EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 12
Issue: 6
Year: 2025

GONADAL DEVELOPMENT CYCLE OF THE CRITICALLY THREATENED PUNTID FRESHWATER FISH PUNTIUS VITTATUS FROM THE RIVER TAMBRAPARANEI

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Article Received on 23/04/2025 **Article Revised on 13/05/2025** **Article Accepted on 03/06/2025**

INTRODUCTION

Maturation refers to cyclic morphological changes, which the male and female gonads undergo to attain full growth and ripeness. Gonads form the microenvironment enabling the germ cells to differentiate into ripe male and female gametes. The development of the piscine gonads has been described in terms of stages of maturity *Puntius filamentosus* (Victor *et al* (2004). The gonadosomatic index increases progressively with the development of the gonads in both sexes until the gonads are ripe, and the index then declines sharply in spawning and spent fish (Arachi, 2023).

Ovarian cells are characterized by their rapid cyclical change of growth, differentiation and regression, which can hardly be found in any other organ of the adult organism *Puntius filamentosus* (Victor *et al* (2004). In some fishes the ovaries increase in volume, not in length, but in most species there is an increase in length, volume and weight of the ovary through development (Ain *et al*.,(2021), Arevalo *et al*.,(2023), Efizon *et al*., (2021), Hasan *et al*.,(2020), and Sidik *et al*.,(2020). The number of ovarian stages varies from five to eight in different fish species. Although some differences occur among species the sequence of oocyte development stages can be generalized among teleost fish species in four stages. The morphological changes in the ovary coincide with the histological changes of the oocytes (Ain *et al*., (2021), Arevalo *et al*.,(2023), Efizon *et al*., (2021), Hasan *et al*.,(2020), and Sidik *et al*.,(2020).

The aim of this study was to provide more information on the gonadal cycles of *P.vittatus*, by observing seasonal changes in ripening and microanatomical changes in gonads during development, and relates these to changes in gonad-body weight ratio. The oocyte size frequency distribution is also analysed to pin-point the spawning season and further to evaluate the size of the mature ova.

MATERIAL AND METHODS

Macroscopic staging of gonads

Fortnightly samples of (N-300) *P.vittatus* were collected from the same pond during the study period of eighteen months. The specimens were measured (SL), weighed, dissected and sexed. The gonad condition was graded visually. The classification is based on position and volume of the gonad in the body cavity and width, length and cross section shape of the gonads, vascularization and size and colour of the oocytes. Identified gonads were categorized on the basis of their morphological appearance, to one of the stages of gonadal development. (Indira *et al*, 2013).

Microscopic staging of oocytes

The dissected ovaries and testes were placed in Bouin's fixative for 24h. After fixation, dehydration was made in increasing ethanol-in-water concentrations and cleared in xylene. After embedding in paraffin (MP=58-60^{0}C) serial sections were cut at 6µm and stained with Mayer's haematoxylin and eosin (HE) using routine histological procedure (Arachi, 2020). Maturity stages were histologically classified on the basis of changes in the diameter of intraovarian oocytes, degree of yolk deposition and proportion of oocytes ready for ovulation. Description of the basic stages in oocyte development was based on Arachi and Sumaiya (2025).

Statistical analysis

The values are expressed as the mean ± SD. Differences were tested by analysis of variance (ANOVA) (Zar, 1984) followed by Duncan's multiple range test. The range of means was tested by students 't' test. Simple regression analysis was used to correlate morphometric characteristics. All analyses were performed with statistical software SPSS.9.0 ver for the microcomputer.

RESULTS AND OBSERVATION

Ovarian development cycle

P.vittatus has paired ovaries of which anterior end is free while the posterior one continues into a oviduct. The ovary of *P.vittatus* is of cystovarian type, enclosed by a fold of peritoneum. Ovaries are usually of the same size in the initial developmental stages. The left and the right ovaries develop into different sizes in advanced stages. The germinal epithelium is projected into the ovarian lumen in the form of freely suspended folds. The ovary had stroma, and the ovarian follicles at different stages of maturation indicating asynchronous oocyte development. The different stages of fishes classified are given in Plate -1.

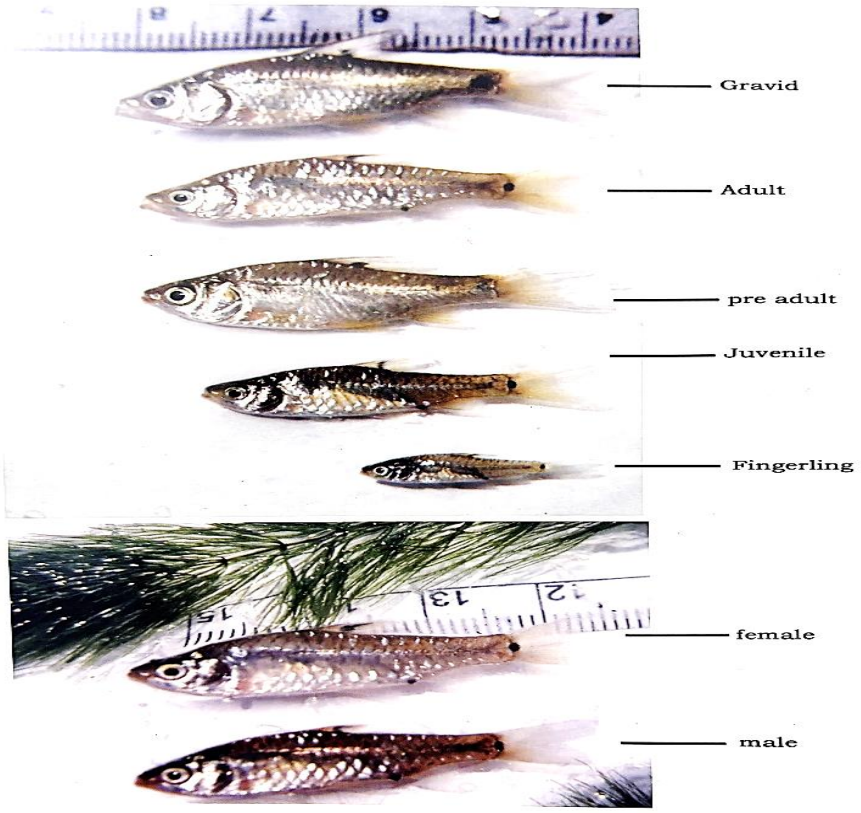

Plate 1 Puntius vittatus : Maturity stages

The development of the ovaries was closely associated with the somatic growth of the fish. The gonadosomatic index (GSI) increased progressively with the development of the ovaries in females until the gonads were ripe, and then GSI declined sharply in spent and recovering spent fish. There is increase in weight of the ovaries from the immature to the ripe condition through well defined intermediate stages of maturation. *P.vittatus* has rhythmic reproductive activity and the breeding phase has been restricted to particular season of the year.

Breeding periodicities depend on the maturation of the oocyte. Based on the gonadal condition of females, six gonadal ovarian stages were graded visually in *P. vittatus* as following

Stage I: Immature: The ovaries were small, tubiform and translucent, measuring about 0.5 mm in length. They occupied only a small portion of the abdominal cavity. The oocytes were of relatively uniform in size. The number of oocytes was small. Virgin ovary was found in almost all months in certain number of fishes.

Stage II: Maturing: The ovaries were larger and less transparent due to more oocyte number. The ovary was still tubiform, measuring about 0.6mm in length. The ovaries occupied nearly one-fourth of the abdominal cavity. This type of ovary was found in March, April and September.

Stage III: Early maturing: The ovaries were cream in colour, which occupied about half of the abdominal cavity. Many oocytes were whitish granular (non - transparent) and the ovary was characterized by a sparsely distributed mature ova. Such ovaries were noticeable during the end of April and early part of October and May.

Stage IV: Late Maturing: The light yellowish coloured ovaries were larger in size, compactly packed with fully-yolked repining ova. The ova were spherical and ivory yellow in colour. Ripening ovaries were found in May, also at the end of October, early November and late December.

Stage V: Gravid: The Ovaries were orange red (or) yellowish in colour, turgid with irregular surface because of the present of large mature oocytes, and numerous blood capillaries ramifying over the surface. The gravid ovaries were traced from the end of October upto middle of November, and also during early June.

Stage VI: Spent: The ovaries were flaccid, shrunken and empty of mature ova but contained oocytes in different states of atresia. Tunica was tough and leathery. Some residual oocytes were visible. This type of ovary was found during late November to February beginning and at the end of July.

Developmental morphology of oocytes

In *P.vittatus* nine developmental stages of oocytes were distinguished by microscopic examination of the ovary. The nomenclature and classification of oocyte stages were based on Arachi and Sumaiya, (2025). The oocyte classification was based on cytomorphological features as given in Table-1. The developing oocytes were arranged in the form of ovigerous cords. The distinctive cytological features of oocyte stages are given below.

Table 1: *P. vittatus*: Cytomorphological characteristics of different developmental stages of oocytes.

Cytological stage	Oocyte stage	Oocyte diameter (µm)	External characteristics		Nucleolus		
			Nucleus	Cytoplasm	No.	Position	Stainability
I	Oogonia (OG)	50 - 30	Large, spherical	Narrow, homogeneous	Single	Centric	Basophilic
II	Chromatin nucleolar oocyte (CNO)	28-45	Large	Thin layer around nucleus	Several	Centric or eccentric	Basophilic
III	Perinucleolar Oocyte(PNO)	38-80	Large, oval	Homogeneous, granulated	Several	Peripheral	Acidophilic
IV	Cortical alveolar oocyte (CAO)	75-130	Large, oval	Appearance of cortical alveoli and cortical granules dispersed in peripheral ooplasm	Many	Peripheral	Acidophilic
V	Early vitellogenic oocyte(EVO)	110 - 315	Irregular periphery	Numerous large acidophilic yolk globules in peripheral ooplasm; lipid bodies near nucleus	Many	Dispersed	Acidophilic
VI	Late vitellogenic oocyte (LVO)	297 - 342	GV near animal pole	Increased appearance of yolk granules, densely packed	Many	Dispersed	Acidophilic
VII	Germinal vesicle migration (GVM) stage	310 - 362	GV migrates to periphery; envelope breaks down	Yolk globules and yolk plates undergo coalescence	Many	Dispersed	Acidophilic
VIII	Germinal vesicle break down (GVBD) stage	345 - 390	Disintegrated nuclear membrane; complete nuclear migration	Hydrated	Many	-	Acidophilic
IX	Atretic oocyte (AO)	395 - 450	Not found	Disorganised vacuolation; collapsed oocytes collapsed inward migration of phagocytic granulosa cells	-	-	Acidophilic

Stage I: Oogonia: Cell diameter ranged between 15-30µm. Oogonia in the immature ovary of *P.vittatus* were small, round cells with a narrow homogeneous cytoplasm. They were characterized by a large nucleus containing a single prominent nucleolus. The oogonia occurred in small nests in the epithelium of the ovarian lamellae.

Stage II: Chromatin Nucleolar oocyte (CNO): Cell diameter ranged 31-45µm. They were characterized by a oval nucleus containing several nucleoli. The nucleus (germinal vesicle) was surrounded by basophilic cytoplasm (Plate2a).

Stage III: Perinucleolar oocyte (PNO): Cell diameter 46-80µm. There was a significant increase in size of the oocytes, due to enlargement of the nucleus as well as the cytoplasm. This stage of oocyte was characterized by numerous, relatively large, basophilic nucleoli at the periphery of the germinal vesicle. The cytoplasm was homogeneous, granulated and stained deeply with haematoxylin (Plate2b -f).

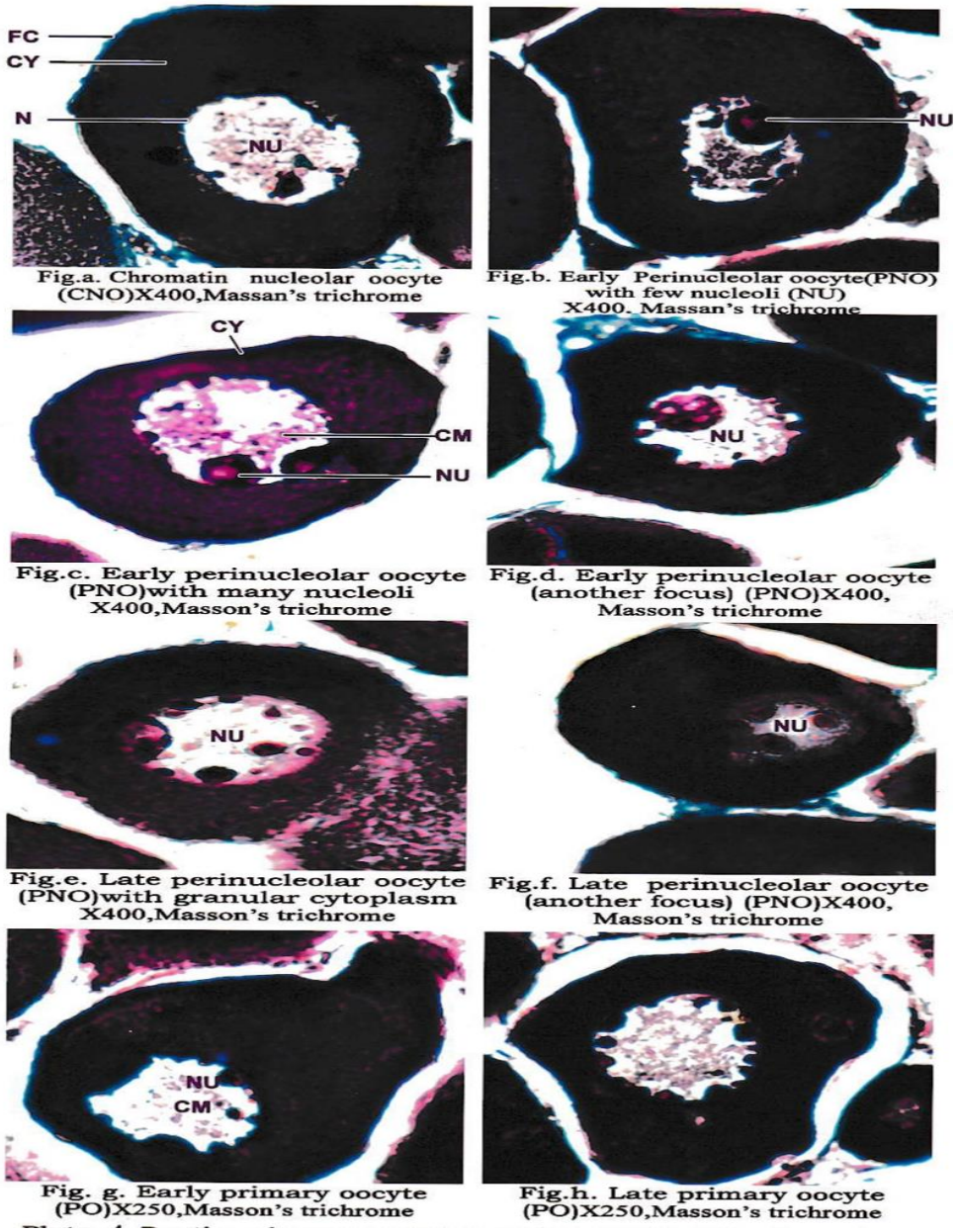

Fig.a. Chromatin nucleolar oocyte (CNO)X400,Massan's trichrome

Fig.b. Early Perinucleolar oocyte(PNO) with few nucleoli (NU) X400. Massan's trichrome

Fig.c. Early perinucleolar oocyte (PNO)with many nucleoli X400,Masson's trichrome

Fig.d. Early perinucleolar oocyte (another focus) (PNO)X400, Masson's trichrome

Fig.e. Late perinucleolar oocyte (PNO)with granular cytoplasm X400,Masson's trichrome

Fig.f. Late perinucleolar oocyte (another focus) (PNO)X400, Masson's trichrome

Fig. g. Early primary oocyte (PO)X250,Masson's trichrome

Fig.h. Late primary oocyte (PO)X250,Masson's trichrome

Plate 2: Puntius vittatus: Developmental morphology of oocytes CY-cytoplasm NU-nucleolus N-nucleus CM-chromatin FC-follicle.

Stage IV: Cortical alveolar oocyte (CAO): Cell diameter 81-130µm. The nucleus was large, oval in shape. Bunches of cortical vacuoles or alveoli of about 5µm in diameter appeared and became randomly dispersed at the peripheral region of the ooplasm (Plate3a).

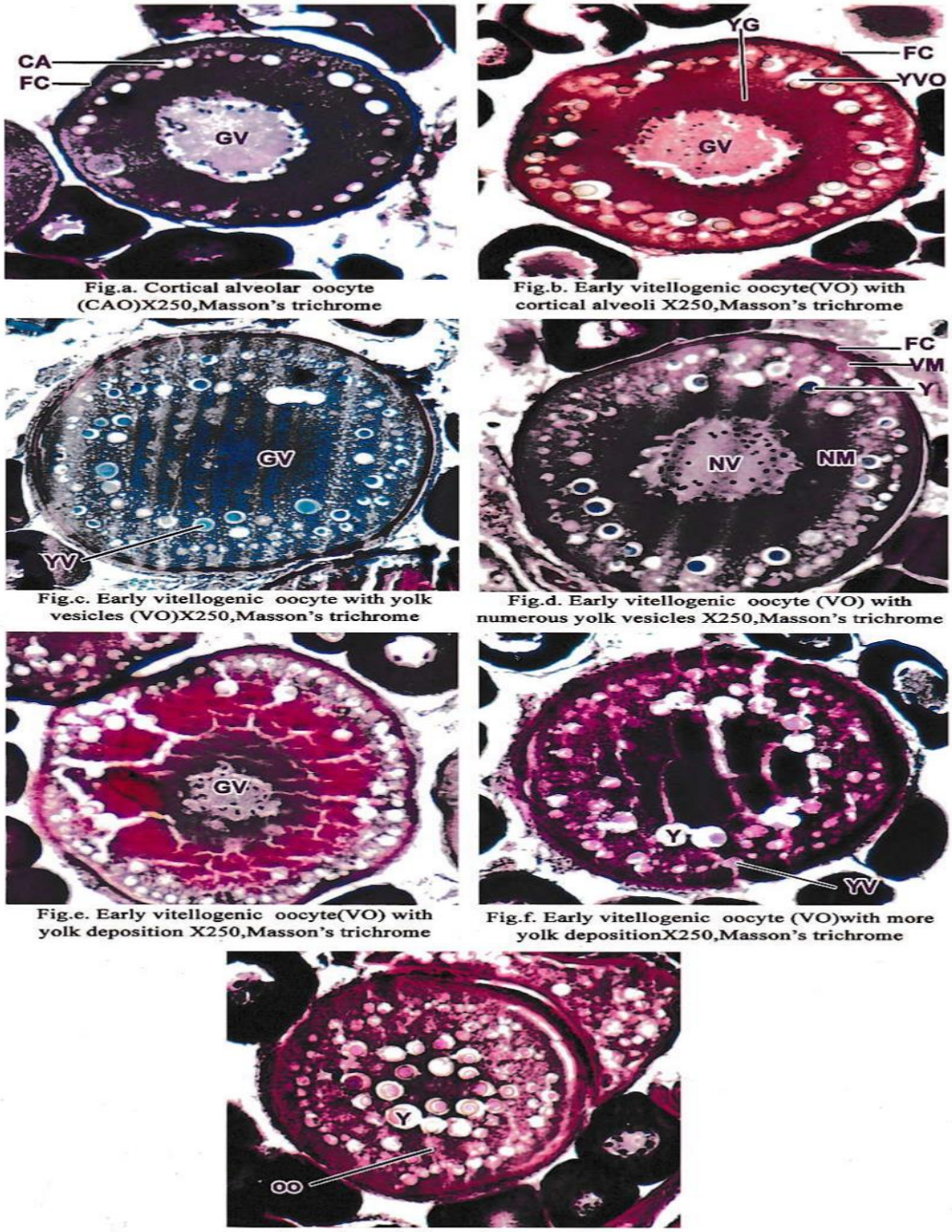

Fig.a. Cortical alveolar oocyte (CAO)X250,Masson's trichrome

Fig.b. Early vitellogenic oocyte(VO) with cortical alveoli X250,Masson's trichrome

Fig.c. Early vitellogenic oocyte with yolk vesicles (VO)X250,Masson's trichrome

Fig.d. Early vitellogenic oocyte (VO) with numerous yolk vesicles X250,Masson's trichrome

Fig.e. Early vitellogenic oocyte(VO) with yolk deposition X250,Masson's trichrome

Fig.f. Early vitellogenic oocyte (VO)with more yolk depositionX250,Masson's trichrome

Fig. g. Late vitellogenic oocyte (VO) X250,Masson's trichrome

Plate 3: Puntius vittatus Developmental morphology of oocytes CA-cortical alveoli GV-germinal vesicle YG-yolk granule YVO-yolk vacuole VM-vitelline membrane NU-nucleus Y-yolk OO-ooplasm.

Stage V: Early vitellogenic oocyte (EVO): Oocyte diameter 131-315µm. The oocytes were in early stages of yolk deposition and were characterized by finely granules perinuclear zone. Nucleus with irregular periphery and numerous large acidophilic yolk globules appeared in peripheral ooplasm (Plate 3b-f).

Stage VI: Late vitellogenic oocyte (LVO) Oocyte diameter 297-342µm: The cytoplasm was packed with acidophilic yolk globules. The oocytes became larger because of accumulation of yolk globules (Plate3g).

Stage VII: Germinal vesicle migration stage (GVM): Ovum diameter 310-362µm. The germinal vesicle was progressively displaced towards the animal pole. The germinal vesicle contained many nucleoli, stained intensively with haematoxylin. Yolk globules and lipid droplets underwent coalescence, which resulted in the formation of a continuous mass of yolk in the egg (Plate4a-c).

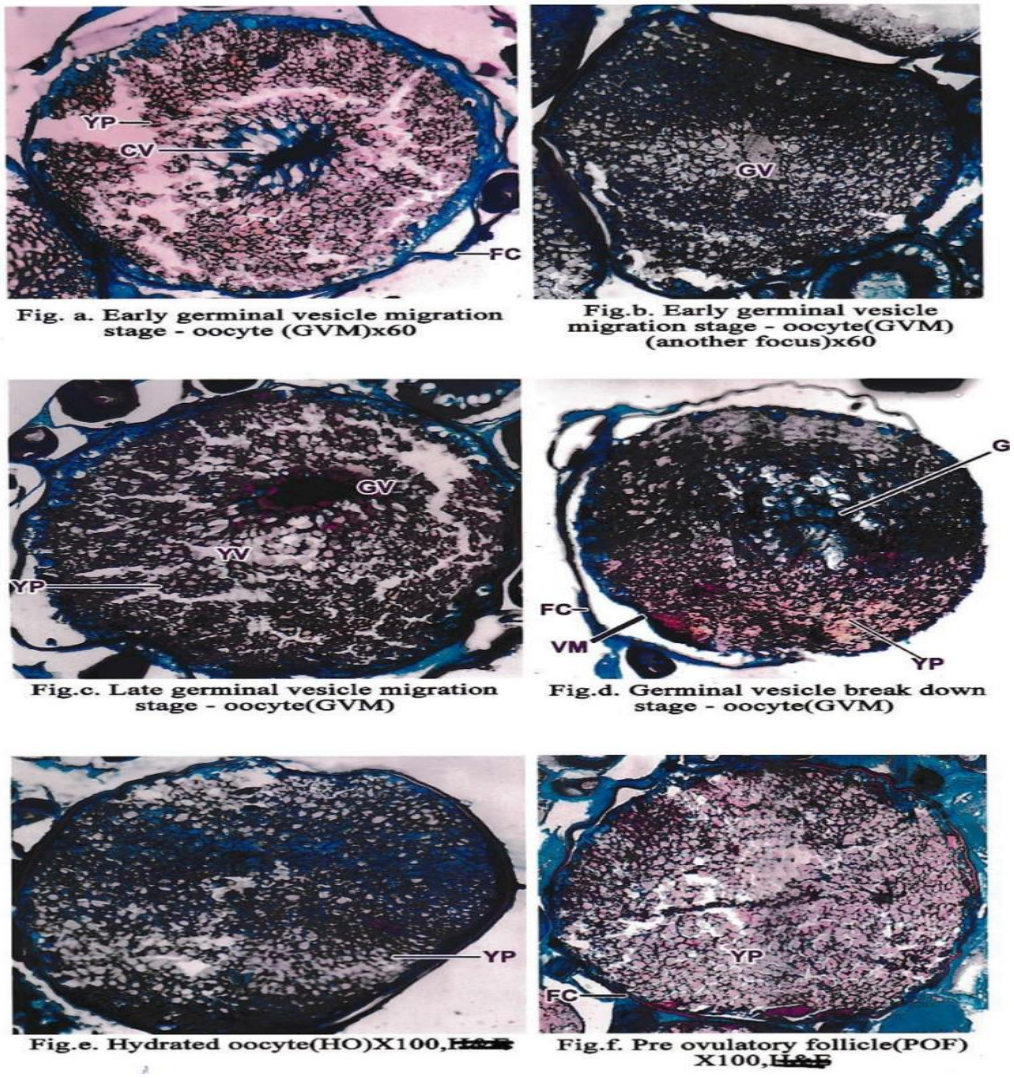

Fig. a. Early germinal vesicle migration stage - oocyte (GVM)x60

Fig.b. Early germinal vesicle migration stage - oocyte(GVM) (another focus)x60

Fig.c. Late germinal vesicle migration stage - oocyte(GVM)

Fig.d. Germinal vesicle break down stage - oocyte(GVM)

Fig.e. Hydrated oocyte(HO)X100,

Fig.f. Pre ovulatory follicle(POF) X100,

Plate 4: Puntius vittatus Developmental morphology of oocytes. GV-germinal vesicle YV-yolk vesicle YP-yolk platelet VM-vitelline membrane.

Stage VIII: Germinal vesicle break down (GVBD) stage: Oocyte diameter 345-390µm. After the migration of germinal vesicle to the periphery the nuclear enveloped became disintegrated (Plate 4d).

Stage IX: Atretic (AO) stage: Oocyte diameter 395-450µm. The unlaid mature oocytes became disorganized, highly vocuolated, collapsed and shrunken inward during the post-spawning period(Plate4e and f). This stage was characterized by the presence of numerous phagocytic granulosa cells (vitellophages) amidst the atretic follicles. The atretic ova were found at the beginning of December and at the start of February.

The period of oogenesis from early November to early January is characterized by an enormous increase in GSI due to strong vitellogenic activity in the ovaries. The resting period from February to May, comprised the stages of atresia and perivitellogenesis. During this period GSI was very low. Atresia was mainly encountered in December and February. Numerous previtellogenic follicles were present during September to October in the ovaries.

Annual testicular cycle

Testes in *P.vittatus* were found in the postero - dorsal part of the abdominal cavity, dorsal to viscera, but ventral to kidneys along the length of the body cavity. Immature testes were two in number, dull white, thin and elongate bodies from the region of liver to rectum. At maturity the testes were long tubular flat ribbon-like structures with a maximum length of 1.6cm and weight of 0.21gram. Spermducts (vasdeferens) opened separately close to the cloacal aperture, which was an elongate, oval shaped slit. The gonadosomatic index increased with progressive development of the testes until became ripe, and the index then declined sharply in spent fish. The testes of *P.vittatus* exhibited marked seasonal changes and the annual testicular cycle was divided into four stages. The morphological features of each stage are presented in Table 2.

Table 2: *Puntius vittatus* Gross anatomical features of testicular maturity stages.

Maturity stage	GSI		Testis colour	Macroscopic features
	Range	Mean		
I Immature	0.95-1.55	1.25	Dull white / pale / flesh-coloured	Very thin, slender and thread-like. About half the length of the visceral cavity
II maturing	2.18-2.85	2.25	Milky white	Uniformly thickened and enlarged, about three-fourth of the visceral cavity.
III Gravid	1.55-2.38	3.0	Milky white	Long, bigger, wide and flat, ribbon-like. Extended full length of the visceral cavity.
IV Spent	0.65-1.25	0.95	Coloured	Shrunken, uniformly thin and tubular

Stage1- Immature: The testes were thin, slender, thread like, translucent and pale or flesh coloured. They extended to about half the length of visceral cavity. The resting phase testes were found from February to March and July – September.

Stage II- Maturing: The testes were white in colour and showed gradual increase in their volume and weight, and became opaque. They extended to about three fourth the length of visceral cavity. It was found during late April and October. During the months, the testes showed a great increase in its size and weight.

Stage III- Ripe: The testes were white in colour, long and flat, ribbon like and narrow behind. They were found to extend the entire length of the visceral cavity. This state was characterized by the onset of spermiation (milt release) on applying slight pressure on the abdomen. Such a stage was found during late May, June, November and January.

Stage IV- Spent: Testes became uniformly thin, slender, flesh coloured shrunken and tubular. It was found in December, February and in July. The size and weight of the testes became reduced considerably.

Spermatogenic cycle

The spermatogenic changes in the testes of *P.vittatus* can be divided into the following five stages on the basis of histomorphological characters. The cytomorphological features of spermatogenic stages are presented in Table3.

Table 3: *P. vittatus*: Cytomorphological features of spermatogenic stages.

Cytological stage	Developmental stage	Developmental features		
		Testicular tubule	Cell differentiation	Interstitial cells
I	Spermatogonia	Small, thick-walled	Multiplication of spermatogonia along periphery; few spermatocytes found.	Few inert cells
II	Spermatocyte	Slightly increased in diameter, thin-walled; tubules are solid	Occurrence of spermatocytes in clusters; a few spermatids.	Proliferation of cells
III	Spematid	Greatly enlarged, wall becomes very thin	Germ cells in various stages of maturation; Numerous spermatids; a few spermatozoa; least number of spermatogonia and spermatocytes	Cells show moderate activity
IV	Spermatozoa	Highly distended	More spermatozoa and seminal fluid; a few spermatids found	Cells are more prominent
V	Post-spermiation	Thick-walled and empty	Sperm resorption; new wave of spermatogonia; spermatocytes absent.	Highly developed and very active.

Stage I. Spermatogonia: The testicular tubules were small, thick walled and contained a large number of primary spermatogonia along the periphery (Plate 5a and b). A few growing spermatocytes were also found.

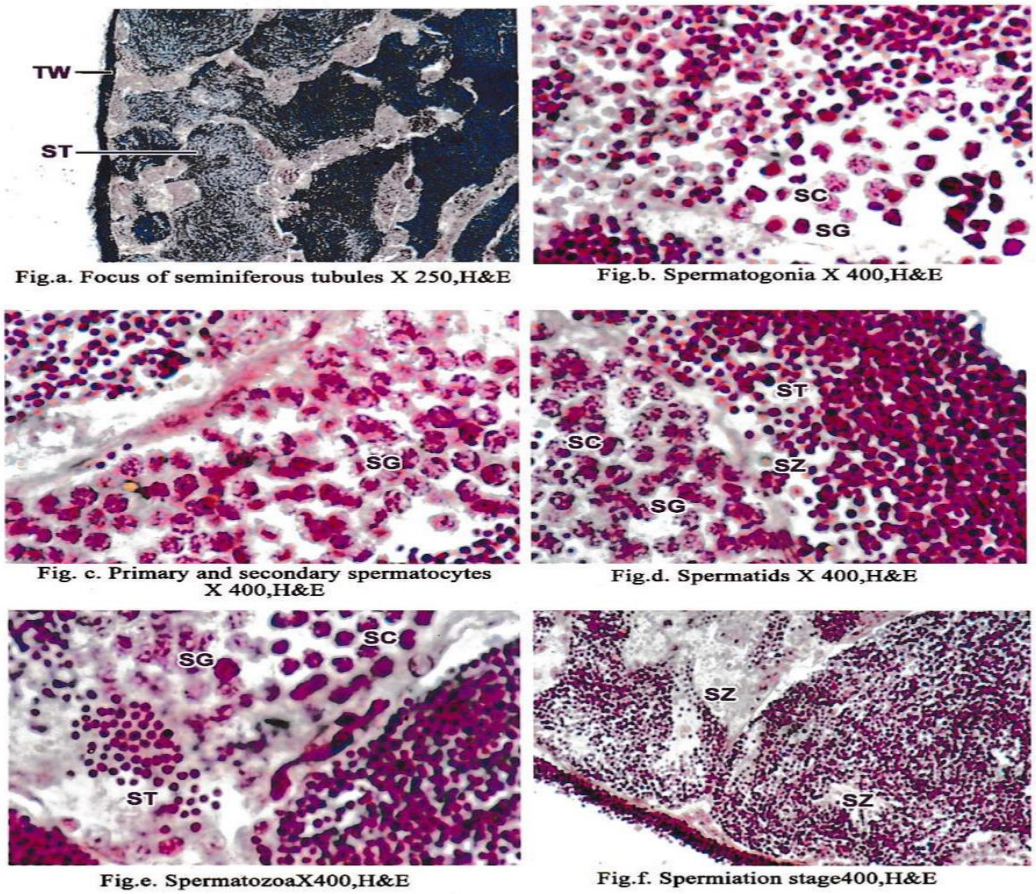

Fig.a. Focus of seminiferous tubules X 250,H&E
Fig.b. Spermatogonia X 400,H&E
Fig. c. Primary and secondary spermatocytes X 400,H&E
Fig.d. Spermatids X 400,H&E
Fig.e. SpermatozoaX400,H&E
Fig.f. Spermiation stage400,H&E

Plate 5: *Puntius vittatus:* Developmental morphology of spermatozoa ST- spermatid SC- spermatocyte SG- spermatogonia SZ –spermatozoan.

Stage II. Spermatocyte: The testicular tubules became increased slightly in diameter. Tubular wall became thin and cysts contained germ cells in varying stages of maturity. The spermatocytes were found in clusters in the tubules, with a few spermatids amidst them (Plate 5c).

Stage III. Spermatid: The tubules were greatly enlarged and their wall becomes thin. The tubules were solid and filled with cysts of germ cells at various stages of active spermatogenesis. Spermatids were found in plenty, with a few spermatozoa in the middle region. Spermatogonial population decreased, and some spermatocytes were also found. The interstitial or Leydig cells showed a moderate activity (Plate 5d).

Stage IV. Spermatozoa: The spermiation-stage testes were characterized by well-formed sperm ducts filled with spermatozoa and seminal fluid. Advanced stages of sperm maturation, spermotocytes and spermatids formed a lining to the ducts (Plate 5e and f). The interstitial cells were slightly more prominent.

Stage V. Post Spermiation: Spent fish exhibited testes of post-spermiation stages showing evidence of sperm resorption. Spermatogonia and spermatocytes were seen abundantly. The presence of a number of empty tubules was an indication that spermiation had already occurred. The interstitial cells were developed to the maximum size and were presumably very active. The cells and nuclei were enlarged and the nucleoli were very prominent. Spermatocytes were almost absent indicating cessation of spermatogenesis. A few spermatids or spermatozoa were also found.

DISCUSSION

Ovarian cycle

The general pattern of ovarian development in *P.vittatus* conformed to that of most teleosts (Ain *et al*.,(2021), Arevalo *et al*.,(2023), Efizon *et al*., (2021), Hasan *et al*.,(2020), and Sidik *et al*.,(2020). The development of the gonads in *P.vittatus* was correlated with general body growth and the maximum gonadosomatic index (GSI) was observed at gravid stage. GSI was found to be directly proportional to the number of mature eggs and inversely proportional to the oocyte atresia, (Ahirro, 2002). GSI values correlated closely with the morphologic changes of gonads as in other *Puntitus* species such as *Puntius filamentosus* (Victor *et al*.,(2004) etc.

The annual ovarian cycle of *P.vittatus* showed a rapid increase in ovary weight, with a ten fold change in the mean GSI from the gravid stage (October-maximum) to the period of spawning (November-January) and also during early June, suggestive of the individuals had a

very short spawning period as reported in *Puntius filamentosus* (Victor *et al.*,2004).

Testicular cycle

The histologic organization of male reproductive organs of *P.vittatus* were same as that of other teleosts as observed in *Puntius filamentosus* (Victor *et al.*, (2004) and as in other teleost, (Ain *et al*.,(2021), Arevalo *et al*.,(2023), Efizon *et al*., (2021), Hasan *et al*.,(2020), and Sidik *et al*.,(2020). The testes were paired elongated structures and showed remarkable seasonal morphological and morphometric changes as in *Puntius filamentosus* (Victor *et al.*, (2004). The testes of *P.vittatus* also exhibited continuous spermatogenic cycle and pass through several successive stages of immature, maturing, gravid and spent during the annual cycle as in *Puntius sarana*, (Ain *et al*.,(2021), Arevalo *et al*.,(2023), Efizon *et al*., (2021), Hasan *et al*.,(2020), and Sidik *et al*.,(2020), in common snook, teleost testis produced and stored sperm during breeding season *Puntius filamentosus* (Victor *et al* (2004). The spermatogenic activity was started in late September, progressed through October and spermiation took place in November and early January, and thereafter spermatogenesis slowed down considerably and almost ceased by the late January. Again spermatogenesis started in April and ended in June. In general the testes of *P.vittatus* showed an intensive phase of spermatogenic activity followed by a period of spermatogenic rest. However in some freshwater fishes spermatogenesis began as soon as the spermiation was terminated, and thence continued throughout the year (Ardelia *et al.*, (2023). Most of the Indian freshwater teleosts attained maturity and bred during monsoon season Ain *et al*.,(2021), Arevalo *et al*.,(2023), Efizon *et al*., (2021), Hasan *et al*.,(2020), and Sidik *et al*.,(2020). In *P.vittatus* also spawning occurred during the monsoon period. In the present study *P.vittatus* showed a gradual increase both in length and weight of the testes from, reproductive stages of immature to gravid and thence declined sharply during the spent stage. During early testicular maturation, there was an increase in both testis height and width this occurred because lobules elongated, and spermatocysts occupied more space than primary spermatogonia (Tamsil and Hasnidar, *et al.*, 2019; 2024).

Both testicular and ovarian germinal epithelia were composed of a somatic and germcell components (Sukarman, 2022), resting on a basement membrane The piscine gonadal germinal epithelium in both testes and ovaries fulfilled all the criteria of an epithelium, (Arachi, 2020; 2023; Arachi and Sumaiya, 2025). In fish testes, two types of germinal epithelia were recognized, continuous and discontinuous (FAO, 2022). Gonad classification based on changes in germinal epithelium, described previously by Ain *et al.*, (2021), may indeed be applicable to a wide range of perciform fishes. Arachi and Sumaiya, (2025), recently used different maturation classes (early maturation, mid maturation, late maturation regression and regressed classes) to describe the reproduction of male spotted seatrout, *Cynoscion rebulosus*. During early maturation, the germinal epithelium was continuous from testicular ducts to the periphery of the testes. In mid maturation, the germinal epithelium near the ducts became discontinuous, but it remained continuous distally. In late maturation a discontinuous germinal epithelium extended all along the lobules to the testicular periphery. The regression class was characterized by a discontinuous epithelium. In regressed testes spermatogonia existed only in a continuous (as discontinuous germinal epithelium). The presence or absence of sperm was not an accurate indicator of reproductive classes, (Pratiwi *et al.*, 2021).

CONCLUSION

Thus investigations on the maturity and spawning of *P.vittatus* were carried out by examining the functional microscopic morphology of gonads, gonadosomatic index of maturity stages and ova morphometry. It is concluded that the species had a short distinct major breeding season falling during the month of November and January and also a brief minor breeding season during June. The reproductive activity of *P.vittatus* coincided with the north-east and south-west monsoon rains respectively. So *P.vittatus* is considered to be a biannual monsoon breeder, having two breeding seasons in a year. The occurrence of the highest diameter range of mature ova in ovaries in each breeding season indicated that there was synchronous and bimodal development of oocytes in *P.vittatus*. The testicular cycle showed that the maturation of males coincided with the maturation of females during the spawning season itself to enable successful fertilization and maintenance of the race. The present study on the reproductive cycle provide knowledge about reproductive patterns, process and capacity of *P.vittatus.*

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