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Reproductive Biology and Histological Pattern of Hilsa Shad (*Tenualosa ilisha*) in Bangladesh

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ABSTRACT

An experiment was carried out to identify the peak breeding season of Hilsa in Bangladesh using gonadal histology through the standard procedure. A total 480 Samples were collected from Meghna River, Chandpur, from July 2012 to June 2013 and Tentulia river, Barisal, from June 2013 to June 2014. Histological sections of gonads were analyzed and ovarian and testicular stages were determined according to the OECD Guidance Document for Diagnosis of Histopathology of Fish Gonads. Maturing germ cells were observed every month where a higher amount of mature germ cells was found in December, January, and September to October, with a high in October. From February to June, the mature germ cells were less. Histological observations of testis suggested that *T. ilisha* spawns throughout the year, but significant spawning takes place in September-October and December-January with a high abundance of SPT (spermatids). During October, mature germ cells were mostly evident, and SPZ (spermatozoa) was in high proportion inside the LU (lumen of tubules). The gradual maturation of germ cells from early to ripened stages inside the testicular LU and peak maturity came during October. Females having ovaries at mature stages were considered those spawning or near to spawn. The occurrence of mature ovaries examined by external characteristics began in January and continued up to December. Percentage (%) occurrence of mature ovaries started to increase in February, peaked in October (75%), and decreased in December, January, and April. In fish ovary, the mature stages are generally observed at the most advanced stage of the ovary when the fishes are ready for spawning and when mean GSI values are comparatively high. In this study, advanced mature oocytes were found from in every month during the study period. The histological study of oocyte development and examination of external characteristics of ovaries suggested that *T. ilisha* spawns throughout the year, but significant spawning takes place in October –November and peaks in October.

INTRODUCTION

Hilsa, being anadromous, lives at sea for most of its life but migrates to freshwater rivers for spawning, after which it returns to the sea. The Hilsa shad occurs in the foreshore areas, estuaries, and freshwater rivers of the western division of the Indo-Pacific region. Its marine distribution extends from Iran and Iraq in the Persian Gulf to the west coast of India, in the Arabian Sea, and in the Bay of Bengal, the coast of Bangladesh and Myanmar. Although it is distributed in large open area, the major percentage of Hilsa (about 95%) is caught by Bangladesh, India and Myanmar (DoF, 2020).

Bangladesh's National fish Hilsa affords the largest single-species fishery in Bangladesh, especially during the monsoon in almost all the primary river systems, estuaries, and the sea area of Bangladesh. The Hilsa fishery supports a commercial fishery, and in the early 1970s, it composed more than 95% of the total commercial catch in Bangladesh (Coad *et al.*, 2003). From the 1970s, the Hilsa fishery began to decline gradually, with outputs reaching a low point of 0.19 million tons in 2001–2002 (BOBLME, 2012). The production of Hilsa declines due to a low discharge of water from the river Ganges and consequently heavy siltation in most of the rivers, the gradual growth of industries, growing urbanization, indiscriminate use of fertilizers, agrochemicals, pesticides, and the discharge of municipal waste are continuously polluting the river system. To conserve the Hilsa fishery, proper management measures should be developed and implemented. One of the most widely practiced fishery management is the identification of peak spawning season and the banning of fishing accordingly. The histology of gonads is the most common and often the most reliable technique to assess the reproductive strategy and tactics of fish species and is therefore applied in a large number of marine laboratories. In this respect, those laboratories aiming to provide management advice use histology for maturity classification to separate the sexually immature component from the sexually mature component, fecundity studies to be used in either recruitment-related studies or in Egg Production Methods (to estimate spawning stock biomass) or to monitor the prevalence

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of any type of reproductive disturbance or failure to spawn regularly. By necessity, these different overall aims require different methodological approaches, although in many cases, the histological protocol as such is reasonably similar. In summary, histology is considered to be an important tool to better understand the productivity of fish stocks and thereby, in the end, contributes to the internationally adopted principles of the precautionary or ecosystem approach to fisheries. McAdam *et al.* (1999) found GSI to be reliable in determining the reproductive status of fish species that spawn once annually but are of limited value when fish are protracted spawners. Alternatively, histology is an effective technique used to identify the reproductive timing of fishes even when species spawn multiple times or have a low reproductive investment (McAdam *et al.*, 1999). Whereas histology is a more reliable technique than GSI, the amount of time required and cost associated with histology diminish the practical advantage of the technique. The present study aimed to identify the peak spawning season of Hilsa by gonadal histology.

MATERIALS AND METHODS

Sampling of fish

Hilsa fish sample were collected from two different location of Bangladesh, upper Meghna River at Chandpur and Tentulia River at Barisal district of Bangladesh. Sampling was conducted during July 2012 to June 2013 in Meghna River and July 2013 to June 2014 in Tentulia river. Samples were collected bought once a month and 20 mature Hilsa samples (10 female and 10 male) were collected at each sampling day. Hilsa were caught in the river during night using gill nets primarily. All specimens were preserved with crushed ice in cool fish boxes and brought to the laboratory as soon as possible. A total of 480 mature fishes (240 samples from each site; 240 female and 240 male) covering various size groups were collected from both sampling sites.

Gonad Collection

The body cavity of fish was opened carefully by scissors and gonads were collected with forceps carefully. Other constitutional units such as muscles, fat tissues, digestive organs and blood veins etc. were taken away properly. Body weight (BW) and Gonad weight (GW; both left and right gonads) were measured to the nearest 0.001g. Total Length (TL) and Gonad Length (GL) were also measured to the nearest 0.01cm. After weighing gonads were fixed with 10% buffered formalin for 24-48 h and preserved with 10% formalin in small vials for further investigation.

Observation of Ovarian External Features

General features and structure, as well as month-wise size, shape, and color of female gonads of *T. ilisha* were studied during sample collection and preservation. In Hilsa gonads, the left gonad was larger than the right one, both in length and width. External features of both ovaries were observed by the naked eye, and under the magnifying glass, the various maturity stages were classified based on external observation of ovaries.

Histological Observation of Hilsa Gonads

For the histological study of Hilsa gonad, the microscopic slides were prepared by the following procedure followed by Agarwal (1996) with slight modification.

First of all, a piece of buffered formalin preserved gonad tissue was dissected randomly from the proximal or medial part of one lobe for histological examination. A transverse segment of about 2-3mm thick from the middle part of the right ovary was taken. After taking a piece of gonad tissue following steps were followed for histological study.

Sample Preparation (Dehydration, Clearing, Infiltration, Embedding, Trimming, Sectioning, Staining, Mounting)

The gonads samples were kept in the dehydration cassette and passed through a graded series of alcohol series kept in glass jars (Figure 1). The dehydration schedule was as follows:

Sl. No.	Solution	Time
1	80% Ethanol	12 hours (overnight)
2	95% Ethanol	1 hour
3	95% Ethanol	1 hour
4	100% Ethanol	1 hour
5	100% Ethanol	1 hour
6	100% Ethanol	1hour

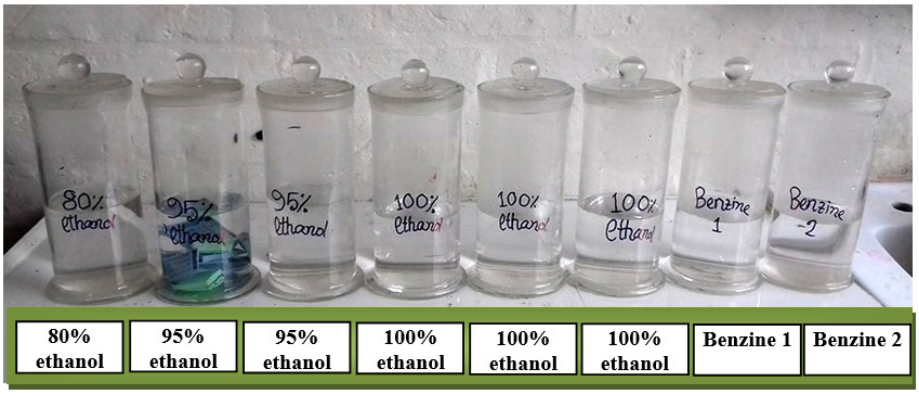


Figure 1: Dehydration using graded alcohol series

Dehydrated gonad samples were then cleared using benzene twice successively for 2 hours to remove traces of alcohol to have consistent paraffin blocks.
The process was as follows:

Sl. No.	Solution	Time
1	Benzine	1 hour
2	Benzine	1 hour

The samples were then infiltrated with paraffin in a paraffin incubator using following steps:

Sl. No.	Solution	Time
1	Paraffin	40 minutes
2	Paraffin	40 minutes

After infiltration, the cassettes were taken out from an automatic tissue processor; being opened, two samples were placed in the middle of the cassette and filled with melted paraffin from the wax dispenser. Then, steel covers were placed on each cassette and allowed to cool to room temperature. After cooling, the cassettes were put into a deep freezer for a few minutes, allowing easy steel plate removal from the cassettes and smooth sectioning (Figure 2).

Figure 2: Embedding using malted paraffin

Trimming is a process in which the undesirable wax layers of the embedded blocks are trimmed by a knife to obtain suitable blocks. Trimming was done by using old microtome blades. Trimming allowed easy sectioning. In this step, both side trimming and surface trimming were conducted (Figure 3).

Figure 3: Trimmed tissue fixed with wooden block

Paraffin-embedded blocks were sectioned by a microtome knife in a microtome machine at 5µm thickness. The sections were placed on previously tagged and prepared glass and dried on a hot plate overnight at 38°C. The prepared section slides were stored in the refrigerator for staining (Figure.4).
Staining is a process by which samples are stained with various dyes and staining materials so that their components are visible under a microscope. The gonad sections were stained through routine H-E staining protocol as mentioned below:

Sl. No.	Solution	Time	Process
1.	Xylene	10 minutes	Clearing (1-3)
2.	Xylene	10 minutes	
3.	Xylene	10 minutes	
4.	100% alcohol	5 minutes	Dehydration (4-8)
5.	100% alcohol	5 minutes	
6.	90% alcohol	3 minutes	
7.	80% alcohol	3 minutes	
8.	70% alcohol	3 minutes	
9.	50% Ethyl alcohol	2 minutes	Staining (9-15)
10.	Distilled water	15 dips	
11.	Haematoxylene (Mayer's)	3 minutes	
12.	Wash in tap water	15 minutes	
13.	50% Ethyl alcohol	10-15 dips	
14.	95% Ethyl alcohol	30 seconds	
15.	Eosin Y	1 minutes	
16.	95% Ethyl alcohol	2 minutes	Rehydration (16-19)
17.	100% Ethyl alcohol	1 minutes	
18.	100% Ethyl alcohol	3 minutes	
19.	100% Ethyl alcohol	1 minutes	
20.	Xylene	20 minutes	Clearing (20-21)
21.	Xylene	20 minutes	
22.	Drying	Over night	
23.	Mounting	Over night	

Figure 4: Staining of sectioned gonad tissues using H-E protocol

D.P.X. was used for mounting as a mounting agent. A jot of D.P.X. is put on each slide, followed by the attachment of the coverslip (22mm×22mm). After mounting, the slides were put for several hours at room temperature.

Microscopic Observation of Gonad Tissue Slides

After mounting, the slides were observed under an electric microscope (Olympus) connected to the computer with a viewer (Magnus viewer). The viewer was also equipped with a camera. With the help of this mechanism, numerous photographs were snapped at different magnifications (Figure.5).

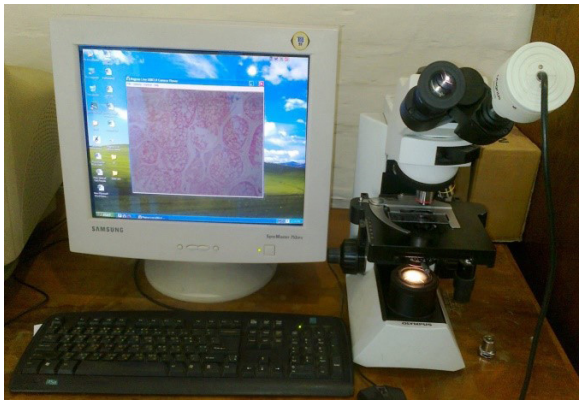


Figure 5: Microscopic observation of stained gonad sections

RESULTS

External Feature of Ovary

The maturation of the ovary could be explained by examination of the external feature of the ovary. Because the shape, size, and color of the ovary varied considerably according to the degree of maturation of the oocytes, the immature ovary is small reddish, and transparent in nature. In mature condition, the ovary's color turns yellowish and becomes larger in size.

Histological study of Ovary of Female Hilsa of Meghna River, Chandpur

Ovarian development is subdivided into distinct developmental stages according to physiological, biochemical, morphological, and histological criteria. Maturity stages were determined following the key outline described by Matsuyama *et al.* 1982 and Shinkafi *et al.*, 2011. Different steps found in the present ovarian histological study are presented in the figures (Figure 7 to Figure 10) below.

GVB (Germinal Vesicle Breakdown) was also seen when female Hilsa partially spent. In the present study, after microscopic observation, the percent occurrence of different stages of oocytes in different months was calculated and presented in Table 1.

Table 1: Percent (%) occurrence of the oocyte stages of ovarian development found in the histological study of Hilsa ovary during the study period July 2012 to June 2013 in the Meghna river, Chandpur.

Month	EPNO	LPNO	YV	YG	PM	M	GVB
July	6	5	8	12	11	58	Seen
August	9	10	-	-	24	47	Seen
September	7	11	12	21	-	49	-
October	4	8	-	8	7	73	Seen
November	2	5	-	9	18	66	Seen
December	27	37	-	-	22	14	-
January	6	7	20	22	16	29	-
February	5	9	16	19	14	37	Seen
March	8	13	13	14	19	33	-
April	12	17	16	18	13	24	-
May	7	8	11	23	12	39	-
June	11	14	10	13	11	41	-

Here, EPNO=Early perinucleolar oocyte, LPNO=Late perinucleolar oocyte, YV=Yolk vesicle, YG=Yolk granule, PM=Pre-mature, M=Mature

From Table 1 and Figure 6, it is extracted that the highest (73%) and lowest (14%) percentage of mature oocytes were found in the month of October and December, respectively. In the month of July, the highest percentage of oocytes was in the mature stage (58%), and the lowest percentage of oocyte was in early perinucleolar oocyte stage (5%) along with all six oocyte developmental stages. In the month of August, no yolk granular stages and yolk vesicle were found; however, 47% of oocyte was in the mature stage, whereas 9% oocyte was in early perinucleolar oocyte stage, besides 10% in late perinucleolar oocyte, 24% pre-mature stage. In September, the percentage of mature oocyte increased little (49%) with 7% early perinucleolar oocyte, 11% late perinucleolar oocyte, 12% yolk vesicle, 21% yolk granular stage; however no pre-mature oocyte was found. In October, the highest percentage (73%) comprised the mature stages oocyte with 7% pre-mature, 8% yolk granule, 8% late perinucleolar oocyte and 4% early perinucleolar oocyte. In November, the percentage of mature oocyte continued in the highest rank (66%) with

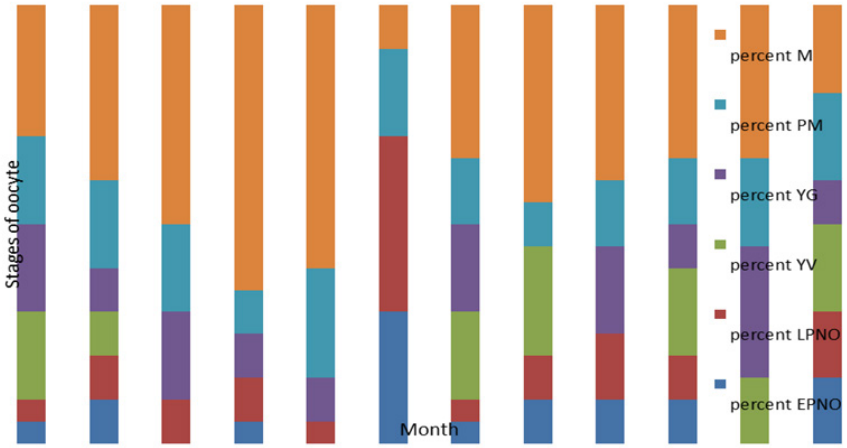
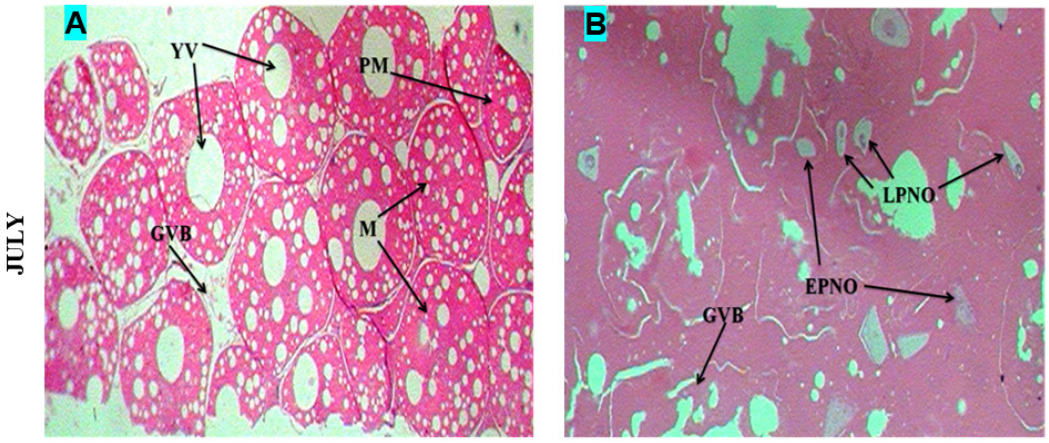


Figure 6: Month-wise occurrence (%) of different stages of oocyte of ovarian development of female Hilsa collected from Meghna river during July 2012 to June 2013. [Here, EPNO=Early perinucleolar oocyte, LPNO=Late perinucleolar oocyte, YV=Yolk vesicle, YG=Yolk granule, PM=Pre-mature, M=Mature]

2% early perinucleolar oocyte stage, 5% late perinucleolar oocyte, 9% yolk granule, 18% pre-mature without having yolk vesicle stage. In December, the percentage of mature oocyte declined considerably. It reached only 14% with no yolk granule and yolk vesicle stage oocyte, 27% early perinucleolar oocyte, 37% late perinucleolar oocyte (highest in number), 22% pre-mature. In January, the percentage of mature stage oocytes increased little by 29% with 6% early perinucleolar oocyte, 7% late perinuclolear oocyte, 20% yolk vesicle, 22% yolk granule, 16% pre-mature stage. In February, the mature stage oocyte increased again by 37% with 5% early perinucleolar oocyte, 9% late perinucleolar oocyte, 16% yolk vesicle stage, 19% yolk granule, 14% pre-mature. In March, the mature stage oocyte again declined and dropped to 33% with 8% early perinucleolar oocyte, 13% late perinucleolar oocyte, 13% yolk vesicle stages, 14% yolk granule, 19% pre-mature stage. In April, the percentage of mature oocyte continued to declining trend, reached to 24% this month other stages of oocyte were 12% early perinucleolar oocyte, 17% late perinucleoar stage, 16% yolk vesicle, 18% yolk granule, 13% pre-mature stage. In May, the percentage of mature oocyte started to rise again and it found 39% with 7% early perinucleolar oocyte, 8% late perinucleoar stage, 11% yolk vesicle, 23% yolk granule, 12% pre-mature stage oocyte. In June, the mature oocyte percentage continued to rise and reached to 41% having 11% in pre-mature, 13% yolk granule and 10% yolk vesicle, 11% early perinucleolar oocyte and 14% late perinucleolar oocyte.

Yolk vesicle stages were found throughout the year except August, October and November, December. Yolk granule stages were absent in August and December. Early perinucleolar oocytes were also observed throughout the year except May, September, and November. Late perinucleolar oocytes were observed throughout the year. Pre-mature oocyte appeared throughout the year except for September and mature oocytes appeared throughout the year during study period.

The advanced oocyte i.e. mature oocyte was found in the very month of the study period. The occurrence of the mature oocyte in every month of the study period confirmed that *T. ilisha* spawns precisely throughout the year, but significant spawning takes place in October.



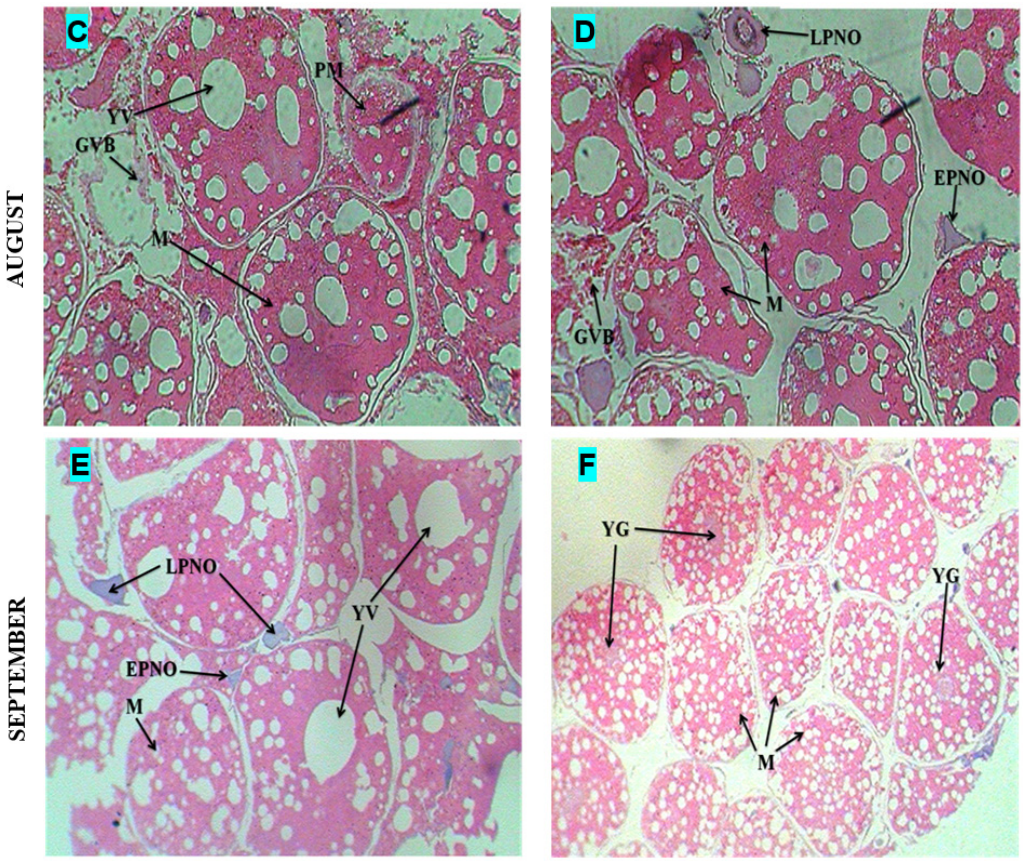
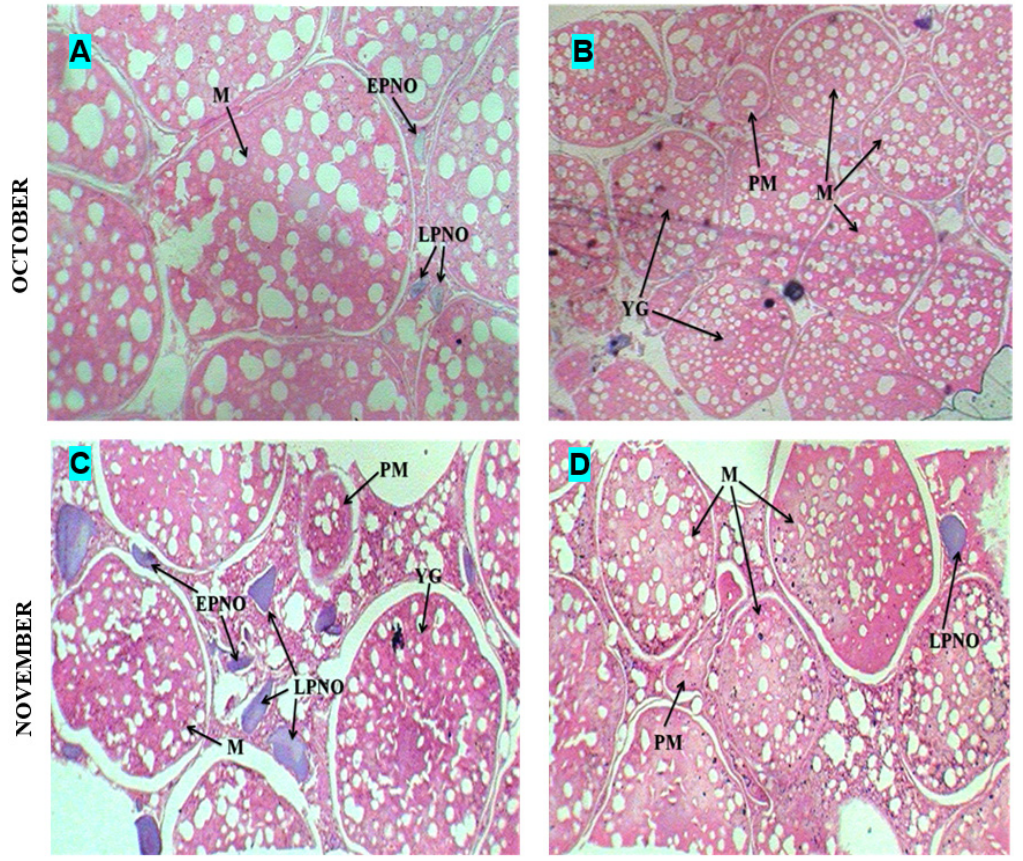


Figure 7: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled in July to September, 2012 [A-B: July; C-D: August; E-F: September]. EPNO, Early perinucleolar oocyte, LPNO; Late perinucleolar oocyte; YV, Yolk vesicle; YG, Yolk granule; PM, Pre-mature; M, Mature stage; GVB, Germinal vesicle breakdown.



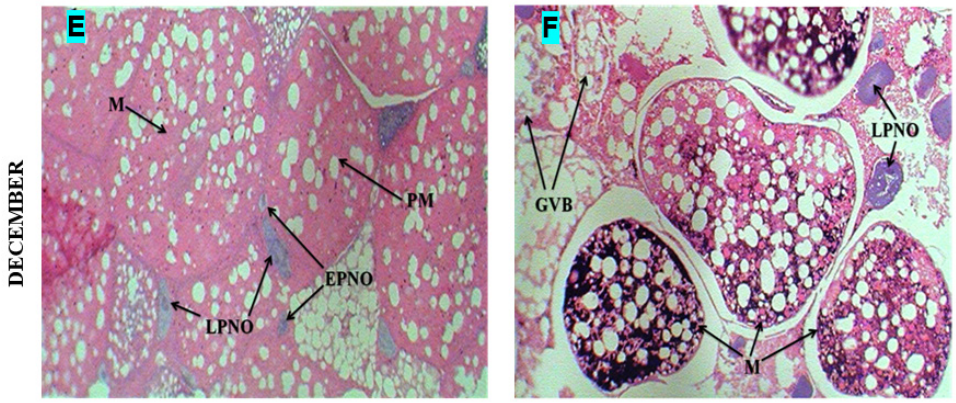


Figure 8: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled in October to December, 2012 [A-B: October; C-D: November; E-F: December]. EPNO, Early perinuclear oocyte; LPNO, Late perinuclear oocyte; YG, Yolk granule; PG, primary growth oocyte; PM, Pre-mature; M, Mature stage.

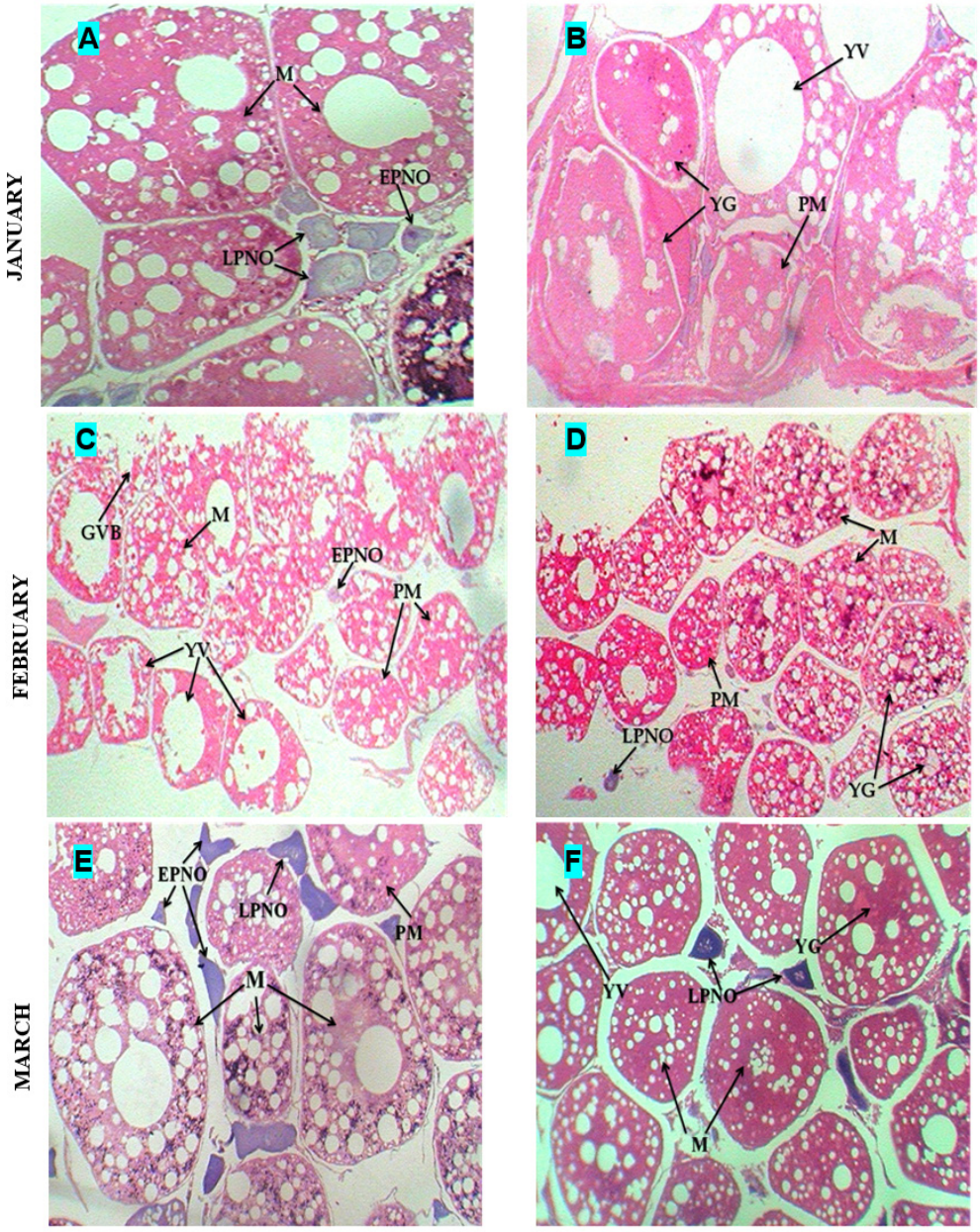


Figure 9: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled in January to March, 2013 [A-B: January; C-D: February; E-F: March]. EPNO, Early perinucleolar oocyte; LPNO, Late perinucleolar oocyte; YV, Yolk vesicle; YG, Yolk granule; PM, Pre-mature; M, Mature stage; GVB, Germinal vesicle breakdown.

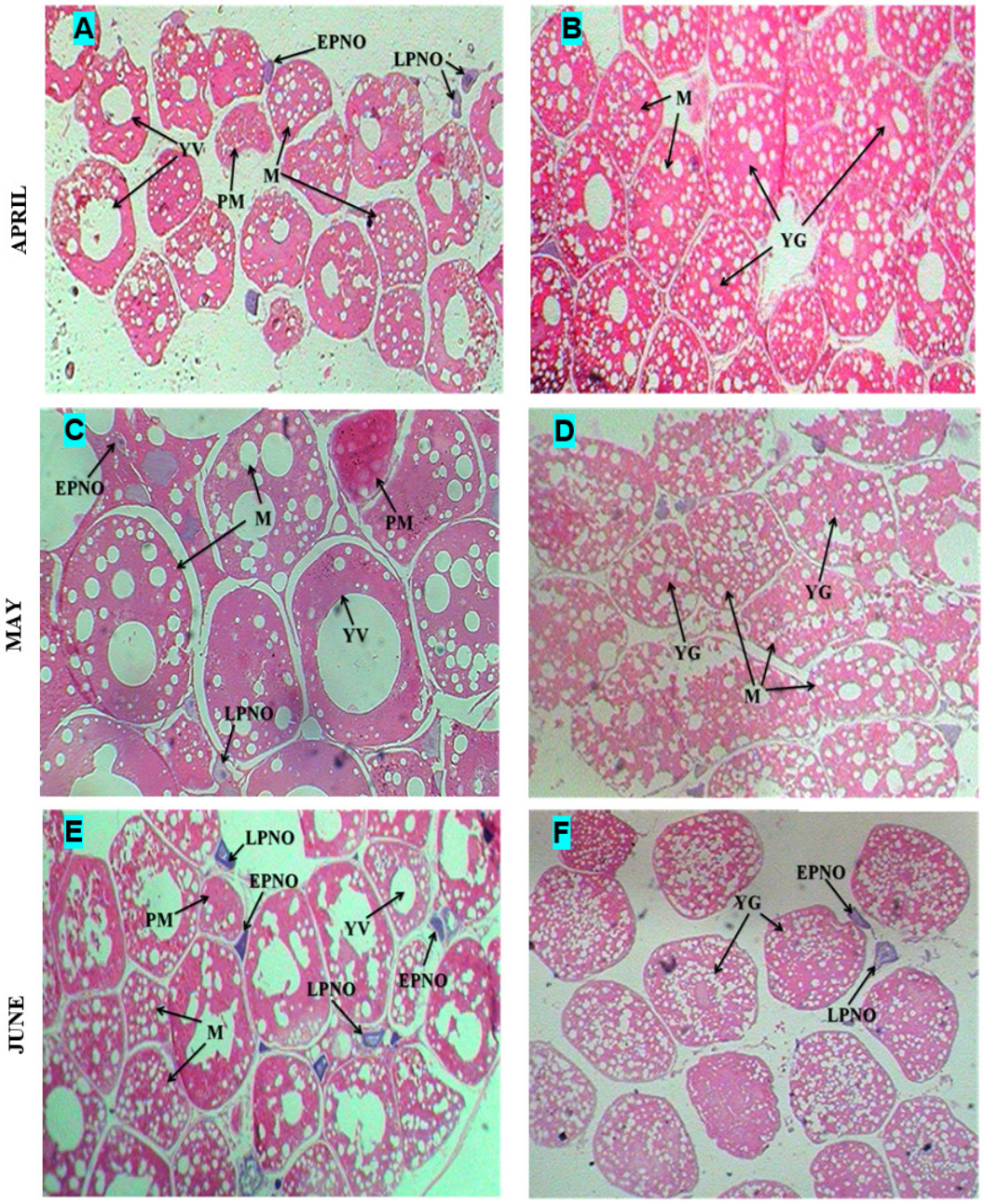


Figure 10: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled in April to June, 2013 [A-B: April; C-D: May; E-F: June]. EPNO, Early perinucleolar oocyte; LPNO, Late perinucleolar oocyte; YV, Yolk vesicle; YG, Yolk granule; PG, primary growth oocytes; PM, Pre-mature, M, Mature stage.

Histological Study Reproductive Cycle of *T. Ilisha*

In the present study, the following stages of testicular germ cells were observed from the month-wise (July 2012 to June 2013) samples of *T. ilisha* testes. Mature germ cells were less evident in July. SC, ST, and SZ were observed in the histo-sections under the microscope. There was appearance of some empty LUs in the slide (Figure 11A). Mature germ cells were not found in August. SC and ST were observed in the histo-sections under the microscope. The testicular LUs were full of SZ (Figure.11B). Mature germ cells were more evident in September. SC, ST and SZ were observed in the histo-sections under the microscope. The testicular LUs were full of SZ and some empty lumens also found (Figure11C).

Maturing germ cells were mostly evident in October. ST, and SZ were observed in the histo-sections under microscope. ST were high in number together with significant accumulation of SZ. Some lumens of tubules (LU) were found empty (Figure. 11D). Early and developing stage germ cells were evident in November. The empty lumen was more evident. SC and ST were less in the histo-sections under microscope (Figure. 11E). Mature germ cells were more evident in December. SZ were observed in the histo-sections under microscope. SZ more in number and some empty lumens also found (Figure.11F). Mature germ cells were evident in

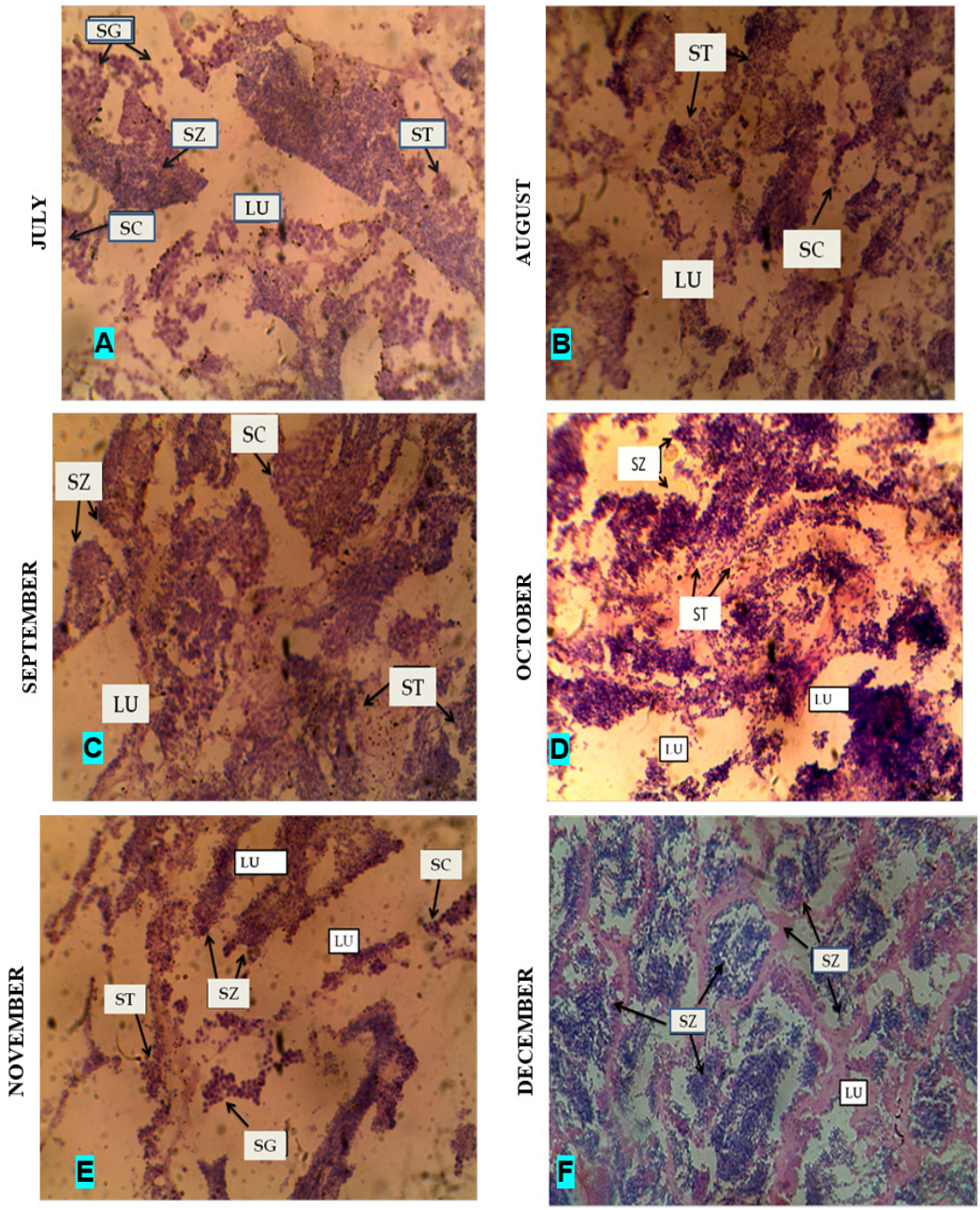
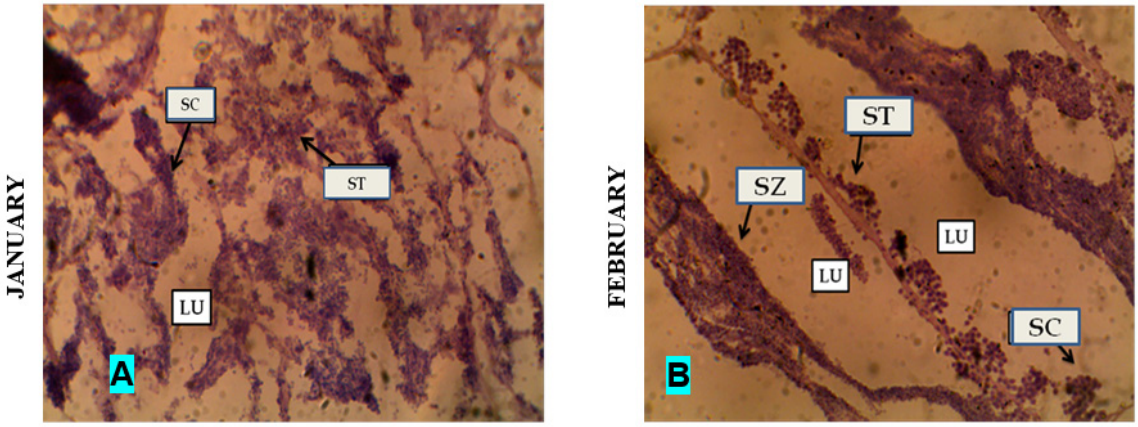


Figure 11: [A-F]-Haematoxylin-eosin (H-E) staining of tissue sections of *T. ilisha* testes (at 40x magnification) sampled in July to December, 2012 [A: July, B: August, C: September, D: October, E: November, F: December]. SG, spermatogonia; ST, spermatids; SC, spermamatocytes; SZ, spermamatozoa; LU, lumen of tubules.



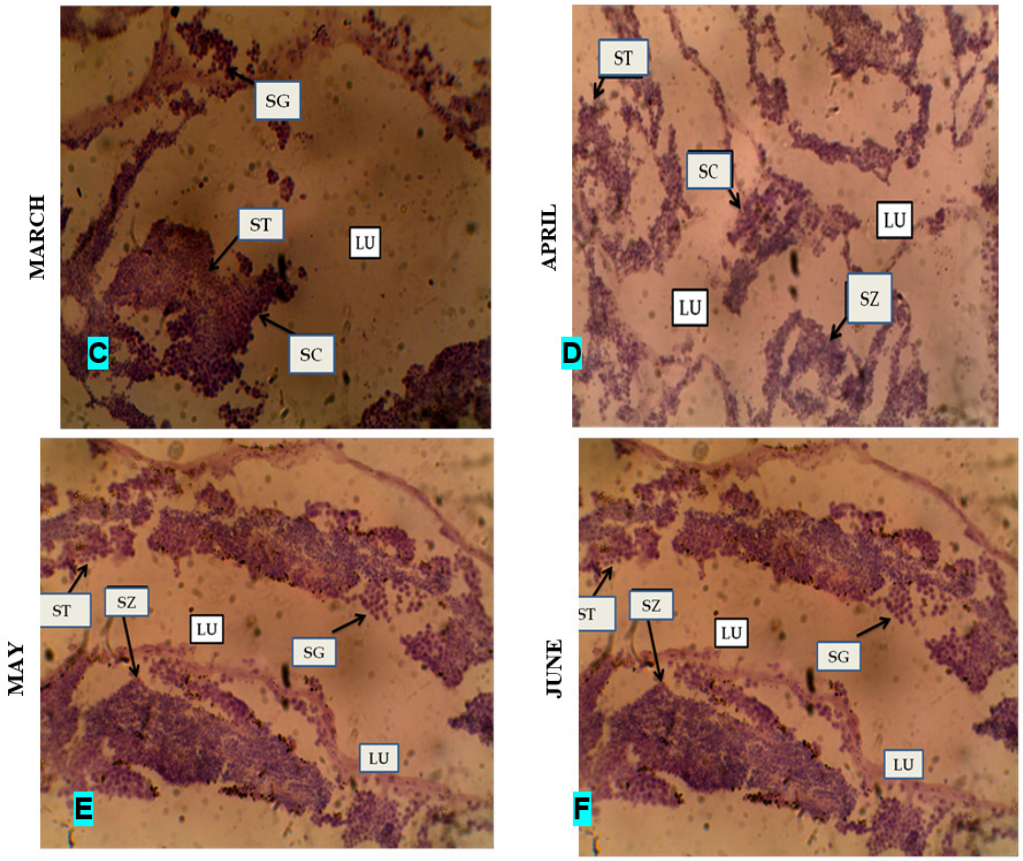


Figure 12: [A-F]-Haematoxylin-eosin (H-E) staining of tissue sections of *T. ilisha* testes (at 40x magnification) sampled in January to June, 2013 [A: January, B: February, C: March, D: April, E: May, F: June]. SZ, spermamatozoa; ST, spermamatides; SC, spermatocytes and SG, spermatogonia; LU, lumen of tubules.

January. SZ and ST were observed in the histo-sections under microscope. SZ more in number and some empty lumens also found (Figure.12A). Empty lumens were more evident in February. SZ, ST and SC were observed in the histo-sections under a microscope. SZ were less in number and empty lumens were more in number (Figure.12B). Early and developing stage germ cells were mostly evident in March. SG, SC and ST were observed in the histological-sections under microscope. SC were less in number (Figure.12C). Early-stage germ cells were more evident in April. SC and ST were observed in the histo-sections under microscope. SC were high in number (Figure.12D). SC and ST were observed in the histo-sections under microscope in May. Abundance of STs was increased although there is a significant amount of SC visible under microscope (Figure.12E). Maturing germ cells were not found in July. SC, ST, and SZ were observed in the histo-sections under a microscope. SPT were high in number together with significant accumulation of SZ. Some lumens of tubules (LU) were found empty (Figure.12F).

Histological Study of the Ovary of Female Hilsa of Tentulia River, Barisal

The ovary of the female Hilsa was bi-lobed elongated and situated in the body cavity. The shape, size, and color of the ovary varied considerably according to the degree of maturation of the oocytes; the immature ovary is small reddish and transparent in nature which turns into yellowish in ripe condition.

Ovarian development is subdivided into distinct developmental stages according to physiological, biochemical, morphological and histological criteria. Maturity stages were determined following the key outline as described by Matsuyama *et al.* 1982. Different stages that found in the present ovarian histological study are presented in the figures (Figure.14 to Figure.17) and described below.

Germinal Vesicle Breakdown (GVB) also occurred when female Hilsa partially spent. In the present study, after microscopic observation, the percent occurrence of different stages of oocytes in different months were calculated and presented in the Table 2.

From the Table 2 and Figure. 13, it is found that the highest (75%) and lowest (10%) percentage of mature oocyte found in the month of October and December, respectively. In the month of July, 60% oocyte was in the mature stage which is the highest in this month having 5% early perinucleolar oocyte, 5% late perinucleolar oocyte, 10% yolk vesicle stage, 20% pre-mature, and no yolk granule stages. In the month of August, no yolk granular stage oocytes were found; however, 40% oocyte was in the mature stage where as 10% oocyte was in early perinucleolar oocyte stage, with 15% late perinucleolar oocyte, 15% yolk vesicle stage, and 20% pre-mature stage.

Table 2: Percent (%) occurrence of the oocyte stages of ovarian development that found in the histological study of Hilsa ovary during the study period July 2013 to June 2014.

Month	EPNO	LPNO	YV	YG	PM	M	GVB
July	5	5	10	-	20	60	Seen
August	10	15	15	-	20	40	-
September	-	10	-	20	20	50	Seen
October	5	5	-	10	5	75	-
November	-	5	-	10	20	65	-
December	30	40	-	-	20	10	Seen
January	5	5	20	20	15	35	-
February	10	10	25	-	10	45	-
March	10	15	20	-	15	40	Seen
April	20	-	-	40	25	15	-
May	-	-	15	30	20	35	-
June	15	15	10	10	20	30	-

Here, EPNO=Early perinucleolar oocyte, LPNO=Late perinucleolar oocyte, YV=Yolk vesicle, YG=Yolk granule, PM=Pre-mature, M=Mature

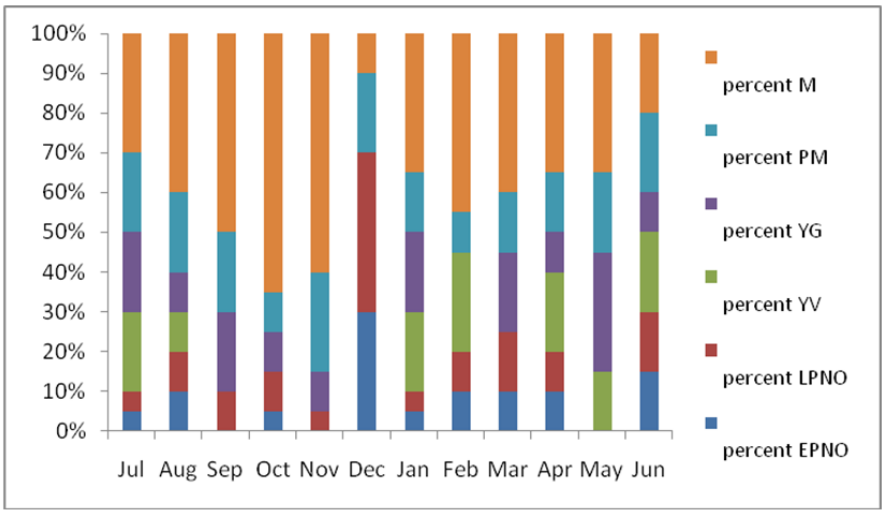


Figure 13: Month-wise occurrence (%) of different stages of oocyte of ovarian development of female Hilsa collected from Tentulia river, Barisal during July 2013 to June 2014. [Here, EPNO=Early perinucleolar oocyte, LPNO=Late perinucleolar oocyte, YV=Yolk vesicle, YG=Yolk granule, PM=Pre-mature, M=Mature]

In September, percentage of mature oocyte increased 10% that the previous month and reached to 50% with no yolk vesicle and early perinucleolar oocyte stage, 10% late perinucleolar oocyte, 20% yolk granular stage, 20% pre-mature stages oocytes. The month of October comprises the highest percentage (75%) of mature stages oocyte among the months with 5% pre-mature, 10% yolk granule, 5% late perinucleolar oocyte and 5% early perinucleolar oocyte. In November, the percentage of mature oocyte was also in highest rank (65%) however start decline, this month's ovary also contained 5% late perinucleolar oocyte, 10% yolk granule, 20% pre-mature oocytes and on early perinucleolar oocyte and yolk vesicle stage oocyte. In December, the percentage of mature oocyte reached in lowest percentage among the month and it was only 10%, the ovary contained 30% early perinucleolar oocyte, 40% late perinucleolar oocyte, 20% pre-mature oocytes and no yolk granule and yolk vesicle stage oocyte. In January, the percentage of mature stage oocyte started to increase reached to 35% having 5% early perinucleolar oocyte, 5% late perinuclolear oocyte, 20% yolk vesicle, 20% yolk granule, 15% pre-mature stage oocytes. In February, the mature stage oocyte increases more 10% than January reached to 45% having 10% early perinucleolar oocyte, 10% late perinucleolar oocyte, 25% yolk vesicle stage, 10% pre-mature oocytes and no yolk granule stages were found. In March, the mature stage oocyte again declines and dropped to 40% having 10% early perinucleolar oocyte, 15% late perinucleolar oocyte, 20% yolk vesicle stages, 15% pre-mature stage oocytes and no yolk granule stages were found. In April, the percentage of mature oocyte continued to declining trend, depleted to 25%, this month other stages of oocyte were found as 20% in early perinucleolar oocyte,

40% in yolk granule, 25% pre-mature stage and no late perinucleolar stages and yolk vesicle stages. In May, the percentage of mature oocyte started to increase again and it found 35% with 15% yolk vesicle stage, 30% yolk granule, 20% pre-mature stage and early perinucleolar oocytes and late perinucleolar oocytes were not found in this month. In June, mature oocyte percentage stayed in higher rate with little bit decreased than previous month valued 30% with 20% pre-mature, 10% yolk granule, 10% yolk vesicle, 15% early perinucleolar oocyte and 15% late perinucleolar oocyte.

Yolk vesicle stages were not found in March, September, October and November, December. Yolk granule stages were found throughout the year except February, July, August, and December. Early perinucleolar oocytes were also observed throughout the year except, May, September, and November. Late perinucleolar oocytes were observed throughout the year except April and May. Pre-mature and mature oocyte appeared throughout the year during study period. The advanced oocyte i.e., mature oocyte was found in every month of the study period. The occurrence of mature oocyte in every month of the study period confirmed that *T. ilisha* spawn precisely throughout the year, but major spawning take place in October.

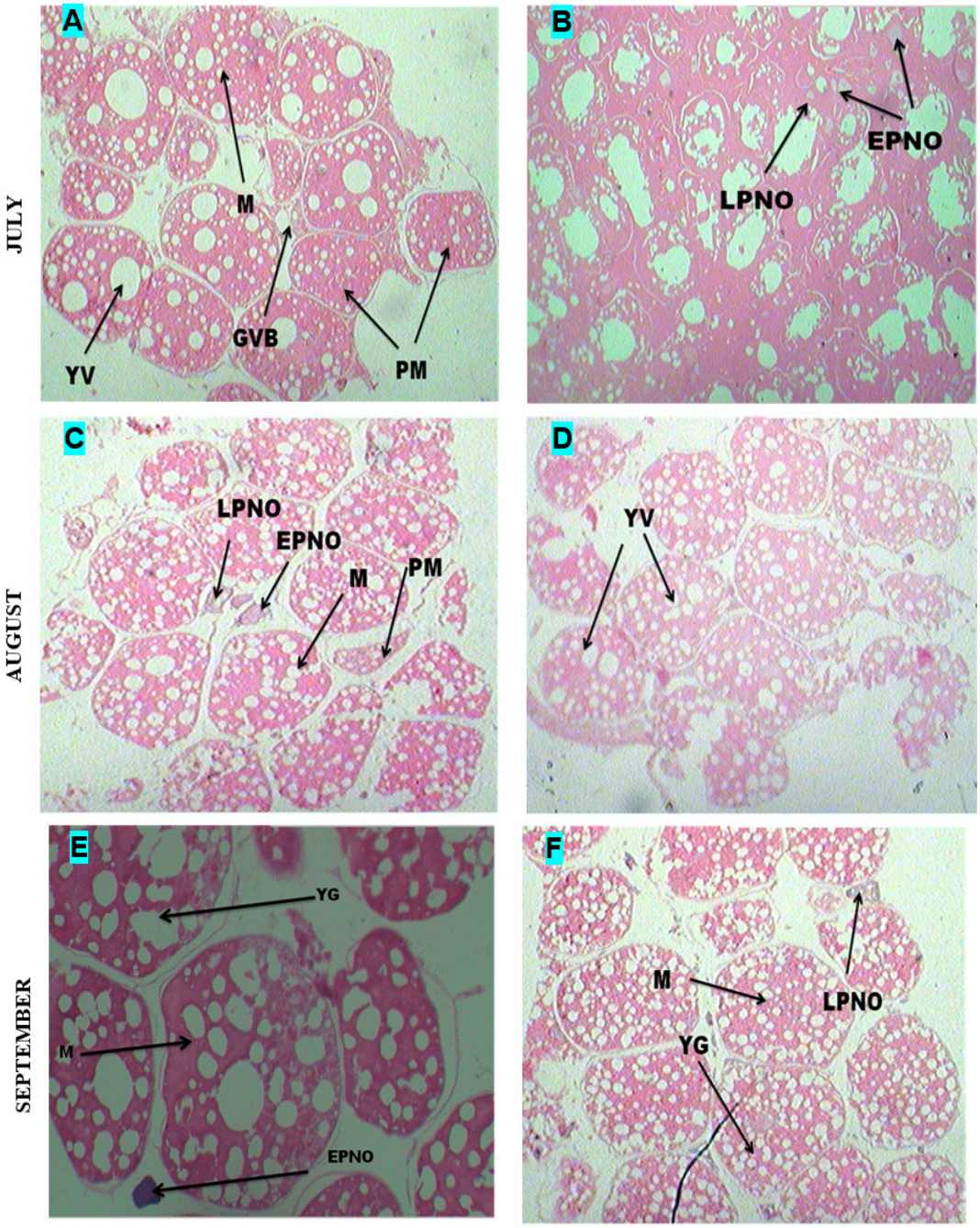


Figure 14: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled from Tentulia river, Barisal in July to September, 2013 [A-B: July; C-D: August; E-F: September]. EPNO, Early perinucleolar oocyte; LPNO, Late perinucleolar oocyte; YV, Yolk vesicle; YG, Yolk granule; PM, Pre-mature; M, Mature; GVB, Germinal vesicle breakdown.

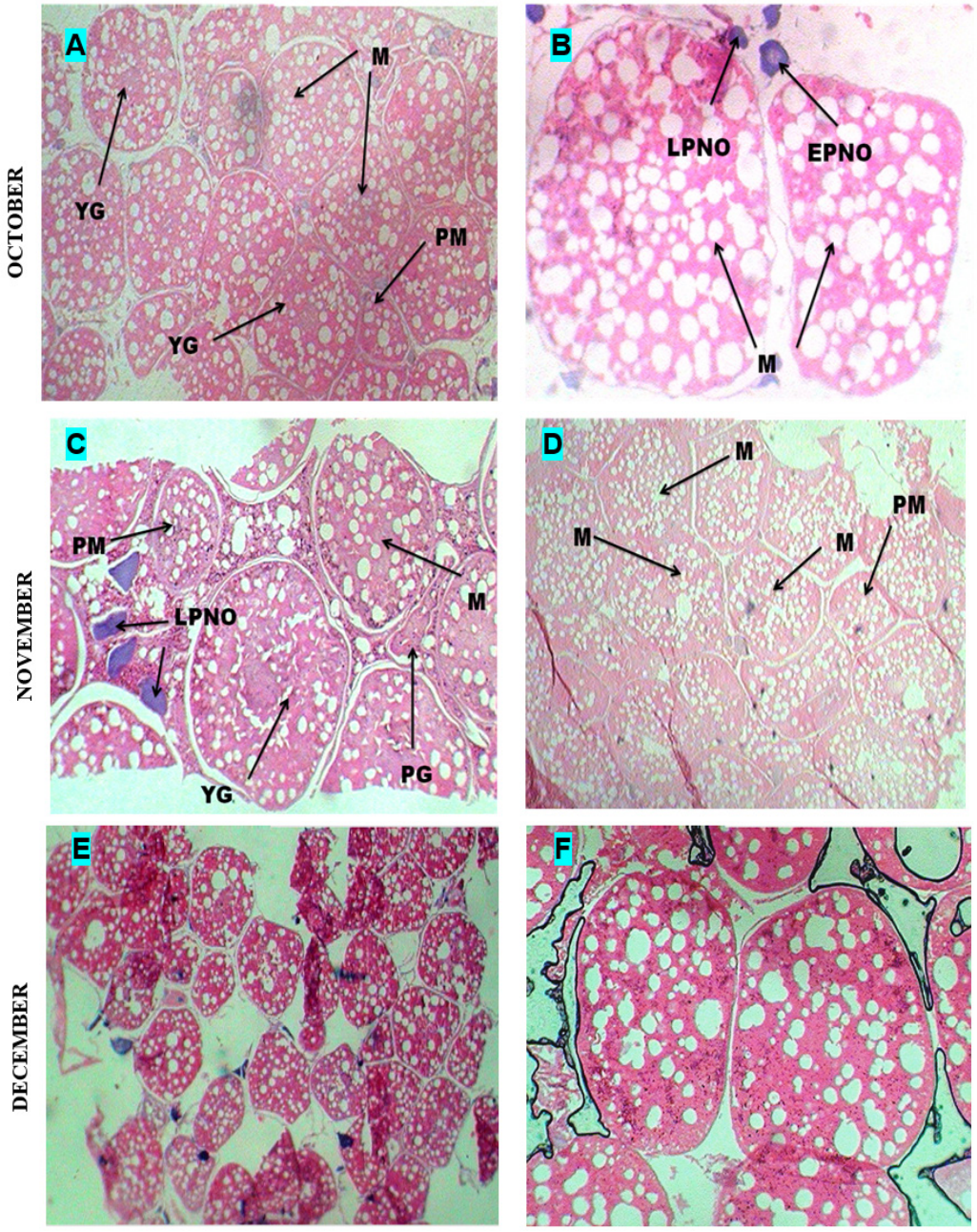
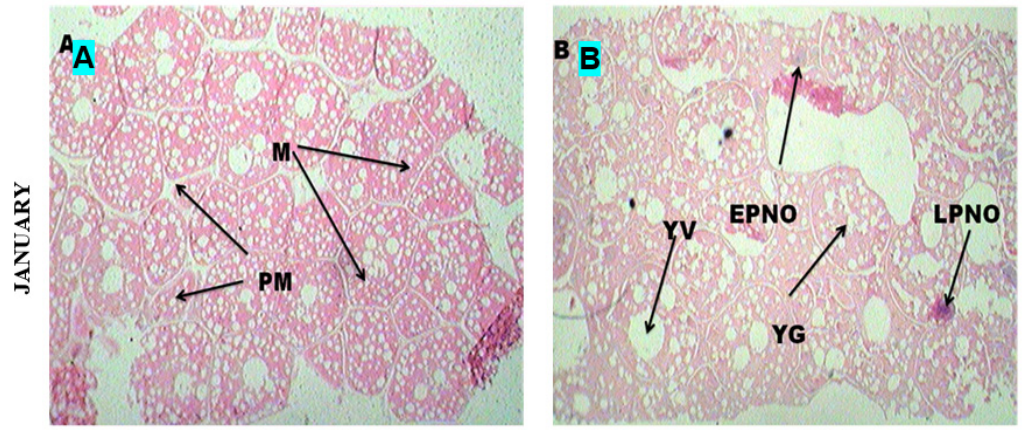


Figure 15: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled from Tentulia river, Barisal in October to December, 2013 [A-B: October; C-D: November; E-F: December]. EPNO, Early perinuclear oocyte; LPNO, Late perinuclear oocyte; YG, Yolk granule; PG, primary growth oocyte; PM, Pre-mature, M, Mature stage; GVB, Germinal vesicle breakdown.



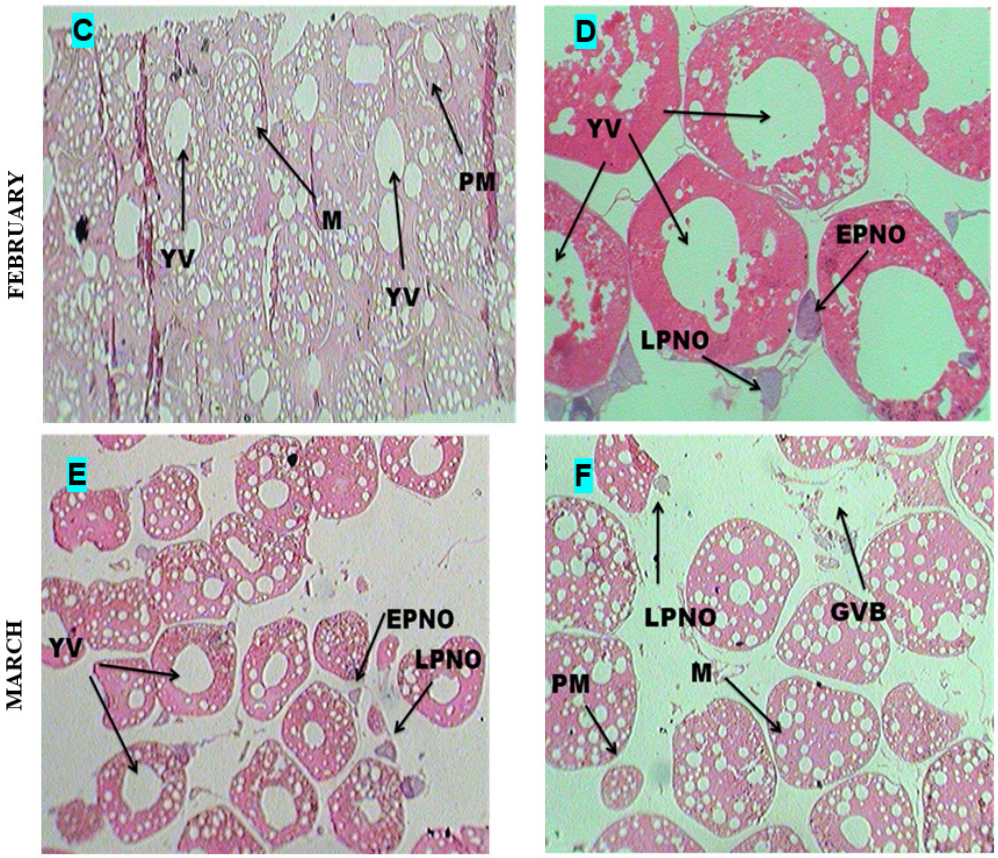
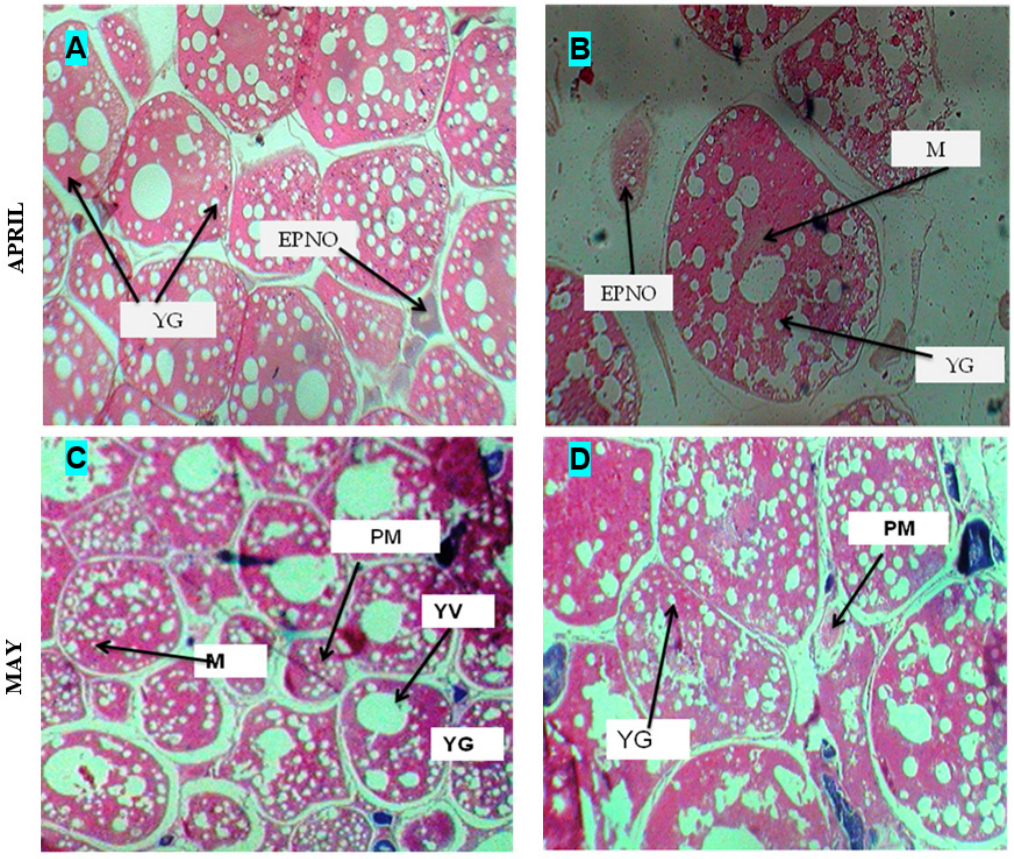


Figure 16: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled from Tentulia river, Barisal in January to March, 2014 [A-B: January; C-D: February; E-F: March]. EPNO, Early perinucleolar oocyte; LPNO, Late perinucleolar oocyte; YV, Yolk vesicle; YG, Yolk granule; PM, Pre-mature; M, Mature stage; GVB, Germinal vesicle breakdown.



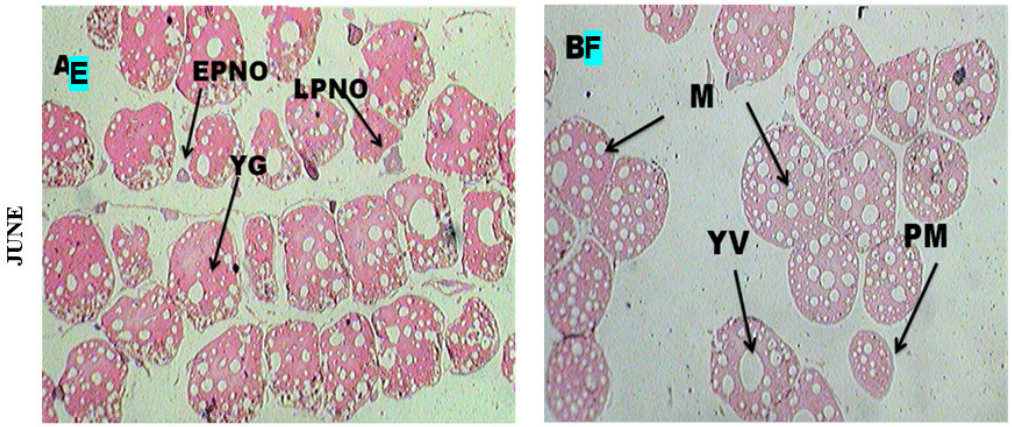


Figure 17: [A-F]-Haematoxylin-eosin staining of tissue section of *T. ilisha* ovary at 4x magnification sampled from Tentulia river, Barisal in April to June, 2014 [A-B: April; C-D: May; E-F: June]. EPNO, Early perinucleolar oocyte; LPNO, Late perinucleolar oocyte; YV, Yolk vesicle; YG, Yolk granule; PM, Pre-mature; M, Mature stages; PG, primary growth oocytes.

DISCUSSION

The reproductive activity in *T. ilisha* is continuous, and the presence of spermatogenic and oogenic cells under their particular development stages was observed in all specimens examined monthly over two years of study (Andrade *et al.*, 2001). This pattern is also described for other teleosts, as a result of tropical water conditions (Andrade *et al.*, 2001; Lowe-McConnell, 1987). Study of vitellogenic stage (VG) of oocyte and increased gonad weight (GW) and histology of gonad are the important aspects of reproductive biology that indicate the maturation level and can estimate approximate spawning time (Brown-Peterson *et al.*, 2011). In the comparison of ovarian development with age from the previous study by Ahmed *et al.* (2020), Hilsa from the Meghna River, the Bay of Bengal, the Padma River, and the Tetulia River showed regular ovarian development in respect to their age. No fish were found with a fully matured ovary at the age below 2 years.

In the teleost fishes, the process of oogenesis may be divided into five to eight stages (Andrade *et al.*, 2001). In the present study, stages of gonadal development in Hilsa shad were identified by histological research according to the scales described by Shinkafi *et al.* (2011) and divided into six stages; such as early perinucleolar oocyte (EPNO), late perinucleolar oocyte (LPNO), yolk vesicle (YV), yolk granule (YG), Pre-mature (PM) and mature (M) stages. PG, primary growth oocytes. Besides these six significant stages, other two stages, such as germinal vesicle breakdown (GVB) and primary growth oocytes (PG) also found in a few cases. Panhwar *et al.* (2011) studied reproductive patterns and some biological features of anadromous fish *T. ilisha* from Pakistan. They identified five stages of oogenesis, nearly ripe, fully developed, running ripe, partially spent, and spent. Lee *et al.* (2005) studied with *Leiognathu sequulus* and they found eight maturity stages of oocytes as chromatin nucleus, perinucleus yolk vesicle, primary yolk, secondary yolk, tertiary yolk, migratory nucleus and ripe stage by histological examination of ovaries.

On the other hand, the four different spermatogenic stages were identified in the *T. ilisha* as described by Andrade *et al.*, 2001. The stages are Spermatogonia (SG), Spermatocytes (SC), Spermatids (ST), Spermamatozoa (SZ). From the histological study, it was found that *T. ilisha* testis contains early and developing stage germ cells from April-June. Maturing germ cells were observed every month where a higher amount of mature germ cells were found in December, January and September to October with a high in October. In the month of February to June, the mature germ cells were less. Histological observations of testis suggested that *T. ilisha* spawns throughout the year, but major spawning occurs in September-October and December-January with a high abundance of ST. During October, mature germ cells were mostly evident and SZ was in high proportion inside the LU. The gradual maturation of germ cells from early to ripened stages inside the testicular LU and peak maturity during October is indicative of the fact that the peak spawning season of *T. ilisha* species is during October. The findings on testicular cycles coincide with that of ovarian cycles of this fish which confirms the fact that *T. ilisha* breed throughout the year and the peak breeding season in October. Some other studies on testicular development have been done on different fishes of Bangladesh, they also identified four testicular developmental stages. Siddiqua *et al.* (2000) observed the testicular development in *O. pabda* and identified three stages of sperm formation, namely spermatocytes, spermatids, and spermatozoa. The percent distribution of spermatozoa was highest in July (about 92%). By analyzing the histology of spermatogenesis, it was established that this species breeds once a year. Akhter (2011) found that early and developing germ cells in *Sperata seenghala* mainly were observed during April- May and mature germ cells found in July-August. Maya (2011) studied on gametogenesis of *Mystus cavasius* and found that mature germ cells (ST and SZ) were observed from July to August samples of

the testis. Alam (2009) observed a large amount of ST, SZ and small amount of SC in testis of *O. pabo* sampled from April to June. Testicular LU was full of SZ in June samples, indicating the peak breeding season of *O. pabo*. In the present study, mature oocytes were observed each month. The percentage of mature oocytes stated to increase in May and continued to November, with peaked in October (73% in the Meghna River and 75% in the Tentulia River) and decreased in December (lowest ever mature oocyte among the months; 13% in the Meghna River and 10% in the Tentulia River), January, and April. Histological examination and presence of mature in oocyte throughout the year suggested that *T. ilisha* spawn throughout the year but major spawning takes place in October. On the other hand, a higher percentage of mature oocytes from May to November suggested the prolonged spawning season of Hilsa shad. This study result agreed with a few previous studies. Pillay *et al.* (1963) studied with *T. ilisha* of Saurashtra coast and stated five ovarian development stages as immature, maturing, mature, partly spent, and spent. Wallac and Selman (1981) noted that the ovary of *T. ilisha* was the synchronous type that contained almost similar oocytes maturation stages. This type of oocyte maturity was the most common strategy among teleosts. In this study, advanced, mature oocytes were found every month during the study period. The study indicated *T. ilisha* breeds throughout the year, with a peak in October.

CONCLUSION

Gonadal histology of teleost fishes is the most common and reliable technique to assess the reproductive strategy of fishes. The present study of the histological study of both female and male gonads suggested that *T. ilisha* has a prolong spawning season with a peak in the month of October.

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