The Indian major carp, *Cirrhinus mrigala*, is a seasonal breeder and spawns during monsoon season. On the basis of ovarian weights and histological studies, the ovarian cycle has been divided into four phases: (i) Postspawning period or the resting phase, (ii) Preparatory period, (iii) Prespawning period and (iv) the spawning period. Three types of primary oocytes have been identified in the stained ovarian sections, viz., stage I non-yolky oocytes, stage II vitellogenic oocytes and stage III yolky oocytes. Maximum ovarian weights were recorded during June-July, while maximum plasma vitellogenin titers were observed during May. Ova diameter was maximum during July and minimum during November-December. Morphological changes observed during follicular development include the appearance of many nucleoli and development of egg envelopes. Yolk nucleus or Balbiani's vitelline body, a highly basophilic structure was observed adjacent to the nuclear envelope, which subsequently moved towards the periphery and finally disintegrated. Following vitellogenesis, the germinal vesicle (GV) migrates from a central position to the oocyte periphery and thereafter where subsequently it disappears. The phenomenon is called oocyte maturation. Theca, follicular epithelium and zona radiata surround the growing egg. A micropyle in zona radiata internus was also identified in the yolky and ovulated oocytes. A progressive decrease in the diameter of zona radiata, thinning and withdrawal of microvilli and stretching of the envelope perhaps appear to expell the egg resulting in ovulation.

**INTRODUCTION**

An understanding of the reproductive cycle and breeding mechanisms of fishes is a basic requirement for their successful propagation, management and maximum exploitation of the fishery resources. It is apparent from the literature that more work on ovarian activity has been carried out in fishes inhabiting temperate regions (de Vlaming, 1972) rather than those inhabiting tropical and sub-tropical regions (Guraya et al., 1975, 1977).

Oocytes pass through several distinct stages during development and complete maturation. They undergo previtellogenic and vitellogenic growth phases, during which the oocytes enlarge, follicular layers develop and yolk accumulates (Gorbman, 1983;
When vitellogenesis is complete, further maturational processes occur prior to ovulation. These are referred to as final maturation (Goetz, 1983; Nagahama, 1987).

Following vitellogenic phase or during this phase, nucleus or germinal vesicle (GV) migrates from the central position to the oocyte periphery. Following migration, germinal vesicle disappears, phenomenon is known as germinal vesicle breakdown (GVBD). It is generally assumed that GVBD marks the resumption of meiosis.

In addition to the nuclear events, several cytoplasmic changes also occur during GV migration and breakdown, these include the coalescence of lipid droplets and yolk globules and an overall increase in oocyte translucency. Changes in oocyte translucency during final maturation are probably related to the fusion of yolk globules that occur in many fish species (Wallace and Selman, 1981).

Present studies examines changes in ovarian activity during the annual reproductive cycle in a seasonal breeder, Cirrhinus miraga. Studies supplement the existing knowledge of ovarian changes especially during oocyte growth, maturation and ovulation.

**MATERIAL AND METHODS**

**Fish**

The fish were collected from the fish farm of the Department of Zoology and Aquaculture. Monthly samples were usually obtained during middle of each month for studying the seasonal cycle of ovarian development. Fish were autopsied within 24 hours of collection and a total of 93 fish were used. On arrival in the laboratory the fish were weighed and then killed with a blow on the head. Blood was obtained by caudal sectioning. The plasma collected after centrifugation and was stored at -30°C until used for the determination of vitellogenin concentrations as alkali-labile phosphorus.

Ovaries were dissected out, weighed and fixed immediately in Bouins fixative (18h) for histological studies.

**Histological technique**

Transverse sections from the middle region of the ovaries were cut at 7 μm and stained in Ehrlich’s haematoxylin-eosin. From the few (4-6) selected sections, number of oocytes in various stages of development were counted.

The average diameter of different types of oocytes was calculated from the transverse sections of the ovaries as follows:
50-100 of all types of oocytes were projected on a projection apparatus at known magnification. Mean greatest width at right angles to the two axis of each oocyte was taken as the diameter and then the average diameter was calculated. The average diameter of ova was divided by the known magnification to obtain the actual average diameter of the oocytes and expressed in μm. The oocytes were divided into different types according to their state of development (see results). The histology of every type of oocyte was observed. To avoid possible errors and to estimate the rate of progression through the major development stages some of the oocyte stages were pooled into one particular group. The chromatin nucleolus and perinucleolar stages were pooled as stage I primary oocytes. All oocytes of the different vitellogenic stages (early or late) were counted and pooled as stage II vitellogenic oocytes. The fully formed oocytes were considered as stage III yolky oocytes.

Since Cirrhinus mirigala spawns only under natural conditions in rivers and streams during rainy seasons, oocytes were cultured (for 18-20h) to observe morphological changes during oocyte maturation/ovulation, under in vitro conditions using human chorionic gonadotropin (HCG) or 17α 20 β-dihydroxy progesterone (Trehan and Garg, 1991, 1993). The oocytes from the culture vials were removed at intervals and fixed for histological studies using scanning and light microscopy.

**Scanning electron microscopy (SEM)**

For scanning electron microscopy, oocytes were fixed in 3 per cent cold glutaraldehyde (pH 7.4) for 4 hours. Using a sharp blade, some oocytes were cut into two pieces after fixation and even the oocytes were thoroughly washed in cold 0.1 M phosphate buffer (pH 7.4) cleared of excess of oozing yolk and dehydrated in acetone series to facilitate scanning of the internal morphology. After silver coating, oocytes were examined using a scanning microscope (Phillips PSEM 501B).

After embedding in Epon-Araldite, two-micrometer thin sections of oocytes were also cut and stained in Alcian blue.

**Estimation of alkali-labile protein phosphorus (vitellogenin)**

Alkali-labile protein phosphorus (vitellogenin) was estimated from the plasma (50-100 μl) according to the methods of Wallace and Jared (1968) and Martin and Doty (1949) and expressed as μg of phosphorus per ml plasma. The amount of inorganic phosphorus in unknown solutions was calculated with reference to a standard curve prepared by using monopotassium dihydrogen phosphate (KH₂PO₄) and expressed as μg/ml plasma. The same procedure was applied for the blank and standards.
RESULTS

On the basis of ovarian weights and histology, ovarian cycle of C. nigra at Hisar (Lat. 28°35' N. Long 70°12' E), Haryana can be divided into four phases:

i) Resting phase-postspawning period (September through January),
ii) Preparatory period (February-March),
iii) Prespawning period (May-June),
iv) Spawning period (July through August)

Following the technique of Sundararaj and Sehgal (1970) in Heteropneustes fossilis and Garg and Jain (1985) in Channa punctatus, three types of primary oocytes were identified in C. nigra as follows:

Stage I (Fig. 1) primary oocytes (mean diameter 140±6.0 μm, range 80-214 μm) are the non-yolky oocytes present in the ovary during all seasons of the year; stage II (Figs. 1&2) primary oocytes (mean diameter 245±8.0 μm, range 184-286 μm) are characterised by the presence of a ring of cortical alveoli, an indication of the onset of vitellogenesis; stage III (Fig. 3), primary oocytes (mean diameter 1750±30.0 μm, range 1600-1875 μm) are the fully formed yolky oocytes.

Postspawning period (September-January)

The ovaries were quiescent, appear translucent and occupy less than one fourth of the body cavity. Ovaries weigh the least of all the phases of the reproductive cycle during this period. Histologically ovaries contained oogonia and stage I primary oocytes (Figs. 17&18). The mean diameter of the oocyte during this period was 226.0 μm (range 107-600 μm).

Preparatory period (February-April)

The gonosomatic index start rising rapidly. The process of vitellogenesis is initiated during the month of February, and thus in addition to stage I (98.0%) primary oocytes, a few stage II vitellogenic oocytes (2.0%) could also be observed. During March the number of stage I primary oocytes fell to 86 per cent, while the per cent vitellogenic oocytes rose to 10 per cent. A few (4%) yolky oocytes were also observed during this month. In April, the yolky oocytes increased to 10 per cent (Figs. 17&18). The average diameter of the oocytes rose to about 410.0 μm (range 192-800 μm).

Prespawning period (May-June)

During this period the ovaries were maximally enlarged and highly distended. The weight of the ovaries reached maximum during June and were fully packed with large stage III yolky oocytes. The average diameter of the ova rose to about 1100.0 μm (range 1000-1200 μm).
Spawning period (July-August)

During July, the GSI remained high which was almost equal to that observed during June (Figs. 17&18). Majority of the oocytes were yolked. In August both spent and unspent ovaries were observed, indicating that ovulation/spawning might have occurred in the fish. The average diameter of the ova was highest (1450.0 μm), during these months (range 1100-1800 μm). At the end of spawning period an abrupt fall in gonosomatic index was observed (Fig. 17).

Alkali-labile phosphorus (Vitellogenin levels)

The results of the assay are given in Fig. 1. Vitellogenin concentration increased very slowly, but a dramatic and many fold increase (30-40 μg/ml) was observed during April-May. After spawning (July-August), plasma vitellogenin levels decreased and seasonal minimum levels were observed during the postspawning period from October onwards.

Morphological changes in oocytes during growth and development

During the winter quiescent phase (postspawning, non-breeding period), the regressed ovaries contained only oogonia. These oogonia persist even in the gravid ovaries and therefore, can be seen at all times in the ovarian sections. During previtellogenesis, oogonia consists of scant, deeply stained cytoplasm with a centrally placed nucleus containing one or two basophilic large nucleoli (early perinucleolus stage). With cytoplasmic and nuclear growth of the primary oocyte, a rapid increase in the number of nucleoli occurs and these are seen lying along the nuclear envelope (Fig. 4). This may be referred to as the late perinucleolus stage. Some nucleoli, however, are also seen scattered here and there in the nucleoplasm. At this stage some extruded nucleoli were also observed in the growing previtellogenic oocytes (Fig. 4). Number of nucleoli varies from 30 to 85. Distinct micro and macronucleoli have also been observed: Invariably the number of macronucleoli was higher (21-62) than micronucleoli (10-22).

As the oocyte grow, the basophilia of the peripheral region of ooplasm starts decreasing and basophilia of ooplasm in the immediate vicinity of the nucleus changes into acidophilia. Ooplasm starts showing granulation. The acidophilia of the nucleus increases. Meanwhile, during the late postspawning and early preparatory period BALBIANI'S vitelline body or the 'yolk nucleus', a basophilic structure, develops adjacent to the nucleus, which subsequently encircles it completely and then move towards the cortical ooplasm (Figs. 5&6). During this movement their basophilia becomes progressively reduced. With further growth of the oocyte, BALBIANI'S vitelline body disintegrates and disappears.

Two distinct cellular layers appear around the previtellogenic oocytes, thus forming the follicle. The inner layer of cubical cells is the granulosalata and the outer layer
Fig. 1. Portion of the ovary showing state I primary and early vitellogenic (Stage II oocytes x 200; Fig. 2. Portion of stage II vitellogenic oocyte (State II). Note wavy nuclear envelope. Note nuclear envelope showing evaginations or pockets n = nucleolus, nu = nucleoli, Ca = cortical alveoli x 800; Fig. 3. Portion of the ovary showing stage III yolky oocytes Y = yolk platelets x 800; Fig. 4. Portion of the ovary showing stage I primary oocytes (late perinucleolus stage). Note nucleus (n) with numerous nucleoli of varying sizes lying along the nuclear envelope (Yn = Yolk nucleus) x800; Fig. 5. Pre-vitellogenic oocytes (Stage I), note the developing yolk nucleus (Yn) lying adjacent to the nucleus (n) x800; Fig. 6. Pre-vitellogenic oocytes (Stage I). Note growing yolk nucleus (Yn), migrating towards the periphery and completely surrounding the nucleus (n) (see also inset) x 800.
of flat cells is the theca. A basement membrane separates the two layers. Later on a thick zona radiata with characteristic striations (the pore canals) (or zona pellucida or vitelline membrane) appears between the oocyte and the granulosa (Figs. 7&8). Once the follicle is formed intra-vacuolar yolk deposition in varying diameter occurs in cytoplasm.

As the vitellogenesis advances, number of unstained cortical alveoli increases and they appear to be distributed uniformly in the ooplasm around the centrally placed nucleus. It is during this stage that the enormous increase in cytoplasmic size occurs due to the uptake and incorporation of an exogenously synthesised yolk. At the end of exogenous vitellogenesis number of nucleoli start decreasing and in fully formed yolky oocytes only 10-15 nucleoli could be observed. Along with these changes, nuclear envelope starts assuming wavy and undulating appearance (Fig. 2). Large undulations, however, were usually observed prior to but also during vitellogenesis. Foldings at the surface of the nucleus perhaps marks the end of vitellogenic phase.

When the deposition of yolk is complete (Fig. 3), the acidophilia of the extravacuolar yolk granules and of the nuclear chromatin increases. The acidophilic zone around the nucleus widens and ultimately reaches the margins of the ovum, ooplasm of the yolky ovum also becomes acidophilic.

**Morphological changes during oocyte maturation and ovulation**

The diameter of yolky oocytes varies from 1600-1875 \( \mu m \) (1750+30.0). Oocytes at this stage in this fish appears to be opaque and possess a centrally placed germinal vesicle (Figs. 3,10,11), which remain arrested at the dictiate stage of meiosis I. On stimulation with hormones (gonadotropins or steroids), maturation divisions and other processes are initiated, and the germinal vesicle (GV) which was initially centrally placed start moving towards the periphery and ultimately it disappears. This is known as germinal vesicle breakdown (GVBD). Following coalescence of lipid droplets and yolk globules, which were earlier in the form of yolk platelets (Fig. 3), and on maturation the ooplasm appears to be more homogenous (Figs. 9&11).

In the fully formed yolky oocytes a very clear micropyle could also be observed (Figs. 9,14,15). Micropyle in C. **mrigala** appears to be consisting of a moderately deep pit formed by zona radiata internus with a shallow and short canal terminating at the oolema. A triangular micropylar cell covering the micropyle can also be seen.

Zona radiata which appeared thick (Fig. 8) (diameter ranging from 2.9 to 3.1 \( \mu m \)), before oocyte maturation, appears to be comparatively thin (1.8 to 2.0 \( \mu m \)) in oocytes which had undergone GVBD (Fig. 9). This perhaps represents, shrinking or withdrawal of microvilli, which results in the separation of oocyte (now egg) from the follicular envelope (Figs. 12-13). With further shrinking and withdrawal of microvilli, cracks appear in the follicular envelope which perhaps facilitates ovulation processes (Figs. 12,13,16).
Fig. 7&8. Photomicrographs of Epon-Araldite oocyte sections, fixed in glutaraldehyde, stained in alcian blue. Zp = Zona pellucida, FW = Follicular wall, L = Lipid droplet, Y = Yolk globules (Fig. 7. X840, Fig. 8. X 2100); Fig. 9. Section through the micropylar region. Note Thin zona pellucida. ZPE = External cortex radiata, ZPI = Internal cortex radiata, a = Follicular epithelium, b = Cone cell, c = Canal of micropyle, y = Yolk x2100.
Fig. 10. Oocyte showing germinal vesicle (GV) and yolk (y) x 1200; Fig. 11. Oocyte showing migration of germinal vesicle (GV) from the centre towards the periphery – 12h in vitro incubation in 17α 20β-Dihydroxy-progesterone, Y = Yolk x 1280; Fig. 12. Note formation of a wide space between the follicular wall (FW) and the zona pellucida (ZP) during ovulation, Y = Yolk x 640; Fig. 13. Cut section of the oocyte. Note thin and slender microvilli (mv) making contact with follicle cells (arrow).
Fig. 14&15. In vitro ovulated oocytes showing micropyle (m). Note microvilli (mv)-24 h after in vitro incubation in 17α 20β-Dihydroxy progesterone x80; Fig. 16. Ovulating oocyte – 12 h after in vitro incubation in 17α 20β-Dihydroxy progesterone x80
DISCUSSION

Present studies on *C. mrigala* suggest that it is a seasonal breeder and only one cycle of oogenesis occur each year. Workers in this field have classified the reproductive cycles of fishes according to various features, such as size and weight of gonads, their general appearance, ova diameter, vitellogenesis and ovulation/spawning. On the basis of cyclic changes in the ovarian structure, the reproductive cycle of *C. mrigala* has been divided into four phases (See results). Sundararaj (1981) in *H. fossilis* and Garg and Jain (1985) in *C. punctatus* have also divided the ovarian cycle into four stages; Bhatti and Javid (1973) in *Colisa fasciata*, Al-Daham and Bhatti (1979) in *Barbus luteus* proposed five stages and Combs (1969) proposed eight stages in *Eucala inconstans*. Guraya et al. (1975) proposed seven stages in *Mystus tengara*.

Several phases of oocyte growth can be distinguished during the annual ovarian cycle. During the spawning period in July-August, fully formed yolky oocytes are shed and only gonadotropin independent follicles could be seen in the ovaries. A resting phase coinciding with low temperature during winter intervenes before the gonadotropin dependent vitellogenic follicles make their appearance in the ovaries during the preparatory period in February. Increasing day length and temperature appears to be the primary factor for gonadal recrudescence in *C. mrigala* (Fig. 17). With further rise in temperature and day length vitellogenic follicles are transformed to full grown yolky oocytes during May-June. Seasonal changes in GSI corresponds closely to the development of various stages of oocytes. Levels of plasma alkali-labile phosphorus (vitellogenin) increased steadily during the late preparatory period and the prespawning period and thereafter during the postspawning period it declined to the minimum,
indicating its synthesis and incorporation into vitellogenic oocytes. Development of foldings and undulations at this stage in the nuclear wall perhaps facilitates exchange of nucleoplasmic molecules during oocyte growth. Enlargement of the oocytes during the breeding period takes place as a result of incorporation of vitellogenin (Wallace, 1985; Tyler, 1991) and its accumulation as yolk in the ooplasm.

Morphological changes occurring in the germinal vesicle during previtellogenesis and vitellogenesis are in accordance with previous observations (Nagahama, 1983; Bromage and Cumaranatunga, 1988). Initially one or two nucleoli are present in the germinal vesicle. As the oocyte grows, the number of nucleoli increases, while their size decreases. Formation of many nucleoli reflect amplification of genes leading to protein synthesis, which perhaps facilitates the synthesis of endogenous vitellogenin.

During the postspawning and preparatory period, the ooplasm of oocytes possesses Balbiani’s Vitelline body or the yolk nucleus. Presence of yolk nucleus have been reported in the oocytes of a number of fish species (Guraya, 1979). The appearance of Balbiani’s vitelline body in the oocytes of \textit{C. mrigala} during the late postspawning period and its development during the preparatory period may indicate its involvement with endogenous vitellogenesis. Its disappearance during the subsequent growth of the oocytes or with the onset of exogenous vitellogenesis, perhaps indicates that this structure is no more required. Movements of Balbiani’s vitelline body to the cortical ooplasm and finally its disintegration has been described by many workers (Kraft and Peters, 1963; Shahi \textit{et al.}, 1979) in a number of fish species. Beams and Kessel (1973) had stated that the exact role of yolk nucleus in vitellogenesis is still not clear but that it probably forms an essential precursor substance needed for oocyte growth and vitellogenesis which corresponds to RNA-rich yolk nucleus substance (Guraya, 1963, 1965). Guraya (1979) has further suggested that the main function of the yolk nucleus is to synthesize and distribute its materials during pre-vitellogenesis thus forming the basic cytoplasmic machinery for various other synthetic activities of the oocyte.

During the breeding period, ovaries remain packed with fully formed large yolky oocytes. These oocytes are nucleated and remain arrested at the dictyate stage of meiosis I (Prophase I). At this stage the oocytes are covered over by a thick follicular envelope consisting of an outer epithelium, theca, follicular epithelium and zona pellucida. Presence of similar types of follicular envelops have also been reported in \textit{Channa punctatus} (Guraya, 1985) and other fish species (Guraya, 1986). As the maturation processes proceed, germinal vesicle which was previously located in the centre of the oocyte now starts migrating and finally disappears.
In fully formed yolky oocytes of *C. mrigala* a micropyle is also seen towards the animal pole in zona radiata internus. In *Gobio gobio* also micropyle is formed only by cortex radiatus internus (Riehl, 1977).

Striations of light microscopy seen in zona radiata have been termed as microvilli by various authors (Kraft and Peters, 1963; Guraya, 1965; Guraya et al., 1975). It has been reported that zona radiata undergoes changes during ovum maturation and ovulation. Initially the layer appears to be thick and during the later stages of oocyte maturation it gets disorganized (Flegler, 1977). A progressive decrease in the diameter of this layer has also been observed in the present studies on *C. mrigala*. Microvilli of the large or mature oocytes are thin and slender units than those of the early stages of oocytes. These observations on *C. mrigala* are similar to those reported in other fish species (Selman and Wallace, 1982; Hart and Donovan, 1983). When the fusion of yolk vesicles is complete and nucleus leaves the center of the oocyte, the microvilli are withdrawn from the pore canals. The zona pellucida decrease in thickness and looses its striated appearance. It appears that thinning of zona pellucida after ovulation is partially a result of stretching of the envelope due to increase in the diameter of the egg. The change in shape constrict the follicle at one end and pushes the oocyte out of the opposite end of the follicle.

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