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# The Ovarian Cycle of the Fish *Leptobotia elongata* Bleeker, Endemic to China

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**Abstract.-** Histological analysis of *Leptobotia elongata* Bleeker ovaries revealed five stages in oocyte development (oogonia, previtellogenic oocytes, vitelline vacuolated oocytes, vitellogenic oocytes and mature oocytes) based on nucleoli, follicle cells and vitellogenesis. Five time-dependent stages in ovarian maturation were characterized based on the appearance and the color of the ovaries and the gonadosomatic index (GSI). Lipid, protein and glycoprotein content varied with the development of the oocytes. Females captured during periods of ovarian ripening had the highest GSI values, their length ranged from 327 to 375 mm, and their body weight between 326 to 483 g. The seasonal variation of GSI indicated that the spawning season of *L. elongate* was from May to July. This study will underpin future work on the population dynamics of *L. elongata* which, in turn, is required for the management of the commercial resource.

Keywords: Ovarian cycle, oogenesis, histological analysis of ovary, *Leptobotia elongate*, spawning activity.

## INTRODUCTION

*Leptobotia elongata* Bleeker, first named by Bleeker in 1870, lives mainly in the upper-middle reaches of the Yangtze River, and is endemic to China (Liang *et al.*, 2000). It is of commercial importance for its appearance, size and taste (Liang and Hu, 2001). However, its population size has dramatically decreased in recent years because of overfishing and destruction of its habitat (Li *et al.*, 2005). *L. elongata* is currently considered an endangered species by the China Red Data Book of Endangered Animals (CRDB) (Wang, 1998; Yuan *et al*., 2010) and is in need of protection.

Some aspects of the biology of this fish have been documented (Liang *et al*., 2000), but its reproduction has never been studied. Investigating fish reproduction can provide useful information to inform conservation programs designed to maintain or improve fish stocks and for management purposes (Chen *et al.*, 2010; Shabanipour and Hossayni, 2010). It also has important implications for preserving species diversity, especially genetic diversity.

Morphological changes in developing oocytes have been described for several species of teleosts (Grau *et al*., 2009; Chen *et al*., 2010; Lubzens *et al*., 2010; Mohamed, 2010) including changes in the size of the gonad and oocyte, the nucleolus (Thiry and Poncin, 2005), follicular epithelium (Quagio-Grassiotto and Guimaraes, 2003) and development of the yolk (Hartling and Kunkel, 1999). For each species of fish, the development of the oocytes may have unique characteristic features, such as GSI and the size of oocytes, which are different in different species. For example, GSI of *Thunnus alalunga* peaks in April (Grau *et al*., 2009), and that of *Sciaena umbra* peaks in May and June (Chen *et al*., 2010).

The main objectives of this study were therefore: 1) to carry out, for the first time, a histological classification of the maturation stages of the gonads of *L. elongata* in order to investigate its spawning activity, based up seasonal variation in the prevalence of the different gonad developmental stages; 2) to estimate size at maturity on the basis of these criteria in order to clarify the structures of the reproductive system, and to analyze the annual reproductive cycle of this fish by studying the gonadosomatic index (GSI).

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## MATERIALS AND METHODS

*Sample collection*

One hundred and fifty-one female specimens of *L. elongata* were collected legally in the Yalong River of Panzhihua, from June to August 2002 and from April to June 2004, and in the Mudong reach of the Yangtze River, from October 2004 to November 2005. The length of each fish ($L_T$) was measured to the nearest 0.1 cm, body weight ($W_T$) and gonad weight ($W_G$) to the nearest 0.1 g. The length of specimens ranged from 105 to 375 mm, and weights ranged from 13.3 to 483 g.

*Maturity index and reproductive mode*

GSI of each fish was calculated as:

$$\text{GSI} = \frac{W_G}{W_T - W_G} \times 100$$

The spawning season was determined from the monthly changes of the GSI.

*Light microscopy observations*

Fishes were euthanized by carnation extract, weighed, measured and dissected. Ovaries were weighed and GSI obtained. For each specimen, the middle part of the left ovary was fixed in Bouin's solution for 12 h, transferred to 70% ethanol as a second fixing solution, dehydrated in series of graded ethanol and cleared in xylene. Samples were embedded in paraffin wax and sectioned at 6 μm thickness on a rotary microtome. The sections were stained with haematoxylin and eosin (H&E), mounted permanently and observed using light microscopy.

Sections were stained with Sudan III for the detection of lipid, with ninhydrin for the detection of protein. For the detection of glycoprotein, the sections were stained and with periodic acid- Schiff reagent for the detection of neutral polysaccharides.

## RESULTS

*Maturation stages of the ovary*

The ovary lay on the dorsal surface of the gut, ventral to the kidney. The maturation of the ovary was divided into five stages according to the GSI, $L_T$, $W_T$ and the morphology and colour of ovaries.

*Stage I*

$L_T$ was less than 125 mm. Gonads were very small, transparent, and in contact with the kidney. They were thin, elongated, and appeared soft and flabby. Gender could not be distinguished from the outward appearance of the gonads.

*Stage II*

$L_T$ ranged from 125 to 348 mm, and $W_T$ from 13.3 to 338 g. Ovaries were broader and less flabby than at stage I. They were peach colored and no oocytes were visible through the ovary wall. GSI values ranged from 0.02 to 1.48.

*Stage III*

$L_T$ ranged from 205 to 360 mm, and $W_T$ from 78 to 456 g. Ovaries appeared turgid and were orange in color. They increased in length and particularly in width. Oocytes were just visible through the ovary wall. GSI values ranged from 0.50 to 3.11.

*Stage IV*

$L_T$ ranged from 327 to 375 mm, and $W_T$ from 326 to 483 g. Ovaries appeared club-like and milky yellow in color. Oocytes were visible, with a large amount of yolk, irregular in shape because all oocytes were squeezed together. The surface of the ovary was vascularized. GSI values ranged from 14.30 to 20.20.

*Stage V*

Oocytes were large, filling the body cavity, with a large amount of yolk. GSI values ranged from 15.13 to 16.14. Oocytes could be extruded from the body by gentle manual pressure.

*Histology of ovarian development*

The terminology used for staging the individual oocytes was based on their histological appearance, following Wallace and Selman (1981) and De Vlaming (1983). The developing oocytes of *L. elongata* were divided into five stages based on size, color and histology, as follows:

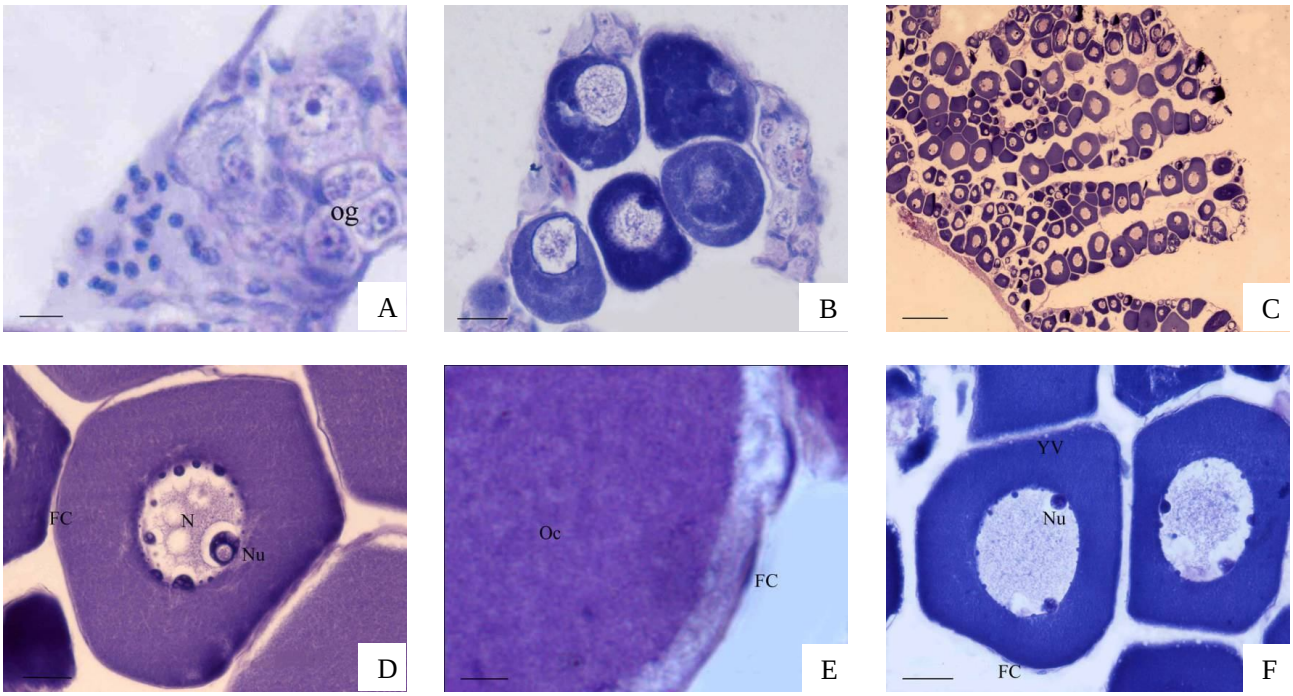


Fig. 1. Histological structure of the oocyte at each developmental stage in *Leptobotia elongata* Bleeker. A, oogonia showing oogonium (Og). B, early phase of previtellogenic oocytes. C, middle phase of previtellogenic oocytes, showing the ovarian wall and germinal epithelium. D, middle phase of previtellogenic oocytes, showing the typical oocytes in this phase. Nu, nucleolus; N, nucleus; FC, follicle cell. E, middle phase of previtellogenic oocytes, showing follicle cell. Oc, oocyte; Nu, nucleolus. F, middle phase of previtellogenic oocytes, showing yolk vesicle (YV). Scale bars: A, 10 µm, E, F, B, 20µm, C, 100 µm, D, 30 µm.

*Oogonia*

The mean diameter of oogonia was 15±2 µm in histological section. A large nucleus approximately 8 µm in diameter occupied the greater part of the oogonia. In the nucleus, a single central nucleolus was visible. Cytoplasm exhibited strong basophily (Fig. 1A).

*Previtellogenic oocytes*

This stage could be divided into early, middle and late phases according to the size and the shape of the oocytes, the appearance of ovarian lamellae, and the size and number of the nucleoli.

*Early phase:* Oocytes displayed a remarkable increase in size, with an intense basophilic ooplasm, a large nucleolus and a high nucleus: cytoplasm ratio (Fig. 1B). The diameters of the oocytes and the nuclei were 43.5-47.9 µm and 21.4-21.9 µm respectively. Ovarian lamellae could not be seen.

*Middle phase:* The germinal epithelium and connective tissue dipped into the ovarian cavity and constituted ovarian lamellae (Fig. 1C). Oocytes were arranged on both sides of this epithelium. The diameters of the oocytes and the nuclei were 72.4-91.1 µm and 41.5-62.1 µm respectively. Two to 21 nucleoli, which were vesicular, adjoined the nuclear envelope (Fig. 1D). The largest nucleolus was ten times larger than the smallest one. In this phase, yolk nucleolus could be found (Fig. 1E). The cytoplasm stained lighter with hematoxylin. Moreover, follicle cells were sporadically arranged around the oocyte (Fig. 1E).

*Late phase:* The oocytes at an advanced stage in this phase displayed a remarkable increase in both size (diameter 91.3 - 124.2 µm) and the number of follicle cells which exhibited a strong nuclear basophily. The diameters of the oocyte nucleoli were 56.4- 93.8 µm. The oocytes in this phase were characterized by the presence of small yolk vacuoles (Fig. 1F).

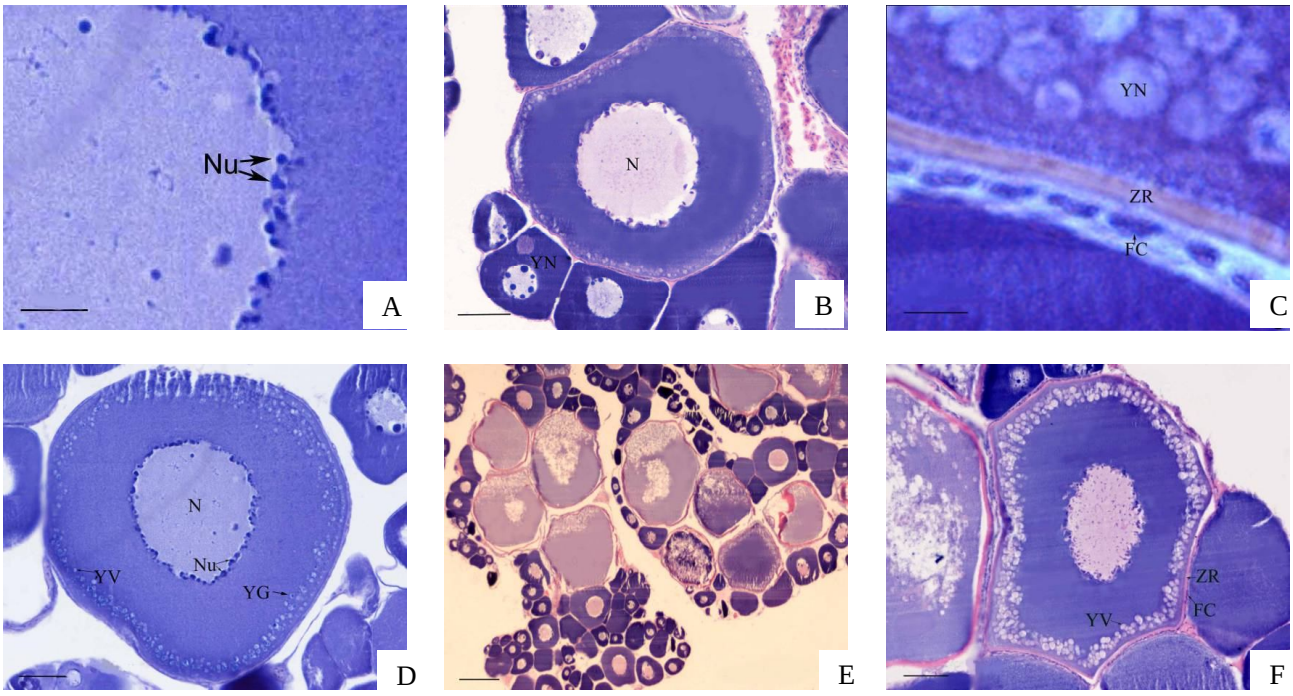


Fig. 2. Histological structure of oocyte at different developmental stages of *Leptobotia elongata.* A, early phase of vitelline vacuole oocytes showing nucleolus located at the periphery of cytoplasm, nucleolus (Nu). B, early phase of vitelline vacuole oocytes, showing one layer of yolk vesicle (YV), yolk nucleolus (YN), nucleolus (N) and yolk vesicle (YV). C, middle phase of vitelline vacuole oocytes, showing 3 layers of vitelline vacuole located in the periphery of the cytoplasm, yolk granule (YG) and yolk vacuole (YV). D, middle phase of vitelline vacuole oocytes a yolk nucleolus (YN), zona radiate (ZR) and follicle cell (FC). E, late phase of vitelline vacuole oocytes, showing more than 3 layers of vitelline vacuole located in the periphery of cytoplasm, follicle cell (FC), yolk vesicle (YV) and zona radiata (ZR). F, late phase of vitelline vacuole oocytes, showing zona radiata with red staining, follicle cell (FC), yolk vesicle (YV) and zona radiata (ZR). Scale bars: A, 20 μm; B, C, E 50 μm; D, 8 μm; E, 200 μm.

*Vitelline vacuolated oocytes*

The oocytes of this period were characterized by the appearance of vitelline vacuoles. The vitelline vacuoles were at first few in number, small in size and appeared at the periphery of the cytoplasm. This stage could be divided into three phases according to the number of the follicle cell layers and the number of the vitelline vacuoles.

*Early phase:* The oocytes displayed a remarkable increase in size (diameter 91.3-124.2 μm), the diameter of nuclei varied from 49.4 to 116.2 μm. The greatest number of nucleoli was 55. The vacuole in the nucleoli was no longer visible (Fig. 2A). One layer of vitelline vacuoles was located at the periphery of the cytoplasm (Fig. 2B). The oocytes exhibited a strong cytoplasmic basophily. A single layer of follicle cells surrounded the oocytes.

*Middle phase:* The diameter of oocytes varied from 281.8 to 423.6 μm with nuclei that varied in diameter between 84.2 and 126.7 μm. The nucleoli were arranged at the periphery of the nucleus. Zona radiata appeared and exhibited a strong acidophilia (Fig. 2). One to three layers of vitelline vacuoles were located in the periphery of the cytoplasm (Fig.2D).

*Late phase:* The oocytes in diameter varied from 644.1 to 701.3 μm with nucleoli that varied in diameter between 236.7 and 306.5 μm. Nuclear membranes were curved. More than three layers of vitelline vacuoles were located at the periphery of the cytoplasm (Fig. 2E,F). There were more yolk granules in the cytoplasm than in the earlier stages. The zona radiata was wider than in the middle phase, and exhibited a strong acidophilia (Fig. 2E). Two layers of follicle cells surrounded the oocytes.

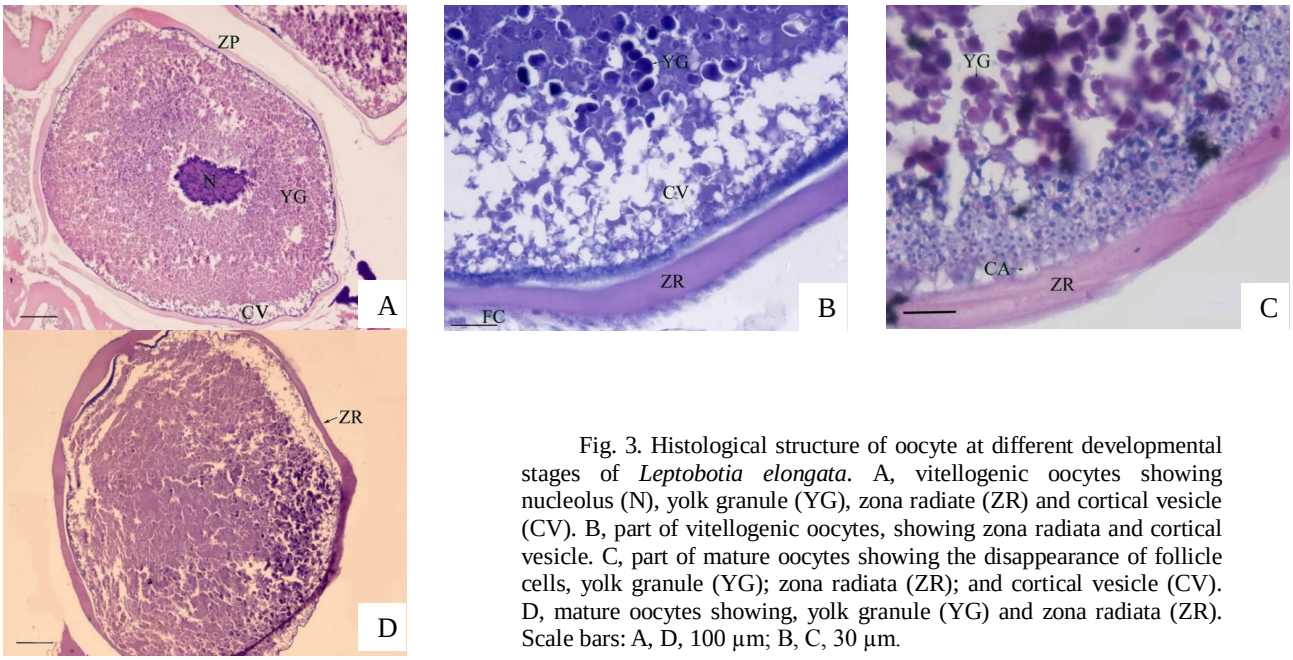


Fig. 3. Histological structure of oocyte at different developmental stages of *Leptobotia elongata*. A, vitellogenic oocytes showing nucleolus (N), yolk granule (YG), zona radiate (ZR) and cortical vesicle (CV). B, part of vitellogenic oocytes, showing zona radiata and cortical vesicle. C, part of mature oocytes showing the disappearance of follicle cells, yolk granule (YG); zona radiata (ZR); and cortical vesicle (CV). D, mature oocytes showing, yolk granule (YG) and zona radiata (ZR). Scale bars: A, D, 100 μm; B, C, 30 μm.

*Vitellogenic oocytes*

Before the onset of vitellogenesis, the oocyte cytoplasm progressively lost its basophilic nature. The diameter of oocytes varied from 780.6 to 947.6 μm. Oocytes were filled with yolk granules, which were scattered in the cytoplasm and varied in diameter from 1 to 7 μm. During this stage the dissolution of the nucleus occurred, and the nucleoli were arranged at the centre of the oocytes (Fig. 3A). Vitelline vacuoles without yolk granules were excluded from the cortical areas and coalesced into a cortical vesicle (Fig. 3B). The oocyte wall consisted of a zona radiata which was 22-32 μm in thickness and coated with three follicular cell layers (Fig. 3B). At this stage the zona radiata layer reached its maximum thickness. The three follicular cell layers were named as the outer thecal layer, the inner thecal layer and the follicular cell layer respectively from outside to inside.

*Mature oocytes*

This period was characterized chiefly by the migration of the nucleus towards the animal pole and the disappearance of follicle cells (Fig. 3C). Oocytes, with diameter ranging from 1012.1 to 1185.8 μm, were at their largest. During this stage, the yolk globules started to coalesce to form yolk plates (Fig. 3D). The zona radiata, remained prominent. Hydrated oocytes were first ovulated, then spawned.

*Gonadosomatic index*

The GSI showed month-to-month variation (Fig. 4), with the mean value ranging from 0.22 to 3.99. GSI increased significantly in May and reached a peak in June, and then dropped sharply in July. The seasonal variation of GSI suggested that the spawning season of *L. elongata* was from May to July.

*Changes of the lipid, protein and glycoprotein*

Cytochemical studies of the oocytes (Table I) revealed that glycoprotein appeared first in the previtellogenic oocytes. Lipid appeared first in the oogonia, increased in the following stages and was most abundant in the mature oocytes. Protein content varied: there was more in oogonia, less in the late phase of previtellogenic oocytes, then more in mature oocytes.

## DISCUSSION

This study represents the first attempt at a detailed histological description of oocyte developmental stages, characterization of the

ovarian cycle and the monthly changes in GSI of *L. elongata*. In addition, the relative levels of substances likely to be of nutrient value were examined.

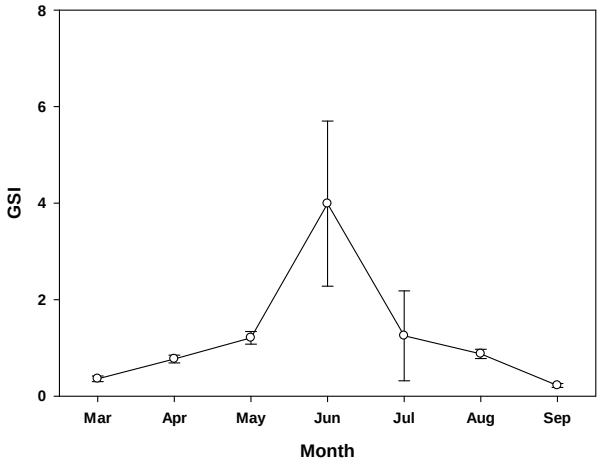


Fig. 4. Monthly changes (mean ± SE) in gonadosomatic index (GSI) of *L. elongata*.

*Patterns of ovarian development*

Ovarian development in most teleosts has been classified as synchronous or asynchronous, according to the growth pattern of the oocytes in the ovary at any one time (Scott, 1987). However, the terms synchronous and asynchronous ovulators describe the two extremes of a continuum, and in teleosts it is likely that all possible strategies exist (Tyler and Sumpter, 1996). *L. elongata* is mainly synchronous.

*Oocyte growth*

In all teleosts studied to date, oocytes undergo the same basic pattern of growth (Tyler and Sumpter, 1996). Developmental events that occur in the oocytes of *L. elongata* are similar to those described for other species (Rickey, 1995; Tyler and Sumpter, 1996; Grau *et al*., 2009; Honji *et al*., 2009; Lubzens *et al*., 2010). In the present study, several criteria were used for characterizing the developing oocytes of *L. elongata*. These included the size, color, as well as the internal morphology, such as the changes of follicle cells, accumulation of yolk granules and the movement of the nucleus. Oogenesis was divided into five stages from oogonia to mature

**Table I.- Histochemistry of staging oocytes.**

| Staining method | Oogonia oocytes | | Previtellogenic oocytes | | | | | | | | Vitelline vacuole oocytes | | | | Vitellogenic oocytes | | Mature oocytes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Early phase | | Middle phase | | Late phase | | Early phase | | Middle phase | | Late phase | | | | |
| | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* |
| Sudan III | + | - | + | - | + | - | + | - | ++ | - | ++ | - | ++ | - | +++ | - | ++++ |
| PAS | - | - | - | - | + | - | + | - | + | - | + | - | + | - | + | + | + |
| Ninhydrin | ++ | + | ++ | + | ++ | + | + | + | + | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ |

*, Cytoplasm; **, Nucleus
Note: "+" represents positive staining. "-" represents negative staining.
PAS, Periodic acid Schiff's reagent.

oocytes. Oogonia grow into immature, previtellogenic oocytes that show signs of vitelline vacuoles in the periphery of the cytoplasm, which will develop into cortical alveoli, and will end when yolk accumulation is completed in the vitellogenic oocyte. Once yolk accumulation is completed, the final maturation process is initiated by migration of the nucleus (De Vlaming, 1983; Redding and Patino, 1993).

*Morphological changes in the nucleolus during oogenesis*

The most prominent features of *L. elongata* nucleoli were the marked vacuolization and changes in their number. The growth and the fragmentation of nucleoli in previtellogenic oocytes are commonly reported in most studied species (Vanganse and Schram, 1972; MacGregor, 1982; Maris and Scheer, 2001; Thiry and Poncin, 2005). The high number of nucleoli observed in early vitelline vacuoleated oocytes of *L. elongata* and their reticulated aspect support the assertion that intense nucleolar transcriptional activity occurs during this oogenetic stage (Thiry and Poncin, 2005). This is in agreement with biochemical and autoradiographical data indicating that massive rDNA transcription occurs during early vitellogenesis in amphibians and teleosts (Vanganse and Schram, 1972; Shah *et al*., 1996). Another prominent feature of the nucleoli of *L. elongata* oocytes is the marked vacuolation occurring in previtellogenic oocytes, also described in another teleosts *Barbus barbus* (Thiry and Poncin, 2005). The vacuolation of the nucleolus appears to be independent of the nucleolar transcription rate.

From a histological perspective, our results indicate that the number of amplified extrachromosomal rRNA genes involved in the transcription increases progressively in previtellogenic oocytes, to reach a maximum level in early vitelline vacuoleated oocytes. It is tempting to speculate that these morphological changes of the nucleolus reflect variations in rRNA synthesis level. At the early phase of previtellogenic oocytes, a few amplified rRNA genes would be transcribed, which give rise to a small number of nucleoli. At the middle phase of previtellogenic oocytes, the activity of these few nucleoli is high, and the nucleoli are vacuolated. Because of the activation of other amplified rRNA genes, the large nucleoli divide into numerous small ones.

*Changes in nutrients of oocytes*

Oocytes accumulate more and more lipid during the whole cycle. Glycoprotein only appears after the follicle cells, indicating that they are actively synthesizing it. Follicle cells absorb low molecular weight substances from the blood, and synthesize higher molecular weight substances which they pass to the oocytes. Oogonia contain abundant protein. Oocytes have no source of protein, but after the follicle cells appear, protein in the oocytes was observed to increase.

*The development of yolk substance*

The development of yolk substance of *L. elongata* is characterized by the following:

1) The yolk nucleolus which is a special structure for the development of yolk substances (Zhang *et al*., 2006). It can be found in the middle phase of previtellogenic oocytes and consists of mitochondria (Browder, 1980).

2) The yolk vesicle, which is first found at the periphery of the cytoplasm in the early phase of vitelline vacuoleated oocytes. After that, a single layer of yolk vesicles form, and then more layers of such vesicles are found.

3) The yolk granules, which are first found in the middle phase of vitelline vacuolated oocytes, accumulating inside the yolk vesicle. More and more yolk granules appear with the development of oocytes and numbers peak at the mature oocyte stage.

*The conservation of* L. elongata

Based on changes in the GSI, *L. elongata* appears to spawn from May to July, with a defined spawning peak between May and June.

As the largest river emptying into the East China Sea, the Yangtze provides habitats and spawning grounds for more than 300 fish species (Zhuang *et al*., 2006). Large dams on the mainstream of the Yangtze River influence the living environments of fishes. Of greater consequence is the fact that more dams and a large water diversion project are planned (Zhang *et al*., 2009). Besides dam constructions, large-scale

reclamation along continental coasts in Asian countries is another serious cause of human impact (Manda and Matsuoka, 2006). In China, tidal flats extending to 723 $km^2$ have disappeared from the estuaries of the Yangtze River in the past 50 years (Tang *et al*., 2003). This could lead to the direct and irrevocable destruction of spawning grounds of fishes. In addition, the number of *L. elongata* has decreased dramatically because of overfishing and damage to feeding place in recent years (Li *et al*., 2005). Consequently, this fish species is in need of protection, especially during the critical spawning season from May to July.

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**Table I,- Histochemistry of staging oocytes.**

| Staining method | Oogonia oocytes | | Previtellogenic oocytes | | | | | | Vitelline vacuole oocytes | | | | | | Vitellogenic oocytes | | Mature oocytes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Early phase | | Middle phase | | Late phase | | Early phase | | Middle phase | | Late phase | | | | |
| | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* | Nuc** | Cyto* |
| Sudan III | + | - | + | - | + | - | + | - | ++ | - | ++ | - | ++ | - | +++ | - | ++++ |
| PAS | - | - | - | - | + | - | + | - | + | - | + | - | + | - | + | + | + |
| Ninhydrin | ++ | + | ++ | + | ++ | + | + | + | + | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ |

*, Cytoplasm; **, Nucleus

Note: “+” represents positive staining. “-” represents negative staining.

PAS, Periodic acid Schiff’s reagent.