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Padmanabha Chakrabarti
Fisheries Laboratory,
Department of Zoology,
The University of Burdwan,
Burdwan – 713104, West Bengal,
India.

Shruti Ghosh
Fisheries Laboratory,
Department of Zoology,
The University of Burdwan,
Burdwan – 713104, West Bengal,
India.

Correspondence
Padmanabha Chakrabarti
Fisheries Laboratory,
Department of Zoology,
The University of Burdwan,
Burdwan – 713104, West Bengal,
India.

Changes in the histological architecture of thyroid follicles and testicular tissues during growth, maturation and spawning phases in *Tenualosa ilisha* (Hamilton)

Padmanabha Chakrabarti, Shruti Ghosh

Abstract
The present investigation dealt with the cytological status of thyroid follicles and correlated them with the changes of testicular activities in male *Tenualosa ilisha* (Hamilton). On the basis of tinctorial properties, each follicle possessed a central lumen either fully or partially filled up with the agranular eosinophilic colloidal materials and the lumen was encircled by a single layer of epithelial cells. The secretory activity of the follicles fluctuated in harmony with the testicular cells during growth, maturation and spawning phases in *T. ilisha*. The low active condition of the thyroid follicles during the growth phase was well coincidence with the increase of spermatogonial and spermatocyte cells. The most active condition of the thyroid follicles were noted during the end of maturation and spawning phases correlated with the highest frequency percentage of spermatids and spermatozoa. It was concluded that the cytological changes in the thyroid follicles and testicular activities correlate well during growth, maturation and spawning phases in male *T. ilisha*.

Keywords: Histology, thyroid follicles, testicular tissues, growth, maturation, spawning, *Tenualosa ilisha.*

1. Introduction
The thyroid is a chief endocrine gland that regulates metabolic rate. The large number of thyroid follicles in teleosts are diffused round the ventral aorta and afferent branchial arteries. Thyroid tissue in teleosts mainly occurs in the pharyngeal region but is often widely distributed anatomically. With the exception of few fishes the scattered nature of thyroid follicles is a ubiquitous feature of all teleosts [1]. Histologically, thyroid tissues are composed of a large number of follicles, each of which is in the form of hollow ball consisting of a single layer of epithelial cells enclosing a fluid filled space. The epithelium surrounded the follicle may be thick or thin and the height of the cells depends upon its secretary activity [2, 3, 4]. The seasonal changes in the activity of thyroid follicles in teleosts have been studied and often been correlated with the gonadal maturation [5, 6, 7]. Many investigators opined that since the large amounts of gonadal steroid hormones are secreted during gonadal maturation in teleosts, some of the apparent thyroid activity during these times might be related to be thyrotrophic effects of the steroid hormones themselves [8, 9, 10]. Chakrabarti [7] conjectured that there is a clear correlation between the gonadal cycle and salinity preference in brackish water teleost *Liza parsia*.
So far as the thyroid follicles in brackish water teleosts and / or anadromous migratory fishes are concerned, no serious attempt has however, been made. It would be interesting to study in details the different functional states of thyroid follicles in correlation with the gonadal status of *Tenualosa ilisha*. This would help in determining the proper maturity stages of gonads and the knowledge of environmental control in reproduction can be manipulated to accelerate or delay gametogenesis in brood fishes to bring forth culture of this species on a sounder line.

2. Materials and Methods
Living mature male fishes of *Tenualosa ilisha* (22 cm in length and 400 to 450 g in weight) were procured from river Ganga at Samudragarh, Tribeni at Hooghly district and from Raidighi during every month from February to August, 2015. Every month data on total body weight and testicular weight of 10 fishes were taken to calculate the mean gonadosomatic index (GSI) using the following formula:

$$GSI = \frac{\text{Weight of the testis}}{\text{Total body weight - testes weight}} \times 100$$

2.1 Histological methods

Immediately after collection for obtaining the thyroid, tissue; lower jaw of fishes were taken out and sternohyoid muscles were removed. The tissues around the ventral aorta and afferent branchial arteries were dissected out and fixed in aqueous Bouin's fluid for 18 hour. Decalcification was made in a mixture of 5% formic acid and formaldehyde (1: 1 volume) for 7 days. Testes were cut into pieces and were fixed in Bouin's fluid for 18 hour. After fixation the testis and decalcified thyroid tissues were placed in 70% ethanol and subsequently dehydrated properly through graded ethanol, followed by acetone and cleared in benzene. Tissues were cut at 4 µm.

Deparaffinized sections were brought to water through graded alcohols and the sections of thyroid were stained with Delafield's haematoxylin – eosin (H & E) and Mallory's triple (MT) stain and that of testis with iron alum haematoxylin (IAH), (MT) and (H & E). Sections were dehydrated through ascending ethanol series, cleared in xylene, mounted permanently with DPX and then examined with binocular microscope. The measurement of colloidal diameter and diameter of thyroid follicles and the diameter of various spermatogenic cells were measured with the help of reticulo-micrometer and ocular micrometer respectively.

3. Results

3.1 Histology of thyroid

In the present investigation, the thyroid follicles in *T. ilisha* is unencapsulated and the follicles are found to be arranged in groups or scattered in loose connective tissue around the regions of middle and posterior part of ventral aorta in between dorsal branchial cartilages and ventral sternohyoid muscles (Figs. 1, 2, 6). The thyroid follicles in *T. ilisha* are mostly spherical to oval shaped and frequently aggregated around the ventral aorta and its final rostral branching in the gill region. Each follicle possesses a central lumen encircled by a single layer of epithelial cells. The lumina of thyroid follicles are either fully or partially filled up with agranular eosinophilic colloid materials but during maturation and spawning phases they are provided with resorption vacuoles (Figs. 5, 6). On the basis of the amount of colloid, presence or absence of colloidal resorption vacuoles and the height of the epithelial cell layer the different states of activities of thyroid follicles have been recognized in *T. ilisha*. In the follicles at the first stage, the non - secretory stage, exhibit the follicular lumen completely filled up with the dense colloid having close contact with the epithelial cell layer. The height of epithelial cell layer is minimum (Fig. 1). The follicles at the second stage of quiescent, the follicular lumen is not completely filled up with the colloid and the resorption vacuoles and peripheral lumen around the central colloid has been encountered. The epithelial cell height appears to be moderate (Figs. 2, 6). Follicles of third stage, the active secretary stage, the number of colloid resorption vacuoles increase considerably and the colloid is liquefied. The height of epithelial cell layer is at its maximum degree having distinct nuclei (Figs. 5, 6). Follicles of the fourth stage, the stage of collapse or atrophied with scanty colloid. Epithelial layer is irregular without any definite arrangement of nuclei (Figs 9, 10).

3.2 Spermatogenesis

The event of spermatogenesis in *T. ilisha* has been divided into five distinct stages *viz.*, spermatogonia, primary spermatocytes, secondary spermatocytes spermatids and spermatozoa.

3.2.1 Spermatogonia

Spermatogonia, the largest spermatogenic cells, more or less spherical in shape occurring singly or in the groups attached to the lobule boundary wall of the testicular lobule with chromophobic cytoplasm. The diameter of these cells vary from 12 to 14 µm with spherical nucleus having diameters of 4 to 5 µm (Figs. 3, 4).

3.2.2 Primary spermatocytes

The primary spermatocytes are comparatively smaller in size than spermatogonia having 5 to 8 µm in diameter with condensed basophilic nucleus measuring from 3 to 4 µm (Fig 4).

3.2.3 Secondary spermatocytes

The secondary spermatocytes are further reduced in size and are more or less spherical in shape with a diameters ranging from 4 to 6 µm. Cytoplasm is hardly seen around the nuclei of those cells (Figs 3, 4).

3.2.4 Spermatids

The spermatids are spherical or oval in shape and further reduced in size with their diameters ranging from 2 to 3.5 µm. Spermatids remain in dense aggregation within the lumen of lobules (Figs 3, 4, 7).

3.2.5 Spermatozoa

Spermatids, the haploid cells and by morphological transformation and metamorphosis, give rise to spermatozoa occurring in the central position of lumen in the lobules (Figs 7, 8, 11, 12). The average diameters ranging from 1.5 to 2.0 µm. The nuclei of spermatozoa exhibit strong affinities towards the basic dyes.

3.3 Changes in the thyroid follicles and testicular cells during growth, maturation and spawning phases

The activities of the thyroid follicles and the frequency of various spermatogenic cells, as revealed by the histological studies are found to undergo changes in harmony with growth, maturation and spawning phases in *T. ilisha*. The activities of the thyroid follicles have been studied by considering the height of the follicular epithelial cells along with the follicular and colloidal diameters.

3.3.1 Growth phase (February to March)

During this phase the thyroid follicles in *T. ilisha* are in non - secretory stage and are surrounded by flat squamous epithelial cell layer. The average diameter of thyroid follicles ranges from 22 ± 0.25 µm in February to 32.46 ± 2.05 µm in March (Figs 1, 2). The height of epithelial cell layers varies from 1.6 ± 0.08 to 2.02 ± 0.08 µm. The oval or rounded follicles are filled up with dense eosinophilic colloids which during early

period are in contact with the epithelial cell layer (Figs. 1). However, during the end of growth phase the height of the epithelial cell layer increase considerably measuring about 1.94±0.88 to 3.5±1.5 µm. The colloids are seen to be unevenly stained and vacuolated structure appears at the outer margin of the colloid (Fig 2).

During growth phase the GSI is recorded from 0.23±0.051 to 0.577±0.03. Spermatogonia are arranged in small groups adjacent to the lobule boundary wall (Fig 3). Large number of primary and secondary spermatocytes having round nuclei are found in the testicular lumen. During early growth phase cyst of spermatids are few but at the end of growth phase the density of spermatids increase considerably within the tubular lumen (Fig 4). Interstitial Leydig cells measuring 2 to 3 µm are noticed in the inter - lobular boundary walls (Figs 3, 4)

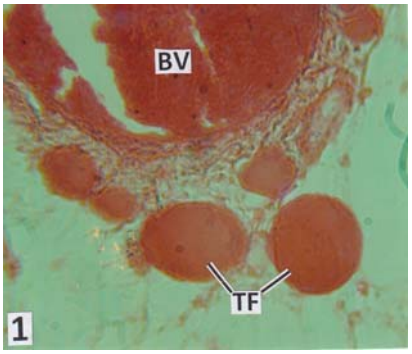

Fig 1: Showing thyroid follicle (TF) having dense colloid during growth phase encircling ventral aorta (BV). (H & E) × 400.

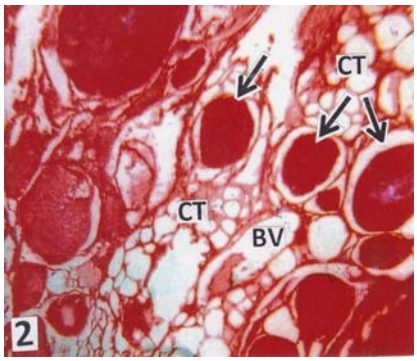

Fig 2: Thyroid follicles (arrows) in between connective tissue (CT) and blood vessels during end of growth phase. Note empty spaces along the periphery of colloid (H & E) × 400.

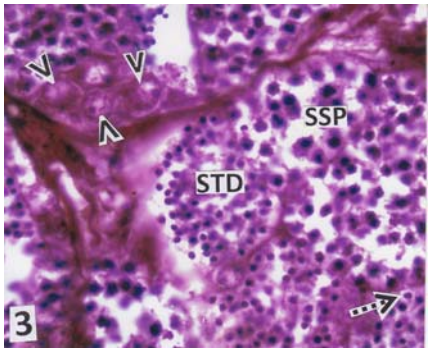

Fig. 3. Showing aggregation of spermatogonial (SPG) cells (arrow heads) along border of testicular lobule, cyst of secondary spermaocytes (SSP) and few spermatids (STD) during growth phase. Broken arrow indicates interstitial Leydig cells (H & E) × 400.

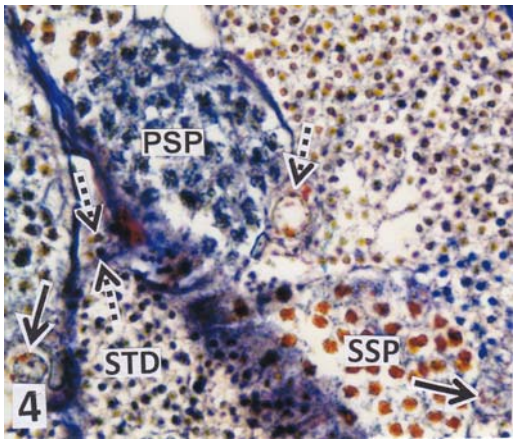

Fig 4: Increased number of primary spermstocytes (PSP), SSP and STD at the end of growth phase. Note the presence of a few SPG along the border (Solid arrows). Broken arrows indicate interstitial Leydig cells (IC) (MT) × 400.

3.3.2 Maturation phase (April to May)

In this phase, the thyroid follicles are in the active secretary stage. An increasing trend in the epithelial cell height having prominent nuclei has been recorded from 2.63±1.12µm in April to 3.5±1.5 µm in May (Fig. 5). The follicles are oval or elongated in shape and closely associated with the blood vessels (Figs. 5 and 6). The follicular diameter measuring about 26.24±1.4×43.84±1.12 µm to 32±1.14 to 41.26±2.16 µm respectively. In this phase third stage follicles are predominate having resorption vacuoles (Fig. 6) and the colloid is liquefied (Fig. 5). An increase of the degree of vascularization around the follicles is also evident (Fig. 5). At the end of this phase the height of the epithelial cell layer increase further having prominent nuclei. The colloids are seen to be faintly stained and vacuolated structures appears at the outer margin of the colloid (Fig. 6).

The GSI is recorded from 1.14±0.09 in April to 1.12±0.106 in May. In April large number of spermatid cysts and spermatozoa are dominated adjacent to few secondary spermatocyte cyst. The interlobular interstitial Leydig cells measuring about 4 to 4.5 µm are seen (Figs. 7, 8). However, at the end of this phase concomitant increase of cysts of spermatids and spermatozoa are discernible (Fig. 8).

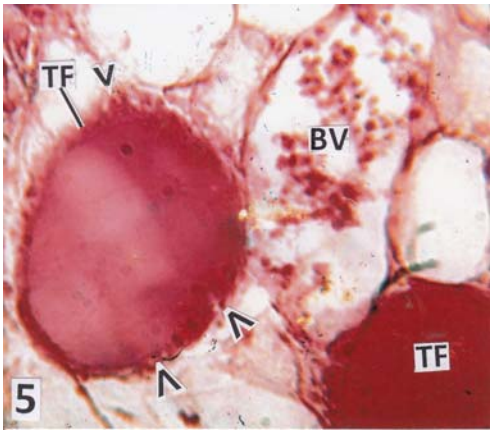

Fig 5: Active scretory stage of thyroid follicle (TF) having thickened boundary with nucleus (arrow heads) and liquification of colloid during maturation phase. BV indicates blood vessels. (H & E) × 600.

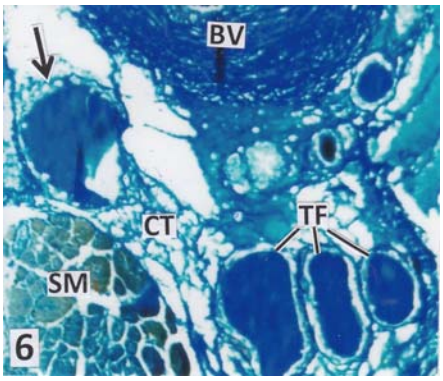

Fig 6: Showing active TF embedded in the connective tissue (CT) with peripheral lumen in between blood vessels (BV) and Sternohyoid muscles (SM). Arrow indicates resorption vacuoles along the periphery of TF (MT) × 400.

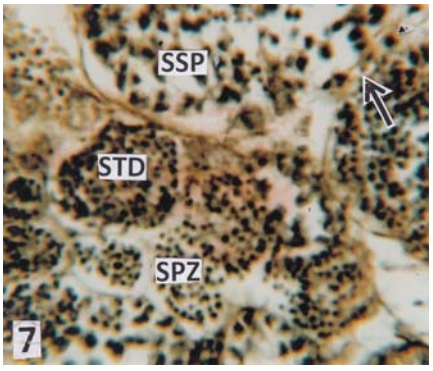

Fig 7: Showing proliferation of STD and spermatozoa (SPZ) during maturation phase. Note scattered SSP and enlargement of IC (arrow) (IA) × 600.

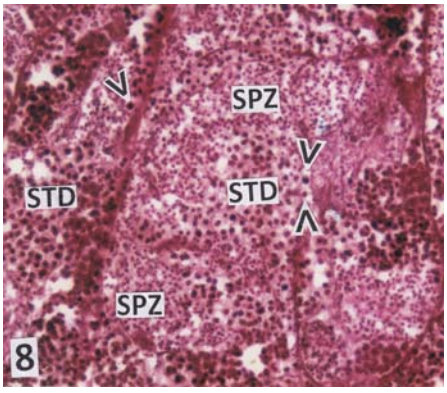

Fig 8: Huge number of SPZ interspersed with cysts of STD within the testicular lobule during end of maturation phase. Arrow head indicate IC (H & E) × 400.

3.3.3 Spawning phase (June to August)

This phase is dominated by the presence of thyroid follicles of fourth stage *i.e.* collapse or atrophied with scanty colloid. The aggregated follicles are closely associated with the blood vessels (Figs 9, 10). The height of epithelial cell layer is maximum measuring about 5.01±0.60 µm to 5.28±0.69 µm. The follicles are of their active functional states as reflected by the empty spaces around the entire margin of colloid (Fig 9). Some of the thyroid follicles are empty (Fig 10). The average follicular diameter has been recorded to be 35.30±1.24 µm × 54.64±1.48 µm.

During this phase the GSI record from 1.49±0.49, 1.55±0.57 and 1.15±0.27 during June, July and August respectively. The testicular lobules are fully packed with mature spermatozoa. Spermatogonia are appear as cyst in the lobular wall (Fig 11) and interlobular spaces are occupied by interstitial cells with densely stained nuclei which are strongly positive to iron alum haematoxylin having diameter 5.0 to 5.5µm (Fig 11). During August the lobule boundary wall gradually becomes thicker and cysts of spermatogonia gradually increase. In the lumen of the testicular lobules sparse residual spermatozoa are noticed adjacent to cysts of spermatids. The interstitial Leydig cells have a tendency to decrease in size (Fig 12).

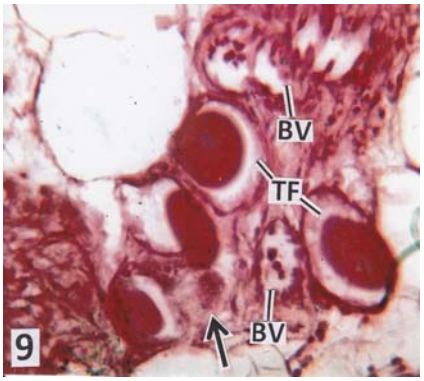

Fig 9: Atrophied TF having thick boundary wall, peripheral lumen and ecentric position of colloid during spawning phase. Note scanty colloid in collapse TF (arrow) and orientation of TF adjacent to blood vessel (BV) (H & E) × 400.

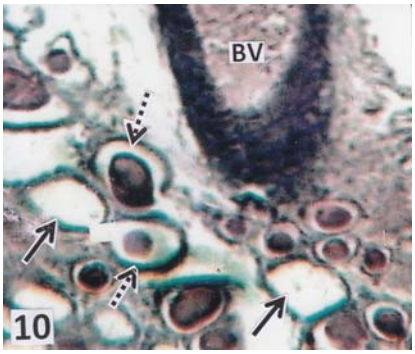

Fig 10: Collapse stage of TF (broken arrows) and irregular empty TF (solid arrows) encircling BV during end of spawning phase (MT) × 400.

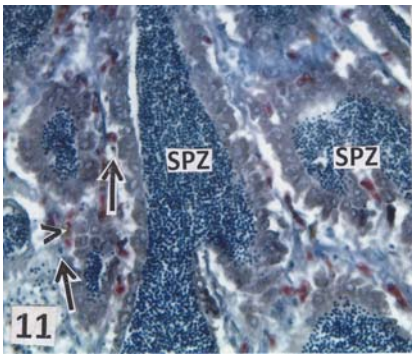

Fig 11: Packed SPZ in the lumen of testicular lobule during spawning phase. Note IC (arrows) adjacent to blood vessels (arroe head) (MT) × 400.

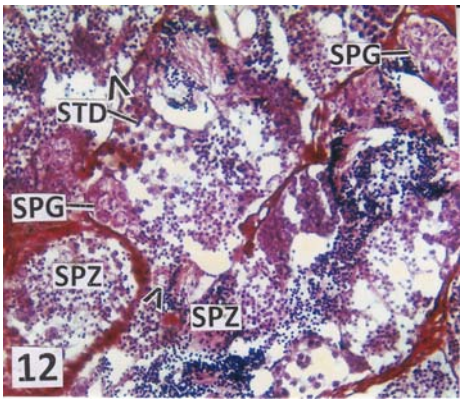

Fig 12: Showing residual SPZ and cyst of STD within the lobules. Note clumped SPG along the lobule boundary wall and IC (arrow heads) (MT) × 400.

4. Discussion

T. ilisha, the prime anadromous species is a major component in the Bhagirathi - Hooghly river system accounting for about 15 to 20% of total fish landings [11]. According to some authors, some stocks of hilsa are permanent resident of the rivers and do not migrate into the sea, spend their life in freshwater and mature and spawn there [12]. The occurrence of two spawning season has also been reported from the Ganga river system by Mathur [13] and Bhaumik and Sharma [14].

The present histological studies reveal that the degree of functional state of thyroid follicles in *T. ilisha* during growth, maturation and spawning phases may be grouped into non - secretary, quiescent, active secretary stage and stage of collapse or atrophied stage on the basis of the amount of colloid material present, epithelial cell height, and the presence or absence of colloidal resorption vacuoles. Mukherjee [15] divided thyroid follicles of *Clarias batrachus* in five stages such as quiescent, non - secretary, secretary, active secretary and atrophied. However, Payne [16] reiterated the opinion that the chromophilic nature of the colloid may not be correct criterion to determine the functional states of thyroid follicles. The scattered nature of thyroid follicles around the ventral aorta in *T. ilisha* has been corroborates with the findings of Srivastava and Sathyanesan [17] in *Mystus vittatus* and Joy and Sathyanesan [18] in *Clarias batrachus* and Abbas *et al.* [19] in *Epinephelus aeneus*. While studying the seasonal changes of thyroid follicles, some workers have reported the occurrence of either one or two annual peaks of thyroid activity [20, 9]. In the present study, however, only one peak of thyroid activity in *T. ilisha* has been detected in June and July which coincides with the spawning phase of the fish. Narejo *et al.* [21] reported that the spawning season in *T. ilisha* is form July to August in Indus river. Panhwar *et al.* [22] observed spawning season in male *T. ilisha* is from May to October with peak in August followed by October and July. In the present study it is apparent that the degree of thyroid activity in *T. ilisha* to have a close relation with testicular maturation. However, it has been found that during spawning phase i.e. June, July and August the thyroid follicles are in their active secretary state followed by atrophied phase at the end of the spawning phase. On the contrary, low activity if the thyroid follicles during growth phase have been encountered in the present study. This is in conformity with the findings of Belsare [23] who while studying the cyclical changes of thyroid follicles in *Channa punctatus* opined that the thyroid activity has got a close relationship with the breeding activity of the fish. The thyroid - gonadal relationship has been established in medaka, *Oryzias latipes* [9]. The thyroid follicles in this fish have been found to be in most active condition during the spawning phase particularly in the month of August and the activity of thyroid follicles become low as soon as the fish enter the post - spawning phase in the month of September.

In the present investigation, it has been found that during the growth phase the low active condition of the thyroid of male, as reveled by the minimum follicular cell height and non - secretary stage of follicular cells are well coincide with the gradual increase of spermatogonial cells and increase of spermatocyte. The GSI remains low at the beginning of the growth phase and then increase slightly during late growth phase due to proliferation of early stages of testicular cells. During the maturation phase the increment of epithelial cell height and resorption vacuoles as liquefied colloid in thyroid follicles adjacent to the blood vessels are well coincide with the increase in spermatids and spermatozoa and also expansion of the testicular lobules. Thyroid follicles lying attached to the blood vessels indicating the pathway of their contents through the blood stream.

The GSI increased considerably due to high frequency of advanced testicular cells. On the other hand during the spawning phase the most active condition of thyroid in male *T. ilisha* as reveled by atrophied follicular cells having maximum epithelial cell height and scanty colloid are correlated with the high frequency percentage of spermatozoa. Thus it can be concluded that the degree of activities of thyroid follicles in *T. ilisha* is in correlation with the gonadal status. Volkoff *et al.* [24] reported that in the Atlantic stingray, *Dasyatis sabina*, the follicular cells varied in size and shape according to the activity of the gland. Salamat *et al.* [25] opined that the epithelial cells bordering the thyroid follicles in *Acanthopagrus latus* are flattened, cuboidal or columnar depending upon their activity. Osborn and Simpson [20] suggested that since the elevation in thyroid hormone level during the time of gonadal development in plaice occurs in both immature and maturing specimens, thyroxine may be regarded as permitting the metabolic changes necessary to save the developing gonad rather than as being directly involved in gametogenesis.

5. Conclusion

Cytological changes in the thyroid follicles were correlated with testicular activities in *T. ilisha* during growth, maturation and spawning phases. It was found that during maturation and spawning phases the density of colloid in thyroid follicles was reduced along with the increased of follicular cell height. These features were well correlated with the proliferation of spermatids and spermatozoa in the testis. Therefore, thyroid follicles may have a direct or indirect role in controlling the event of spermatogenesis of the fish under study.

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