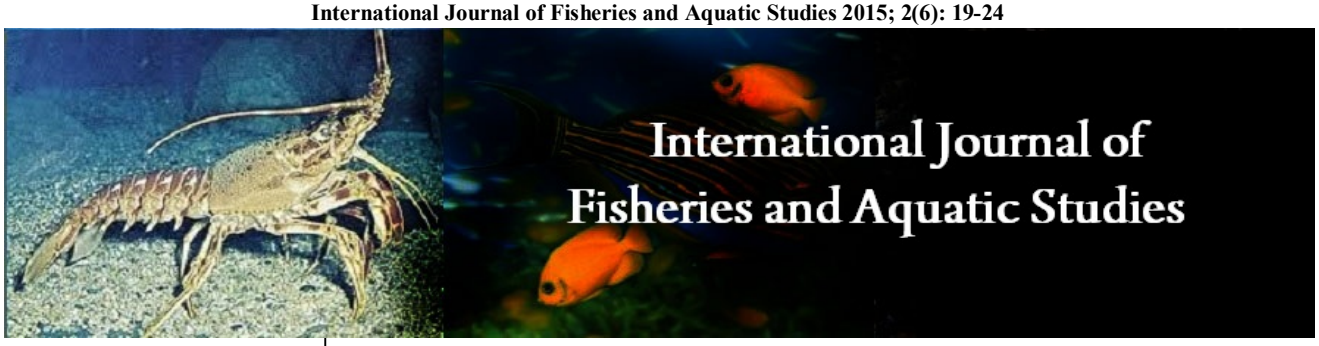

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Maturity stages of ovary of a minor carp, *Labeo bata* (Hamilton-Buchanon, 1822)

Kousik Roy, Dipak Kumar Mandal

Abstract
Histological studies of the ovary of a carp, *Labeo bata* demonstrate the annual events of its ovarian function. The month wise studies reveal that oogonial proliferation and recruitment of primary oocytes occur during resting (November-December) and preparatory phase (January- February). Ovarian growth is coincided with the enhancement of temperature and photoperiod from the month of March when oocytes are characterised with the inclusion of yolk vesicles and yolk granules. These oocytes are transformed to yolky mature follicles during maturation phase (April to May) and attain the maximum oocyte diameter during spawning phase (June to August) after germinal vesicle breakdown. Ovary undergoes regression with decline of water temperature during September-October. Hypertrophid granulosa cells take active part in resorption of yolky oocytes. The new oogonia appear only after complete resorption of yolky oocytes. This study shows that photo-thermal conditions are the major regulator of the ovarian function in *L. bata.*

Keywords: Oocyte, GSI, Histology, Growth phase, Ovary development, *Labeo bata*

1. Introduction
Reproduction in most of the tropical subtropical fish species is periodic and the peak reproductive event, spawning occurs in the most suitable time of the year to ensure maximum survival and growth of the young. Annual fluctuation in photoperiod and its dependant variable temperature are considered as the primary environmental factors regulating reproductive cycle of fish [1, 2, 3]
Fish reproduction, especially teleost has achieved more attention among fisheries scientists during recent few years due to economic interest and nutritional requirements among increasing population. The peak period of spawning evaluation, biological characteristic and life cycle of species determine by the histological studies [4]. The histological description of gametogenesis is the most important for macroscopic staging if errors in the estimation of maturity and reproductive seasonality are to be minimised [5]. The reproductive cycle in female fish was determined by the most suitable method to observe seasonal developmental changes in the gonads [6].
The literatures revealed that the mechanism of oogenesis and oocyte maturation among teleosts seem to be similar except variations in the timing of recruitment and maturation of oocytes [7, 8, 9, 10, 11].
Labeo bata is a popular food fish in Indian subcontinent and gets importance for it potentiality to be included in carp culture system. It breeds in the river during monsoon months [12]. However pattern of the annual ovarian cycle of this fish is not known.
Accordingly, the purpose of this study is to perform month wise study of different histo-morphological features of the ovarian activities (oogenesis, oocyte maturation) and thereby demonstrate the pattern of an annual reproductive cycle of an understudied food fish, *Labeo bata*.

2. Materials and methods
2.1. Materials
The study was conducted on the adult female Indian minor carp, *Labeo bata* (Class Teleostomi; Order Cypriniformes; Family Cyprinidae). It is distributed throughout the Indian subcontinent and the main habitat of this carp includes rivers, reservoir, freshwater lakes and ponds. The fish is a popular food fish comprising good amount of protein [13]. This fish is benthopelagic, herbivorous, column feeder and cultivable in carp culture system.

2.2. Procurement of Fish
Adult *Labeo bata* weighing 75-100g and length 20-25 cm were procured from the nearby freshwater pond located adjacent to the University campus of Visva-Bharati, Santiniketan, West Bengal (Latitude N 23°67", Longitude E 87°72") on every second week of each month from June 2010 to May 2011. Immediately after collection fish were transported to the laboratory and body weight of each individual of at least 10 fish every month were recorded. The fish were deeply anaesthetized with MS 222 exposure at 100 mg/L until would be senseless and sacrificed following the guideline of the departmental animal ethics committee.

2.3. Procurement and preparation of ovaries
After scarifying the fish both the ovaries of each fish dissected out, soaked in blotting paper and weighed with the help of an electronics balance. The weight of the ovaries was used for calculation of Gonadosomatic index (GSI) using the formula [14]: GSI = Gonad weight (g) x 100 / Total body weight (g). GSI represented as mean of 10 fish ± standard deviation. The macroscopic characters viz. colour, texture, shape, size of the ovary and its position in the body cavity were considered to determine different developing phase of ovary [15].

2.4. Histological preparation
Ovaries were cut into small pieces and fixed in Bouin's fixative for overnight; dehydrated through graded ethanol; cleared with benzene and embedded in paraffin wax (56-58°C, Emarck). Routine microtomy was followed to obtain 5µ thin sections of the tissue with the help of a rotary microtome (Weswox). Sections were stretched on albuminized glass slides and stained with Haematoxylin and Eosin.

2.5. Microscopic evaluation
Stained slides were studied under a research microscope (Olympus BX 52) and the oocytes were classified into six different stages on the basis of cytoplasmic inclusion and characteristics of nucleus. Month wise percentage frequencies of different stages of ova were calculated using the formula: Number of ova of a particular stage x 100 / Total Ova counted. The oocyte diameters were measured by the ocular micrometre (Erma, Japan). Average oocyte diameters for each month were calculated by counting oocytes of at least five stained slides and represented as mean ± SD.

2.6. Statistical evaluation
The month wise variation in the relative abundance of different oocytes in the ovary of *Labeo bata* was evaluated through one-way analysis of variance (ANOVA). The mean and standard deviation (±SD) value of each data was calculated. The values of both F and P were calculated at the 1% or less ($p \leq 0.001$) level of significant (using computer programme MINI TAB).

3. Results
3.1. Gonadosomatic index (GSI)
Monthly variations in GSI of female *L. bata* were quite distinct (Figure 1). Gonado-somatic indices were high from the month of April to August. Maximum value was recorded in June (24.14) when ovary attained full maturation. GSI declined slightly in the month of July and August (12.25 and 10.86) and sharply drop in September. The lowest GSI value was found during winter months (October to January). GSI corresponded to the average oocyte diameters of oocytes (Figure 2). The average diameter of the ova varied from 20 µm to 700 µm and reached to the peak in the month of June and the minimum was found in October (Figure 2)

3.2. Developing stages of oocytes
The process of oogenesis and oocyte maturation was a complicated process and took year long time in *Labeo bata*. Oogonia proliferated from the germ mother cells in the ovigerous fold and transformed to oocytes by the process of oogenesis. Oocytes initially grew slowly during its primary growth phase and then rapidly with the incorporation of yolk protein during its secondary growth phase and finally attained maturation with the germinal vesicle breakdown. On the basis of size, morphology and cytoplasmic inclusion the growing oocytes were categorised into seven stages

Oogonia: Oogonia were small, round, 20-30 µm in diameter and each possessed a nucleus of 12-18 µm. Nucleus to cytoplasmic (N/C) index is nearly 70%. Nucleoli indistinct, Cytoplasm appeared as thin rim around nucleus (Figure 3A).

Stage-I: Early perinucleolus oocyte (EPO): EPO were spherical and small (50-70 µm) and it possessed a large round nucleus (25-40 µm) with Nucleus to cytoplasmic (N/C) index 55-65%. Cytoplasm was dense, compact and strongly basophilic and the nucleus contained 6-15 peripheral nucleoli (Figure 3A, 3B).

Stage-II: Late perinucleolus oocyte (LPO): LPO were spherical and large (80- 220 µm) with large nucleus (40-60 µm) and N/C index 48 to 60%. Cytoplasm was strongly basophilic and possessed yolk nucleus (Balbiany body). Nucleus comprised of 15-20 peripheral nucleoli. Primary theca appeared around the oocyte (Figure 3B, 3C).

Stage-III: Alveolar stage of Early maturing oocytes (EMO): EMO were large (180-250 µm) and round. It possessed a large round nucleus (60-80 µm) showing the N/C ratio 35-50%. Cytoplasm was slightly acidophilic and numerous vesicles appeared in the cytoplasm. Nucleus contained dispersed chromatin and numerous small nucleoli. Distinct zona radiata (ZR) and zona granulosa (ZG) layers appeared around the oocytes. ZG contained cuboid epithelial cells (Figure 3D, 3E).

Stage-IV: Late maturing oocytes (LMO): LMO were vitellogenic oocytes of pre germinal vesicle breakdown (GVBD) stage, round and large (330-450 µm). Cytoplasm filled up with yolk granules and yolk vesicles were pushed to the periphery. Nucleus was large (70-85 µm) and round with distinct nuclear membrane, compact chromatin and numerous small nucleoli. ZR, ZG and theca layers were prominent (3F).

Stage-V: Mature follicle (MF): MFs were vitellogenic oocytes of post GVBD stage, round or elliptical and 550 to 750 µm in diameter. Germinal vesicle without nuclear membrane migrates toward the periphery and nuclear membrane disappears (Figure 4A). Oocyte hydrated and yolk globules coalesced to form a translucent yolk mass. Oocytes were covered by thick ZR and follicular cells of ZG and theca (Figure 4B).

Stage-VI: Atretic follicle (AF): AFs were large post ovulatory follicles or degenerating yolky oocytes (Figure 3D, 4C) with irregular and collapsed ZR and hypertrophied granulosa cells.

3.3. Size frequency distribution pattern of oocytes
Immature ova (stage-I and stage-II oocytes) was found from September to April as dominating oocytes showing the peak abundance during January. Some of the immature ova were transformed to early maturing oocyte (Stage-III) in the month of February but maximum maturing ova (stage-III and stage-IV oocytes) was observed from March to April. Mature ova

(stage-V) were observed from May to August showing the maximum in the month of June-July was listed in table 2.
The mean monthly average oocyte diameter was recorded to be low during winter months, October to January and began to increase in spring (February-March) and rapidly increase with the onset of summer (April-May) and reached to the peak during Monsoon (June-August) (Figure 2)

3.4. Annual maturation cycle of ovary

In the annual reproductive cycle of *Labeo bata,* development of ovary were categorised into seven maturation phases on the basis of its macroscopic condition and relative abundances of different maturity stages of oocytes in the ovary is listed in Table 1. The criteria of the division of different maturity stages of ovary is summarised in Table 2.
Resting phase: The ovary was long thread like structure lying on the left and right side of the swim bladder. The oocytes could not be seen with naked eye. Immature ovary (Figure 3A) was found from the month of November to December. The major event in this phase was proliferation of oogonia and recruitment of oocytes. Oogonia were found in clusters throughout the germinal epithelium of ovigerous fold. The immature oocytes were of EPO and LPO stages and small in size. The average oocyte diameters were less than 100 µm.
Preparatory phase: In early preparatory phase ovary was elongated and transparent. Oocytes were not distinctly visible in naked eye. The thickness of the ovary was slightly greater than that of resting phase. This phase of ovary was found from the month of December to January (Figure 3B). Oocytes were of primary growth phase, spherical, small (90µm-120µm) with dense, compact and strongly basophilic cytoplasm (Figure 3C). Late preparatory phase or developing ovary was found during February to March. In this phase ovary was pale yellow in colour and oocytes were visible by a hand lens. Histologically, the primary oocytes were dominant but some of the oocytes entered into the secondary growth phase with the appearance of alveolar oocytes (Figure 3D). The oocytes were almost round in shape and size (120 µm -220 µm) was increased due to the yolk vesicles and deposition of yolk droplets (Figure 3E).
Maturation phase: In this phase, ovary was enlarged in volume and was occupied by yolky oocytes. The oocytes were visible with the naked eye. The maturing stage was found in April to May (Figure 3F). Three types of oocytes were found viz. few primary oocytes, some secondary oocytes and the largest group was tertiary oocytes of stage-IV, V and VI. Oocytes continue to enlarge with incorporation of yolk. The yolk globules increased in number and displaced most of the cortical alveoli toward the periphery. The lipid droplets enlarged and scattered between the yolk granules. The average diameter of the oocytes at this stage was reached up to 612 µm. Later in the ripening stage, germinal vesicle breakdown (GVBD) and migration of germinal vesicle occurred. The tertiary oocytes were hydrated, enlarged in size and yolk globules coalesces to form yolk mass.
Spawning phase: Ovaries were full in mature yolky follicles. This phase of ovary was found during June to August. Two types of oocytes were found as primary and tertiary oocytes. During this phase oocyte size was increased occurred due to hydration and vitellogenesis. The oocyte diameters varied from 600µm to 700µm. Germinal vesicle breakdown (GVBD) was occurred in most of the follicles (Figure 4A). Follicular layers around the oocyte were prominent (Figure 4B).
Post-spawning phase: The ovary of post spawning phase (September and October) were shrunken containing a few atretic oocytes and residual primary oocytes (Figure 4C, 4D). In this stage, weight of the ovary was decreased. Follicular cells of the atretic follicles were hypertrophid. Average oocyte diameter sharply declined.

Table 1: Relative abundance (%) of different stages of oocytes in the ovary and month-wise changes of Gonadosomatic index (GSI) (Mean ±SD) of *Labeo bata* during an annual reproductive cycle.

Months	GSI	Oogonia	Stage-I	Stage-II	Stage-III	Stage-IV	Stage-V	Stage-VI
Jan	0.41±0.04	28.35±1.84	22.44±2.86	35.18±2.48	0	0	0	0
Feb	0.82±0.03	25.24±2.71	29.21±2.09	37.13±4.21	4.28±1.69	0	0	4.30±1.70
Mar	2.61±0.71	17.09±1.41	18.38±3.48	38.36±1.59	21.34±3.27	0	0	5.39±1.53
Apr	6.93±2.96	0	10.91±2.26	46.29±2.94	6.53±0.92	28.42±2.06	3.43±1.47	4.38±1.35
May	11.65±2.86	0	8.50±2.11	46.71±2.24	2.49±0.61	12.26±0.59	28.17±1.77	2.65±1.26
Jun	24.13±3.94	0	6.98±1.90	3.77±1.65	1.38±0.52	18.35±0.91	66.19±3.54	2.60±0.72
Jul	12.46±2.25	0	14.58±2.04	13.71±1.91	0	17.44±2.48	54.32±1.99	2.06±0.89
Aug	10.70±2.12	0	23.54±1.37	3.49±1.84	2.63±0.68	14.49±2.36	52.50±3.64	2.74±2.03
Sep	0.70±0.07	41.73±3.70	26.94±2.96	25.50±2.59	0	0	0	3.66±0.76
Oct	0.46±0.03	48.62±2.79	24.57±2.41	26.44±2.10	0	0	0	0
Nov	0.46±0.06	54.52±5.04	18.20±2.87	28.44±2.30	0	0	0	0
Dec	0.52±0.02	49.38±4.68	29.27±3.09	18.39±2.73	0	0	0	0

The table shows Gonadosomatic index (GSI) as well as month wise percentage of ovarian follicles vary significantly as found in the one-way analysis of variance (ANOVA) test at the respective *p*-level, $p < 0.001$.
F Values:
GSI—$F_{11,48}$ 78.76, ($p < 0.001$)
Oogonia—$F_{11,48}$ 361.87 ($p < 0.001$)
Stage-I—$F_{11,48}$ 48.22 ($p < 0.001$)
Stage-II—$F_{11,48}$ 177.98 ($p < 0.001$)
Stage-III—$F_{11,48}$ 143.54 ($p < 0.001$)
Stage-IV—$F_{11,48}$ 356.52 ($p < 0.001$)
Stage-V—$F_{11,48}$ 1154.63 ($p < 0.001$)
Stage-VI—$F_{11,48}$ 15.41 ($p < 0.001$)

Table 2: Macroscopic and histological description of the maturity stages of the ovary of *Labeo bata*.

Developing stages of ovary		Macroscopic description	Histological description
Resting phase (November to December)	Oogonia	Thread like, transparent, no oocyte visible with naked eye.	Well-developed ovigerous fold, oogonia dominant in this stage, found in nests.
Preparatory phase (early) (January to February	Stage-I	Whitish colour, thickness just higher than resting phase ovary.	Perinucleolar oocytes dominant. Yolk nucleus in cytoplasm, nucleolus was found in the periphery of nuclear membrane.
Preparatory phase (Late) (March)	Stage-II	Ovaries were pale yellow colour, blood vessel prominent on the surface of ovary.	Cortical alveoli stage oocyte appeared containing cortical alveoli and yolk granules in cytoplasm.
Maturation phase (early) (April)	Stage-III	Longer, thicker, fully yolked ova were ivory yellow colour. Eggs were visible with naked eye.	Large vitellogenic oocyte dominant containing well-developed zona radiata and yolk globule.
Maturation phase (late)(May)	Stage-IV	Ovary larger, deep yellowish colour, round oocytes were present in the gonad.	Mature follicles (MF) showing germinal vesicle migration (GVM) and yolk granules in cytoplasm were found.
Spawning phase (June-August)	Stage-V	Ovary larger, deep yellowish colour, round oocytes were present in the gonad Ovary shrunken and transparent oocyte visible.	MF with fused yolk globules, hydrated and large. Nuclear membrane disappear (GVBD) in most of the MF and some oocyte collapsed to atresia.
Post-spawning phase (September-October)	Stage-VI	Ovary long and transparent colour.	Residual primary oocytes are dominant. Some atresia follicles found and empty space between ovigerous folds.

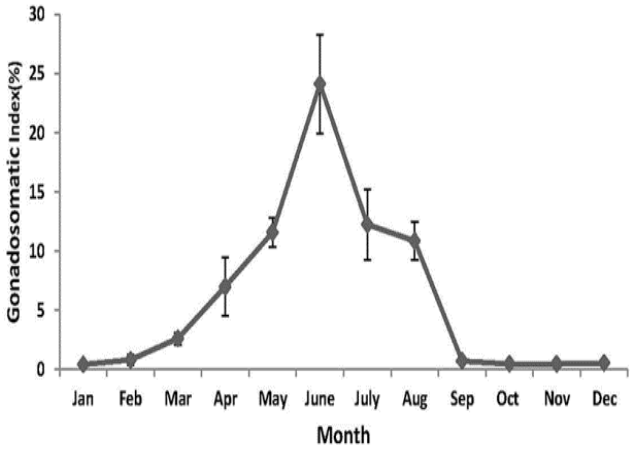

Fig 1: Annual variation of gonado-somatic indices (GSI) mean (±SE) in female *Labeo bata*

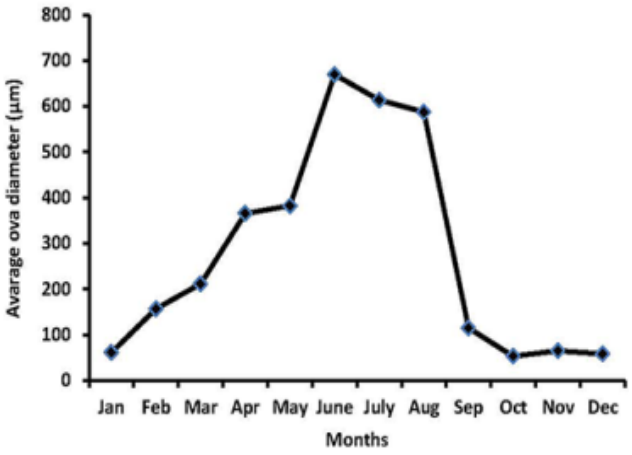

Fig 2: Monthly variation of average ova diameter of *Labeo bata*

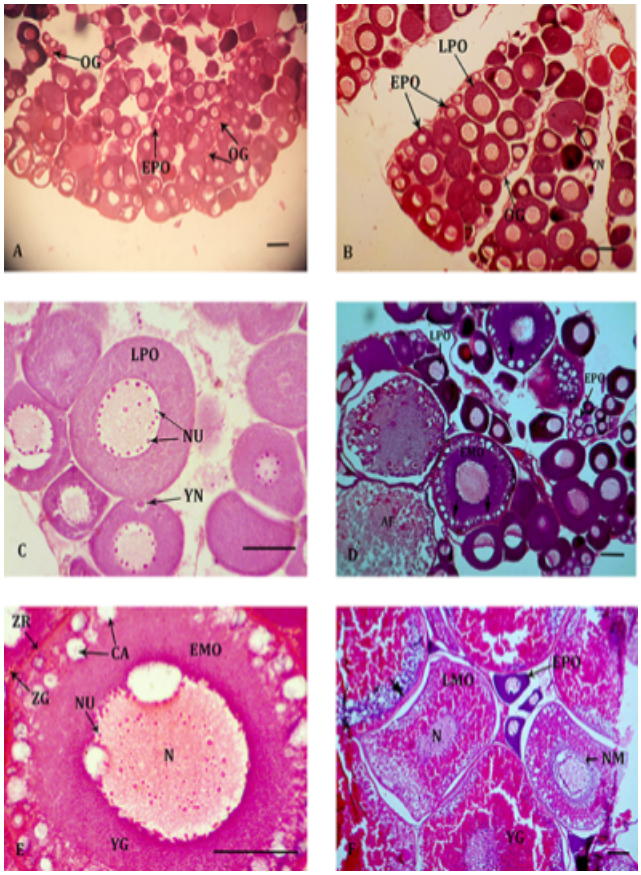

Fig 3: Histological section showing different developing stages of Ovary of *Labeo bata*, Hematoxylin eosin stained, Bar=100 µm (A) Resting phase of ovary showing recrudescence of oogonia(OG) and early perinucleolar oocytes (EPO) (B) Early preparatory phase of ovary shows ovigerous fold packed with EPO and late perinucleolar oocytes (LPO) (C) LPO with large central nucleus containing numerous peripheral nucleoli (NU) and granular cytoplasm with yolk nucleus (YN). (D) Late preparatory phase ovary shows early maturing oocytes (EMO) with cortical alveoli (arrows) in cytoplasm and zona radiata, many perinucleolar oocytes (EPO and LPO) and a few atretic follicles (AF). (E) EMO or cortical alveoli stage oocyte shows large nucleus (N) containing chromatin materials and numerous nucleoli (NU). Cytoplasm filled with yolk granule (YG) and cortical alveoli (CA) pushed to periphery. Oocyte covered with Zona radiata (ZR) and Zona granulosa (ZG) layers. (F) Maturation phase ovary shows late maturing oocyte (LMO) with considerable increase in size, Nucleus (N) with distinct nuclear membrane (NM) and cytoplasm filled with yolk granules (YG) and vesicles.

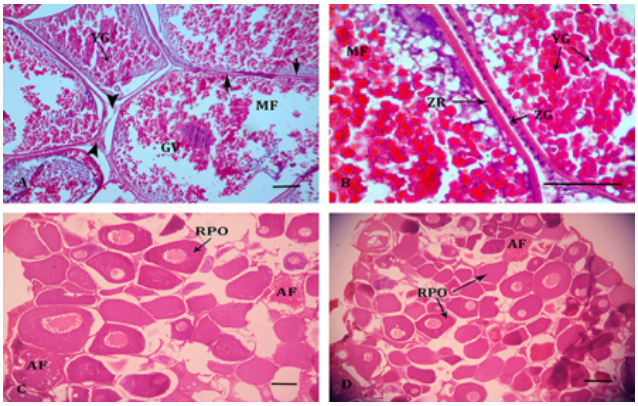

Fig 4: Histological section showing different developing stages of ovary of *Labeo bata* Hematoxylin eosin stained, Bar=100 µm (A) Spawning phase ovary shows mature follicles (MF) of post germinal vesicle breakdown and migration of germinal vesicle (GV) containing yolk globules (YG) and follicular layers (arrow heads). (B) Mature follicles shows yolk globules (YG) in cytoplasm, zona radiate (ZR) and zona granulose (ZG). (C) Post-spawning phase of ovary shows Atretic follicle (AF) and residual primary oocytes (RPO) and empty spaces. (D) Post- spawning phase ovary shows RPO and AF.

4. Discussion

Reproduction of all Indian freshwater fish exhibit annual periodicity and maturation of their gonads and spawning occurs in a particular period of the year. Photoperiod, temperature, rainfall and food availability are the important external factors influencing breeding periodicity [16, 17, 18, 19]. This study showed that *Labeo bata* is an annual breeder and its gonads reached to the peak maturity stage during monsoon months, June-July. The major events like recrudescence of oogonia, oogenesis and maturation during annual reproductive cycle correlated with the fluctuation of environmental parameters [20].

Labeo bata showed only one GSI peak in the month of June and high GSI values were observed from May to August. GSI increased with the maturation of gonads at the onset of breeding period. GSI decreased abruptly as soon as breeding period was over. The month wise variations of GSI were highly significant ($p< 0.001$). Similar pattern of the annual variations in GSI were observed in other teleosts [18, 21, 22, 23].

The study of ova diameter was a necessary tool for evaluation of the spawning periodicity of fish [24]. Average monthly ova diameter exhibits the highest value during spawning months when the ovary was full of mature ova. In this fish, the highest average oocyte diameters were found during June to August suggesting the period as breeding season. The changes in the average monthly ova diameter corresponded with the percentage frequency of different size ova describing a synchronous development of oocytes as in other fish [25, 26].

In this study, the development of oocytes of *L. bata* was divided into seven maturity stages on the basis of size, growth cytoplasmic inclusions and characteristic of nucleus. The criteria of staging of oocytes are in conformities with the description in other fish [27, 21].

The process of the ovary development has been described in many fish and divided into seven to eight maturity stages [28, 21, 29, 30, 31, 32, 33]. However, in many of the Indian teleosts annual changes in ovary are divided into four to six stages [16, 34, 20].

The annual breeding cycle of the female *Labeo bata* could be described into five different phases, namely the resting phase (November-December), the preparatory phase (January-February), the maturation phase (March to May), the spawning phase (June to August) and the post spawning phase (September to October) on the basis of macroscopic and microscopic features of the ovary.

The period October to January was noted as the period of recrudescence of oogonia and primary oocytes. Oogonia proliferated from the germ mother cells of the ovigerous fold and found in clusters. Oogenesis began and primary oocytes were recruited during this period. The period February to May indicated as the growth phase of oocytes. Primary oocytes grew rapidly with the incorporation of yolk. Yolk vesicles contained endogenously synthesized lipids and glycoprotein and increased the space for incorporation of exogenously synthesized yolk protein [11]. Zona radiata and follicular cell layers as zona granulosa and theca appeared in this stage. Zona granulosa and theca was the site for steroid hormone synthesis and played important role in the synthesis and incorporation of yolk precursors [35]. The important event of oocyte maturation was the germinal vesicle breakdown (GVBD) and its migration toward periphery. Yolk globules coalesced and formed a translucent yolk mass. Hydration diluted the cytoplasmic content resulting translucent in appearance and maximum size of oocytes [36].

The period June to August was the breeding season of *Labeo bata* because during this period ovary attained maximum size and the ovary was full of mature follicles. Gonadosomatic indics and mean average oocyte diameters reached to the peak in this period. The absence mature and maturing follicles in the month of September marked the completion of spawning period.

The atretic follicles and post ovulatory follicles were found with residual primary oocytes during post-spawning phase (September-October). Same observations were reported in different fish species [37, 6]. Mature follicles became atretic with the withdrawal of gonadotrophin [16, 38, 39]. The hypertrophid granulosa cells of the atretic follicles perhaps took the active role in reasorbtion of yolk. Oogonial proliferation and oogenesis began after completion of degeneration of yolky oocytes for recovery and repeat the cycle.

5. Conclusion

The annual histological feature of ovary in *L. bata* varies with the fluctuation of the photo-thermal condition of the environment. The recrudescence of oogonia and primary oocyte take place from October to February and thereafter oocyte grow with vitellogenesis during March to June and spawning takes place from June to August when ovary attains the maximum size and contains maximum mature follicles. After spawning period the ovary undergoes regression. New batch of oogonia appears only after complete resorption of yolky oocytes.

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