Alterations in Histoarchitecture and Ultrastructure of the Ovary of Brackish-water Grey Mullet *Liza parsia* (Hamilton) During Different Reproductive Phases

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ABSTRACT: *The brackishwater teleost Liza parsia is an important food fish. Variations in the GSI, diameter of the oocytes were studied in different months of the year in this fish. Seasonal variations in the structure, morphology and histological characteristics of the cystovarian and synchronous ovary of the wild Liza parsia, are similar with majority of the teleosts. Oocytes of the fish were classified into six stages (Oogonium, early and late perinucleolus, yolk vesicle and granule and mature oocytes) of development. Postovulatory follicles are exclusive in spent phase and atretic oocytes are present in all the phases (growth, maturation, spawning and post-spawning). The minimum GSI occurred in the month of July and maximum in the month of February. The GSI data and histological and ultrastructural observations revealed that the spawning season is from December to February. The seasonal histological and ultrastructural changes in the ovary have been elaborated along with the variation in the gonado-somatic index and cytological changes in the different stages of oocyte.*

Key Words: *Annual cyclical changes- histology-ultrastructure- ovary -Liza parsia.*

1. Introduction:

Liza parsia (common name- Parse) forms a good fishery in the estuaries of India (Jhingran, 1991) and is commercially very important fish in the Indian subcontinent. The reproductive biology of fishes were studied by many authors: EL-Gharabawy (1996) for *Lithognathus mormyrus*; Zaki *et al.* (1998) for *Sparus aurata*; Assem (2000 and 2003) for *Caranx crysos and Pagellus erythrinus;* Honji *et al*. (2006) for *Merluccius hubbsi* and Garcia-Diaz *et al*. (2006) for *Serranus atricauda*. Several authors have described the process of oogenesis in many teleost fish. This included Portela *et al*. (1994) for *Loligo*, *Illex argentines* and *Merluccius hubbsi*; Louge (1996) and Honji *et al.* (2006). Spawning of the teleost fishes occur once or twice in a year or after regular time interval throughout the year. To increase the effective methods for increasing efficiency of ovulation and reproductive pattern of fishes and increasing fish production, it is necessary to determine the exact spawning period of the fish. Success of reproduction depends upon the normal gonadal development, which is stimulated by favorable environmental conditions. The oogenesis is a dynamic process in the ovaries, in which the oocyte passes through various phases of the development that are more or less similar in different fish species. Gonadosomatic index (GSI) is a good parameter for the measurement of gonadal development and reproductive effort in fishes (Calow, 1979; Saksena, 1987). Maturation of egg is fairly a long process that involves relatively complex and biochemical process. Vitellogenesis is a process by which liver produces yolk proteins and these are further transported to the ovary and stored in the egg, results the abrupt enlargement of the egg. At the optimum condition for the final maturation, nuclear development resumes and germinal vesicle migrate to the one side. At the end, the walls of the germinal vesicle brake down and the egg is liberated. Histological studies are able to provide the necessary and appropriate strategies for optimum utilization and maintain supplies of this valuable and palatable species harvesting process.

2. Material and Methods:

Adult specimens of *L. parsia* (15 to 20 cm in total length and 50-75 gm. in total body weight) were procured from Junput brackish water fish farm, (21°43'27. 1"N, 87°49'17. 5"E), West Bengal and near Digha Mohana region (21°38'. 004"N, 87°32'55. 4"E) of West Bengal. Fishes were sacrificed within a few second of capture by decapitation. Total body length and body and ovary weights of each fish were recorded immediately in the fresh condition. Ovaries were then carefully dissected out from the abdominal cavity and were fixed in aqueous Bouin's fluid for 16-18 hrs. After fixation the tissues were washed repeatedly in 70% ethanol and dehydrated properly through ascending series of ethanol. Then the tissues were cleaned with xylene and embedded in paraffin wax of 56-58°C under a thermostat vacuum paraffin-embedding bath for a period of 1

h and 30 min. Sections were cut at 4 µm thick using a rotary microtome (Weswox). Data on total body weight and ovary weight of 30 fishes were taken monthly to calculate the mean gonadosomatic index (GSI) from the following formula:

$$GSI = \frac{\text{Total ovary weight}}{\text{Total fish body weight -total ovary weight}} X \ 100$$

Sections were stained with Mallory's triple stain, Iron-alum haematoxylin and Delafield's haematoxylin followed by alcoholic eosin. The annual reproductive cycle of the ovary and its cells like oogonia, various stages of oocytes and ovum in the ovarian sections were identified. Percentage frequency of various cell types was calculated by observation of the diameters of various stages of oocytes along with their nuclei to determine the nuclear cytoplasmic volume index [N/C index = diameter of nucleus/ (total cellular diameter-diameter of nucleus)] were measured by the reticulo-micrometer (magnification x 40X) and ocular micrometer. For ultrastructural study small pieces of ovary were dissected out and were immersed with 2.5 % glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 20 minutes. The tissues were carefully washed repeatedly in heparinised saline (heparin sodium salt 10 000 IU dissolved in 0.67 % NaCl solution). Then the samples were rinsed in 0.1 M phosphate buffer (pH 7.4), again fixed with 2.5 % glutaraldehyde solution for 24 hours at 4 ℃ and secondarily fixed with 1 % osmium tetroxide (OsO_4) in 0.1 M phosphate buffer (pH 7.4) for further 2 hours at room temperature. Fixed tissues were washed repeatedly in the same buffer and dehydrated in ascending series of acetone followed by isoamyl acetate. The tissues were dried with critical point drier (Hitachi 8CP2), mounted on metal stubs, coated with gold palladium (20 nm thick) and observed under scanning electron microscope, Hitachi S-530.

3. Results:

3.1. Ovarian morphology: The ovaries of the female fish are paired, located in the abdominal cavity just ventral to the kidneys and surrounded by a layer of thick connective tissue (Fig. 1). The ovary of the brackish-water teleost *Liza parsia* is a bi-lobed solid sac, suspended in the body cavity by a vascularized mesovarium. The anterior ends of the two ovaries are free but the caudal ends are united. The posterior end of each ovary empties into a short oviduct and the two oviducts ultimately fuse and open to the exterior through a common urinogenital aperture. Apart from maturation and spawning phase, the wall of the ovary is fairly thick and gradually becomes thinner during those phases. The outer wall consists of an outermost thinner peritoneum, a thicker tunica albugenea and the innermost germinal epithelium which projects its lamellae deep inside the ovary. The ovigerous lamellae anchor the different stages of oocytes during course of their development. The lamellae lose its structure during spawning phase.

3.2. Gonadosomatic Index (GSI):

In the present study, it has been observed that the values of gonadosomatic index (GSI) in *Liza parsia* follow regular cyclical changes in the different months of the year, due to variations in different ecological factors and food availability. However, maximum GSI values are noticed during the months of December, January and February while the GSI values reduce to a minimum during the months of May and June (Fig. 12). During the annual cyclical changes of the ovary, the GSI values vary from minimum 0.33 to maximum 15.76. However, the lowest mean GSI value (0.33 ± 0.06) has been noticed during resting phase in June. In July and August when preparatory or growth phase begins the mean GSI values increases and the values are 0.52± 0.11 and 0.85± 0.14 respectively. During September to October, the GSI values rises from 1.29± 0.09 to 5.74± 0.28. However, from November onward when the ovary is in the late maturation phase, GSI increases sharply showing the value of 9.07± 0.43. In December, the GSI recorded to 13.87± 0.87. In January GSI slightly falls resulting 12.03± 0.75. In the spawning period, *i.e.,* in February the ovaries constituted with full of mature follicles and GSI rises upto the peak value 15.76± 1.34. In the post-spawning period *i.e.,* March the values are recorded 4.59 ± 0.14, in April the GSI value drops to 1.31± 0.08. From May onwards yolky follicles reabsorbed and the ovaries suffer from a regression showing the value 0.42± 0.05.

3.3. Ovarian histology:

Stages of the female gonad: The developing oocytes present in the ovary classified into various stages according to their size, appearance of nucleus, nucleolus and cytoplasm distribution in the ovary. The overall stages of different oocytes can be divided into 6 stages of development.

Stage I or Oogonium stage or Chromatin nucleolus stage:

The germ cells or oogonia are found in the oogonial nests of the lamellae in clusters of 7-9 cells and originating from the germinal epithelium layer. These cells are 15-32µ in diameter and consist of a large nucleus occupying most of the cellular volume and a thin layer of chromophobic or basophilic cytoplasm. Oogonium consists of an eccentrically large nucleus (11 to 24µm), which occupies three-fourth portion of the cell and provided with chromatin threads. This stage is characterized by the presence of conspicuous nucleolus associated with chromatin threads. Some highly basophilic nuclei are randomly distributed in the nucleus (Fig. 2 & 3).

Stage II or early perinucleolar stage:

This stage can be subdivided into early and late stages. These types of oocytes are small, diverse shaped and oocytes diameter ranges from 35-55µ. These oocytes are the product of the mitotic division of the oogonium and majority of them have oval shaped nucleus with numerous spherical nuclei at the peripheral nuclear boundary (Figs. 2, 3 & 10). N/C index ranges from 70 to 80%. Cytoplasmic materials are compact, homogeneously distributed, strongly basophilic in nature and take deep haematoxylin stain.

Stage III or late perinucleolar stage:

The oocytes in this stage are rounded in shape and 55-90µ in diameter. In the early stage oocytes grow in volume, nucleus increases in size and multiple nucleoli become located around the periphery of the nucleus (Figs. 4 &7). The nucleoplasm is rough and presents an average of 18-28 nuclei at the periphery (Figs. 4 &7). Nucleus to cytoplasmic volume index ranges from 50-70%. The cytoplasm in this stage is basophilic in nature. The late perinucleolus stage is characterized by the increment of the oocytes. The besophilia of the cytoplasm shifts from the cytoplasm to nucleoli at the end of this stage.

Stage IV or yolk vesicle stage:

The oocytes are larger than the previous stage and approximately 90-130µ in diameter. This stage is marked by the formation of large number of small vacuoles called yolk vesicles and cortical alveoli. The vesicles that appear empty accumulate near the periphery of ooplasm at first stage and later these enlarge in size distributing homogeneously inside the cytoplasm (Figs. 5 & 6). The yolk vesicles make their appearance as minute circular bodies having diameter from 6 to 10µ.Early maturing oocytes contain larger nucleus having irregular outline. Nucleus to cytoplasmic volume index ranges from 40 to 55% in these oocytes. Thin layers of follicular cells are visible in this stage (Fig. 5). Many oocytes show an undulated nuclear membrane and nucleoli enter inside the pockets of nuclear membrane to pass out into the cytoplasm (Fig. 7).

Stage V or yolk granule stage or late maturing oocytes:

The oocytes in this stage undergo an increase in size (approximate diameter range 130-190µ) and proper vitellogenesis begins in this stage due to the appearance of small rounded granules of yolk. These granules appear in the outer cortex of oocytes and among the cortical alveoli (Fig. 7). The yolk granules are lager toward the center and smaller at the periphery of the ooplasm. Until the whole ooplasm is filled, the accumulation of yolk granules continues and smaller granules fuse to form larger granules. The follicular layer of the oocytes increases in thickness and can be distinguishable and differentiable into zona radiata, zona granulosa and thecal layer (Figs. 5, 7 & 9).The nucleus starts to migrate gradually to the periphery. In this stage due to incorporation of yolk materials, cytoplasm transforms basophilic to acidophilic nature.

Stage VI or mature follicle or ripe egg stage:

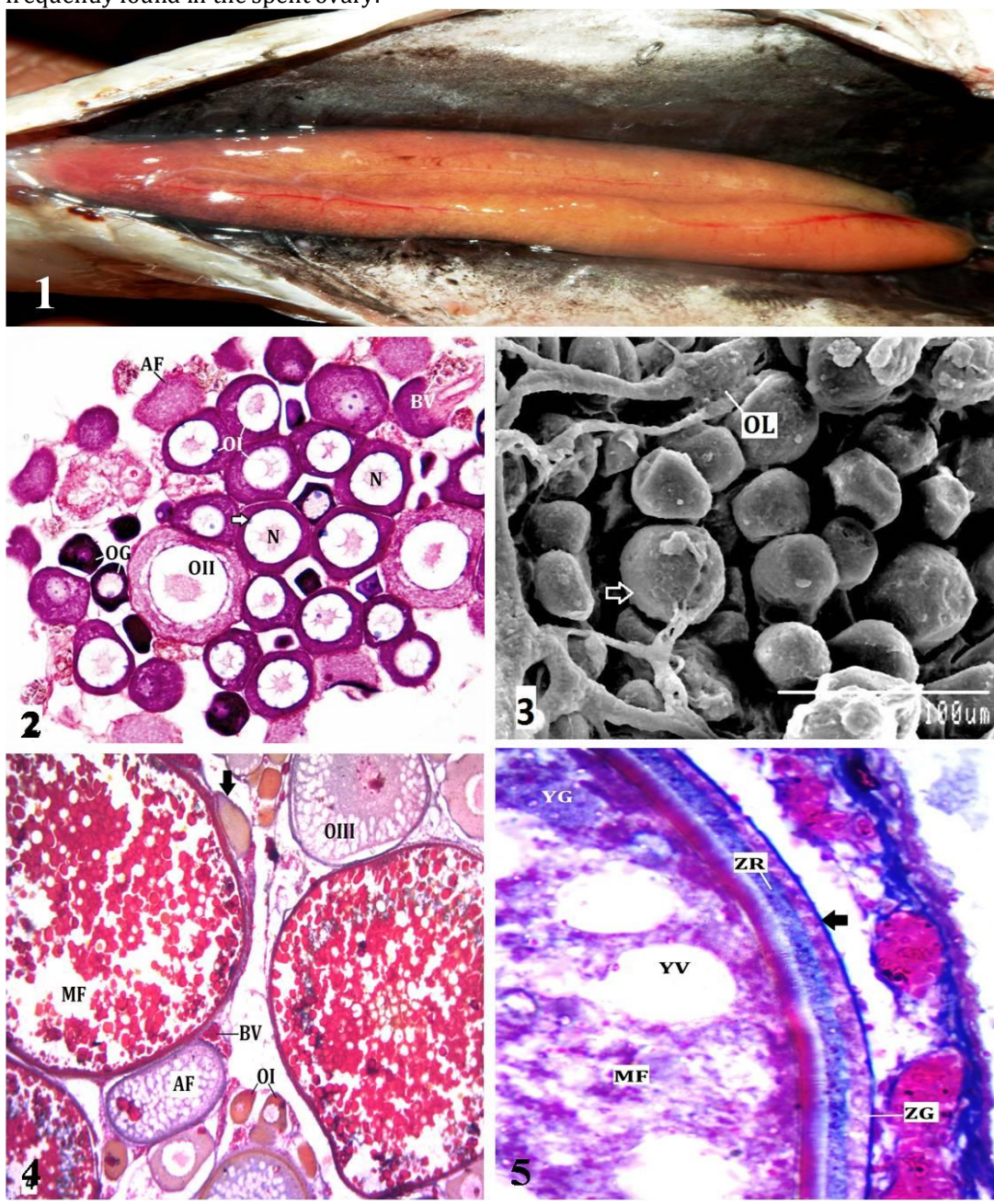
The mature follicle or ripe egg is the largest in size among oocytes having a heavy deposition of yolk that are also fairly large in size (Figs. 7, 8 & 9).The diameter of the oocytes reached a whapping 190-260µm. This stage is marked by the resumption of meiosis, which begins with the nuclear movement towards the animal pole of the oocyte boundary, and this is followed by the breakdown of the germinal vesicle. The outer connective tissue layer or theca separates the oocytes from each other. The Theca layer sometimes can be distinguishing into outer theca externa and inner theca interna. The zona radiata is very prominent and becomes much thicker. The nucleus is hardly seen in this stage, if found having an irregular outline and eccentric in position. Zona granulosa layer composed of cuboidal epithelial cells having deeply staining nuclei (Figs. 5 & 9).

Atretic oocytes:

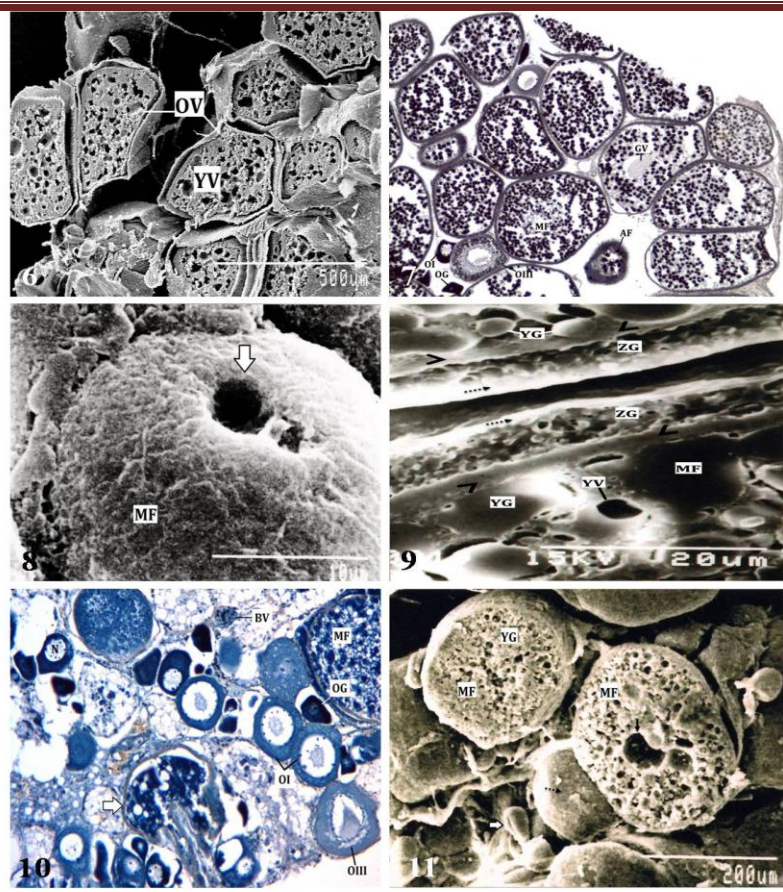
The developing oocytes and matured oocytes of advanced condition sometimes fail to attain maturity, resulting the absorption of the oocytes and are designated as atretic follicles and the process of resorption is called as follicular atresia. The follicular atresia is frequent during the pre-spawning and immediately after the spawning phases (Figs. 2, 4 & 7). At the beginning atretic follicles exhibit the hypertrophy of zona granulosa. The zona radiata deviates from the granulosa layer and make finger-like projections in the ooplasm (Figs. 2, 4 & 7). The zona radiata becomes fragmented and granulosa cells invade the cell at several points. The follicles lose their original shapes and the liquefaction of the yolk in the follicle is apparent. The

last stage is characterized by the complete absence of yolk masses. The whole follicle is filled up with the zona granulosa layer and the follicle becomes more compact totally (Figs. 2, 4 & 7).

Apart from all these stages post-ovulatory follicles are the leftovers after the shedding of mature oocytes, can be frequently found in the spent ovary.



Figs (1-5); 1:- Photomicrograph of smooth walled paired ovary inside the peritoneum cavity. **2:-** Showing ovigerous lamellae provided with oogonia (OG), oocyte I (OI) and oocyte II (OII) and few atretic follicles (AF) during early growth phase. Note the presence of prominent nucleus (N) with nucleolus (white arrow) in the oocytes (HE) × 100. **3:-** Showing primary oocyte encircling one mature follicle (arrow) and ovigerous lamellae (OL). (SEM) × 1000. **4:-**Showing mature follicle (MF) with yolk granules and follicular layers during maturation phase. Note the presence of oocytes I (OI), oocyte III (OIII) with prominent cortical alveoli, atretic follicles (AF) and blood vessels (BV). Solid arrow indicates early oocyte stage (H & E) × 400. **5**:- Showing mature follicle (MF) with full of prominent yolk globules (YG) during maturation period. Note eccentric yolk vesicle (YV), zona radiata (ZR), granulosa cells (ZG) and, theca layer (black arrow). (H & E) × 1000.



Figs (6-11); 6:- SEM photomicrograph of oocytes V (OV) having yolk vesicles (YV) during maturation phase. (SEM) × 1000. **7:-** Showing mature oocyte (MF) stage during spawning phase having yolk granules (YG) and germinal vesicle (GV). Note the presence of oogonia (OG), early oocytes (OI & OIII) atretic follicles (AF) adjacent to mature oocyte. (H & E) × 100. **8:-**Scanning electron microscopic (SEM) photomicrograph of ovary showing depressed micropyle (white arrow) on the surface of mature oocyte covered by membrane. (SEM) × 1000. **9:-** Scanning electron microscopic (SEM) photomicrograph showing mature follicles (MF) with yolk granules (YG), yolk vesicles (YV) during spawning phase. Note the presence of zona radiata (black arrow head), zona granulosa (ZG) and theca (broken arrow). (SEM) × 1000. **10:-** Scanning electron microscopic (SEM) photomicrograph of ovary showing various oocytes during early post-spawning stage of development. Mature follicle (MF) showing uniform yolk mass in the entire oocyte. Note eccentric position of germinal vesicle (solid black arrow). Note also oogonial cell (solid white arrow) and oocyte III (broken arrow) stage in between mature follicles (MF). (SEM) × 600. **11:-** Light microscopic (LM) photomicrograph of ovary showing various oocytes during different stages of development. Post-spawning phase showing the proliferation of oogonia (OG) and presence of primary oocytes (OI) with nucleus (N) containing numerous nucleolous. Note the presence of blood vessels in between the oocytes (BV), oocyte III (OIII) stage and discharging mature follicle (white arrow) in between primary oocytes. (IA) × 100.

3.4. Seasonal Changes in the ovary:

Based upon the histological observations and the frequency distribution of the various stages of the oocytes development, annual seasonal changes of the ovary can be classified into four phases *viz.* growth phase, maturation phase, spawning phase and post-spawning phase.

Growth phase:

Oogonia, perinucleolus stage and cortical alveoli stage present in this phase (Figs. 2& 3). Ovigerous lamellae occupy the cavity of the ovary. The frequency percentages of different stages of oocytes are StageI – 18%, StageII - 34%, Stage III – 44% and Stage IV- 4% (Fig. 13). The oocytes of Stage V&VI are completely absent in this phase.

Maturation phase:

In this phase all the stages of oocytes can be observed but their percentages of occurrence are variable. Cortical alveoli and yolk granule oocytes are predominant in this phase (Figs. 4, 5 & 6). The oocytes of late perinucleolus and vitellogenic stages are present. The frequency percentages of all stages of oocytes are as

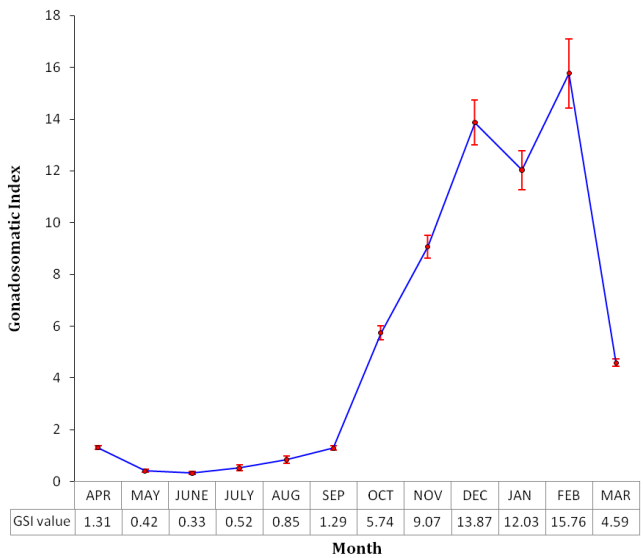
follows: Stage I – 11%, Stage II - 13%, Stage III – 28%, Stage IV –21%, Stage V – 17% and Stage VI – 12% (Fig. 13).

Spawning Phase:

Oocytes observed in the previous stage can also be seen here but the predominance of the mature follicle stage oocytes. The frequency percentages of the studied oocytes are Stage I – 12%, Stage II - 5%, Stage III – 1%, Stage IV – 1%, Stage V – 16% and Stage VI – 65% (Fig. 13).

Post-spawning or spent phase:

Convoluted ovigerous folds can be seen. Ovigerous lamellae are widely spaced containing nests of oogonia; Chromatin nucleolus and early perinucleolus stage oocytes. The lamellae are pointed towards the center of the ovary. Oocytes of all the stages can be found sparsely in this stage, the stage II oocytes & I predominate. Postovulatory follicles and conspicuous spaces inside the lumen of the ovary and atretic follicles can be observed (Figs. 10 & 11). The frequency percentages of all stages of oocytes are as follows: Stage I – 23%, Stage II - 53%, Stage III – 9%, Stage IV – 2%, Stage V – 3% and Stage VI – 10% (Fig. 13).



	APR	MAY	JUNE	JULY	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR
GSI value	1.31	0.42	0.33	0.52	0.85	1.29	5.74	9.07	13.87	12.03	15.76	4.59

Fig. 12: Seasonal variations in the Gonadosomatic index (GSI) of female *L. parsia.*

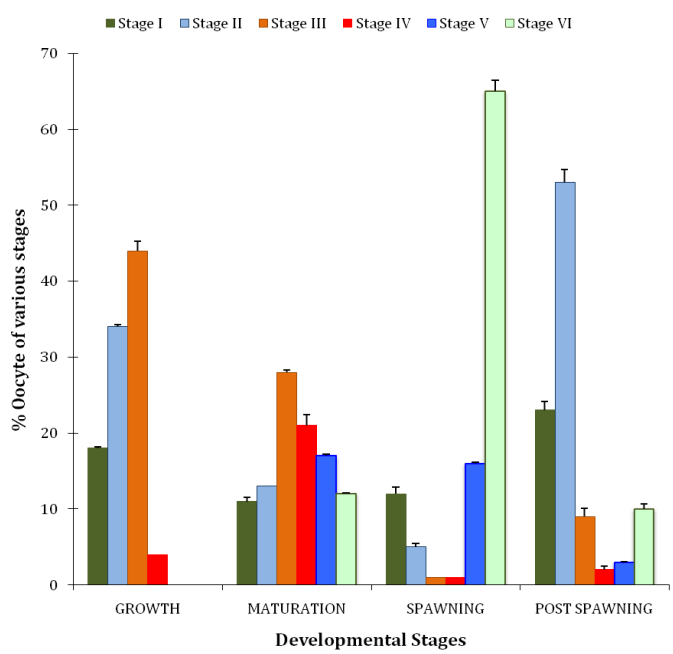


Fig. 13: Occurrence of various stages of oocytes (percentage variation) during different reproductive phases in female *L. parsia.*

4. Discussion:

This study represents the first attempt at a detailed histological and ultrastructural description of oocyte developmental stages, characterization of the ovarian cycle of *L. parsia.* The variety seen in the morphology of the ovaries in female teleost is immense. The annual reproductive cycle of female *Liza parsia* was studied on the basis of gross morphology, weight of ovaries and histological observations. The ovaries of this teleost are cystovarian type and both the ovaries are equally functional. Cystovaries are the condition that characterizes most of the teleosts, where the ovary lumen has continuity with the oviduct (Hoar 1969, Connaughton & Katsumi 1998).

The GSI of the fish seems to provide a clear indication of the reproductive status of *L. parsia* females. It shows a gradual increment with the increment of the mature females towards the spawning season. The highest values of the GSI obtained in the month of December to February clearly indicating the spawning months. *L. parsia* is an annual breeder showing a single peak value in a year. From March onwards GSI decreases dramatically indicating the onset of post-spawning or spent phase and attain its lowest level in the month of June, when the fish remain in resting phase.

According to the pattern of the oocyte development, (Selman and Wallace, 1989) the ovaries of the fishes have been classified into three types. In the case of synchronic oogenesis, all the oocytes develop at the same time and ovulation is simultaneous as found in *Oncorhyncus masou* (Yamamoto *et al*., 1959). In the group synchronous ovary at least two populations of the oocytes remain at different developmental stages: teleosts like Herrings and Speckled trout having this type of ovary generally spawn once a year with a relatively short breeding season (Hickling & Rutenberg, 1936; Vladykov, 1956). In asynchronic type of ovulation, different development stages of the oocyte maturation and ovulation in groups may be found within the ovaries (Nagahama, 1983; Nejedli *et al,* 2004). The development of oocytes in *L. parsia* ovary is synchronous type where all the oocytes remain at the same developmental stage in a given period of time.

On Histological perspective, the study was based on oogenesis, size of oocytes, size and behaviour of nucleus, nuclear membrane, number and location of nucleoli, appearance and distribution of yolk vesicles, yolk granules, appearance of oil droplets (if any), final maturation of oocytes and the GSI values of the ovaries. Developmental events that occur in the oocytes of *L. parsia* are more or less similar to those described for other species of teleosts (Rickey, 1995; Tyler and Sumpter, 1996; Grau *et al*., 2009; Honji *et al*., 2009; Lubzens *et al*., 2010). In this study the development of the oocytes in *L. parsia* was divided into six stages. In most of the teleost fishes five, six or eight stages of oocytes have been observed during the oogenesis process (Fishelson *et al*., 1996; Nagahama, 1983; Ünal *et al*., 1999; West, 1990; Gökçe *et al*., 2003; İşisağ, 1996; Arockıaraj *et al*., 2004).According to Munoz *et al*., 2002, in *Trigla lyra* ovaries contain oocytes in different synchronous groups of development are discharged as they mature. Seven stages of development were described depending on the histological and ultrastructural characteristics of the oocytes, in addition to the postovulatory follicles and atretic oocytes. Arockıaraj *et al*. (2004) mentioned the overall morphological and histological changes in the gonad of *Mystus montanus* and divided into five stages. According to Nejedli *et al*. (2004), in the European pilchard (*Sardina pilchardus* Walbaum) in the Northern Adriatic Sea, the process of oogenesis can be divided basically into four groups depending on the histological changes in the oocytes. Fishelson*et al*. (1996) and West (1990) observed that in the primary growth phase of oogenesis, the oocyte nucleus was large, with approximately 2-4 nucleoli. In the cortical alveolus phase, yolk vesicles can be observed inside the ooplasm. In the mature oocyte stage, at first the nuclear membrane dissolves andthen the migration towards periphery of the oocyte proceeds. In the present study, all the stages were identified in similar manner. Fishelson *et al*., 1996, also mentioned that the number of nucleolus might vary between the species depending on the sizes of the oocytes.

The developmental process of ovary is much similar with the other teleostean species (Yamamoto, 1956; Dadzie, 1974; Forberg, 1982; Mayer *et al*., 1988; Abou-Seedo & Al- Khatib, 1995; Coward & Bromage, 1998;Dadzie & Owiti, 1998; Maddock & Burton 1999).The sequential changes in the ovary of the fish in different reproductive phases are immense. Ovarian development of *Liza parsia* is divided mainly into four developmental stages. (1) The developing stage or growth phase is marked by the presence of oocytes with chromatin nucleoli and perinucleolus type of oocytes. vitellogenic oocytes were predominant in the ovary. Oocytes at the yolk vesicle, early and late vitellogenesis stage can be visible. (3) In the maturation stage, the compositions of oocytes were similar to that of the developing stage, but more mature oocytes were present. (4) The spawning stage was characterized by the presence of postovulatory follicles. The overall appearances were much similar to the ripe stage but mature and post ovulatory oocytes were majority in number. (5) In the post-spawning stage, the abundance of early oocytes had increased. Vitellogenic and mature oocytes had been reabsorbed, and only abundant follicles remained. In growth and maturation phases Stage III oocytes predominates in case of *L. parsia* but the percentage may vary. The maturation

phase shows the greater variety of oocytes and yolk vesicle oocytes can be observed. According to Yueh and Chang (2000), changes of the oocytes of black porgy during maturation were similar to that of other teleosts. From the GSI data and as per the distribution of oocytes, it can be implied that *L. parsia* is an annual breeder fish and follows the synchronous mode of development.

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