Chapter - I

Circannual ovarian cycle with associated changes in gonadal and extra-gonadal hormones and blood glucose level in the grey mullet *Mugil cephalus* L.
INTRODUCTION

A majority of teleost fishes are seasonal breeders, while a few breed continuously. Among seasonal breeders, there is a wide variation in the timing of breeding. Fresh water temperate zone fishes spawn in spring and early summer, while others such as the salmonids do so in autumn. In the Indian subcontinent, a vast majority of fresh water fishes breed during the monsoon season when rainfall is heaviest (Jhingran, 1975). The time of breeding of each species is so precisely timed that fingerlings are produced in an environment in which the chances of survival are maximal. Natural selection possibly favours genomes of individuals that produce their young ones at a time most suitable for survival.

The circannual reproductive cycle has been extensively studied in the freshwater teleost fishes such as the catfish, *Heteropneustes fossilis*. Many other teleost species of the genus *Sarotherodon* (*Tilapia*) of the equatorial zone, *Tilapia leucostiata*, *Tilapia aurea*, *Tilapia nigra* and *Tilapia zilli* and of tropics, *Tilapia mosambica* and *Tilapia nigra* have also been studied. Others include the common carp, *Cyprinus carpio*, Indian major carps, *Labeo, Catla catla* and *Cirrhina mrigala*, *Cirrhina reba* and Chinese carps. Relatively less information is available in marine and estuarine teleost fishes, such as the rain-bow trout *Salmo gairdneri*, the brook trout, *Salvelinus fontinalis*, mullet, *Mugil cephalus*, snappers (Order / Family : Lutjanidae) and groupers (Order / Family : Serranidae) in the carribean *Prochilodus* and *Brycon* of the central Amazonian flood-plain lakes (Schwassmann, 1978) and milkfish *Chanos chanos* of Hawaiian waters (Kuo and Nash, 1979).

The catfish, a native of India, Burma and Sri Lanka, is a seasonal breeder. Ghosh and Kar (1952) have reported for the first time that the gonadal activity varies
annually in one of the species of the catfish, *Heteropneutes fossilis*, available in the vicinity of Calcutta, India. On the basis of the changes in gravimetry and oocyte diameter, three phases, such as preparatory (February to early April), active (late April to July) and quiescence phases (August to January) have been recognized in the annual ovarian activity of *Heteropneustes fossilis*. But the *Heteropneustes fossilis*, available in the vicinity of Delhi (latitude 28°35'N and longitude 77°12'E) (900 km away from Calcutta) shows 4 phases in the annual ovarian cycle such as, preparatory (February to March), pre-spawning (May to June), spawning (July to August) and post spawning (September to January) (Nayyar and Sundararaj, 1970; Sundararaj and Vasal, 1976).

Subhedar and Rao (1979) have shown a correlation between the gonadosomatic (testis and ovary) index (GSI) and histological features of the corpuscles of stannious (CS) in *H. fossilis*. Breeding is highly variable in carps. In Israel, *Cyprinus carpio* normally breeds in April and May (Moav and Wohlfarth, 1976), but the common carps in India show two main peaks of breeding activity in a year, once in spring and later in autumn when optimal thermal conditions prevail in nature. Whereas in France, the common carp spawns in summer (Billard and Breton, 1978). Bieniarz and others (1978) have reported that carps in Poland attain sexual maturity in 4-5 years and spawn in late May when water temperature reaches 18°C. Atresia of oocyte is associated with the lowest levels of gonadotropin when water temperature is below 14°C.

In an Indian major carp, *Labeo rohita* of Tarai region of Uttaranchal the ovarian cycle passes through five phases i.e. resting, preparatory, pre-spawning, spawning and post-spawning / regression; the resting and preparatory phases are characterized by the Chromatin-nucleolar and yolk-vesicle stage, yolk globule stage with cortical alveoli and differentiating follicular wall in the pre-spawning phase, densely packed yolk-
granules with large masses of yolk vesicles and germinal vesicle movement (GVM) in
the spawning phase, and pre-ovulatory atretic oocytes with significant number of
oogonia in the regression phase. There are four phases of the ovarian or testicular cycle
found both in Notopterus notopterus and Colisa fasciatus (Dalela et al., 1977). The
ovary is enlarged due to accumulation of yolk in the egg. The spawning in both the
fishes occurs during late May and August. In mountain stream teleost, Schizothorax
richardsonii (Grey and Hard), the whole of the ovary, is fertile unlike testis. The
ovarian cycle is divided into five stages. The ovary becomes much enlarged and
distended in the month of October and delicate as well as thin in March. The spawning
season extends from late October to December and the ovary becomes asynchronous
subsequently. The grey mullet of Hawaiian waters is a seasonal breeder and spawns
during January and February. In nature, vitellogenesis starts shortly before attainment
of minimal day-length (Kuo and Nash, 1975). Constant short photoperiod regime of LD
6:18 is effective in stimulating vitellogenic oocytes within 49-62 days, whereas in
controls, vitellogenic oocytes do not appear until 235 days. Thus, ovarian
recrudescence can be triggered out of season by a photoperiod-temperature
combination of LD 6:18 at 21°C (Kuo and Nash, 1975) and consequently the breeding
season of mullet can be prolonged to obtain ripe eggs throughout the year (Kuo et al.,
1974).

Hormonal and other changes

In the female catfish, Heteropneustes fossilis, testosterone (T), estradiol-17 beta
(E2), and estrone (E1) are detectable in the plasma only during the reproductively
active (preparatory through spawning) period and their levels increase during
vitellogenesis. In the fully gravid catfish, when vitellogenesis is nearly complete, levels of E2 decline but those of T continue to increase suggesting a product-precursor relationship between the two steroids (Lamba et al., 1983). Subsequently, Mishra and Joy (2006) have reported that in *Heteropneustes fossilis*, the estradiol-17 beta (E2) concentration is highest in the preparatory phase (April) with active vitellogenic activity and declines significantly across pre-spawning and spawning phases. But the concentration of 2,4-hydroxy estradiol (OHE2) which remains lowest in the preparatory phase, is increased significantly to the peak level in the spawning phase. The steroids are not detectable in the resting and post-spawning phases. E2 and 2,4-OHE2 maintain an inverse relationship in the recrudescent phase. Lamba et al. (1983) have further reported that the gradual enlargement of the gonads with concomitant vitellogenesis or spermatogenesis occur during the late preparatory – pre-spawning period (March to June), when in nature both the daily photoperiod and mean environmental temperature increase progressively. The ovulation and spawning of oocytes or spermiation occur in the monsoon season (July to August), the prime time for breeding in the environmental niche. Seasonal changes in gonadal maturation is evident from gonadosomatic index and diameter of the testicular lobules and egg diameter with the morphometric alterations in the basophilic cell area (BCA) of the pars distalis. The maximum BCA values are preceded with the time of greatest atresia of the eggs indicating the influence of the pituitary gondotropins in the follicular resorption in the test fish.

In another catfish, *Clarias batrachus*, plasma testosterone levels are increased first in the previtellogenic phase (March) and the second in the early vitellogenic phase (May). The levels remain elevated till the early postvitellogenic phase (July) and reach basal levels in the regressed phase (September). Ovarian testosterone levels are very
low without any significant change from the late previtellogenic to the postvitellogenic phases (April to August). But estradiol-17 beta and estrone levels in the ovary are increased significantly in the previtellogenic phase (April), reach a peak in the vitellogenic phase (June) and decline in the postvitellogenic period (July). Ovarian levels of sex steroids are always lower than plasma levels, indicating low storage and rapid secretion. Gonadosomatic index (GSI) is low in the regressed and previtellogenic phases and increased in the vitellogenic and postvitellogenic phases, with a peak in July. Plasma estradiol-17 beta levels correlate with GSI from May to July, and plasma estrone and testosterone levels with GSI in early June and July, respectively (Singh and Singh, 1987). The neurons of pars medialis (pm) and pars ventrocaudalis (pvc) attain their maximum size during spermiation phase in August indicating a possible link between the hypothalamic nucleus (pm and pvc) and the testicular activity in *Clarias batrachus* (Kumar, 1982). The serum calcium level is maximum during the prespawning phase, whereas the prolactin cells show minimum activity during this phase indicating an inverse correlation between the serum calcium level and prolactin cells during the annual ovarian cycle of *Clarias batrachus*. In male specimens there is no change in the serum calcium level and prolactin cells in relation to the testicular cycle (Srivastava et al., 1985). In *Channa striatus* (Bloch) the cyanophils of the promixal pars distalis of the pituitary gland are conspicuous during the reproductive cycle. The cyanophils undergo a process of hypertrophy and granulation, followed by degranulation and vacuolization, emptying for their contents during the post-spawning period. The concentration of glycoproteinaceous contents is poor during the resting phase and higher during the maturation phase (Srivastava and Swarup, 1979). In exotic carp *Carassius carassius*, the activity of adenohypophysial GTH cells are altered with
the annual reproductive cycle (Bhatt and Negi, 1986). The immunoreactivity in the LH cells is moderate in the resting phase, increased in the preparatory and pre-spawning phases, and reduced in the spawning. Furthermore, the beta-endorphin immunoreactivity (bEP-im) is reduced in the nucleus lateralis tuberalis (median) (NLTm) neurons during the preparatory through spawning phases; the neurons of the (NLT) is negatively linked to the seasonal gonadal cycle and show correlation with the number of luteinising hormone (LH) cells in the pituitary gland and ovaries in the teleost, Cirrhina mrigala. They also suggest that the peptide in the NLTm and NLT may show functional duality during the spawning phase (Sakharkar et al., 2006). Expression of LH-beta and FSH-beta mRNA and the status of gonads and secondary sexual characters have been investigated in the annual gonadal cycle in male and female three-spined sticklebacks, Gasterosteus aculeatus a species in which the development of male secondary sexual characters and spermatogenesis are separated in time. In males, 11-ketotestosterone (11KT) level reaches a peak in May. Both testosterone (T) level and the gonadosomatic index in females increase in April and peak in May as well. Later in summer, after the breeding season, these features are declined. In females, LH-beta expression, followed the GSI and T levels closely, increases to a peak in late May and declines to low levels in July through winter and early spring. FSH-beta expression peaks earlier in January and declines slowly over spring. In males, LH-beta expression peaks in May. During June-September, when spermatogenesis is active, LH-beta levels are very low. FSH-beta expression peaks in January, earlier than LH-beta expression and reaches lowest levels in July. Thus, when spermatogenesis starts at the end of summer, the expression of both GTH-beta mRNAs and circulating 11KT, are at their lowest levels. Moreover, seasonal fluctuations in lipid
contents of the ovary and liver, and cholesterol contents of the ovary, liver and serum in relation to annual reproductive cycle in *H. fossilis* has been noticed (Singh and Singh, 1979). They also have reported that clomid, sexovid, prostaglandin E₁ (PGE₁) and prostaglandin E₂ (PGE₂) alpha are seen to affect cholesterol level indirectly and trigger gonadotropin secretion which in turn probably accelerates cholesterol utilization in the ovary for increased steroid hormone output. In *Labeo rohita*, histophysiologically the biosynthetic activity of the liver is augmented during the preparatory and pre-spawning phases that well correlates with the gonadal development (Singh *et al.*, 2005). The increasing water temperature and day-length seem to be favourable for the gonadal growth (Singh *et al.*, 2005). In *Cirrhina mrigala* the ovary grows rapidly in the preparatory (February to April) and pre-spawning (May to June) phases, attains maximum size during spawning (July to August) when ovulation occurs, and is regressed subsequently in the resting phase (December to January) (Sakharkar *et al.*, 2006). In female gudgeon *Gobio gobio* L. (Teleostei, cyprinidae), a fish which has an asynchronous-type ovary containing oocytes at various stages of development and spawns several times during the reproductive period. Plasma levels of estradiol-17 beta, testosterone, and 17 alpha-20 beta-dihydroxy-4-pregnen-3-one (DHP) remain low from October to April and increase rapidly in May and are maintained during spawning when vitellogenic oocytes are alongside oocytes in final maturation. Fish in the regressive phase also shows high steroid levels. The vitellogenic oocytes in prevulatory atresia and the postovulatory follicles may be responsible for these events (Rinchard *et al.*, 1993). In *Glossogobius giuris* (Ham) degranulation of the basophils from the proximal pars distalis of the pituitary gland indicates release of hormones (GTH) during the spawning period (June to September) unlike in the regressed gonads. In absence of
natural spawning due to scanty monsoon rains, gonadal regression is delayed and the sex steroids persist in the plasma well beyond the normal spawning season (Saksena, 1980). In the female spotted-seatrout, the mean serum binding protein (SBP) level changes with mean gonadosomatic index, hepatosomatic index, and plasma estradiol concentration, suggesting that the plasma sex-hormone binding protein (SBP) may have an important role in the reproductive cycle (Laidley and Thomas, 1997). In the spotted seatrout, *Cynoscion nebulosus* there are significant correlations between plasma estradiol and ERc and between ERn and plasma vitellogenin during the period of ovarian recrudescence (January-May), but not during the remainder of the reproductive season (June-September). Estrogen-receptor concentrations and plasma vitellogenin titers do not fluctuate during the remaining stages of the ovulatory cycle (hydration, ovulation, and spawning). In addition, both hepatic ERc and ERn are elevated for the rest of the seasonal reproductive cycle, during which several ovulatory cycles may occur in this multiple-spawning species. Plasma estradiol titers decline in August, near the end of the reproductive season, prior to the decline in estrogen-receptor concentrations. The persistence of the estrogen receptor during the ovulatory periods, the hepatic responsiveness to estrogenic stimuli is not diminished and thus vitellogenin synthesis, if interrupted at all, could be resumed soon after spawning (Smith and Thomas, 1990). The Lusitanian toadfish has group synchronous oocytes, which grow from November until June-July when they are released probably as a single batch. Blood plasma levels of estradiol-17 β(E2) and testosterone (T) increase during vitellogenesis and drop rapidly during final maturation and ovulation, when 17α-, 20β-, 21-trihydroxy-4-pregnen-3-one (17α, 20β, 21-P) levels are increased. Testosterone, 11-ketotestosterone (11KT) and 17, 20 alpha-dihydroxy-4-pregnen-3-one (17α, 20 alpha-
P) are generally low except for a sharp peak in June. 17, 20 beta, 21-P also peaks in June and then declines slowly. 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-P) is undetectable in males and females (Modesto and Canario, 2003).

Female Korean spotted sea bass *Lateolabrax maculatus* does not mature and spawn in net culture, but spermiation occurs spontaneously in natural spawning season. However, hCG treatment can induce oocyte maturation and ovulation in the cultured fish. GSI begins to increase in September, reaches peak in mid November and sharply declines in late November. Tertiary yolk stage oocyte persists longer in the cultured fish than the wild fish and continues even after breeding period in the cultured fish. Serum estradiol 17-β (E₂) correlates with ovarian development, increases in September, reaches peak in October and early November, and decreases in mid-November and late November. Serum 17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-P) maintains low level and is not significantly altered during the experimental period (Lee and Yans, 2002).

**Environmental influence**

A significant increase in ovarian weight and in the formation of yolky oocytes occurs during the preparatory phase (February-April) by exposing the catfish *H. fossilis* to photperiod regime of LD, 12:12 or 14:10 for 36 days with the water temperature at 25°C or 34°C. But ovarian recrudescence with the increase of ovarian weight and concomitant vitellogenesis occurs even in the post spawning catfish in November exposed to 30°C for 60 days, irrespective of photoperiod (Vasal and Sundararaj, 1976). Exposure of males and females to long photoperiod (LD, 14:10 or 18:6) at 19° to 30°C causes gonadal recrudescence in males that attain maturity earlier than females. In *Catla catla*, long photoperiod (LD : 16:8) during the preparatory phase (February-
March) increases serum vitellogenin and 3β- and 17β-hydroxysteroid dehydrogenase activities without any change in oocytes stages or ovary weight, and stimulates them further up to the pre-spawning phase (April-May), without any change during spawning or post-spawning phase. But short photoperiod (LD : 8:16) inhibits ovarian growth or maturation during pre-spawning or spawning without any change in other phases in *Catla catla* (Dey et al., 2005). Circannual ovarian rhythm persists even after exposure of mature female catfish to continuous illumination (L:L) or darkness (D:D) at 25°C, but the acrophase (period when ovarian weight is maximum) occurs earlier in LL than in DD or NL (natural light) (Sundararaj et al., 1978). Interruption of the scotophase (dark period) of a short day (LD, 6:18) by 1 hour of light induces ovarian recrudescence to a greater extent than that of the control group exposed to LD 7:17, indicating circadian rhythm of photosensitivity with two sensitive periods occurring between 16-17 h and 20-21 h after the onset of the daily photoperiod (Vasal and Sundararaj, 1975).

Precociously gravid catfish, obtained by photothermal treatment as early as in April, have been induced to spawn in the laboratory by administering ovine LH. The spent fish when subjected to the same photothermal treatment develops fresh crop of yolky eggs within one month. Consequently, the same set of fish can spawn four times between April and July of the same year (Anand and Sundararaj, 1974; Sundararaj and Vasal, 1973). Increasing photoperiod from LD 4:20 to 14:10 and finally to 20:4 causes female fish to mature earlier than males, whereas at elevated temperatures (27.1° to 31°C), males mature earlier than females (Verghese, 1970). But in autumn-spawning rainbow trout *Salmo gairdneiri* and brook trout *Salvelinus fontinalis*, photoperiod appears to be the major environmental factors involved in gonadal development. In brook trout, accelerated light regime of increasing photoperiod followed by decreasing
photoperiod results in precocious functional maturity (Htun-Han, 1977). A few marine and estuarine temperate-zone fishes have been studied. In the viviparous seaperch, *Cymatogaster aggregata*, males and females respond differently to different environmental cues (Wiebe, 1968). Warm temperatures and short photoperiods of late summer accelerate ovarian recrudescence, while final oocyte maturation occurs under cold temperatures. But in males, cold temperatures favour spermatogonia formation, while warm temperatures and long photoperiod stimulate later stages of spermatogenesis. In the winter-spawning estuarine gobid fish, *Gillichthys mirabilis*, temperature is the primary environmental cue; cool temperatures stimulate gonadal recrudescence, whereas warm temperatures induce gonadal regression (de Vlaming, 1972). In the dab, *Limanda limanda*, exposure to accelerated photoperiod at 11°C in six months, similar in pattern to a normal year’s cycle, results in the formation of ripe eggs 2-3 months ahead of the season, while in turbot, *Scophthalmus maximus* and *Solea solea*, long photoperiod (LD 18:6 or 20:4) induces formation of ripe eggs (Htun-Han, 1977). In the rabbit fish, *Siganus caniculatus*, long photoperiod (LD, 18:6 at 20°C) retards gonadal development in both sexes, while short photoperiod (LD, 12:12) does not (Lam and Soh, 1975). In the common carp, *Cyprinus carpio*, the pattern of reproductive cycle is dependent more on temperature than on photoperiod. Warm temperature (23°C) accelerates ovarian recrudescence and spawning (Gupta, 1975). In India, the common carp shows two main peaks of breeding activity in a year, once during spring and again in autumn where optimal thermal conditions prevail in nature. Whereas in France, the common carp spawns in the summer and pituitary gonadotropin concentration increases in spring at the time of gonadal recrudescence and in the spawning season (Billard and Breton, 1978). But plasma gonadotropin levels increase
in late winter and decrease in spring when ovarian weights are highest (Billard et al., 1978). Thus, in the carp, the gonadal development is associated with increasing temperature and spawning occurs when the temperature is at its maximum (Billard and Breton, 1978). In Poland, carps attain sexual maturity in 4-5 years and spawn in late May when water temperature reaches 18°C. Fresh vitellogenesis starts within 2 months after spawning and is associated with high serum level of gonadotropin in July and September. Thus, the serum gonadotropin level remains low but increases at the time of spawning. Atresia of oocyte is associated with the lowest level of gonadotropins when water temperatures are below 14°C. Indian major carps, *Labeo rohita*, *Catla catla* and *Cirrhina mrigala* show gonadal recrudescence, as early as March in Assam (Latitude ca 25°N), June in Orissa (Latitude ca 20°N) and July in other parts of India depending on their geographical location (Jhingran, 1975). Indian major carps show gonadal recrudescence from March to June, at a time when both photoperiod and temperature are increasing. Chinese carps reared in temperate and tropical climates attain sexual maturity earlier in the tropical (2 years) than in the temperate waters (10 years). Warm temperatures and long photoperiod coupled with good diet are responsible for accelerated growth and gonadal maturity. In West Bengal and Madhya Pradesh, Indian major and Chinese carps spawn in wet ‘bundh’ type tanks where fluviatile conditions are simulated during monsoon rains (Jhingran, 1975). Indian major carps, *Labeo rohita*, *Catla catla* and *Cirrhina mrigala*, breed only once a year during the monsoon season, spawning can be induced in the spent fish by hypophysation in June (Bhowmick et al., 1977). Chen and others (1969) have reported multiple spawning cycles in a single year in Chinese carps in Malaysia. Bighead carp and silver carp have been spawned almost at monthly intervals in Malacca (Harvey and Hoar, 1979).
Objectives

Literature review reveals that substantial information is available on the seasonal gonadal activity studied mostly in fresh water teleosts including common carps. Such information is rather inadequate in marine or estuarine teleosts, especially in migratory marine teleosts like the grey mullet, *Mugil cephalus*. Furthermore, the earlier studies were carried out mostly with GSI, oocyte diameter and gonadotroph cells together with vitellogenin and few plasma hormones (mainly sex steroids), but not all the reproductive hormones in any single fish species. Additionally, none of the earlier investigators studied extra-gonadal hormonal concentrations during the seasonal gonadal cycle in any single fish species including *Mugil*. Consequently, the interrelationship of reproductive hormones with the extra-gonadal hormones during the circannual ovarian cycle is not clearly known in fish including marine teleosts. Ultrastructural changes of the vitellogenic follicles are lacking in teleosts especially in grey mullets. In the current thesis, these topics were investigated by studying GSI, oocyte size and oocyte population, histology, electron microscopy, gonadal and extra-gonadal hormones and blood glucose level (barring vitellogenin) during the circannual ovarian cycle in a migratory marine teleost grey mullet *Mugil cephalus*.
MATERIALS AND METHODS

(a) Specimen collection

Female grey mullets *Mugil cephalus* L. (Fig. 1) were available plenty from the Alampur estuarine Fishery station, located 13 km away from the Bay of Bengal at Digha, East Midnapore, West Bengal, India (Fig. 2). Ten specimens were collected on day 15 of each month, once, round the year for two consecutive years. Water temperature, dissolved O₂, salinity, pH, hardness, PO₄ and suspended particles of the huge estuarine water bodies are presented in Fig. 3.

(b) Blood and tissue collection

Specimens were freshly caught from the Alampur Fishery station by the drag net and anaesthetized with intraperitoneal injection of sodium barbital. Blood was collected from heart of each specimen. Serum was collected and stored at -20°C until assayed for hormones and blood glucose.

(c) Gravimetry

Body weight of the specimens was recorded by a single pan balance (Yamato, Japan) each month throughout the year for 2 consecutive years. Paired ovary weight was recorded by a single pan Mettler balance (Switzerland) month-wise throughout the year for 2 consecutive years. Gonadosomatic index (GSI) was calculated by the method of Render *et al.* (1995) (*Ovary wt* / *Body wt* x 100) and determined in percent.
(d) Ovarian follicles

Five mg of the freshly collected ovary was weighed by a single pan Mettler balance (Switzerland). Follicles were immersed in 0.65% NaCl solution and were separated by needles under binocular. Unfixed and unstained follicles were counted under microscope.

i) Follicle size: Largest follicular size was measured under microscope (x 100 magnification) by oculometer. At least 100 such follicles selected at random from different area of the ovary of each specimen were measured. Five specimens were considered for each month. Follicular diameter (µm) was calculated using stage micrometer.

ii) Follicle population:

(a) Follicle population (per 5 mg of ovary): Total number of follicles present in 5 mg of ovary (left side) were counted for each month round the year. Five specimens were considered for each month.

(b) Total follicle population (per paired ovary of each specimen): Total follicle population per paired ovary of each specimen was calculated from the population present in 5 mg of the ovary multiplied by paired ovary wt in mg. Five specimens were considered for each month.

(c) Total follicle population (per microscopic field): Follicular populations were counted from pre-breeding, breeding and post-breeding phases of the mullet. Ten random sections of the ovary of each specimen from five specimens were considered for each phase.
Follicle populations of 5 specimens were averaged and mean value with standard error of the mean were determined.

(e) Histology

Ovary was quickly dissected out and fixed in Bouin’s fluid and processed for routine microtomy. Five μm thick paraffin sections were cut by Microtome (Reichert-Jung, model 2030 BIOCUT, Cambridge Instruments, West Germany). Paraffin sections were deparaffinized and stained by haematoxylin and eosin solutions for histological study during pre-breeding (October), breeding (November) and post-breeding (June) phases of the ovarian cycle of the mullet.

(f) Transmission Electron microscopy

Ovarian follicles were studied during pre-breeding (October) and breeding (November) phases of the ovarian cycle of the grey mullet.

The mullets were anaesthetized with intra-peritoneal injection of sodium barbital and perfused intra-cardinally first with normal saline (0.65% NaCl) for 10 minutes and then with 2.5% paraformaldehyde for 20 minutes. Ovary was removed, cut into small pieces (1x1 mm²) and fixed by immersing in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 8 hours at 4°C. After washing in the buffer, the tissue samples were post-fixed in 1% osmium tetraoxide (OSO₄) for 1 hour at 4°C. Tissues were dehydrated through ascending grades of acetone, infiltrated and embedded in araldite CY 212. Thick sections (1 μm) were cut by ultracut E (Reichert, Austria), stained with toluidine blue and observed under a light microscope. Ultrathin sections (60-80 mm), cut by the ultratome E, were contrasted with uranyl
acetate and alkaline lead citrate, and viewed under a Morgagni 268D transmission electron microscope (Fei Company, Eindoven, The Netherlands) at an operating voltage of 80 KV. The total width of the zona interna and zona externa of the late vitellogenic and post-vitellogenic follicles were measured using the soft-wave (“Soft-Imaging System” GmbH, Münster, West Germany) equipped with the microscope.

Quantitation of Hormones:

(a) Reproductive hormones:

The following hormones were estimated once a month (15th) throughout the year for 2 consecutive years. All the hormones were assayed by ELISA, except estradiol 17β, testosterone and DHP which were assayed by RIA.

FSH

Serum FSH was quantitated by a microplate immunoenzymometric assay (IEMA/ELISA) kit (Eliscan FSH, RANBAXY, India) following the method of Odell et al. (1981). In this assay, microtitration wells were coated with high affinity monoclonal antibodies in excess. 50 µl of the appropriate serum reference and test samples containing FSH were added to the assigned wells. 100 µl of FSH-enzyme conjugate solution was added to each well. The microplate was swirled gently for 20-30 seconds, covered and incubated for 60 minutes at room temperature (25°C). The contents of the microplate were discarded and blotted with absorbent paper. 300 µl of wash buffer was added and discarded, and this procedure was repeated thrice. 100 µl of working substrate solution, containing tetramethyl benzidine (TMB) and hydrogen peroxide, was added to each well and incubated at room temperature for 15 minutes. 50 µl of stop
solution (1N HCl) was added to each well and gently mixed for 15-20 seconds. The absorbance of the content of each well was recorded at 450 nm using a reference wavelength of the colour developed in each well indicated the presence of FSH. Using standard FSH, a standard curve of the concentration of FSH against OD was plotted from which the levels of FSH (µIU/ml) present in the test samples were calculated. Specific cross reactivity was obtained at 75% level. Coefficients of intra-assay and inter-assay variations of hormones were noted at 6% and 10% respectively.

**LH**

Serum LH (µIU/ml) was quantitated by IEMA/ELISA using an assay kit (Eliscan LH, RANBAXY, India) as followed for FSH (Kosasa, 1980). Specific cross reactivity was observed at 75% level. Coefficients of intra and inter-assay variations were recorded at 5% and 8% respectively.

**Prolactin (PRL)**

Prolactin (PRL) was quantitated from blood serum by a microplate immunoenzymometric assay (IEMA) (Eliscan prolactin (PRL), RFCL Ltd. SIDCUL, Hardwar, Uttarakhand, India) following the method of Balagura et al. (1979).

50 µl each of the calibrators and blood serum samples were added to the assigned wells of the microplate. 100 µl of the enzyme conjugate was added to each well. The microplate wells were shaken gently for 30 seconds to mix and cover, and was incubated at room temperature (25°C) for 60 minutes. The contents of each well were aspirated and the wells were filled completely (300 µl) with the diluted washing solution. This procedure was repeated thrice. After the last wash, all the wells of the
microplate were blotted on absorbent tissue to remove excess liquid from the wells. 100 μl of working substrate solution was added to all the wells. The microplate wells were incubated at room temperature (25°C) for 15 minutes. 50 μl of stop solution (1 N HCl) was added to each well and mixed for 20 seconds. All the additions of reagents/others were followed in the same order to minimize error due to time difference between the wells. The absorbance of each well was read at 450 nm (using a reference wave length of 620-630 nm to minimize well imputations) in a microplate reader. The absorbance values of the calibrators and test samples were calculated and plotted a point to point curve by plotting the absorbance of each calibrator of Y axis against concentration of each calibrator on the X axis. Using the absorbance value for each sample, the corresponding concentration of prolactin in ng/ml was determined from the standard. Specific cross reactivity was observed at 73% level. Coefficients of intra-assay and inter-assay variations were 7% and 9% respectively.

Extraction and assay of estradiol 17β, testosterone and DHP :

**Steroid extraction procedure** :

Steroid from plasma was extracted with diethylether (DEE). 200 μl of plasma were taken and 3 ml of DEE were added to it. All the tubes were then vortexed vigorously. The aqueous layer was then frozen by keeping the tube overnight at -20°C. The ether solution was then decanted into tubes and the solvent were dried in air. This process was repeated thrice for maximum extraction. Extraction efficiency, averaging 85% was monitored with the addition of 500 cpm of tritiated steroid prior to extraction. Steroid in tubes were diluted by adding 1 ml steroid assay buffer (SAB) [0.01 M sodium phosphate buffer with 0.6% sodium chloride, 0.1% sodium azide and 0.1%
gelatin]. Tubes were kept for 15 minutes at 60°C followed by vortex for 30 seconds to bring the steroid completely in the solution.

*Borohydride Reduction for DHP:*

The method for extraction and estimation of DHP from incubation medium was same as described earlier (Scott *et al.*, 1984; Sen *et al.*, 2002; Mukherjee *et al.*, 2006). A 25 µl aliquot of 17α-hydroxy [1,2,6,7-3H] progesterone (1.88 µCi, dissolved in toluene-ethanol; 9:1 v/v) was dried under a stream of nitrogen in a glass tube. The steroid was dissolved in 200 µl of a solution containing 4 mg sodium borohydride in 5 ml distilled chilled methanol followed by shaking for 1 hr at 4°C. In the next phase 1 ml of 1% acetic acid was added to the tube and dried under a stream of nitrogen to evaporate methanol. This was followed by the addition of 5 ml dichloromethane (DCM). The organic phase was washed sequentially with 200 µl of distilled water, 1.0 M sodium hydrogen carbonate and further three times with water. The product was then extracted with DEE (2x2.5 ml) under a stream of nitrogen. The extracted steroid was redissolved in n-hexane and applied to one channel of silica gel G-coated (0.25 mm thickness) TLC plate. In other two channels of TLC plate, cold 17α-hydroxy-progesterone (17α-p4) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) were applied. Plate was then developed in benzene-acetone (4:1, v/v) solvent system. The positions of the steroids and their metabolites were detected by UV absorption at 254 nm, visualized in iodine vapour, or spraying with 10% molybdophosphoric acid in ethanol and heating for 5 minutes at 100°C (Kime *et al.*, 1992).

Two radioactive spots corresponding to position of 17αP4 and DHP were found on channel where steroid extracted after borohydride reduction was spotted. These were
scrapped off separately and steroid from the silica gel was eluted with a mixture of 4 ml DEE and 500 µl distilled water. After separating and drying down the diethylether phase, the steroid was redissolved in 1 ml of absolute toluene-methanol (1:1, v/v) mixture. Under this condition almost 70% [3H]-17αP4 was converted to [3H]-DHP and the tracer was stable for at least 6 months.

The antiserum of DHP was highly specific. It cross reacted with DHP, 5β-pregnen-3α,17α,20β-triol, 5α,3α,17α,20β-P, progesterone, 17α-hydroxyprogesterone, testosterone and 17β-estradiol at 100%, 42%, 4%, 0.001%, 0.01%, 0.25% and 0.01% respectively.

The specificities of the DHP antiserum, as determined by their cross reaction with a wide range of synthetic steroid, was characterized by Professor John Y. L. Yu, Institute of Zoology, Academia Sinica, Nankang, Taipei, Taiwan R.O.C. This antiserum is highly specific and cross-reacted in less than 0.01% with most of the steroids tested so far. Cross reactivity was noted at 82.80% for 5α-pregnen-3β,17α,20β-triol and 22.63% for 5-pregnen-3β,17α,20β-triol and 9.93% for 5α-pregnen-3α,17α,20β-triol. The sensitivity of the assay was 6 pg/tube. The intra-assay and inter-assay co-efficient of variations were 5.25% and 6.5% respectively.

**Determination of 50% binding:**

To determine the precise amount of antibodies necessary for 50% binding, a fixed concentrations of [3H]T or [3H]E₂ or [3H]DHP was incubated in duplicate with serial dilution of respective antibodies 1:100 – 1:40,000) in polypropylene test tube. Each tube contained 100 µl of incubation buffer, 100 µl of [3H] steroid (cpm 10,000 approximately) and 100 µl of steroid antibodies of different dilutions except that tubes
kept for total count and non-specific counts. The final volume of assay mixture was 300 µl in each tube. The tubes were then vortexed gently and incubated at 40°C for 18 hr followed by 30 min incubation in ice bath.

Separation of bound and free steroids:

Using dextran-coated charcoal suspension (0.625% charcoal and 0.0635% dextran T-70) bound and free steroids were separated. To ensure thorough particulate suspension, the flask containing dextran-coated charcoal was stirred for half an hour at 40°C and 200 µl of chilled homogeneous charcoal suspension was added rapidly to the tubes kept for total count, where the similar volume of assay buffer was added. Tubes were quickly vortexed and kept under ice for 15 minutes, followed by a centrifugation at 3000 x g in a refrigerated centrifuge for 10 minutes. Total 500 µl of the supernatant was taken carefully into a counting vial containing 5 ml of scintillation fluid and counted in a liquid scintillation counter (Beckman, LS 6000 SC). Approximately 50% binding of added count (total count) were observed at dilution of antibody 1:3000 for T, 1:5000 for E$_2$ and 1:10,000 for DHP. Using the above mentioned protocol, antibody dilution standard curves for each steroid were prepared. Testosterone, E$_2$ and DHP contents of unknown samples were determined from standard curve by using the following formula: $B - bo / Bo - bo \times 100$ where B is the binding with the inhibitor, bo is the non-specific binding and Bo is the binding without inhibitor.

Assay Procedure:

Testosterone (T) and estradiol-17β (E$_2$) levels were quantitated by RIA as described by Korenman et al. (1974) and Mukherjee et al. (1994). Briefly, T and E$_2$
contents of the extracted steroid materials were incubated with phosphate buffer saline (10 mM sodium phosphate and 0.15 mM NaCl, pH 7) containing 0.1% gelatine, T or E\textsubscript{2} antiserum and \[^{3}\text{H}]T or \[^{3}\text{H}]E\textsubscript{2} for 18 hour at 40°C. Bound and free \[^{3}\text{H}]T or \[^{3}\text{H}]E\textsubscript{2} were separated by using dextran coated charcoal (0.6% activated charcoal, Norit A and 0.06% dextran). An aliquot of bound \[^{3}\text{H}]T or \[^{3}\text{H}]E\textsubscript{2} was added to 5 ml scintillation fluid and then counted in a liquid scintillation counter. The sensitivity of the assay was 8 pg/ml for T and 5 pg/ml for E\textsubscript{2}. The intra-assay co-efficient of variation was 5% for T and 9% of E\textsubscript{2}, and the inter-assay co-efficient of variation was 8% for T and 12% for E\textsubscript{2}. Results were expressed as pg of T or E\textsubscript{2} per ml of plasma or T or E\textsubscript{2} released per ml of the medium.

The basic procedure for the estimation of DHP was same as T and E\textsubscript{2} using 17\alpha,20\beta\textsubscript{[1,2,6,7,\textsuperscript{3}H]-dihydroxy-4-pregnen-3-one and an antiserum against 17\alpha,20\beta-dihydroxy-4-pregnen-3-one at 10000 dilution.

Intra- and inter-assay coefficients of variations were recorded at 8% and 10% respectively for E\textsubscript{2}, 9% and 11% respectively for testosterone, and 9% and 12% respectively for DHP.

(b) Pineal indoleamines (Serotonin, N-acetyl serotonin and melatonin) (by fluorometric method):

Serum indolamines (serotonin, N-acetylserotonin and melatonin) were measured fluorometrically by the method of Miller and Maickel (1970). 1 ml of serum was taken and added with 1 ml of 0.05 N NaOH, 6 ml chloroform and vortexed. The organic phase contained 5-methoxy tryptamine and melatonin, and the aqueous phase contained serotonin, NAS, 5-hydroxyindole acetic acid and 5-methoxyindole acetic acid. 2.5 ml
of the aliquot (chloroform phase) containing melatonin was added with 8.0 ml of $N$-heptane, 0.3 ml of 5 N HCl and vortexed. Melatonin was transferred to the acid phase. 0.2 ml of the aliquot with the acid phase containing melatonin was added with 0.67 ml of 10 N HCl (containing 15 mg of ortho-phthal aldehyde (OPA) as fluorescent compound in 100 ml of 10 N HCl) and vortexed. The mixture was heated in a boiling water bath for 10 min, cooled and its fluorescence was measured in a Hitachi spectrofluorometer (Model 650-10M) at 360/470 nm wavelength. For serotonin 0.6 ml of the aliquot in aqueous phase containing serotonin and $N$-acetyl serotonin was added with 10 ml of ethyl acetate and 0.2 ml of 1.2 N HCl. The ethylacetate phase contained $N$-acetyl serotonin (NAS), and the aqueous phase contained serotonin. The serotonin – OPT reaction was carried out in a 0.2 ml aliquot of the aqueous phase and measured as described for melatonin. For NAS 4 ml of ethyl acetate phase was added with 6 ml of $N$-heptane and 0.3 ml of 1 N HCl and vortexed. NAS was transferred to the acid phase. 0.2 ml aliquot of the acid phase was reacted with OPT as mentioned earlier and was measured as described for melatonin. Coefficients of intra- and inter-assay variations were 8% and 10% respectively for serotonin, 7% and 12% respectively for NAS and 9% and 12% for respectively for melatonin.

(c) **Thyroid hormones ($T_3$, $T_4$) and TSH:**

Total triiodothyronine ($T_3$) (Walker, 1977) and total thyroxine ($T_4$) (Schurrs and van Weeman, 1977) and TSH (Soos and Siddle, 1982), were quantitated by EIA kit (PATHOZYME $T_3$, $T_4$ and TSH) of OMEGA, UK (Product No. OD387).
**T3:**

For T₃, Goat anti-Mouse IgG antibody was coated onto microtitration wells. Test sera were applied along with antibody reagent coating T₃ to bind to the wells. T₃ enzyme conjugate was added which competed with the released serum T₃ for available binding sites on the solid phase. After incubation, the wells were washed with wash buffer to remove any unbound T₃ or T₃ enzyme conjugate. On addition of the substrate tetramethyl benzidine (TMB), a colour developed only in those wells in which enzyme was present, indicating a lack of serum T₃. The reaction was stopped by the addition of dilute (0.2 M) sulphuric acid and the absorbance was measured by an ELISA reader (Qualigen plate reader PR-601, U.K.) at 450 nm. Specific cross reactivity was recorded at 85% level.

**T₄:**

For T₄, specific anti-T₄ antibodies were coated onto microtitration wells. Test sera were applied. T₄ with horseradish peroxide enzyme conjugate was added which competed with the released serum T₄ for available binding sites on the solid phase. After incubation, the wells were washed with wash buffer to remove any unbound T₄ or T₄ enzyme conjugate. On addition of the substrate (TMB), a colour developed only in those wells in which enzyme was present, indicating a lack of serum T₄. The reaction was stopped by the addition of dilute (0.2 M) sulphuric acid and the absorbance was measured by an ELISA reader (Qualigen plate reader, PR-601, U.K.) at 450 nm. Specific cross reactivity was observed at 85% level.
TSH:

Specific anti-TSH antibodies were coated onto microtitration wells. Test sera were applied. Then Goat anti-TSH labelled with horseradish peroxidase enzyme conjugate was added. If TSH was present in the sample it will combine with the antibody on the well and the enzyme conjugate, resulting in the TSH molecule being sandwiched between the solid phase and the enzyme linked antibodies. After incubation, the wells were washed with wash buffer to remove unbound labelled antibodies. On addition of the substrate tetramethyl benzidine (TMB), a colour developed only in those wells in which the enzyme conjugate was present, indicating the presence of TSH. The enzyme reaction was stopped by the addition of dilute (0.2 M) sulphuric acid and the absorbance was measured at 450 nm by an ELISA reader (Qualigen plate reader PR-601, U.K.). Specific cross reactivity was observed at 75% level. Coefficients of intra- and inter-assay of variations were 7% and 9% respectively for T₃, 8% and 10% respectively for T₄ and 9% and 12% respectively for TSH.

(d) Adrenal hormones:

Cortisol:

Serum cortisol was estimated by EIA following the methods of Longs cope (1996) and Friess et al. (2000). 1 ml of blood serum was added to 1 ml of diethyl ether and vortexed. The top ether layer was pipetted and placed in a clean test tube. This procedure was repeated twice. All samples were dried under nitrogen. Samples were dissolved in 250 μl of assay buffer and vortexed well. This procedure was repeated twice. 100 μl of assay buffer was pipetted and placed into the NSB and the BO wells.
100 μl of the standard was placed into the appropriate wells. 100 μl of the sample was pipetted and placed into the appropriate wells. 50 μl of assay buffer was pipetted into the NSB wells. 50 μl of the blue conjugate was added into each well, except the total activity and blank wells. 50 μl of yellow antibody was added to each well, except the blank, total activity and NSB wells. The plate was incubated at room temperature on a plate shaker for 2 hours at ~ 500 rpm. The plate was covered with a plate scaler provided. The contents of the wells were emptied and washed by adding 400 μl of wash solution to each well. This procedure was repeated thrice. After final wash, the wells were emptied and blotted by a lint-free paper towel to remove any remaining wash buffer. 5 μl of the blue conjugate was added to the total activity wells. 200 μl of the pNpp substrate solution was added to each well and incubated at room temperature for 1 hour without shaking. 50 μl of stop solution was added to each well to stop the reaction. The plate was read immediately at 405 nm. Sensitivity was at the level of 56.72 pg/ml. Specific cross reactivity was observed at 90% level. Coefficients of intra-assay variations within the group was observed at 10% and inter-assay variations between the groups in any month of the year were recorded at 12%.

**Norepinephrine and epinephrine (by fluorometric method):**

Norepinephrine and epinephrine hormones were extracted with acidified n-butanol and purified with activated alumina that separated catecholamines from other amines (Cox and Perhach, 1973). Purified samples were oxidized with NaI-I₂ solution at pH 7.0 for norepinephrine and at pH 4.0 for epinephrine, essential for complete oxidation, using Sorenson's M/15 phosphate buffer (pH 7.0) for norepinephrine and McIlvaine's citrate-phosphate buffer (pH 4.0) for epinephrine, following the method of
Laverty and Taylor (1968), resulting in the formation of adrenochromes. Oxidation was
stopped using sodium sulfite as an antioxidant to form the fluorescent product. The
oxidized product was then exposed to strong alkali (NaOH and Na₂-EDTA) for
tautomerization of the adrenochrome to the corresponding lutins. To achieve peak
fluorescence, sulfite and alkali were added together and left for 5 min for
norepinephrine and 1 min for epinephrine. Oxidation of lutins was prevented by adding
glacial acetic acid which stabilized the lutins and increased the fluorescence.
Noradrenolutin fluorescence was read at 380/480 nm 30 min after adding acetic acid
and adrenolutin fluorescence was recorded at 410/500 nm immediately after adding acetic
acid; both were measured by Hitachi Fluorescence Spectrophotometer (Model 650-10
M) with excitation slit at 10 nm, emission slit at 2 nm, and sensitivity at 0.1.
Coefficients of intra- and inter-assay variations were 6% and 8% respectively for
epinephrine and 7% and 12% respectively for norepinephrine.

(e) Pancreatic hormone:

Insulin

Serum insulin was assayed by ELISA (O’Rahilly and Moller, 1992) Kit
(ELISA-DSL-10-1600, DSL, U.K. Ltd.). The DSL-10-1600 ACTIVE insulin ELISA is
an enzymatically amplified “one-step” sandwich-type immunoassay. In the assay,
standards, controls and unknown serum samples were incubated with anti-insulin
 antibody in microtitration wells which were coated with another anti-insulin antibody.
After incubation and washing, the wells were incubated with the substrate tetramethyl
benzidine (TMB). The enzymatic reaction was stopped by adding a dilute (0.2 M)
sulphuric acid solution. The absorbance of the solution was measured in the wells
within 30 minutes using a microplate ELISA reader set at 450 nm (Qualigen plate reader PR-601, U.K.).

The absorbance measured is directly proportional to the concentration of insulin present. A set of insulin standards was used to plot a standard curve of absorbance versus insulin concentration and insulin concentrations in unknown samples were calculated. Specific cross reactivity was observed at 70% level for insulin. Coefficients of intra- and inter-assay variations were recorded at 6% and 9% respectively.

**Blood glucose**

Serum glucose level was measured by the glucose oxidase-peroxidase (GOD-POD) enzymatic method of Trinder (1969) using the AUTOSPAN kit (Span Diagnostic Lt. Surat, India). Glucose was oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. In a subsequent peroxidase catalyzed reaction, the oxygen liberated was accepted by the chromogen system to give a red coloured quinoneimine compound. The red colour so developed was measured by a Spectrophotometer (Smart spec 3000, BIORAD, Australia) at 505 nm (490-550 nm) against a reagent blank and was directly proportional to the glucose concentration. Coefficients of intra- and inter-assay variations were 9% and 13% respectively.

**Statistical analysis**

Data were analyzed statistically by one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1989). Recoveries of the standard hormones or glucose were estimated at the levels of 95% followed by Duncan’s multiple range tests. Coefficients of intra-assay and interassay variations of hormones or blood glucose were recorded and shown against each hormone. P-value <0.05 were considered significant.
RESULTS

Gross morphology

The freshly captured grey mullet *Mugil cephalus* L. looked greyish on dorsal side with bright silvery white colour on lateral and ventral sides of the body in the breeding phase (December) (Fig. 1). The body weight was higher during the breeding phase (November-December) than that was in the post-breeding (January-August) or pre-breeding (recrudescence) (September-October) phases. The ovary in the breeding phase occupied the entire viscera antero-posteriorly and the abdomen was extremely swollen. The ovary was paired, lobular of unequal length, the left (fish) being longer than the right, containing 100% intensely yellow, yolky vitellogenic follicles (Fig. 5a), unlike the extremely reduced, transparent, ovary with narrow lobes seen in the post-breeding phase (January-August) (Fig. 5b). In the pre-breeding phase (September-October), the ovary was large, looked reddish, contained few relatively larger yolky follicles. Each lobe of the ovary was covered by a thin tough transparent membrane, mesovarium, which joined posteriorly with that of the other ovary to form a single tube as uterus that opened outside through cloacal aperture. Yolky follicles were loosely connected with each other by thin thread-like fibres (connective tissue ?). During the breeding phase the cloacal opening was wide and looked reddish unlike the appearance of cloaca of the non-breeding phase, the post-breeding or pre-breeding phase of the seasonal ovarian cycle of the mullet.
Gravimetry

The ovary weight began to increase gradually from the pre-breeding phase (September-October), increased maximally in the breeding phase (November-December) and drastically reduced in the post-breeding phase (January to August). The gonadosomatic index (GSI) \(\frac{\text{Ovary wt.}}{\text{Body wt.}} \times 100\) was also altered in the same pattern as seen in the ovary weight. The GSI index was moderately high in the pre-breeding or recrudescence phase reached peak in the breeding phase and remained low in the post-breeding phase (Fig. 6).

Follicles (oocytes)

(a) *Intact non-yolky and yolky follicles (under microscope)*:

Non-yolky follicles were abundant in pre-breeding, negligible in breeding and highest in post-breeding mullets. Yolky follicles were low in pre-breeding; abundant in breeding (Fig. 5a) and were absent in post-breeding phase especially after spawning (Fig. 5b).

(b) *Follicle size*:

The follicle (with oocyte) size (diameter) remained small from January through July, began to increase from August and became maximum in December (Fig. 7).
(c) **Follicle (oocyte) population :**

Total follicle population of each specimen varied drastically during the circannual ovarian cycle of the grey mullet. Total follicular population per paired ovary of each specimen began to increase from pre-breeding especially in September, became maximum in breeding especially in December and began to decrease in post-breeding from January till August (Fig. 8a). Ovarian follicular (oocyte) population per mg of the ovary was high in pre-breeding (September-October), least in breeding (November-December) and highest in post-breeding (January to August) (Fig. 8b). Two types of follicles (healthy and atrophic) were identified from the histological sections of the ovary. The healthy type was characterized by distinctly stained intact oocyte with the intact nucleus and the other type by the degenerated tissue mass without any distinction between the oocyte and follicular cells (Figs. 9A, B and C). Total follicle population (healthy and atrophic types) per microscopic field was moderately high in pre-breeding, least in breeding and highest in post-breeding phases of the circannual ovarian cycle of the mullet (Fig. 8c). But there was no significant change in healthy or atrophic follicular populations (%) in any phase of the ovarian cycle (Fig. 8d).

(5) **Histology**

The histology of the ovary was studied in pre-breeding (October) (Fig. 9A), breeding (November) (Fig. 9B) and post-breeding (June) (Fig. 9C) phases. The ovary was covered by tunica albuginea (mesovarium) consisting of outer stroma, middle circular muscle layer and inner longitudinal muscle layer. The ovary consists of numerous lobules, surrounded by a thick basement membrane that encloses numerous differentiating ovarian follicles. In the pre-breeding phase (recrudescence) (October),
the ovary was large and consisted of numerous moderately wide lobules, lined by linearly arranged double or multiple rows of many large developing follicles of different size (Fig. 9A). The ovary contained many small deeply-stained primordial follicles and lightly-stained large developing follicles characterized by centrally located nucleus in the oocyte. But in the primordial follicles, the nucleus was eccentrically located in the oocyte (Fig. 9A). Few atretic follicles characterized by degenerated oocyte and follicular cell mass with pycnotic nuclei were noticed (Fig. 9C). Follicular thecal cells were not clearly distinguishable in the pre-breeding phase. During the breeding phase, the ovary showed numerous, extremely wide lobules with abundance of extremely large well developed yolky follicles, represented by yolk droplets in the oocyte cytoplasm containing very large centrally located nucleus. Many small primordial follicles were present even in the breeding phase. Several atretic follicles were also noticed which were intermingled with very large healthy follicles. In the post-breeding phase (June), the ovary became extremely small, narrow, looked reddish and were loaded with mostly undifferentiated small primordial follicles with small oocytes (Fig. 9C). Follicular thecal or granulosa cells were indistinguishable.

**Electron microscopy**

(a) *Oocyte:*

In the pre-breeding stage, three categories of oocytes were recognized, viz. early primordial, pre-vitellogenic and early vitellogenic. Numerous developing follicles were arranged in double rows with a central lumen (Fig. 10A). The early primordial oocyte was surrounded by a thin plasma membrane and subsequently by follicular cell layer with nucleus. Oocyte cytoplasm showed electron dense cytoplasm (Figs. 11A and B).
Fig. 5a. Huge size of the intact paired ovary with two lobes, the left being larger than the right, showing deep yellow vitellogenic follicles with oocytes seen in the breeding phase (December) of the mullet.

b. The post-breeding ovary (June) showing extremely narrow, lobes of the ovary.
Fig. 6. Circannual changes of the gonadosomatic index (GSI) (%) showing maximum value in the breeding phase (November and December) in the grey mullet. Each point represents mean value ± SE. (ANOVA, P < 0.001).

Fig. 7. Circannual changes in diameter (µm) showing largest size of the oocytes in the breeding phase of the mullet. (For other legends see Fig. 6, ANOVA, P < 0.001).
Fig. 8.  

a) Circannual variations in total follicular (oocyte) population (per paired ovary per specimen) showing huge number in the breeding phase of the mullet. (For other legends see Fig. 6, ANOVA, $P < 0.005$).

b) Circannual variations follicular (oocyte) population (number per mg of ovary) showing reduced oocyte number in the breeding phase compared to other phases. (For other legends see Fig. 6, ANOVA, $P < 0.001$).
Fig. 8. c) Histograms showing significant changes in total follicle population per microscopic field with healthy and atrophic types during pre-breeding, breeding and post-breeding phases of the mullet (ANOVA, p < 0.001).

d) No significant changes in healthy and atrophic follicular population percents are seen in any phase of the circannual ovarian cycle.
Fig. 9A. Light microscopic photograph of the mullet ovary of the pre-breeding phase (October) showing moderately broad lobules (bl) consisting of rows of developing follicles (df) separated by central lumen (l) with many small primordial (pf) and few large developing (df) follicles (X100).

B. Abundance of large very well developed follicles (f) (oocytes) with atretic follicles (af) are seen in extremely broad lobules (bl) of the ovary in the breeding mullets (November) (X 100).

C. Very small undeveloped primordial follicles (pf) (oocytes) are abundant seen in the narrow lobules (nl) of the ovary in the post-breeding mullet (January) (X 100).
The oocyte nucleus was hyperchromatic, few with large and small, round prominent electron dense nucleoli located near the periphery of the nucleus (Figs. 11C and D). The pre-vitellogenic oocytes were relatively larger than the early primordial type, characterized by the presence of abundant lipid droplets, accumulated in the central part of the oocyte (Fig. 10B). Few early vitellogenic oocytes (arrows) containing numerous yolk globules and lipid droplets were seen. Few atrophic follicles, characterized by unidentified degenerated cell mass, were also noticed in the pre-breeding phase. The early vitellogenic oocyte cytoplasm (asterisks) showed microvilli (arrow) (Fig. 12A) which were subsequently found to be well developed, elongated and penetrated into the thick vitelline membrane and projected towards the follicular cell layer (Figs. 12B and C). The cytoplasm contained large yolk globules. Desmosomes (arrow) were seen between the follicular cells (Fig. 12C). Microvilli from the follicular cells were projected into the oocyte (arrows). Thecal (t) cell was also seen (Fig. 12D) in pre-breeding phase.

In the breeding phase, two types oocytes, late vitellogenic and post-vitellogenic types, were noticed. The late vitellogenic oocytes (o) showed clear differentiation of thick electron dense zona externa and a relatively thin multilaminar zona interna the vitelline membrane (Fig. 13A). The microvilli from the oocyte became very long and penetrated across the very thick zona interna (zi) (862.66 nm) and zona externa (za) (1713.19 nm) (Figs. 13A and B, 14A). Abundance of lipid droplets, mostly of oval shape with many electron dense yolk globules of different size were seen in the cytoplasm of the oocyte (Fig. 13C). The oocyte showed undulated nuclear membrane. A large electron dense nucleolus was seen in the nucleus (Fig. 13D). The late vitellogenic oocyte also showed long microvilli that run from the zona interna (zi) to
Fig. 10. A. From pre-breeding stage showing early primordial follicles and oocytes (asterisks) arranged in double rows with central lumen (l). B. Pre-vitellogenic oocytes (arrows), characterized by the appearance of abundant lipid droplets are seen. Asterisks denote primordial oocytes. C. Early vitellogenic oocytes (arrows) showing numerous yolk globules, lipid droplets and primordial oocyte (asterisk). Few atretic follicles (af) characterized by unidentified degenerated cell mass are seen. D. A late vitellogenic oocyte with abundance of yolk granules and thick vitelline membrane (arrow) are seen.
Fig. 11. A,B: A magnified view of the early primordial oocyte surrounded by a thin plasma membrane (arrows). Asterisks indicate electron dense cytoplasm of the oocyte. B: The oocyte is surrounded by a follicular cell (fc) layer. n, nucleus of a follicular cell. C, D: Huge nuclei of the oocyte showing few prominently large (l) and small (s) round electron dense nucleoli towards the periphery of the nucleus (arrows).
Fig. 12A-D. Early vitellogenic oocytes. A. shows the appearance of the external layer of the vitelline membrane with short microvilli (arrows). B. Microvilli (arrows) elongate from the oocyte and project into the follicular cell layer (fc) across the vitelline membrane. C. Desmosomes seen between the follicular cells (arrow). D. Microvilli (arrows) from the follicular cell layer (fc) also project into the oocytes. A thecal (f) cell layer is seen. Asterisks denote oocyte cytoplasm.
Fig. 13. A & B. from breeding stage. Late vitellogenic oocytes (o) showing clear differentiation of a thick electron-dense zona externa (ze) and a thin multilaminar zona interna (zi) (arrow) of the vitelline membrane. The microvilli (arrow) from the oocytes (o) are long and penetrate deep into the cytoplasm of the follicular cell layer (fc) across the very wide zona interna and zona externa. Arrows showing widths of zona interna. C. Numerous lipid droplets (l) and many electron dense yolk globules (asterisk) are seen in the late vitellogenic oocyte cytoplasm. D. Undulated membrane of an oocyte (o) nucleus (arrow). A large electron dense nucleolus (nl) is seen in the nucleus (n).
Fig. 14. A. Late vitellogenic oocyte (o) showing long microvilli that run from the zona interna (zi) to zona externa (ze) of the vitelline membrane. One microvillus from the follicular cell (fc) was penetrated into the zona externa and zona interna to reach to the oocyte cytoplasm (arrow). The thickness (diameter) of both zona externa (1713.19 nm) and interna (862.66 nm) is indicated. Follicular cell (fc) layer is seen. B. Rough endoplasmic reticulum (arrow) seen abundantly in the oocyte cytoplasm. C. Numerous electron dense mostly round and large yolk globules (asterisks) are seen in the oocyte cytoplasm. D. Several mostly elongated mitochondria (arrow) of the oocyte cytoplasm are noticed.
Fig. 15.  A. Post-vitellogenic oocyte showing an extremely thick zona interna (zi) and relatively thin zona externa (ze) of the vitelline membrane. The pore canals of the microvilli are closed both in the zona interna and zona externa. B. The multilaminar nature of the zona interna (zi) is negligible, but still evident. The zona externa (ze) is drastically regressed. Note: retraction of microvilli (arrows) especially in the zona interna (zi) and closure of pore canals.
the zona externa (ze) of the vitelline membrane and reached to follicular cell (fc) cytoplasm (Fig. 14A). Another microvillus was projected from the follicular cell and penetrated into the zona externa and zona interna to reach upto the oocyte cytoplasm (Fig. 14A). Abundance of rough endoplasmic reticulum (RER) was seen in the oocyte cytoplasm (Fig. 14B). Numerous electron dense, mostly large and round yolk globules (asterisks) of different size were seen in the oocyte cytoplasm (Fig. 14C). Several mostly elongated mitochondria were scatteredly distributed in the cytoplasm of the mullet oocyte (Fig. 14D). The post-vitellogenic oocyte showed an extremely thick zona interna (zi), and relatively thin zona externa (ze) in the vitelline membrane of the oocyte (Fig. 15A). The pore canals of the microvilli were found to be closed both in the zona interna and zona externa. But the multilaminar nature of the thick zona interna (zi) was till evident in the post-vitellogenic oocyte (Fig. 15B). Subsequently both the zona externa and zona interna (zi) became thin and were regressed. The zona externa, compared to zona interna, was drastically regressed. The microvilli were retracted and the pore canals were closed located especially in the zona interna (Fig. 15B).

**Hormones** (Table - 1, Fig. 22)

*FSH, LH and prolactin:*

FSH level was very low during January-June, and then began to rise from late post-breeding phase (July-August), reached peak in pre-breeding (October) and was declined in the breeding phase (November-December) (Fig. 16a). LH level also began to rise from pre-breeding (September-October), reached peak in breeding phase (December) and declined subsequently (Fig. 16b). Prolactin (PRL) concentration
showed two peaks, one in pre-breeding (October) and another in early post-breeding phase (January) (Fig. 16c).

**Estradiol 17β (E₂) and testosterone :**

Estradiol 17β (Fig. 17a) and testosterone (Fig. 17b) concentrations began to rise from late post-breeding (July-August), reached peak in pre-breeding (October), declined in the breeding phase, and subsequently remained low (Figs. 17a,b).

**Dihydroxy progesterone (DHP) (17α,20β-dihydroxy-4-pregnen-3-one) :**

Serum DHP level began to rise slowly from pre-breeding (September-October), sharply reached the peak in December of the breeding phase, then drastically declined in January and remained low till August of the post-breeding phase (Fig. 17c).

**T₃, T₄ and TSH :**

The concentrations of both T₃ (Fig. 18a) and T₄ (Fig. 18b) showed two peaks, one in April (post-breeding) and the other in August-September of the early pre-breeding phase (Figs. 18a,b). But TSH (Fig. 18c) level altered reversely to that of T₃ and T₄, being low during two peak periods of T₃ and T₄ (one in April and the other in August-September) (Fig. 18c).
Adrenal hormones:

Serum cortisol (Fig. 19a), epinephrine (Fig. 19b) and norepinephrine (Fig. 19c) concentrations began to rise from September of the pre-breeding phase, reached peak in November of the breeding phase and declined subsequently from December of the early post-breeding phase (Figs. 19a,b,c).

Pineal indoleamines (serotonin, N-acetyl serotonin and melatonin):

Serum serotonin level (Fig. 20a) began to increase gradually from the pre-breeding phase, reached peak in the breeding phase (peak in December) and declined subsequently in the post-breeding phase (precisely from March to September). But N-acetyl serotonin (NAS) (Fig. 20b) and melatonin (MEL) (Fig. 20c) levels altered reversely to that of serotonin, being high in the post-breeding (peak in June) and low subsequently in pre-breeding and breeding phases (Figs. 20a,b,c).

Insulin:

Serum insulin level remained low in the post-breeding phase (January to August), began to rise from the pre-breeding phase (September) and reached peak in the breeding phase (November-December) (Fig. 21a).

Blood glucose:

Serum glucose level was reversely altered to that of insulin; the level remained high from post-breeding to pre-breeding phase (January to October) and drastically declined in the breeding phase (November-December) (Fig. 21b).
Fig. 16. Circannual changes in (a) serum FSH, (b) LH and (c) prolactin (PRL) showing peak level of FSH in pre-breeding (October), and LH in breeding (December) with two peak levels of PRL, one in pre-breeding (October) and the other in early post-breeding (January) phase during the circannual ovarian cycle of the grey mullet. Each point represents mean ± SE (ANOVA: P < 0.005).
Fig. 17. Annual changes in serum estradiol (a) 17β (E$_2$), (b) testosterone (T) and (c) 17α,20β-dihydroxy-4 pregnen-3 one (DHP) concentrations showing peak E$_2$ and T levels in pre-breeding in October with peak level of DHP in December of the breeding phase seen during the circannual ovarian cycle of the grey mullet. Each point represents mean ± SE (ANOVA : P < 0.005).
Fig. 18. Circannual changes in serum (a) $T_3$ and (b) $T_4$ and (c) TSH concentrations showing two peaks of $T_3$ and $T_4$ with lower level of TSH, one in April (post-breeding phase) and the other in August-September (early pre-breeding phase) of the circannual ovarian cycle of the mullet. Each point represents mean±SE (ANOVA : P-value <0.005).
Fig. 19. Circannual variations in (a) serum cortisol, (b) epinephrine (E) and (c) norepinephrine (NE) levels with their peaks seen in the breeding (November) mullet. Each point represents mean value ± SE (ANOVA: P-value < 0.001).
Fig. 20. Circannual variations in serum pineal indoleamines, (a) serotonin, (b) N-acetyl serotonin (NAS) and (c) melatonin (MEL) with peak levels of serotonin in the breeding phase (November-December) and of NAS and MEL in the post-breeding phase (peak in June) in the circannual ovarian cycle of the mullet. Each point represents mean value ± SE (ANOVA: P-value < 0.005).
Fig. 21. Circannual variations in serum (a) insulin and (b) blood glucose levels with peak insulin and declined blood glucose levels seen in the breeding phase (November-December) of the mullet. Each point represents mean value ± SE (ANOVA : P-value < 0.005).
Table 1. Summary of the findings of the ovarian activity associated with hormonal and blood glucose profiles during different phases of the circannual ovarian cycle of the Indian grey mullet

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PRE-BREEDING (September-October)</th>
<th>BREEDING (November-December)</th>
<th>POST-BREEDING (January-August)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Ovary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSI</td>
<td>Moderate</td>
<td>Highest</td>
<td>Lowest</td>
<td>Maximum follicular (oocyte) growth in breeding</td>
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<tr>
<td>Follicle size (diameter)</td>
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<td>Same</td>
<td>Same</td>
<td></td>
</tr>
<tr>
<td>Follicle population (per paired ovary/specimen)</td>
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<td>Same</td>
<td>Same</td>
<td></td>
</tr>
<tr>
<td>Follicle population (per mg of ovary)</td>
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<td>Least</td>
<td>Highest</td>
<td></td>
</tr>
<tr>
<td><strong>B. Hormones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>Highest</td>
<td>High</td>
<td>Low</td>
<td>E₂⁺, T⁺ (?)</td>
</tr>
<tr>
<td>LH</td>
<td>High</td>
<td>Highest</td>
<td>Low</td>
<td>DHP⁺, T⁺</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Very high</td>
<td>Very high</td>
<td>High initially low subsequently</td>
<td>Oocyte growth and maturation</td>
</tr>
<tr>
<td>E₂</td>
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<td>Low</td>
<td>Vitellogenesis</td>
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<td>T</td>
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<td>High</td>
<td>Low</td>
<td>Vitellogenesis</td>
</tr>
<tr>
<td>DHP</td>
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<td>Very low</td>
<td>Oocyte maturation</td>
</tr>
<tr>
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<td>Low</td>
<td>High</td>
<td>Oocyte growth initiation</td>
</tr>
<tr>
<td>T₄</td>
<td>High</td>
<td>Low</td>
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<td>Lowest</td>
<td>Negative feedback control of T₃, T₄</td>
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<td>Low</td>
<td>Oocyte growth</td>
</tr>
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<td>Epinephrine</td>
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<td>Low</td>
<td>Same</td>
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Continued

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<th>Parameters</th>
<th>PRE-BREEDING (September-October)</th>
<th>BREEDING (November-December)</th>
<th>POST-BREEDING (January-August)</th>
<th>Remarks</th>
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</thead>
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<td></td>
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<tr>
<td>Norepinephrine</td>
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<td>Low</td>
<td>Same</td>
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<td>Highest</td>
<td>Low</td>
<td>Oocyte maturation (?)</td>
</tr>
<tr>
<td>N-acetyl serotonin</td>
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<td>Highest</td>
<td>Antigonadal (oocyte growth inhibition ?)</td>
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<tr>
<td>Melatonin</td>
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<td>Lowest</td>
<td>Highest</td>
<td>Antigonadal (oocyte growth inhibition ?)</td>
</tr>
<tr>
<td>Insulin</td>
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<td>Highest</td>
<td>Low</td>
<td>Oocyte growth (?) and maturation (?)</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>Low</td>
<td>Lowest</td>
<td>High</td>
<td>(a) Huge energy utilization during breeding</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(b) Hypoglycemia due to huge insulin production during breeding</td>
</tr>
</tbody>
</table>
Fig. 22. Endocrine (gonadal and extra-gonadal) influence of the circannual ovarian cycle of Indian estuarine grey mullet *Mugil cephalus* L.

*Evaluated by GSI%, follicle size, follicle population per microscopic field or per mg of ovary, follicle population per specimen, and light and electron microscopic structures

Legends, + : low; ++ : moderate; +++ : high
DISCUSSION

A month-wise round the year study of the ovary of the grey mullet *Mugil cephalus* reveals that ovarian activity varies annually (Table 1). It is evident from the changes in ovarian weight, gonadosomatic index (GSI), follicle size and population of follicles, light and electron microscopy of the oocyte and follicular cells those were marked during the circannual ovarian cycle of the mullet. Simultaneously, concentrations of sex hormones [(estradiol (E₂), testosterone and 17α,20β-dihydroxy-4 pregnen-3 one (DHP)], gonadotropins [(follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (PRL)] and extra-gonadal [pineal melatonin including other indoleamines such as serotonin and N-acetyl serotonin), thyroid (T₃ and T₄) and thyroid stimulating hormone (TSH), adrenal (cortisol, epinephrine and norepinephrine) and pancreatic (insulin)] hormones are altered with the circannual ovarian cycle of the mullet. Moreover, the blood glucose level is altered with the annual ovarian cycle. On the basis of the changes in the above-cited parameters, the circannual ovarian cycle of the grey mullet is divided in 3 phases viz., pre-breeding (recrudescence) (September and October), breeding (November and December) and post-breeding (regressive) (January to August). The ovary weight, GSI, follicular size and total follicular population (per paired ovary) per specimen began to increase in pre-breeding, attained maximum value during breeding and were decreased thereafter in the post-breeding phase. But the follicular populations per mg of the ovary or per microscopic field were reversely altered to that of the total ovarian follicular population, because the follicular population per mg of the ovary or per microscopic field of the ovary section was moderately high in pre-breeding, least in breeding and maximum in the post-breeding phase. The latter findings reflect the changes in follicular size which is moderately
large in pre-breeding, maximally large in breeding and subsequently become small in
the post-breeding mullets. Gradual increment in the size of follicle (follicular growth)
was evident from the follicle diameter because it was highest in breeding compared to
pre-breeding or post-breeding mullets. These findings explain the changes in follicular
population per mg of ovary per microscopic field observed during the different phases
of the circannual ovarian cycle.

Circannual ovarian follicular growth is also evident from light microscopic and
ultrastructural studies of the follicles and the oocytes of the mullet. Histologically
ovarian lobules were longitudinally oriented with double rows of mostly small and few
large follicles. Long central lumen was observed throughout the length of the lobule in
the pre-breeding phase. But in the breeding phase, the ovarian lobules were extremely
enlarged containing very large differentiated follicles with very large oocytes. But such
larger follicles or oocytes were not found in the narrow lobules of the post-breeding
ovary. Healthy and atretic follicles were present in all the phases of the ovarian cycle,
but their populations were not significantly altered in the circannual ovarian cycle of
the mullet. Ultrastructural studies revealed various categories of oocytes during
different phases of the circannual ovarian cycle in the mullet. With the onset of the pre-
breeding phase three categories of oocytes appeared viz., early primordial (surrounded
by thin plasma membrane), pre-vitellogenic (abundance of numerous lipid droplets),
early vitellogenic (yolk globules with thick vitelline membrane) oocytes were marked.
In the breeding phase, two types of oocytes, late vitellogenic and post-vitellogenic were
observed. Differentiation of vitelline membrane into zona externa and zona interna,
penetration of long microvilli from the oocyte cytoplasm to the cytoplasm of the
follicular cells and from follicular cells to oocyte cytoplasm were also observed.
Furthermore, abundance of RER and enlarged mitochondria suggest active protein synthesis in the vitellogenic oocytes during the breeding phase. Presence of huge quantity of yolk globules may be due to their huge accumulation causing enlarged size (growth) of the vitellogenic oocytes in the breeding phase. But in the post-vitellogenic oocytes, vitelline membrane was regressed with thin zona interna and zona externa, retraction of microvilli and obliteration of the pore canals of the microvilli were seen in the breeding phase. Hormonal studies revealed that FSH, PRL, E$_2$, testosterone, T$_3$ and T$_4$ concentrations were at peak in the pre-breeding phase, whereas other hormones such as LH, DHP, cortisol, epinephrine and norepinephrine and insulin levels attained peak in the breeding phase suggesting that hormonal titres of the pre-breeding phase are involved in the initiation of ovarian growth (Fig. 22). Other hormones of breeding phase probably help in the final oocyte and follicular growth, and subsequently the oocyte maturation in the Indian grey mullet. Whereas pineal hormones, especially melatonin including N-acetyl serotonin probably do not contribute to the ovarian follicular and oocyte growth in the circannual ovarian activity of the mullet. Furthermore, very high amount of insulin is also required for active follicular growth resulting in the drastic reduction of blood glucose, probably due to extensive utilization of glucose for rapid follicular and oocyte growth in the breeding phase of the mullet.

Vitellogenin is synthesized in the liver and is transported to the developing oocytes during follicular growth in fish (Reinecke et al., 2006). Estradiol induces hepatocytes to produce vitellogenin which is sequestered by the oocytes in a receptor-mediated process enhanced by GtH-I (FSH) (Arukwe and Goksoyr, 2003). Androgens and GH can also induce hepatic vitellogenesis, probably as a result of their (androgen) conversion to estrogens by the hepatic aromatase (Peyon et al., 1996). Earlier study in
several teleosts also shows accumulation of true yolk, formation of microvilli originating from the oocyte, penetrating into the granulosa cells with gap junctions for metabolic exchange. During final growth, the oocytes becomes flat and it shows formation of animal pole, production of maturation inducing hormone MIS by the granulosa cells with resumption of first meiotic division and ovulation, followed by atresia with disorganized theca, granulosa and oocyte cytoplasm, phagocytosed by macrophages and granulocytes (Rocha and Rocha, 2006). E2 level increases with the increase of vitellogenin and both reach the peak level during breeding (spawning) and are declined in post-spawning and pre-gametogenesis (Pavlidis et al., 2000). Sen et al. (2002) have also reported the influence of E2 on vitellogenesis in teleost fishes. In our ultrastructural study, huge accumulation of yolk (vitellogenin) globules were seen in the vitellogenic oocytes during the breeding phase of the mullet. E2 and testosterone levels were also maximally increased in pre-breeding but declined in breeding mullets, unlike in other teleosts. In the mullet, the peak levels of E2 and testosterone in the pre-breeding phase indicates that E2 and testosterone are required in huge quantity for initiation of vitellogenesis and their peak levels are no longer required for vitellogenesis in the mullet. Ovarian levels of E2 and DHP are also elevated near the time of ovulation compared with the non-breeding zebra fish. Ovarian level of PGF2α is also raised near the time of ovulation in zebra fish (Lister and Kraak, 2008). Difference in timing of peak E2 and testosterone levels may be due to the species difference. Testosterone is the precursor of E2 which is synthesized by the follicular cells (granulosa) of the ovary (Larsen et al., 2003). As both testosterone and E2 levels were simultaneously increased in the pre-breeding phase, probable involvement of testosterone in E2 production cannot be ruled out in the mullet. GtH-II (LH) stimulates testosterone production by the thecal
cells and GtH-I (FSH) by its aromatization converts testosterone into 17β-estradiol (E\textsubscript{2}) in the granulosa cells of the ovarian follicles (Peter and Yu, 1997). Thus higher testosterone level might have been required to synthesize huge quantity of E\textsubscript{2} which is eventually required for vitellogenin production by the liver in the mullet. It is known that estrogen and estrogen receptors (Er) in the cytosol (Erc) and nucleus (En), and vitellogenin levels are elevated throughout the ovarian cycle and a significant correlation between plasma estradiol and estrogen receptor, and plasma vitellogenin have been noted especially during the period of ovarian recrudescence, but not during other phases of the reproductive cycle, because hepatic Erc and En concentrations remain elevated for prompt resumption of vitellogenin synthesis in the liver even when plasma E\textsubscript{2} and vitellogenin levels are declined as reported in a marine teleost Cynoscion nebulosus (Smith and Thomas, 1999). Nevertheless, such a correlation between E\textsubscript{2}, Erc and En and vitellogenin levels need to be elucidated during the circannual ovarian cycle of the mullet.

Extra-gonadal hormones are known to contribute to oocyte growth. Growth hormone (GH) is also known to be involved in hepatic vitellogenesis (Peyon et al., 1996). Cortisol is the principal adrenocortical hormone of the interrenal gland in teleosts (McCormick 2001; Reinecke et al., 2006) and it may have some role in vitellogenesis, because first and second phases of cortisol secretion or peak coincide with vitellogenesis and spawning respectively in female catfish, Heteropneustes fossilis (Lamba et al., 1983). There is a steady rise in plasma cortisol associated with maturation and spawning in salmonid (Onuma et al., 2003). Cortisol may also play some metabolic role in respect of energy production by stimulating glucose formation through gluconeogenesis from amino acid and fatty acids (Bloom et al., 2000). In the
rainbow trout, cortisol affects gluconeogenic enzymes suggesting a gluconeogenic role of cortisol in fish (Freeman and Idler, 1973). Cortisol also elicits hyperglycemia in wide varities of fish (Vijayan et al. 1997). Epinephrine and norepinephrine are also potent hyperglycemia agents in vertebrates (Larsen et al., 2003; Ray et al., 2008). In the mullet. Cortisol may also be involved in vitellogenesis and maturation of oocytes because cortisol level was elevated in the breeding phase. Epinephrine and norepinephrine profiles also attain peak in breeding mullets and thus, their participation in oocyte growth and maturation cannot be ignored in mullet. Apart from their involvement in reproduction, adrenal hormones may contribute to energy production. But blood glucose level was low when adrenal hormonal levels were high. Simultaneously, insulin level was also high along with adrenal hormones. Thus, increased glucose production by adrenal hormones might be decreased by insulin-induced enhanced glucose uptake by the developing oocyte, resulting in hypoglycemia in the breeding mullet.

There are evidences that thyroid status is increased during early oogenesis or spermatogenesis. Thyroid status is correlated with various stages of vitellogenesis and oocyte development in viviparous rock fish (Kwon et al., 1999). Thyroid hormone level is increased during vitellogenesis in some iteroparous fishes (Eales, 2006). TSH β subunits have high degree of amino acid sequence homology with the human (h) TSH β subunit and cross react with fish TSH. It varies seasonally and regulated by negative feedback action of thyroid hormones (Reinecke et al., 2006). Thyroid hormone level is increased in spawning and remains universally low after spawning. Thyroid hormone level is associated with the increase of testosterone level, and androgens can increase T3 production and plasma T3 level (Cyr and Eales, 1996). Thyroid hormone has a
permissive role to facilitate GtH action. Additionally thyroid hormones may also participate in energy supply which is required during gametogenesis (Wiens and Eales, 2005, Reineke et al., 2006). In the present study, both $T_3$ and $T_4$ and testosterone levels were increased in the pre-breeding phase. Thus involvement of $T_3$ and $T_4$ with testosterone production cannot be ignored in the mullet. Thyroid hormones may also participate in energy production in the mullet, because thyroid hormone levels were high when blood glucose level was elevated in pre-breeding mullets. Thus, thyroid hormones may play a critical permissive signal in timing of final gonadal development, by facilitating GtH action, testosterone production, vitellogenesis and oocyte growth in Indian grey mullets.

Fish FSH and LH have structural and functional similarities with tetrapod gonadotropins (Querat et al., 2000; Weltzein et al., 2003). Mostly FSH cells are present in the pituitary during early vitellogenesis whereas LH cells are predominant prior to spawning (Yu et al., 1997). Presence of both FSH and LH are confirmed by isolation and cloning of the encoding cDNA in most of the teleosts studied (salmonids, perciformes, and many other fish species). Population of gonadotrophs also vary seasonally with the sexual cycle (Querat et al., 2000; Weltzein et al., 2003). Both FSH and LH are synthesized synchronously during gametogenesis in some teleosts (Mateos et al., 2003) and their concentrations vary during the reproductive cycle in the rainbow trout (Prat et al., 1996). But their functions are different. FSH (GtH-I) regulates gonadal growth, vitellogenesis, spermatogenesis and steroidogenesis from early gonadal development to puberty. Whereas LH (GtH-II), in addition to steroidogenesis, is involved in regulating final stage of oocyte maturation and ovulation, spawning and spermiation in teleosts including salmonids (Swanson et al., 1991; Tyler et al., 1991;
Rocha and Rocha, 2006). Concentrations of GnRH, as well as FSH and LH vary seasonally with the reproductive cycle in the male rainbow trout (Prat et al., 1996). Moreover, seasonal variation of the expression of genes encoding GnRH has also been reported in red seabream. Neuropeptide Y (NPY) increases GtH-II (LH) release by direct actions on the pituitary or by increasing GnRH release (Pang et al., 1994). Additionally, pituitary adenylate cyclase-activating polypeptide (PACAP) also stimulates GtH-II (LH) release both in vivo and in vitro in goldfish and European eel Anguilla anguilla (Wang et al., 2000). Mathews et al. (2002) suggest that GtH-II secretion is regulated by a negative feedback action of maturation inducing steroid (MIS). In the current study, FSH level is maximally increased during pre-breeding and LH in breeding phase, probably by accelerating their release into circulation involving beta endorphin and NPY-GnRH in the hypothalamus and gamma amino butyric acid (GABA), PACAP/DA in the pituitary during the breeding phase of the ovarian cycle of the mullet, but it needs to be confirmed. Somatolactin (SL) is structurally related to growth hormone (GH) and prolactin (PRL) in teleosts (Takayama et al., 1991) and is maximally increased at spawning in coho salmon, Oncorhynchus kisutch and rainbow trout, oncorhynchus mykiss (Rand-weaver et al., 1995). FSH (GtH-I) and LH (GtH-II) receptors have been detected in the ovarian follicles of zebra fish Brachydonia revio (Kwok et al., 2005) indicating direct evidence of gonadotropin involvement in ovarian follicular growth in zebra fish.

IGF-I and IGF-II are present mainly in the liver in teleost fishes (perciforms, common carps Cyprinus carpio and some cultured aquarium fishes) (Reinecke et al., 2006). IGF-I stimulates E2 and DHP productions by the granulosa cells in the preovulatory coho salmon ovary (Maestro et al., 1997). IGF-I varies with the
reproductive cycle and increases GnRH stimulated FSH release in vitro (Baker et al., 2000). IGF-I promotes proliferation of follicular cells, maturation of oocytes and increases germinal vesicle migration and breakdown, a marker for resumption of meiosis, in oocytes of striped bass, *Morone saxatilis* (Weber and Sullivan, 2000). It also increases the density of gap junctions between granulosa cells or granulose cell and oocytes in red seabream (Patino and Kagawa, 1999). Insulin and IGF-I bindings are increased during various stages of primary oocyte growth and early vitellogenesis, decreases as vitellogenesis advances and again increase in preovulatory stage (Maestro et al., 1997). In the current study of the grey mullet, serum insulin and DHP levels reached peak in breeding phase as compared to pre-breeding and post-breeding phases indicating their involvement in breeding like that of E₂ and other hormones. Both insulin and DHP may have synergistic action in the induction of oocyte maturation in the breeding phase of the grey mullets. Our in vitro study also demonstrated that addition of IGF-I or DHP in the culture medium increased germinal vesicle breakdown (GVBD) of the post vitellogenic oocytes indicating their role in oocyte maturation in the mullet (Chapter-III).

Pituitary adenylate cyclase activating polypeptide (PACAP) is known to be an effective stimulant of GTH-II release, both in vivo and in vitro in fish (European eel, *Anguilla anguilla* and oldfish, *C. auratus*) (Montero et al., 1998; Wang et al., 2000). Gamma-aminobutyric acid (GABA) is also involved in the regulation of GtH-II secretion in Atlantic croaker (Khan and Thomas, 1999). Thus, PACAP and GABA may have been involved in LH (GTH-II) release during the circannual ovarian cycle of the mullet. Furthermore, GtH-II release is inhibited by the negative feedback action of maturation inducing steroids (MIS) in fish (Mathews et al., 2002). In the mullet, FSH
and prolactin levels were highest in pre-breeding suggesting involvement of both the
tropic hormones in oocyte growth and only FSH on E$_2$ production. Whereas LH was at
peak in breeding when testosterone level was declining despite the fact that LH
regulates testosterone production. It may so happen that LH may initiate testosterone
production in pre-breeding and further stimulation of LH may not be necessary to reach
peak testosterone level. But rise of LH level needs to be continued upto breeding phase
probably to stimulate DHP production that may be essential for oocyte maturation.
These findings suggest the dual role of LH on testosterone production in pre-breeding
and subsequent role on DHP production in the mullet.

There are evidences that environmental cues like photoperiod and temperature
stimulate hypothalamic GnRH which in turn cause release of FSH (GtH-I) and LH
(GtH-II) that eventually lead to synthesis and release of E$_2$ and T in the circulation.
GnRH in the hypothalamus is known to regulate GtH synthesis by the pituitary which
in turn regulates synthesis of all the three sex steroids viz., E$_2$, T and DHP. But actions
of GtH on these three steroids are different because LH (GtH-II) stimulates testosterone
synthesis by the thecal cells and FSH (GtH-I) produces E$_2$ by the granulosa cells
(Bhattacharyya et al., 2000), but FSH is more potent than LH in steroid hormone
production in salmonid fish. In the Indian grey mullet as FSH and prolactin (PRL)
levels reach peak in pre-breeding (October) and LH in breeding (December), it is likely
that these tropic hormones may have independent role on oocyte growth and oocyte
maturation during the circannual ovarian cycle of the mullet.

In addition to oocyte growth, oocyte maturation is essential for breeding. The
17α,20β-dihydroxy-4-pregnen-3-one (17, 20β-P) (DHP) (Nagahama and Adachi, 1985;
Leva-vizermonsky and Yaron, 1986; Bhattacharyya et al., 2002) and 17α,20β,21-
trihydroxy-4 pregnen-3 one (17, 20β-P) (Trant and Thomas, 1989; Thomas, 1994) are the most potent and effective maturation inducing hormones (MIS), identified in many teleost species, and essential for oocyte maturation (Nath, 1999; Paul et al., 2009). The thecal cells produce 17α-hydroxyprogesterone (17-P) which is converted to MIS in the granulosa cells by the action of 20β-hydroxysteroid dehydrogenase (20β-HSD). With regard to the pituitary control of DHP, it has been reported that LH, not FSH, stimulates DHP production in the granulosa cells that cause germinal vesicle breakdown (GVBD) indicating oocyte maturation. Increase in MIH in salmonid ovarian follicles is stimulated by the interaction of LH and gonadotropin receptor-II found in granulosa cells (Planas et al., 2000; Reinecke et al., 2006). The receptor-ligand interaction results in the upregulation of 20-hydroxysteroid dehydrogenase, the enzyme that catalyses the final step of MIH synthesis (Nagahama, 1997). The preovulatory surge of GtH-II (LH) is responsible for the rapid expression of 20β-HSD mRNA transcripts in the granulosa cells leading to increased production of MIS (DHP) during oocyte maturation (Kanamori et al., 1988). In the current study in the grey mullet, LH surge occurred in pre-breeding (October) and DHP in breeding phase (December) subsequently indicating influence of LH on DHP secretion, which in turn induced oocyte maturation in the mullet.

The time of breeding varies even in the same fish species of different geographical distribution. Mugil cephalus of Hawaii spawn in January and February (Kuo and Nash, 1975), those of South Carolina estuary in November and December (McDonough et al., 2003), and Tanshni estuary of North West Taiwan in November to January (Chang et al., 2000), unlike the Indian grey mullet that breeds in November and December as observed in the current study. The difference in timing of breeding
including spawning may be related to the climatic changes due to difference in latitude and longitude of the countries. Nevertheless, all the grey or stripped mullets of 4 widely distributed countries of the world breed (spawn) in short day length and low temperature as recorded in Indian (LD, 10 : 14) and Hawaiian (LD, 6:18) mullets. Moreover, constant short photoperiod (LD, 6 : 18) stimulates vitellogenesis within 49-62 days unlike the control whose vitellogenesis does not occur until 235 days in the mullet. Even exposure of short photoperiod (LD, 6 : 18) with low temperature (21°C) can prolong the breeding period to obtain eggs throughout the year in the mullet (Kuo et al., 1974; Kuo and Nash, 1975).

Certain other proximal environmental cues (oxygen content, electrolyte concentration, photoperiod, rainfall, flood, lunar cycle, pH) and food availability also provide favourable season for timing of reproduction in the circannual gonadal cycle in fish, (Rocha and Rocha, 2006), But photothermal cycle is most important in the induction of the breeding cycle in fish (Rocha and Rocha, 2006) and other wild vertebrates (Bentley, 1998). But their influence is different in different teleosts. It has been suggested that day length and temperature act as synchronizers to bring the ovarian rhythm into phase (Sundararaj and Vassal, 1976). Such proximal environmental factors, in addition to hormones, may have been responsible for the initiation and running of the circannual ovarian cycle in the Indian grey mullet. These environmental factors perceived by the exteroceptors, CNS, pituitary and finally by the gonads, cause endogenous rhythm which is brought for the precise breeding time (Rocha and Rocha, 2006). Environmental photothermal cues are known to mediate its action via hypothalamo-hypophysial ovarian axis in inducing circannual ovarian cycle. Environmental cues are converted to neural outputs by the sensory receptors and this
neural information is transduced into a hormonal output via the hypothalamic releasing hormones and finally the pituitary which releases the gonadotropic hormone(s). Such a mechanism might have operated in the induction of the circannual ovarian cycle in the mullet.

Recently some new studies clearly suggested that melatonin interacts with the reproductive cascade at a number of key steps such as through the dopaminergic system in the brain or the synchronization of the final oocyte maturation in the gonad. Search for additional pathways for a fish photoneuroendocrine system indicated that there are four main potential regulatory systems (light perception, melatonin, clock-gene network and kisspeptin signaling) which are involved in fish reproduction (Migaud et al., 2010). Zhang et al. (2009) reported that global genes (873), identified in the hypothalamus, are associated with the seasonal gonadal cycle in gold fish. Several genes including isotocin, ependymin II, GABA Gamma² receptor, calmodulin and aromatase b are differently expressed during sexual maturity (pre-spawning), sexual regression and early gonadal development in gold fish. Expression of these identified genes appears regulated by photoperiod, a major factor controlling vertebrate reproductive cyclicity. In the current study, global gene expression patterns may be responsible for induction of the circannual variation of the ovarian cycle regulated by photo neuroendocrine system including clock gene network and kisspeptin signalling in the estuarine gray mullet, but it needs to be confirmed in future.

The findings are highlighted in the following. (a) There is a distinct circannual ovarian cycle that passes through 3 phases viz., pre-breeding (September to October), breeding (November to December) and post-breeding (January to August), recognized on the basis of the changes in GSI, follicle (oocyte) size and population, light and
ultrastructural findings, and hormonal [FSH, LH, prolactin (PRL), E₂, T, dihydro progesterone (DHP), T₃, T₄, TSH, insulin, cortisol, epinephrine, norepinephrine, N-acetyl serotonin (NAS) and melatonin] and blood glucose profiles. (b) In the pre-breeding phase, the GSI and follicle size (diameter) and population (per paired ovary/specimen) were low, with higher follicle population per mg of ovary and per microscopic field, because of small size of the follicles. Ovarian lobules were small with abundance of small follicles. Ultrastructurally primordial, pre-vitellogenic and early vitellogenic oocytes surrounded by follicular cells, showed numerous lipid droplets and many yolk globules.

During the breeding phase, values of all these parameters were increased with decreased value of follicle population per mg or per microscopic field of the ovary. The ovarian lobules were maximally enlarged containing mostly well developed larger follicles. Ultrastructurally vitelline membrane with zona externa and zona interna, and long microvilli penetrated between the oocytes and follicular cells, abundance of RER and mitochondria were seen in the vitellogenic oocytes. In post-breeding phase, values of these parameters were significantly decreased with increased number of follicle population per mg or per microscopic field of the ovary. The ovarian lobules became extremely narrow and contained mostly undeveloped small follicles. (c) Hormonal profiles (FSH, PRL, E₂, testosterone, T₃ and T₄) were elevated with decrease of TSH in pre-breeding. Insulin level was low with high blood glucose level seen in the pre-breeding phase. Whereas, LH, DHP, cortisol, epinephrine and norepinephrine levels were greatly increased with low T₃ and T₄ levels and high TSH levels in breeding phase. Insulin level was highest with lowest blood glucose level in the breeding phase. But pineal hormone (NAS and melatonin) levels were declined in breeding and were
elevated in pre-breeding and post-breeding. Additionally, values of all other parameters, except TSH and blood glucose, were decreased in post-breeding mullets. (d) The findings suggest that FSH, PRL, E₂, testosterone, T₃ and T₄ are probably involved in oocyte growth including vitellogenesis; whereas LH, DHP, cortisol, epinephrine and norepinephrine and insulin may be involved in final oocytes growth and oocyte maturation. Energy requirement was high in the breeding phase. (g) Pineal hormones (NAS and melatonin) probably do not participate in the induction of circannual ovarian cycle in the mullet. (f) In addition to hormonal influence, low temperature with short photoperiod, may also be required to induce the circannual ovarian cycle in the Indian grey mullet.
SUMMARY

The circannual ovarian cycle has been extensively studied in freshwater teleosts. Such information, especially at ultrastructural, hormonal and metabolic levels is inadequate in marine and estuarine teleosts. In the current thesis, this problem has been resolved in an estuarine teleost, the Indian grey mullet *Mugil cephalus* L. The investigation was carried out month-wise round the year consecutively for 2 years. The parameters included gravimetry, gonadosomatic index, follicle size and follicle (oocyte) population, histology and transmission electron microscopy (TEM) of the ovary. Histology and ultrastructure of the ovarian follicles including the oocyte were studied only in pre-breeding (October) and breeding (November) phases. Histological study of the ovary was also carried out in the post-breeding phase (January). Serum hormones (gonadal and extra-gonadal) and blood glucose levels were quantitated in each month round the year for 2 consecutive years. The findings revealed that ovary weight and gonadosomatic index (GSI) (%) began to rise from September to October (pre-breeding), reached peak in November and December (breeding) and were declined subsequently from January to August (post-breeding) phase of the circannual ovarian cycle. Follicle (oocyte) size (diameter) began to increase from pre-breeding (September-October), attained maximum size in the breeding and was drastically reduced in the post-breeding mullets. Likewise, the follicular population (per paired ovary per specimen) of the ovary was increased in pre-breeding (October), attained peak in breeding and was drastically declined in the post-breeding phase. But the follicular populations per mg of ovary and per microscopic field were moderately high in pre-breeding, least in breeding and highest in post-breeding. But there were no significant changes in healthy and atrophic follicular population percent during pre-breeding, breeding and post-breeding mullet. Histologically, the ovary showed numerous lobules, each lined by double rows of small and large follicles with central
lumen seen in the pre-breeding phase. Ovarian lobules were enlarged and lined by mostly very large well differentiated follicles and oocytes in the breeding phase. The lobules became narrow, mostly small and with undeveloped follicles in the post-breeding mullet. Follicle (oocyte) size, follicle populations per mg of ovary and per microscopic field were high in pre-breeding, least in breeding and highest in post-breeding phase of the mullet. But total ovarian follicular population per specimen was moderately high in pre-breeding, highest in breeding and least in the post-breeding phase. Ultrastructurally, three types of oocytes were seen in the pre-breeding phase. (a) Small early primordial oocytes were characterized by a thin plasma membrane. (b) Large pre-vitellogenic type showed abundance of lipid droplets in the cytoplasm. (c) The early vitellogenic oocyte showed appearance of the vitelline membrane with the microvilli from the oocyte surrounded by follicular cell layer. In the breeding phase, the late vitellogenic and post-vitellogenic oocytes were recognized. The late vitellogenic oocytes were characterized by zona interna and zona externa seen in the vitelline membrane and long microvilli from the oocyte were penetrated into the follicular cell across the zona interna and zona externa. Microvilli from the follicular cells were also penetrated into the oocyte across the zona externa and zona interna. Subsequently in the post-vitellogenic oocytes, the zona externa and zona interna and microvilli were regressed and pore canals of the microvilli were closed.

Serum FSH level reached peak in pre-breeding (October), LH in breeding (December) and prolactin in pre-breeding phases (October). Whereas estradiol and testosterone levels reached peak in pre-breeding (October) and DHP in the breeding (December) phase.

Thyroid hormones (T₃ and T₄) showed peak levels with lowest TSH level in pre-breeding (September), with another earlier peak in April of the post-breeding phase. Serum cortisol, epinephrine and norepinephrine concentrations were highest in
breeding phase (November). Pineal serotonin level was higher in breeding phase with a peak in breeding phase (December) unlike N-acetyl serotonin and melatonin which were highest in post-breeding phase (June). Insulin level was highest with lowest blood glucose level in the breeding phase (November-December). The findings suggest that (a) the Indian grey mullet breeds once a year and the annual ovarian cycle passes through 3 phases, pre-breeding (September-October), breeding (November-December) and post-breeding (January-August). Rapid oocyte growth occurs in the breeding phase supported by GSI, oocyte population and morphometric and ultrastructural findings. (b) FSH, PRL (?), E₂, testosterone, T₃ and T₄ probably trigger oocyte growth and vitellogenesis as their levels reach peak in pre-breeding phase, but LH, DHP, cortisol, epinephrine, norepinephrine and insulin levels attain peak in the breeding phase indicating further oocyte growth of ovulable size (?). Peak DHP level in the breeding phase suggest its involvement in oocyte maturation. But pineal hormones (NAS and MEL) are not required for oocyte growth or maturation, because their levels were declined in the breeding phase in the Indian grey mullet. Thus, all the gonadal and extra-gonadal hormones, except pineal, are involved in the induction of circannual ovarian cycle in the Indian grey mullet. (c) As the Indian grey mullet breeds only once in winter months (November-December) it is likely that in addition to the hormonal (gonadal and extra-gonadal) factors, some environmental factors (viz., low temperature, short photoperiod and many other) may also contribute to the induction of circannual ovarian cycle in the Indian grey mullet.