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Histological status of thyroid follicles and their impact on ovarian tissues in *Puntius sarana* (Hamilton) during growth, maturation and spawning phases

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Abstract
In the present study, it was found that the thyroid follicles of *Puntius sarana* (Hamilton) were in the form of groups of follicles around the anterior median extension of the ventral aorta in the sub-pharyngeal region between the connective tissues in close association with blood capillaries. The number of follicles was extremely low. Two types of follicles have been identified – the smaller follicles and the large follicles. The thyroid follicles in *P. sarana* was unencapsulated and therefore, capable of widespread ectopic growth as evidenced by active migration of the follicles in head kidney adjacent to blood vessels. The lumen of the follicles were either fully or partially filled up with the agranular eosinophilic or aniline blue positive colloidal materials or encircled by a single layer of epithelial cells. The low active condition of the thyroid follicles during the growth phase i.e. December to February was well in coincidence with the increase of early and late perinucleolar oocytes. During maturation phase i.e. March to May, the colloids were faintly stained having resorption vacuoles as well as vacuolated structure at the outer margin of the colloid. These features were well correlated with the occurrence of cortical alveolus and yolk granule stages in the ovary. The most active condition of the thyroid follicles were noted during spawning phase i.e. June to August as revealed by the maximum follicular cell diameter and also correlated with the dynamic cytological activities during vitellogenesis and highest frequency percentage of mature oocytes in the ovary. It was concluded that the cytological changes in the thyroid follicles and ovarian activities correlate well during growth, maturation and spawning phases in *Puntius sarana*.

Keywords: Thyroid follicles, ovarian tissues, *Puntius sarana,* growth, maturation, spawning

1. Introduction
The thyroid is the chief endocrine gland that regulates metabolic rates and energy balance. The thyroid follicles have also been recognized as an important modulators of reproductive functions. The thyroid follicles of fish are loosely scattered in the gill region along the ventral aorta [1, 2]. 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 diameter of cells depends upon its secretory activity [3-5]. In fish the central control of thyroid hormone is limited to the production and secretion of T_4, which is transformed into the biologically active T_3 in peripheral tissues, mainly the liver which is essential for reproductive activities [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 [8-10].
The present study is designed to identify the histological changes, that is, distribution of the thyroid follicles, hyperplasia, homogeneity of the follicles in correlation with the gonadal status of *Puntius sarana*. This would help in determining the proper maturity stages of gonads very precisely and the knowledge of environmental control in reproduction that can be manipulated to accelerate gametogenesis in brood fishes to enhance the culture practice and stock management of the said species.

2. Materials and methods
2.1. Gonadosomatic Index (GSI)
Living mature female fishes of *Puntius sarana* (length 21 to 24 cm and weight 100 to 150 gm) were procured from river Khari, Monteswar, Burdwan (23° 25′ 21″ N, 88° 6′ 27″ E) West Bengal during the second week of every month from January to December 2014.

The fishes were acclimatized for 5 days period by feeding artificial diet daily. Data on total body weight and ovarian weight of 10 fishes were taken to calculate the mean gonadosomatic index (GSI) using the formula

$$GSI = \frac{\text{Total ovary weight}}{\text{Body weight} - \text{weight of the ovary}} \times 100$$

2.2. Histological methods

After decapitation of the fish 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 hours. Decalcification was made in a mixture of 5% formic acid and formaldehyde (1:1 volume) for 7 days. Ovaries were cut into pieces and were fixed in Bouin's fluid for 18 hours for histological studies. After fixation the ovaries 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 embedded in paraffin and serial sections of the tissues were cut at 4µm.

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

3. Results

Thyroid follicles of *P. sarana* are not encapsulated but appear to be highly diffused. The group of follicles which seems to be scattered around the regions of anterior and middle part of the ventral aorta (Fig. 1). The spherically to oval shaped thyroid follicles, consisting of cuboid to flat epithelia surrounding a homogeneously stained colloid, were loosely distributed in the connective tissue adjacent to ventral aorta (Figs. 2, 7, 8). The lumina of thyroid follicles are either fully or partially filled up with agranular eosinophilic colloid but during maturation and spawning phases they are provided with resorption vacuoles or peripheral empty spaces (Figs. 7, 8). 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 *P. sarana*. 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 the epithelial cell layer is minimum (Figs. 1, 2, 3). The quiescent second stage, 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 increasing trend in the epithelial cell height with prominent nuclei has been recorded and are closely associated with the blood vessels (Figs. 8, 9, 13). In the active secretory stage the third stage follicles contains liquefied colloid and the height of the epithelial cell layer is at it's maximity (Figs. 10, 14, 16). Follicles of the fourth stage, the stage of collapse or atrophied with scanty colloid. Epithelial layer is irregular without any definite arrangement (Figs.10, 14, 15).

3.1. Oogenesis

The sequence of oocyte maturation in *P. sarana* has been divided into six developmental stages based upon the cytological characteristics of the cells, viz., Oogonia (stage I), early and late perinucleolus stage (stage II and stage III), yolk vesicle stage (stage IV), yolk granule stage (stage V) and mature follicle (stage VI).

3.1.1. Oogonia (6 µm × 8 µm to 12 µm × 10 µm)

Oogonia are present either singly or in small nests within the lamellar epithelium. An oogonium is made up of a large nucleus (4 µm to 5 µm) with chromatin threads (Figs. 5, 6).

3.1.2. Early perinucleolus oocyte (23 µm × 28 µm to 50 µm × 60 µm)

This stage consisted of a large oval centrally placed nucleus and contained about 2 to 5 basophilic nuclei together with fragmented chromatin (Fig. 5).

3.1.3. Late perinucleolus oocyte (84 µm × 100 µm to 90 µm × 105 µm)

The late perinucleolus oocytes are comparatively larger in size than early perinucleolus stage and are characterized by the appearance of cortical alveoli along the periphery of the ooplasm (Figs.4, 7). Rounded nucleoli having variable sizes with condensed chromatin materials are seen within the nuclei (Figs. 6, 11).

3.1.4. Yolk vesicle oocyte (112 µm × 132 µm to 124 µm × 145 µm)

In this vitellogenic oocyte stage formation of yolk globules take place and as a result the cell volume and diameter increase considerably. Migration of germinal vesicle from the centre of the egg towards the periphery is started (Fig. 11). The granulosa cells are more prominent with distinct nucleus (Fig. 12).

3.1.5. Mature follicle (380 µm × 400 µm to 450 µm × 500 µm)

The yolk granules coalesce and remains packed to form homogenous yolk mass. The nuclear membrane loses its identity and the nucleus (germinal vesicle) is eccentric in position. The thickness of the theca, zona granulosa and zona radiata reduced considerably (Figs.17, 18).

3.1.6. Atretic oocytes

Sometimes the developing oocytes fail to attain maturity are called atretic oocytes. These are characterized by disintegrated nuclei and liquefied yolk granules (Figs.11, 17).

3.2. Sequential changes in thyroid follicles and ovarian cells during growth, maturation and spawning phases:

In the present investigation the histoarchitecture of thyroid follicles, GSI, morpho-histological characteristics of various oogenetic cells are found to undergo changes during growth, maturation and spawning phases. The activities of the thyroid follicles have been investigated by considering the height of the follicular epithelial cells along with the follicular and colloidal diameters.

3.2.1. Growth phase (December to February)

In this phase the thyroid follicles in *P. sarana* are in non-secretory stage and are surrounded by flat squamous epithelial cell layer. The average diameter of thyroid follicles ranges from 20± 0.28 µm in December to 31.46 ± 0.16 µm in

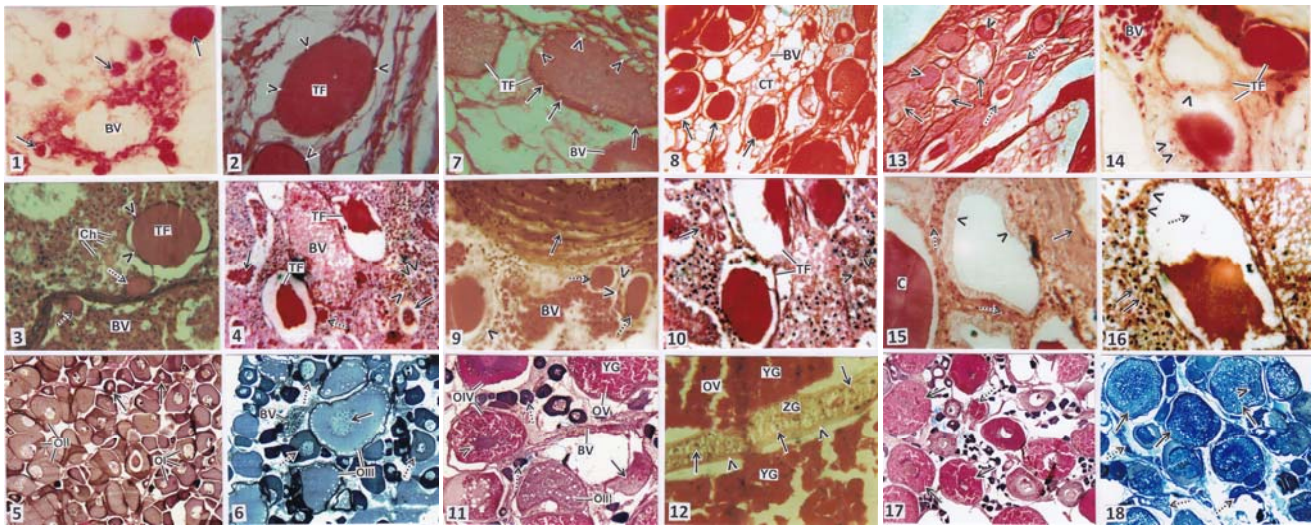
February (Figs.1, 2). The diameter of the epithelial cell layers varies from 1.5 ± 0.08 to 2.12 ± 0.26. The oval or rounded follicles are filled up with dense eosinophilic colloids which are in contact with the epithelial cell layer (Fig. 2). Possibly due to the lack of connective tissue barrier, thyroid follicles with full of colloids can have widespread ectopic distribution in between the haemopoietic tissue and renal tubules (Fig. 3).
During growth phase the GSI is recorded from 1.48 ± 0.25 to 2.19. In December and January the GSI value increases gradually from 1.48 ± 0.25 to 1.86 ± 0.22. Gradual increment of GSI is noticed in February and recorded to 2.19 ± 1.15. Primary oocytes (stage I and stage II) are present in the ovary (Fig. 5). However, during the end of growth phase percentage of stage III oocytes increases which show cortical alveoli (Fig. 6).

3.2.2. Maturation phase (March to May)

The thyroid follicles are in the active secretory stage in this phase. An increasing trend in the epithelial cell height having prominent nuclei has been recorded from 2.60±1.12 µm in March to 3.2 ±1.6 µm in May (Figs. 7, 9). The follicles are oval or elongated in shape and closely associated with the blood vessels (Figs. 8, 9). The follicular diameter measuring about 25.20±1.4 × 42.54 ± 1.12 µm to 32 ± 1.14 × 41.26 ± 2.16 µm respectively. In this phase third stage follicles are predominating having resorption vacuoles (Fig. 7). At the end of this phase the colloids are seen to be faintly stained vacuolated structures appear at the outer margin of the colloid (Fig. 10).
The highest oogenetic activity is found to occur during this phase. Different stages of vitellogenic oocytes are present. However, majority of the developing oocytes are of stage IV and stage V respectively. At the end of this phase the yolk granules of stage V continued to coalesce. An increase of the degree of vascularization around the vitellogenic oocytes is also evident (Fig. 11). Prominent zona granulosa and zona radiata are present (Fig. 12). The immature oocytes are found to be decreased in number. Small number of atretic follicles are also found at this stage in between yolk vesicle and yolk granule oocytes (Fig. 11). During the onset of maturation phase in March onwards when the ovary entered into the maturation, GSI gradually increase from 3.12 ± 0.68 in March, followed by 6.52 ± 1.60 in April and 12.01 ± 2.15 in May respectively.

3.2.3. 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. 14, 15). The follicles are of their active functional states as reflected by the empty spaces around the entire margin of colloid (Figs. 14, 16). Some of the thyroid follicles are empty (Figs. 14, 15). The average follicular diameter has been recorded to be 30.16 ± 1.24 µm x 42.64±1.48 µm.
Ovary at this stage is full of mature follicles (stage VI) which are larger and irregular in shape, yolk globules condensed and provided with eccentric germinal vesicles (Figs. 17, 18). A few discharged follicles are also observed. In June the ovary is with full of mature follicles and GSI attained the peak value (16.73 ± 186) but in July and August the GSI value shows a declining trend (13.44 ± 2.10 and 8.60 ± 2.32).



Figs 1 to 18: Photomicrographs of different stages of thyroid follicles and various oogonial cells during growth, maturation and spawning phases in *P. sarana*. (Haematoxylin-Eosin: HE; Mallory's triple: MT)
Fig 1: Thyroid follicles having compact colloid and thin epithelial layer (arrows) encircling blood vessels (BV) during growth phase; (HE) × 150.
Fig 2: Enlarged view of thyroid follicle (TF) during growth phase having dense colloid intimately contact with epithelial cell layer (arrow heads); (HE) × 400.
Fig 3: Growth phase showing smaller thyroid follicles (broken arrows) inside and adjacent to wall of the blood vessel (BV) and larger one (TF) within the haemopoietic tissue surrounded by epithelial cell layer (arrow heads). Ch indicates chromaffin cells; (MT) × 400.
Fig 4: End of growth phase showing secretory stage of TF adjacent to blood vessels (BV) having peripheral lumen within the head kidney. Solid arrows indicate renal tubules and arrow heads indicate interrenal cells; (HE) × 400.
Fig 5: Showing Oogonia (arrows), Oocyte I (OI) and Oocyte II (OII) during early growth phase; (HE) × 150.
Fig 6: End of growth phase showing gradual reduction in number of oogonia and appearance of oocyte III stage having cortical alveoli and prominent nucleus with fragmented nucleoli (solid arrows). Broken arrows indicate oocyte II stage and BV indicates blood vessels; (MT) × 400.
Fig 7: Thyroid follicle (TF) adjacent to blood vessel (BV) during maturation phase showing resorption vacuoles (arrow heads) and epithelial cells (arrows) in the thick epithelial layer; (HE) × 600.

Fig 8: Thyroid follicles (arrows) within the connective tissue (CT) and adjacent to BV during maturation phase showing empty space along the outer margin of colloid; (HE) × 400.
Fig 9: Smaller thyroid follicles (broken arrows) showing empty space and bordered by prominent epithelial cells (arrow heads) adjacent to wall of the blood vessels (solid arrow). BV indicates blood cells of blood vessel; (HE) × 600
Fig 10: End of the maturation phase showing active secretory follicles (TF) in the head kidney. Note empty space and scanty colloid within the TF. Arrow heads indicate interrenal cells and arrow indicates renal tubule; (HE) × 400.
Fig 11: Showing oocyte IV (OIV) and oocyte V (OV) stages with full of yolk granules (YG) and prominent germinal vesicle (arrow head) during maturation phase. Note blood vessel (BV); atretic follicle (solid arrow), oogonia (broken arrows) and oocyte III (OIII) in between mature oocytes; (HE) × 400.
Fig 12: Showing oocyte V (OV) during maturation phase with prominent granulosa cells (solid arrows) in zona granulosa (ZG). Note zona radiata (arrow heads) adjacent to yolk granules (YG); (HE) × 600.
Fig 13: Showing different stages of non-secretory (arrow heads), secretory (broken arrows) and spent (solid arrows) thyroid follicles during spawning phase; (HE) × 150.
Fig 14: Showing increased height of the epithelial layer with epithelial cells (arrow heads) of the active secretory and spent thyroid follicles (TF) adjacent to blood vessel (BV) during spawning phase; (HE) × 400.
Fig 15: Spawning phase showing presence of empty thyroid follicle adjacent to wall of the blood vessel (solid arrow) bordered by epithelial cells (arrow heads) encircled by cords of blood cells (broken arrows). C indicates dense colloid of secretory thyroid follicle; (HE) × 600.
Fig 16: Spawning phase showing active secretory thyroid follicle in head kidney having empty space (broken arrow) and scanty amorphous colloid bordered by active epithelial cells (arrow heads). Arrows indicate interrenal cells; (HE) × 600.
Fig 17: Showing large number of mature follicles (solid arrows) during spawning phase. Note atretic oocyte (broken arrow) and primary oocytes in between mature follicles; (HE) × 400.
Fig 18: Showing mature follicles (arrows) during spawning phase. Note migration of germinal vesicle of mature follicle towards the periphery of oocyte (arrow head). Broken arrows indicate blood vessels in between oocytes; (MT) × 400.

4. Discussion

In the present investigation the thyroid follicles in *P. sarana* consisting of cuboid or squamous epithelia with homogeneously stained colloid were loosely distributed in the connective tissue adjacent to the blood vessels corroborates the findings of Srivastava and Sathyanesan [11] in *Mystus vittatus*, Joy and Sathyanesan [12] in *Clarias batrachus* and Schmidt and Braunbeck [13] in *Danio rerio*. In *P. sarana* widespread ectopic growth either/or secretory and non-secretory stages of follicles extended in the head kidney. Harshbarger [14] opined that in some cases, hyperplasia may be extensive and thyroid follicles may extend into normal tissues. Thyroid tissue in teleosts mainly occurs in the pharyngeal region but is often widely distributed anatomically [15]. The present histological studies reveal that the degree of functional state of thyroid follicles in *P. sarana* during growth, maturation and spawning phases may be grouped into non-secretory, quiescent, active secretory stage and stage of collapse or atrophied on the basis of the amount of colloid material present, epithelial cell height and the presence or absence of colloidal resorption vacuoles. Schmidt and Braunbeck [13] emphasized that a very interesting endpoint was the observation of changes in the quality of the colloid as the main site of thyroid hormone synthesis and storage. Mukherjee [16] has further divided thyroid follicles of *Clarias batrachus* in five stages such as quiescent, non-secretory, secretory, active secretory and atrophied. While studying the seasonal changes of thyroid follicles, some workers have reported the occurrence of either one or two annual peaks of thyroid activity [9, 17]. In the present study, however, only one peak of thyroid activity in *P. sarana* has been detected in June/July which coincides with the spawning phase of the fish. In the present investigation it is apparent that the degrees of thyroid activities in *P. sarana* have a close relationship with ovarian maturation. However, it has been found that during spawning phase i.e. June and July the thyroid follicles are in their active secretory state coincides with the increase GSI value and proliferation of vitellogenic oocytes. On the contrary, low activity of the thyroid follicles during growth phase have been encountered in the present study may be due to the gradual proliferation of the late perinucleolar oocytes in the ovary. This is in conformity with the finding of Belsare [18] 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. A thyroid-gonadal relationship has been established in medaka, *Oryzias latipes* [9]. In this fish, the thyroid follicles have been found to be in most active condition during the spawning phase particularly in the month of August and the activity of the thyroid follicles become low as soon as the fish enter the post-spawning phase in the month of September. The activity of thyroid remains thereafter at minimum level but again an abrupt increase in the month of May is noticed the onset of active breeding period. In the present observation in *P. sarana* the thyroid follicles in the head kidney also contribute cellular changes during growth, maturation and spawning phases which corroborates with the finding of Agrawala and Dixit [19] in *P. sophore*.

Epithelial cell height of thyroid follicle represents a classical parameter to detect thyroid activation [20-22]. In the present investigation, it has been found that during the growth phase the low active condition of the thyroid of female as revealed by the minimum follicular epithelial cell height is well coincidence with the gradual increase of early and late perinucleolus oocyte. During the maturation phase the increment of epithelial cell height along with resorption vacuoles as well as liquefied colloid in thyroid follicles adjacent to blood vessels are also well coincidence with the increase in yolk vesicle and yolk granule stages. Thyroid follicles lying attached to the blood vessels indicating the pathway of their contents through the blood stream. During the spawning phase the most active condition of thyroid in female *P. sarana* as revealed by atrophied follicular cells having maximum epithelial cell height are correlated with the high frequency percentage of mature oocytes. Thus it may be conjectured that the degree of activities of thyroid follicles in *P. sarana* is in correlation with the gonadal status. Salamat *et.al*. [23] have stated that the epithelial cells bordering the thyroid follicles in *Acanthopagrus latus* are flattened, cuboidal or columnar depending upon their activity. However, the participation of the thyroid follicles in harmony with the gonadal status in fish under study may or may not be a direct one as the teleostean thyroid is reported to have a capacity to regulate the metabolic rates [6, 20]. Osborn and Simpson [17] have 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.

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