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Cyclical rhythms in the cytomorphology of testis of brackish water grey Mullet *Liza parsia* (Hamilton, 1822) inhabiting South-Eastern coast of India

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ABSTRACT

The cytomorphological changes occurring in the testicular tissues of the brackish water teleost fish *Liza parsia* (Hamilton, 1822) was elaborated during different seasonal conditions. The testes were synchronously arranged with various stages of germ cells such as primary and secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. The seasonal changes in the testis were described into five reproductive phases namely growth, maturation, spawning, post spawning and resting according to the variations in the gonadosomatic index and the occurrence of different developmental stages. However, the first highest spawning peak occurred in February and second highest peak in December. The sequential proliferative pattern of the germ cells during different reproductive phases was correlated with the gonadosomatic index and testicular morphology.

Keywords: Cyclical changes, Histology, Testis, *Liza parsia*.

1. Introduction

The knowledge of the reproductive activities of a fish together with its functional mechanisms is of prime importance concerning the successful management of fisheries and mobilization of seed resources. The testicular cycles in majority of freshwater teleosts which are seasonal breeders undergo remarkable changes during various periods of the season [1, 2, 3]. The reproductive biology of the various species of mullets have been observed by different researchers in India in different periods [4, 5, 6, 7, 8, 9]. Histological studies offer the scope to understand the cellular kinetics of gonad, recruitment, development and reabsorption of gonadal cells and finally in staging the maturity state of the gonads. However, there is dearth of histological studies on testicular activities of brackish water mullets. For a proper sustainability of a fish species, a thorough study of maturation cycles and alterations of gonads are important, since such a study is aimed in understanding and predicting the annual changes of the population [10, 11, 12, 13]. Therefore, an attempt has been made to study the event of spermatogenesis and the annual cyclical changes in the testes of *Liza parsia* (Hamilton, 1822). This is an endemic fish species and therefore studies on its development are very important for the preservation and population dynamics.

2. Materials and Methods

Adult live male samples of *L. parsia* (14 to 25 cm in total length and weight 25 to 90 g) were collected round the year from the Junput brackish water fish farm, Purba Midnapore and also from the sea coast of Sankarpur and Digha of West Bengal, India. The brackish water is actually the natural habitat for the species but they mostly breed in the sea. After sacrificed the fish, the testes were dissected out and fixed in aqueous Bouin's fluid for 18-24 h.

Data on total body weight and testes weight of 30 fishes were taken to calculate the mean gonadosomatic index (GSI) from the following formula:

$$GSI = \frac{weight\ of\ the\ testes}{weight\ of\ the\ fish} \times 100$$

2.1. Histological methods
After fixation in the Bouin's fluid, the tissues were dehydrated in graded series of ethanol and finally embedded in paraffin (melting point 56-58 °C). All the tissue samples were serially sectioned at 4 µm. Deparaffinised sections were brought to water through downgraded ethanol series and were stained with Delafield's haematoxylin-eosin, Mallory's triple stain and Iron alum haematoxylin. The sections were dehydrated through upgraded ethanol series, cleared in xylene and mounted in D.P.X. The diameter of various spermatogenic cells along with their nuclei were measured by the reticulo-micrometer and ocular micrometer.

3. Results
3.1. Morpho-histology of testis
The testes of *L. parsia* are paired organs and are enclosed by an outer thin peritoneum and an inner thick tunica albuginea which eventually composed of dense connective tissue. Each testis is composed of numerous seminiferous tubules surrounded by a lobule boundary wall and containing nests of various germ cells. The sizes of the lobules enlarge during spawning season for the accommodation of spermatozoa and spermatids. The spaces between the lobules are filled with connective tissues and interstitial cells associated with blood vessels (Fig. 2).

3.2. Gonadosomatic Index (GSI)
The present study enlightens the seasonal fluctuations in the gonadosomatic index of *L. parsia* as illustrated in Fig. 11. The value of GSI showed remarkable changes in different season and month. The GSI follows a regular cyclical change in the different months of the year.

3.3. Histological architecture of testis
In the testis of *L. parsia*, five types of germ cells viz., spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa are identified during different reproductive phases (Fig. 12).

3.3.1. Spermatogonia
In the present investigation, two types of spermatogonia are most commonly found in the inner margin of the seminiferous tubule. The primary spermatogonia are the largest cells and occur singly without making any groups. The nucleus is relatively large in size with a prominent deeply stained nucleolus but without any well-defined nuclear membrane. The cytoplasm is also large in volume but does not take much stain. The diameter range of these cells varies from 15.2 × 11.67 µ to 25.5 × 17.02 µ (Figs. 1, 8, 9 and 10).
The primary spermatogonia divide mitotically to give rise secondary spermatogonia which have slightly smaller nuclei than its precursor and can be seen in groups called cysts. The nucleus becomes dense and darker and having chromophobic cytoplasm. The diameter ranges from 7.5 × 5.6 µ to 11.3 × 8.2 µ whereas the nucleus varies from 1.8 to 3.2 µ in diameter (Figs. 1, 2, 3 and 5).

3.3.2. Primary spermatocyte
The primary spermatocytes contain relatively lesser amount of chromophobic cytoplasm and the nucleus is deeply stained with haematoxylin. The diameter of the nucleus approximately measuring 4.75 × 5.10 µ whereas, cell diameter approximately measuring 9.2 × 7.6 µ (Fig. 2).

3.3.3. Secondary spermatocyte
The secondary spermatocytes arising from the division of the primary spermatocytes are smaller in diameter and present in large numbers within the cysts. Cytoplasms of these cells are difficult to observe. The chromatin materials are seen to form a thick clump. The diameter of the nucleus approximately measuring 3.42 × 4.23 µ in size (Figs. 2 and 4).

3.3.4. Spermatids
The spermatids are further reduced in size with crescent or elliptical nucleus that takes a deep stain with haematoxylin. The diameter of nucleus approximately measuring 2.91 × 3.87 µ in size (Figs. 2, 3 and 5).

3.3.5. Spermatozoa
These are eventual consequence of spermatogenesis and the smallest one of all the spermatogenic cells with an average diameter of 1.93 × 1.72 µ. It has strong affinity to haematoxylin (Figs. 3, 5 and 7).

Interstitial cells
The interstitial cells are round or oval in shape and reside singly or in small groups between the lobular spaces and associated with the blood vessels (Figs. 2, 3, 4, 5 and 7). The interstitial cells undergo changes in morphology in different seasons.

3.4. Cyclical changes during spermatogenesis
The activities of the testis are found to undergo correlative seasonal changes along with the different reproductive phases. On the basis of gonadosomatic index (GSI) (Fig. 11) and the occurrence of various spermatogenic cells (Fig. 12), the reproductive phases of *L. parsia* can be divided into 5 phases: growth (July to September), maturation (October to November), spawning (December to February), post-spawning (March to April) and resting (May to June).

3.4.1. Growth or Rebuilding Phase (July to September)
During this phase the GSI in testis ranges from 0.45±0.11, 0.37±0.14 and 0.42±0.09 for the months of July, August and September respectively. During early growth phase the predominant primary spermatogonia are arranged in a definite pattern and a few primary spermatocytes are also present in between them (Fig. 1). In the late growth phase, the testes possess thick lobule boundary wall. This phase is characterized by the presence of all stages of the spermatogenic cells (Figs. 2 and 3). The primary and secondary spermatocytes are gradually increase in number, the lumen of the seminiferous tubule becomes expanded, cysts of spermatids and few spermatozoa can be seen inside the lumen (Fig. 3).

3.4.2. Maturation Phase (October to November)
The GSI value gradually increases from early part of October (0.46±0.13) and attained 0.86±0.14 in the month of November. The lobule boundary wall of the testes has become considerably thin (Fig. 4) and the spermatogonia are reduced in number and restricted along the boundary wall of the lobules (Fig. 5). The primary and secondary spermatocytes are reduced considerably and gradually transformed into the spermatid with the condensation of the chromatin material. During the late maturation phase maximum lobules are packed with spermatozoa (Fig. 5). The active interstitial

cells are noticed in between the lobules and the diameter varies from 5-7 µ (Figs. 4 and 5).

3.4.3. Spawning Phase (December to February)
This phase has been characterized by the extensive spermiation. The mean GSI value ranges from 1.08±0.06, 0.98±0.17 and 1.19±0.2 respectively for the months of December, January and February. The germinal epithelium is very thin (Fig. 6) and the spermatogonia are further reduced in number (Fig. 7). The testicular lobules with full of spermatozoa and the spermatocytes are rare (Fig. 6). The spermatozoa are consisted of deeply stained head and a long single flagellum (Fig. 7). The maximum activity of the interstitial cells can be seen in this stage as they increase in number and size (5.5 × 6.6 µ to 7.9 × 9.3 µ) (Fig. 7).

3.4.4. Post Spawning or Spent Phase (March to April):
In this phase the GSI value recorded as the lowest among all the months, 0.12±0.08 and 0.18±0.11 respectively for March and April. The lumen of the seminiferous tubules greatly reduce in diameter due to release of sperms and this represent the onset of post spawning or spent phase of the testis. The lobule boundary gradually becomes thicker and the lobules contain residual spermatozoa and few cysts of spermatids (Fig. 8). The interstitial cells are small in size (Figs. 8 and 9). The primary spermatogonial cells becomes increase in number along the border of few lobules (Fig. 8) which later become prominent and gradually occupied in the inner margin of the lobules (Fig. 9).

3.4.5. Resting Phase (May to June)
During this phase the GSI values ranging from 0.27±0.12 to 0.33±0.06 in the months of May and June. The lobule boundary wall becomes thicker and the seminiferous tubules are occupied by cysts of primary and secondary spermatogonia (Fig. 10). Some of the testicular lobules are still packed with residual spermatozoa (Fig. 10).

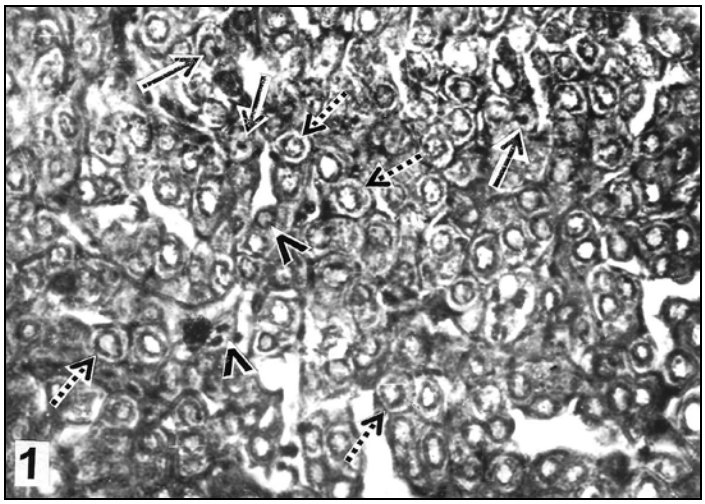


Fig 1: Large number of primary spermatogonial (PSPG) (broken arrows) during early growth phase having large volume of chromophobic cytoplasm. Note the presence of secondary spermatogonial cells (SSPG) with basophilic nucleus (solid arrows). Arrow heads indicate few primary spermatocytes (PSP) (H&E) × 400 X.

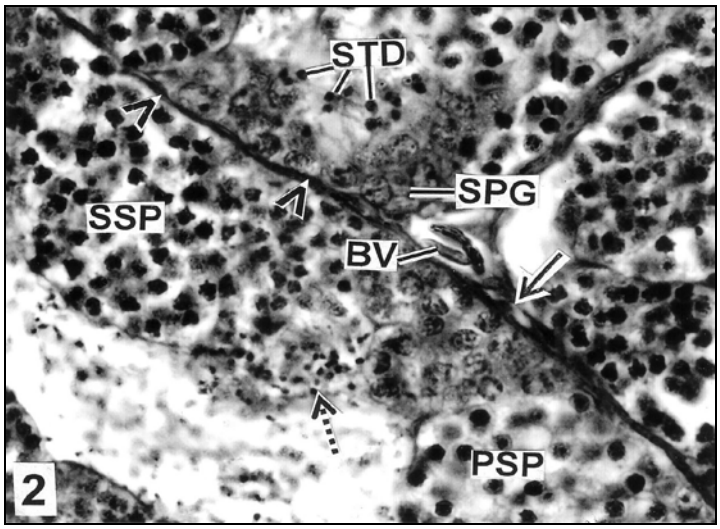


Fig 2: Spermatogonial cells (SPG), PSP, secondary spermatocytes (SSP), spermatids (STD) and few spermatozoa (SPZ) (broken arrow) during late growth phase. Note the thick lobule boundary wall (arrow heads) and interstitial cells (solid arrow) adjacent to blood vessel (BV) (H&E) × 1000 X.

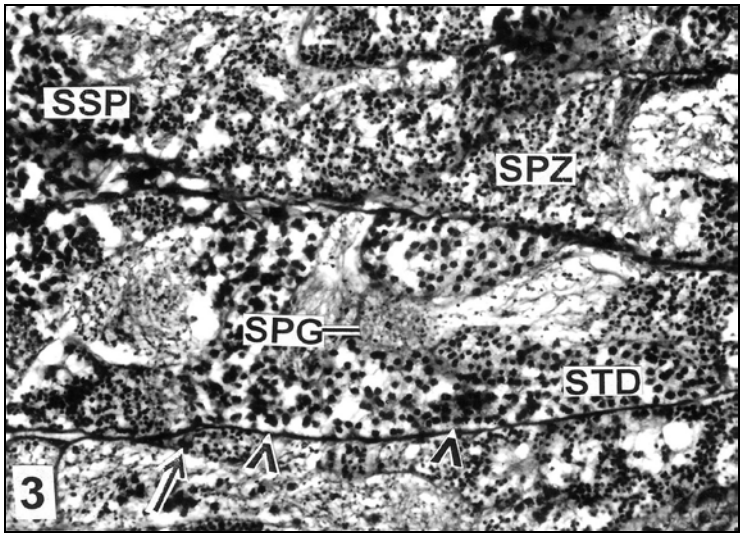


Fig 3: Showing cysts of SSP, STD and SPZ in the lumen of the seminiferous lobules in the late growth phase. Note thin lobule boundary wall (arrow heads) and interstitial cells (arrow) in inter lobular spaces (H&E) × 400 X.

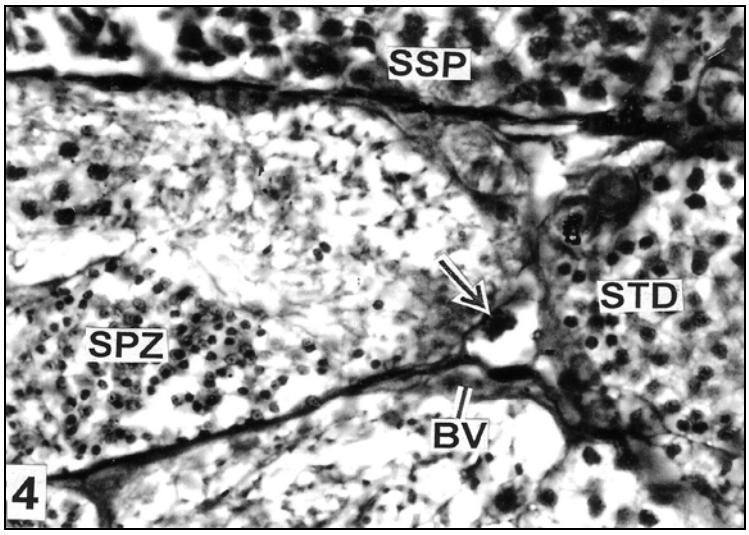


Fig 4: Increased number of STD and SPZ and few SSP in the lumen of the testicular lobules during maturation phase, having thin boundary wall. Note the active interstitial cells (arrow) adjacent to BV (MT) × 1000 X.

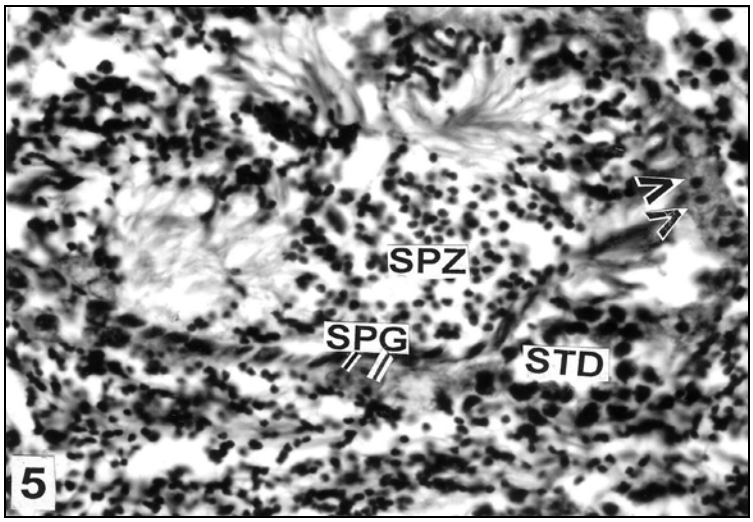


Fig 5: Showing reduced number of SPG and significant increment of STD and SPZ in the testicular lobules during late maturation phase. Arrow heads indicate interstitial cells (H&E) × 1000 X.

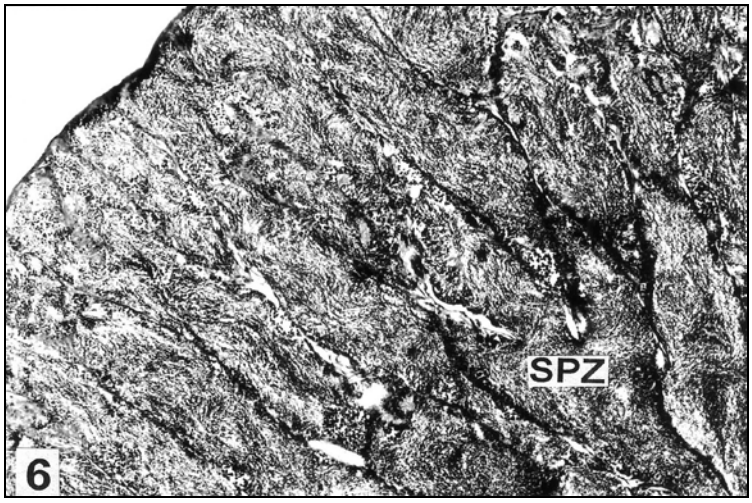


Fig 6: Showing different stages of testicular lobules having thin boundary wall and packed with SPZ during spawning phase (IAH) × 100 X.

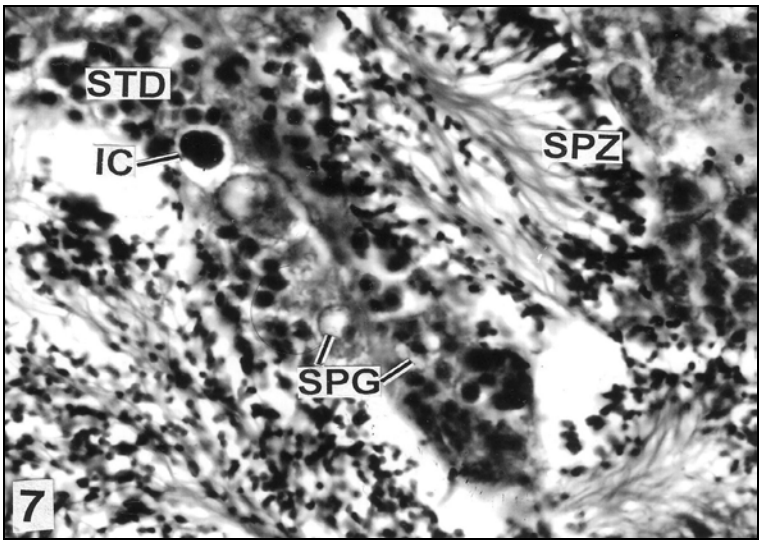


Fig 7: Maximum population of SPZ having deeply stained head and a long flagellum during spawning phase. Note the presence of hypertrophied interstitial cells (IC), cysts of STD and few SPG (IAH) × 1000 X.

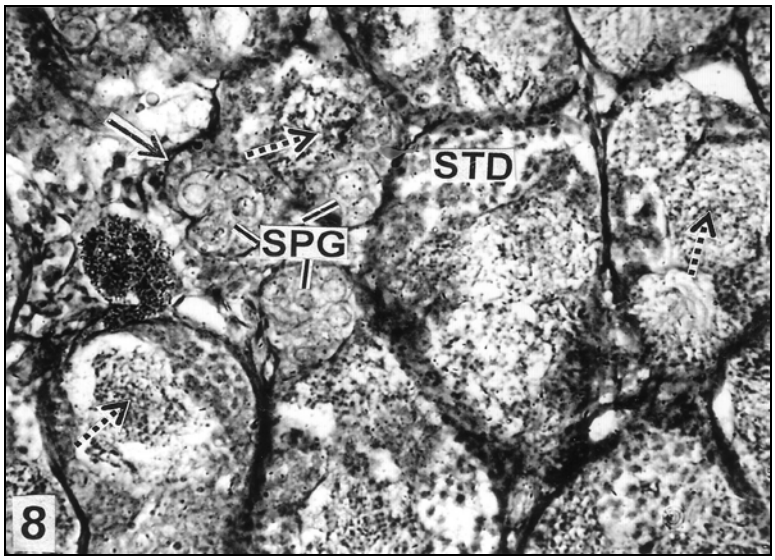


Fig 8: Different sizes of reduced, thick walled testicular lobules during post spawning phase containing residual SPZ (broken arrows) and cysts of STD. Note the considerable number of SPG in the lobules. Solid arrow indicates interstitial cell (H&E) × 400 X.

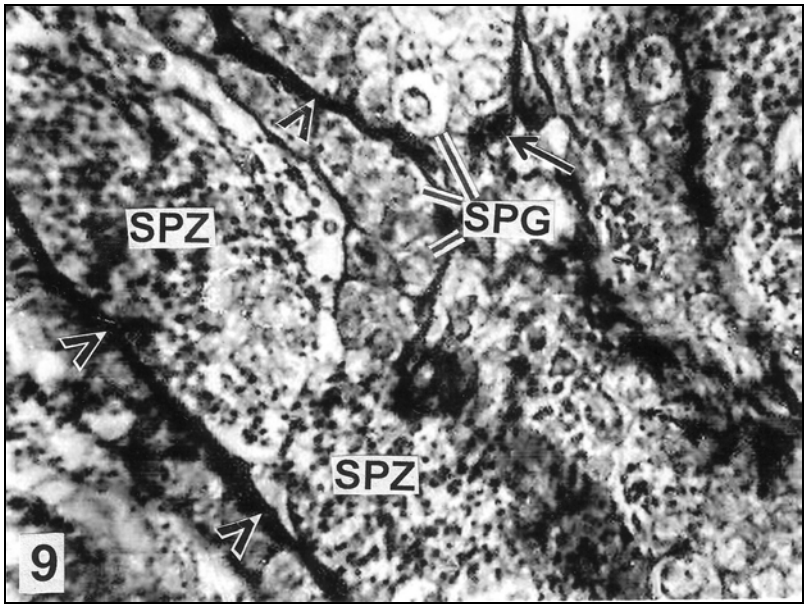


Fig 9: Thick walled testicular lobules (arrow heads) showing residual SPZ in the lumen during post spawning phase. Note the increased number of primary SPG and reduced size of interstitial cell (arrow) (H&E) × 400 X.

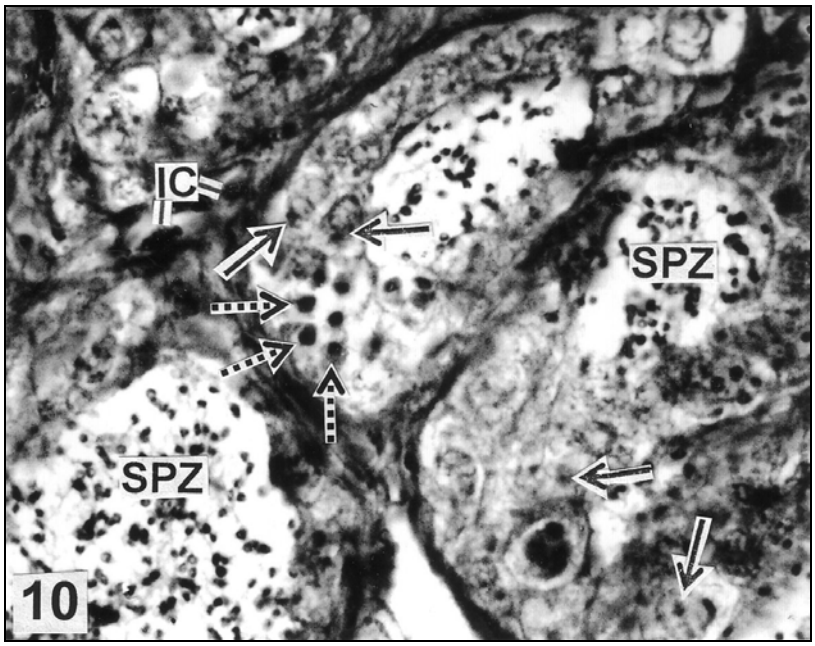
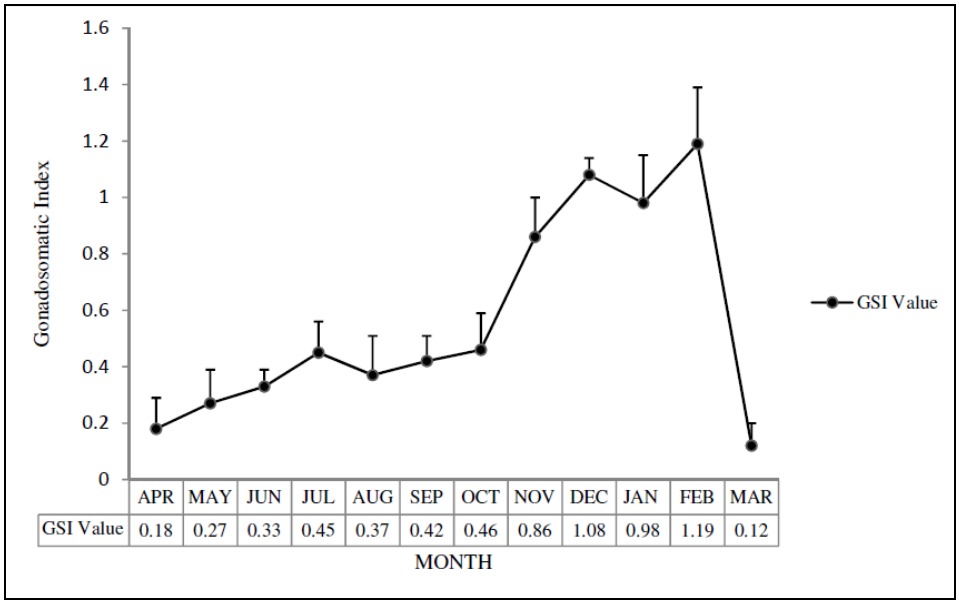
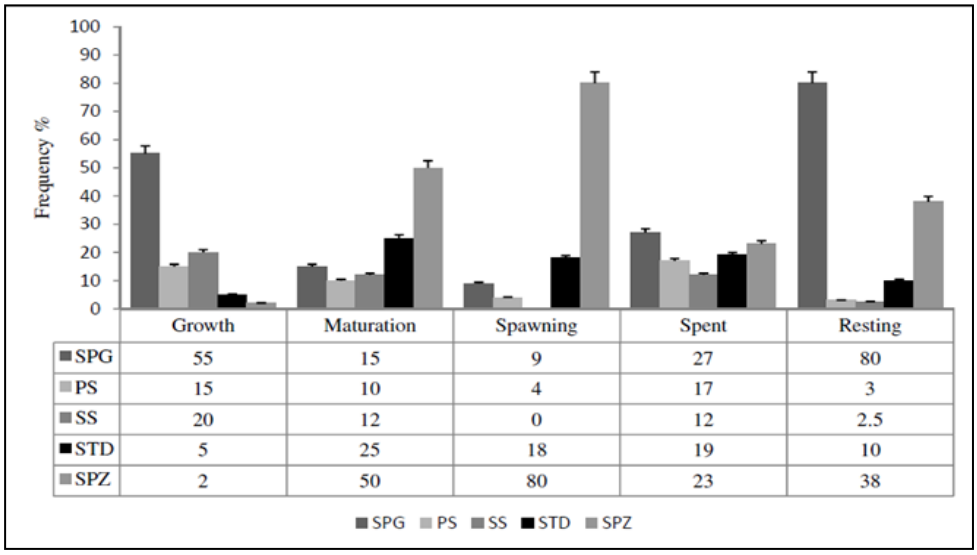


Fig 10: Showing reduced number of residual SPZ within the thick walled testicular lobules. Note the presence of primary SPG (solid arrows) and secondary SPG (broken arrows) within the lobules. Note the presence of IC in the inter lobular space (H&E) × 600 X.



	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR
GSI Value	0.18	0.27	0.33	0.45	0.37	0.42	0.46	0.86	1.08	0.98	1.19	0.12

Fig 11: Seasonal variations in the Gonadosomatic index (GSI) of male *L. parsia*



	Growth	Maturation	Spawning	Spent	Resting
SPG	55	15	9	27	80
PS	15	10	4	17	3
SS	20	12	0	12	2.5
STD	5	25	18	19	10
SPZ	2	50	80	23	38

Fig 12: Occurrence of various spermatogenic cells during different reproductive phases in *L. parsia* (SPG = Spermatogonial cells; PS = Primary spermatocyte; SS = Secondary spermatocyte; STD = Spermatid; SPZ = Spermatozoa).

Figs 1-10: Photomicrographs of the histological sections of testis of *Liza parsia* showing architecture of cells during different reproductive phases. (Haematoxylin and Eosin: H&E; Mallory's triple: MT; Iron Alum Haematoxylin: IAH)

4. Discussion

In the present investigation, it has been established that *L. parsia* is a seasonal breeder and it migrates towards the sea for spawning in the spawning season i.e. December to February. The migratory pathways of mullets from estuaries towards the sea were earlier reported by Jhingran [14] and Thompson [15]. Prior to spawn, the testes undergo preparatory stages during the remaining part of the season, which includes various degrees of histological and cytological changes in relation to spermatogenetic activity. In the present study the testes of *L. parsia* exhibit phenomenal variations in their shape, size and volume during different seasons. It has also been observed that the GSI values vary greatly during the different months of the year. It remains very low throughout the entire post-spawning phase when spermatogenetic activities are almost ceases. However, because of the proliferation of the spermatogonial cells and subsequent formation of spermatocytes in the testis, gradual increase of GSI has been noticed. The highest GSI value in the late maturation and spawning phases is due to the active proliferation of the later stages of the spermatogenetic cells causing the relative increase of the testes weight. Similar changes of the GSI values in relation to spermatogenetic activity in the testes of different teleosts have also been observed by Sanwal and Khanna [16], Jayaprakash and Nair [17], Chakrabarti and Gupta [18] and Rheman *et al.* [19]. It has been observed that the fish spawn only after gaining the highest GSI value during December and February. Therefore the GSI is closely related with the maturity and spermiation of the fish under study. In the present investigation the dormant nests of spermatogonia occurring in the post-spawning and resting periods. However, these spermatogonia appear to be responsible for their reconstruction and subsequent formation of

various spermatogenetic cells of the different periods of the year. Similar type of dormant spermatogonia has also been reported in some other teleosts by different authors [20, 21, 22]. In *L. parsia*, two types of spermatogonial cells, primary and secondary have been recognized. The cellular boundary of secondary spermatogonial cells is more prominent than that of the primary spermatogonial cells. Some authors also categorized primary and secondary spermatogonial cells in other teleosts [23, 24]. During the process of differentiation of spermatogonia into spermatozoa through the subsequent intermediate stages, the cytoplasm and nuclei of spermatogonia progressively decrease in size and volume. Chromatin materials in the nuclei of latter stages of spermatogenetic cells are relatively more condensed when compared with those of spermatogonia and primary spermatocytes. These identifying characters of male germ cells of different teleosts have also been followed by various authors [25, 21]. In the present study it has been observed that in *L. parsia* the spermatids are finally metamorphosed into spermatozoa which comprises of a rounded but darkly stained head having dense chromatin material and a long flagellum. But, Brusle [26] pointed out that the mature spermatozoa of the teleost, *Liza aurora* comprises of a round nucleus and a pseudo middle piece.

According to the meiotic and spermatogenetic activity of the testicular lobules as well as the fluctuations in GSI values obtained in the present study, the stages of the reproductive cycle of male grey mullet *Liza parsia* has been divided into five distinct stages viz; developing, mature, spawning, spent and resting [27, 28]. During growth phase, due to the formation of maximum numbers of secondary spermatogonial cells from the primary ones by the process of active mitosis the frequency percentage of secondary spermatogonia increases. However, gradual cellular activity is found to be associated with the testicular lobules during the latter part of the growth phase which in turn is characterized by an increased activity in the conversion of spermatogonia to primary, secondary spermatocytes, few spermatids and spermatozoa. The frequency percentage of primary spermatocytes increase gradually, reaching maximum during early part of the maturation phase. However, during the late maturation period enormous number of cysts of spermatids and spermatozoa almost completely filled up the entire lumen of testicular lobules. Therefore, early spawning phase is characterized by the presence of maximum number of spermatozoa and spermatids in the testicular lobules of *Liza parsia*. This is due to the rapid transformation of spermatids into mature spermatozoa by the process of spermiation during the said phase. The spermatogenetic activity decreased sharply following the regressive period and the testes finally enters into the post-spawning and resting phase (March to June). This phase is characterized by the presence of majority of empty follicles containing residual spermatozoa and few cysts of spermatids along with few dormant spermatogonial cells. The percentage of residual spermatogenetic cells also has been reported by Davis [29], Sinha and Mandal [21] in some teleosts during the resting phase. Similar testicular cycles have been reported in different teleosts [30, 31, 32, 33]. Interstitial cells are present within the interlobular spaces in *L. parsia*. These cells undergo seasonal variation in structure and function. The number of size of the cells as well as the size of the nucleus of the interstitial cells, undergo enlargement during maturation and early spawning period thus indicating their role in spermatogenesis. Chung *et al*. [34] opined that the leydig cells are typical steroidogenic cells exhibiting several cytoplasmic characteristics and are actively involved in spermatogenesis in male *Pampus argenteus*.

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