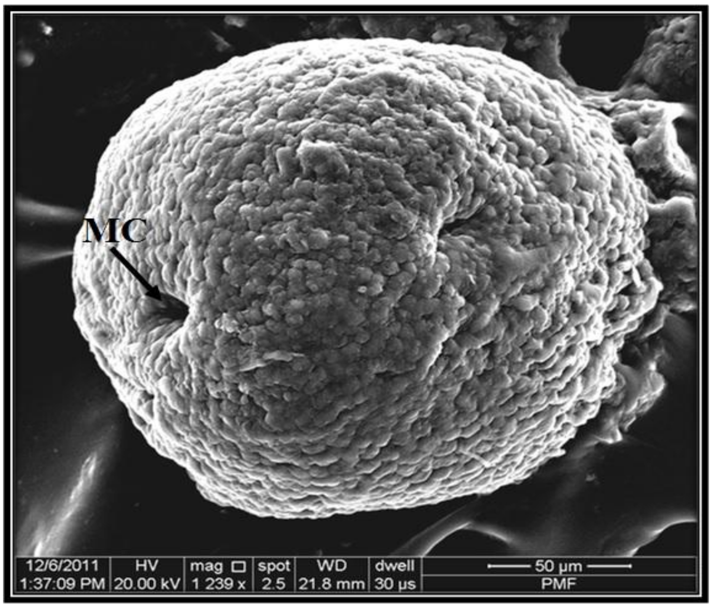

Figure 1: Scanning electron micrographs (SEM) of non-polluted L. equulus showing whole view of ripe ova having a granular surface and micro pylar opening (MC).

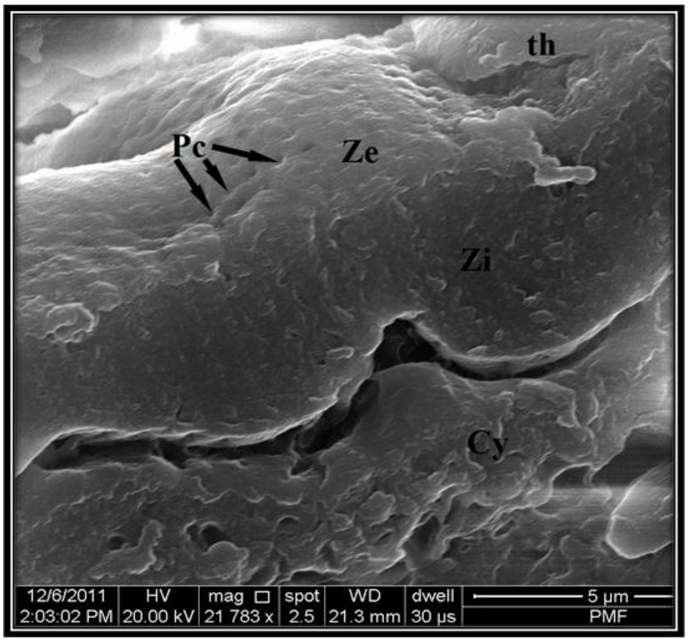

Figure 2: Scanning electron micrographs (SEM) of ova of non-polluted L. equulus showing two layers of zona radiata: zona radiata externa (Ze); zona radiata interna (Zi); cytoplasm (Cy); theca layer (th) and the distribution of pores (Pc) in zona radiata externa.

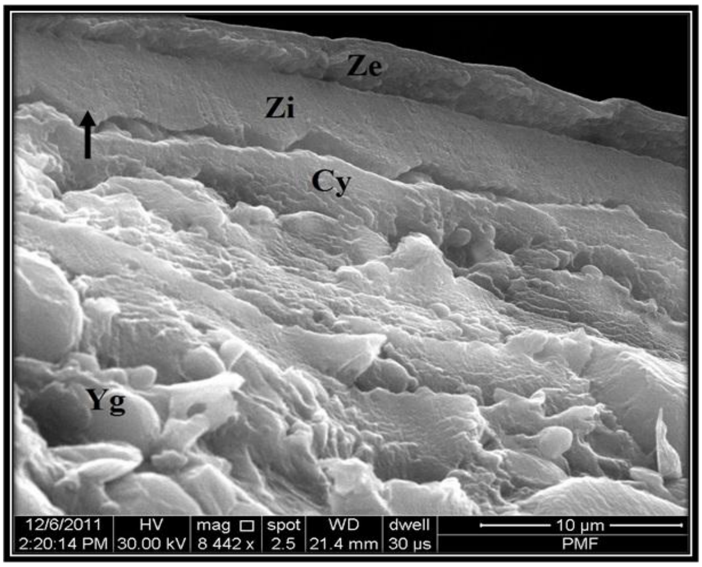

Figure 3: Scanning electron micrographs (SEM) of ova of non-polluted L. equulus showing the zona radiata interna (Zi) having 6 layers (arrow); cytoplasm (Cy); yolk globules (Yg) and zona radiata externa (Ze).

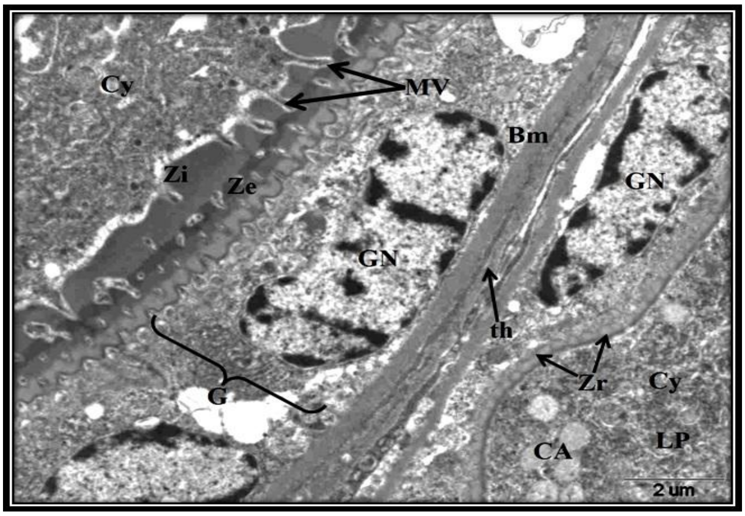

Figure 4: Transmission electron micrographs (TEM) of ova of non-polluted L. equulus showing late peri-nucleolus oocyte stage (LP) and vaculized stage, the appearance of zona radiata (Zr) and microvilli (MV) and differentiation of zona radiata into extrena and interna zona radiata (Ze & Zi); Basement membrane (Bm); cortical alveoli (CA); cytoplasm (Cy); granulosa (G); granulosa nucleus (GN) and theca(th).

In the late peri-nucleolus oocyte stage, The follicular layer is formed by flat cells, with oval-shaped nuclei and condensed chromatin mainly close to the nuclear envelope and scattered in the nucleoplasm and granulosa cells have flattened nuclei. The plasma-membranes of the cytoplasm shows project stumpy microvilli. During this stage of development, zona radiata interna is formed (Fig. 4).

The ovarian oocyte of L. equulus had three layered chorion: an outermost layer (theca + granulosa layer), zona radiata externa and zona radiata interna. The early vacuolized stage in which a flat follicular cell layer is formed, that is composed of granulosa layer, a thick basal consisted lamina and a squamous coat of thecal cells surround the oocyte. This thecal cell layer is composed of fibroblast-like cells, collagen fibrils and blood capillaries (Fig. 4).

Concomitant with the growth of the oocyte (vaculized stage), and considered to be the first sign of specialization of the Oolemma, is the formation of the microvilli in the space between the growing oocyte and the follicular layer, called perivitelline space (extracellular space). In this stage the surface of both the oocyte and follicular epithelial cells, no longer run in smooth and in parallel. A lot of ovular microvilli protrude into the perivitelline space as tubular micro-projections. The granulosa cells develop microvillar processes, which are smaller in number and size to the ovular microvilli, which will penetrate the perivitelline space to a variable distance depending on the stage of the oocyte growth. Later on, within the same stage, oocytes show the presence of a second layer, the zona radiata interna, under the former one (zona redaiata externa). This second layer is more coarsely aggregated and has less electron density than the zona radiata externa (Fig. 4).

In mature oocyte (Fig. 5) a significant increase in volume of the oocytes. Due to the great accumulation of yolk granules the cytoplasmatic organelles are displaced towards the peripheral ooplasm. Follicular cells of mature oocytes show an increased number of organelles mitochondria, rough endoplasmic reticulum. The cytoplasm of oocyte is progressively filled with a variety of vesicular elements. Lipid droplets, yolk globules of different sizes can be seen (Fig. 5). The zona radiata interna gets thicker (about 0.91 µm ± 0.199) and displays a multi laminar structure with alternating layers of different density. Up to 6 different layers can be distinguished. The zona radiata externa is still about 0.69 µm ± 0.077, and maintains a compact homogeneous structure. The pores in the viteline envelope increase both their diameter and the distance between them.

Ultra structure of polluted ova:

Ova of fish Al-Shabab lagoon showed completely disappearance of zona radiata externa layer, hypertrophied follicular epithelial cells with pyknotic nuclei. Granulosa and follicular epithelial layers became hypertrophied measuring 6.084 µm ± 0.521 and 1.337 µm ± 0.47, while these two layers in non-polluted ova measured 4.321 µm ± 0.604 and 0.563 µm ± 0.249 respectively (Fig. 6 & 7).

The zona radiata externa and interna decrease in thickness to be 0.659 µm ± 0.069 and 0.833 µm ± 0.1 respectively losing its irregular shape and laminations and beginning of disintegration (Fig. 6 & 7). Follicular epithelial layer showed cells hypertrophy and hyperplasia, increased number of mitochondria and appearance of vacuoles of variable size in granulosa layer and shrinkage of its nuclei losing its normal shape (Fig. 6).

Dilation in granulose layer, increasing of mitochondria and completely disintegration and degeneration of granulosa cell were observed in figure (7).

Table (1) illustrates the measurement of both non-polluted and polluted ova chorion of L. equulus. It is obvious that there were increasing in the thickness of both theca and granulosa layers of polluted egg. Also, it is cleared that the thickness of wall and the both layers zona radiata externa and interna is decreased in polluted ova. While in non-polluted ones the thickness of wall, and zona radiata externa and interna is 6.653 µm ± 0.803, 0.686 µm ± 0.077 and 0.911 µm ± 0.199 respectively.

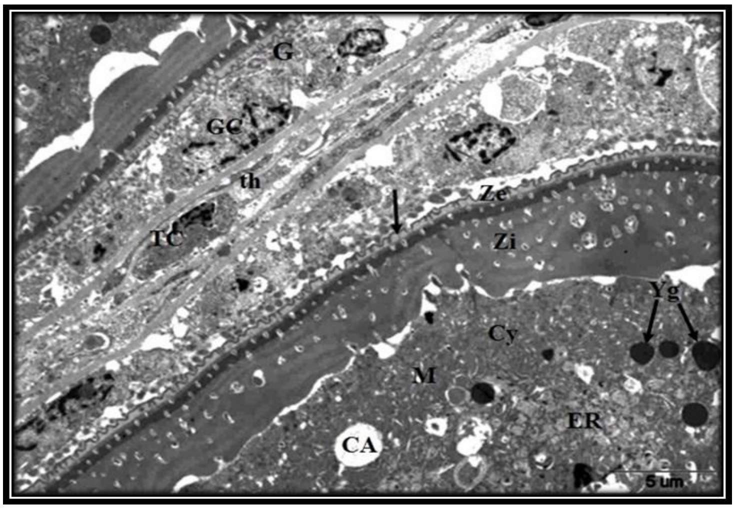

Figure 5: Transmission electron micrographs (TEM) of ovary of non- polluted L. equulus showing the wall of mature oocyte; chorion pores (Arrows); cortical alveoli (CA); Cytoplasm (Cy); Endoplasmic reticulum (ER); granulosa cell (GC); granulosa layer (G); mitochondria (M); Theca layer (th); theca cell (TC); yolk globules (Yg); zona radiata externa (Ze) and zona radiata interna (Zi).

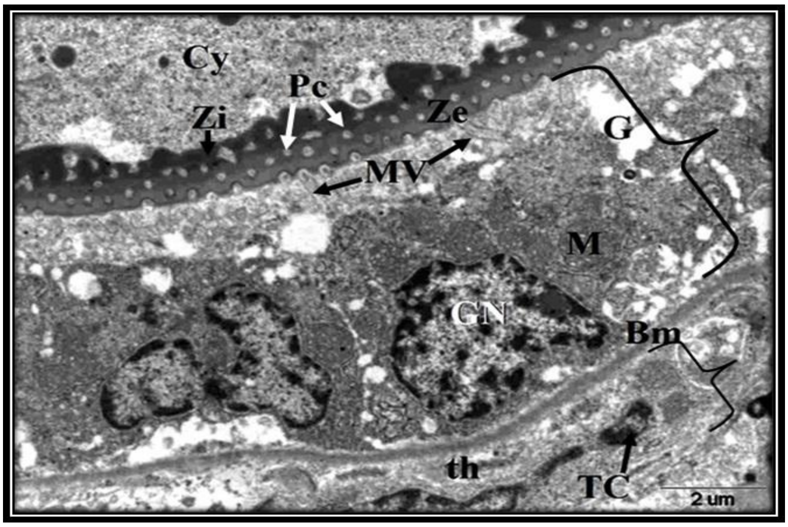

Figure 6: Transmission electron micrographs (TEM) of ovary of polluted L. equulus showing hypertrophy of granulosa (G) and theca layers (th); Basement membrane (Bm); cytoplasm (Cy); granulosa nucleus (GN); microvilli (MV); mitochondria (M); pore canal (Pc); theca cell (TC); zona radiate externa (Ze) and zona radiata interna (Zi).

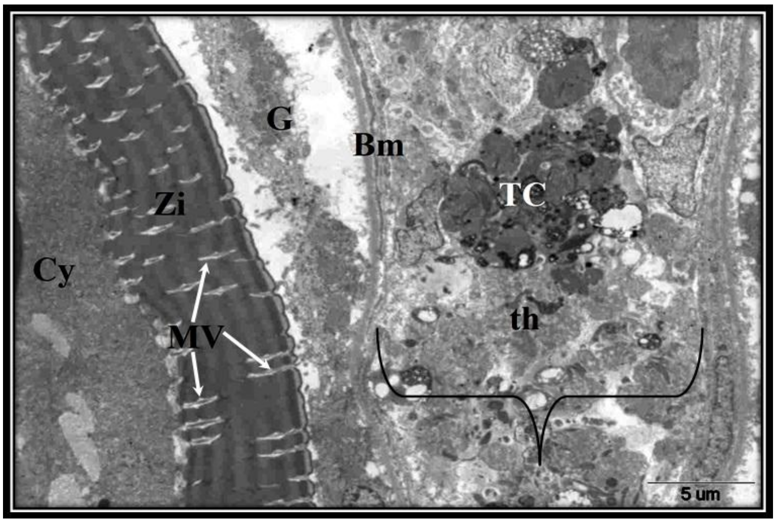

Figure 7: Transmission electron micrographs (TEM) of ovary of polluted L. equulus showing loss of zona radiata externa (Ze); degeneration of granulosa layer (G) and disintegration of granulosa cells; Basement membrane (Bm); cytoplasm (Cy); microvilli (MV); theca cell (TC); theca layer (th) and zona radiata interna (Zi).

Table 1: Ova measurements of non-polluted and polluted of L. equulus.

Thickness of ova chorion	Non-polluted		Polluted	
	Mean	Standard deviation	Mean	Standard deviation
Theca thickness	0.5630 µm	± 0.2493	1.3366 µm	± 0.4698
Granulosa thickness	4.3206 µm	± 0.6042	6.0838 µm	± 0.5210
Zona radiata thickness	1.5976 µm	± 0.2458	1.4918 µm	± 0.0948
Ze thickness	0.6864 µm	± 0.0774	0.6593 µm	± 0.0691
Zi thickness	0.9112 µm	± 0.1986	0.8326 µm	± 0.0997
Wall thickness	6.6529 µm	± 0.8027	4.8666 µm	± 0.2046

IV. Discussion:

The study area receives different pollutants from four sources: untreated domestic sewage wastes, oil from the oil refinery, fish wastes from the big fish market (El-Bangalah) and desalination plant effluents which contribute both organic and metallic contaminants (Behairy and Saad, 1984; Basaham, et al., 1990 & Mohorjy and Khan, 2006).

As in many teleosts, the development of the vitelline envelope in L. equulus first becomes apparent with the appearance of the zona radiata externa, which is dense with homogenous structure, reduced thickness and structurally similar to that described in Synbranchus marmoratus (Ravaglia and Maggese, 2003). As in other teleosts, the zona pellucida of L. equulus begins to be formed in the late peri-nucleolus (previtelognic oocyte, Andrade, et al., 2001). The zona radiata interna is less dense and generally has a fibrilar aspect. It has up to 10 dense layers. In L. equulus (present study) the zona radiata interna consists of six lamellae. The present observation was consistent with the finding in Pagrus major, where the vitelline envelope (VE) has a similar structure composed of 2 major layers Zona radiata externa (Ze) and Zona radiata interna (Zi) and it becomes more complex with seven membranous reticular lamellae for Zi when the oocyte enters vitellogenic phase (Matsuyama, et al., 1991).

The surface of zona radiata of L. equulus was wavy and uneven and many round pores having no lips were scattered uniformly on the egg envelopes (Fig. 1). The granulated egg envelope of L. equulus could increase the surface of the egg, and thus the number of the pores. This may increase the gas exchange between egg and water. The presence of pores has been reported on the egg envelopes of E. malabericus and E. coiodes, while only pore-traces have been observed on the egg envelope of S. ocellatus and no pores have been found on the envelopes of M. cephalus (Li, et al., 2000). According to Li, et al. (2000) and Chen, et al. (1999), pore was significantly different for different genera, even when the genera were in the same family.

Ovarian atresia characterized by, proliferation of follicular theca cells are detected. Similar findings were reported by Wahbi and El-Greisy (2007) for S. rivulatus exposed to different pollutants.

According to polluted L. equulus in lagoon water changes in follicular epithelial layer showed cells hypertrophy and hyperplasis, shrinkaged nuclei, increased number of mitochondria and appearance of vacuoles of variable size in Cytoplasm. Deformed zona radiata are highly present and clearly observed. Most of zona radiata are broken down in several sites invade the oocyte. Polluted showed hypertrophied follicular epithelial cells with picnotic nuclei and degenerated cytoplasmic organellae.

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