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Original Article

Evaluation of the reproductive cycle and gonadal development in the climbing perch, *Anabas testudineus* (Bloch, 1792) in captivity

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Abstract
The climbing perch *Anabas testudineus*, the most demanded indigenous fish species of India, were used for the evaluation of reproductive strategy and gonadal development at first maturation under the controlled laboratory condition. In both sexes, four distinct gonadal developmental stages namely preparatory, pre-spawning, spawning, and post-spawning were identified based on the gonadosomatic index (GSI), hepatosomatic index (HSI), gonadal morphology, and the levels of serum sex hormones. The spawning stage was identified from July and August and the fecundity ranged from 6500–11000 eggs. The GSI exhibited a significant increase from preparatory to pre-spawning and spawning stages, and maintained high until August. The HSI was maintained the same during preparatory, pre-spawning, and spawning stages while increased in post-spawning stage in both sexes. Serum estrogen and testosterone levels increased significantly during pre-spawning, and spawning stages in both sexes. Fecundity showed a positive correlation with the length, weight, and ovary weight. Histological sections showed that male *A. testudineus* were group-synchronous spawners. Both sexes showed discrete reproductive cycles characterized by distinct changes in gonadal size, sex hormones and gonadal indices. This study will bridge the gap of insufficient knowledge to support the best practices suitable for the *A. testudineus* culture and survival under captivity.

Keywords: *Anabas testudineus*; gonadosomatic index; hepatosomatic index; histology; serum sex hormones

1 | INTRODUCTION

Most of the teleost fishes are seasonal breeders adapting themselves strategically to seasonal changes for successful reproduction while a few breeds throughout the year are influenced by several environmental cues (Juntti and Fernald 2016). The variations in the environmental cues are sensed by the fish and stimulate the hypothalamo-pituitary-gonadal axis for the regulation of gonadal development, maturation, and spawning (Pham *et al.* 2012). Gonadosomatic index (GSI) is one of the biological indices widely used as a good indicator to determine the different stages of gonadal maturation (Hismayasari *et al.* 2015). On account of the gonadal development, the size and the weight of gonads increases, consequently leading to an increase in GSI during reproduction (Taranger *et al.* 2010). The liver, the storehouse of nutrients such as lipids and glycogen, supplies energy for gonadal development to maintain gametogenesis and vitellogenesis under the influence of sex steroid hormones (Nagahama 1994; Nagahama and Yamashita 2008). Thus hepatosomatic index (HSI) is also assessed during the reproductive period of fish as an indicator the reproductive activity (Chellappa *et*

al. 1995). The reproductive strategy concerning fecundity is also an important factor in fish stock management where it determines the maximum reproductive potential of a stock, and its survival from egg to the first spawning stage (Zworykin 2012). Gonadal steroid hormones play a tremendous role in sexual differentiation and reproduction where it directly influences the gonadal development, maturation, and sexual behaviour in fish (Nagahama and Yamashita 2008; Yaron and Levavi-Sivan 2011). Besides, histology of gonads offers a powerful tool and good indicator to study the reproductive health of fish and can be used for the identification of sex, development, differentiation, and maturation stages thereby assist other reproductive indices at the cellular level (Blazer 2002).

The climbing perch, *Anabas testudineus* (Teleostei: Anabantidae) is an economically important, obligatory air-breathing teleost fish native to south and Southeast Asia (Galib *et al.* 2010; Samad *et al.* 2010; Ara and Nabi 2018; Ndobe *et al.* 2020). International Union for Conservation of Nature and Natural Resources (IUCN) has categorized this species as Least Concern (IUCN 2019). This species inhabits in a wide range of water bodies of fresh and brackish waters and can withstand adverse ecological condition with the help of an accessory air-breathing organ (Froese and Pauly 2014). The natural food for the fry of this fish includes phytoplankton and zooplankton, while the adults are carnivorous that feed on algae, worms, crustaceans, molluscs, macrophytes, insects, and organic debris (Bhattacharjee and Chandra 2016). In India as well as various part of tropical Asia, *A. testudineus* is one of the most preferred food fish species in inland rural communities. However, the increased rate of habitat destruction and extreme agricultural practices threatens its survivability in the natural habitats. Hence, the availability of artificially propagated seeds of *A. testudineus* is indispensable for the artificial cultivation of these important food species. Besides, the understanding of gonadal development, breeding nature, seasonality, and development of mass seed culture technology of *A. testudineus* under captive conditions could augment the local economy and employment opportunities to the rural population (Rajbongshi *et al.* 2020).

In fisheries research, reproductive biology is an important aspect since it represents stock assessment and management simultaneously, based on sound biological information (Jakobsen *et al.* 2009). In the present study, the reproductive cycle and gonadal development of *A. testudineus* was assessed at first maturation in captivity by examining various indices of seasonal reproduction like GSI, HSI, serum hormones, and histological examination of the gonads. Some reports have stated that the breeding of this species in captivity is practiced at a low level in India, Vietnam, and Thailand (Bhattacharyya and Homechaudhuri 2009; Morioka *et al.* 2009). However, the data related to the reproductive biology of *A. testudineus* are relatively insufficient to support the best practices suitable for the species culture and survival, and the present study provides the fundamental data necessary for aquaculture and conservation management practitioners to strengthen the availability of information to avoid overexploitation of the stocks and failure of management.

2 | METHODOLOGY

2.1 Collection and housing of the animal

A total of 500 individuals of *A. testudineus* (7 ± 1 g and 7 ± 1.5 cm) were collected from Pulimugham Aquafarms and hatcheries, near the backwaters of river Pampa, Alappuzha district of Kerala, India. Fish were transported to the laboratory and acclimatized in dechlorinated, aerated water under semi-static conditions in two tanks (250 L; 0.5 m × 1 m × 0.5 m; stocking density was 1 individual per litre) at 12 h: 12 h photoperiod for two weeks. Fish were fed twice daily at 10 am and 5 pm with pelleted feed (at the rate of 3% of the body weight) containing 28% crude protein, 3% crude fat, 4% crude fibre and 10% moisture. The water quality parameters such as temperature (28 ± 2°C), salinity (less than 0.1%), pH (7.4 – 7.6), and dissolved oxygen (DO, 12.64 ± 1.3 mg L^{-1}) were monitored following standard method (APHA 1998). As specified in OECD guideline 203 (OECD 2019), other physico-chemical parameters including alkalinity and hardness were measured by analysing the concentrations of particulate matter, unionised ammonia, nitrate, chlorine, total organic carbon and other metallic impurities, and retained throughout the study period (from February 2017 to January 2018) to ensure the best fit for the survival and growth of the fish.

2.2 Preparation of samples

Four stages of gonadal development namely preparatory, pre-spawning, spawning, and post-spawning were classified following Chakraborti and Bhattacharya (1984) and Pal *et al.* (2018). The length and weight of the fish measured in each month of the experiment were represented as the growth performance during different stages of reproduction. Total length was measured using a standard fish measurement board to get the nearest millimeter, and the total weight was measured using digital weighing apparatus (to the nearest 0.1 g).

Gonads and liver, dissected out at the end of each month, were weighed, the relative weight of gonads (testes and ovary) and GSI, the relative weight of liver tissues for both sexes and HSI were evaluated using the following formula; GSI or HSI = (Tissue weight (g) / Fish weight (g)) × 100 and expressed in percentage (King 1995; Sulistyo *et al.* 2000).

The fecundity was estimated for 30 – 35 invdividuals by the gravimetric method using the ovaries from pre-spawning and spawning stages. In brief, after 48 h of

preservation in modified Gilson's fluid, the eggs were completely liberated, washed thoroughly, and spread on the blotting paper to air dry, divived into subsamples and counted. Fecundity was calculated usuing the following formula, $F = nG/g$; where F = fecundity, n = number of eggs in the subsample, G = total weight of the ovaries, g = weight of the subsample in the same units (Grimes and Huntsman 1980).

2.3 Histological analysis

Gonadal tissues were dissected, rinsed in physiological saline to remove the tissue debris and clotted blood, and then fixed in 10% buffered formalin for 24 – 48 h. The tissues were dehydrated in ascending series of alcohol and cleared in xylene until they became translucent. The tissues were embedded in molten paraffin wax for an hour for the complete impregnation to make the tissue blocks. Sections were made using rotary microtome at 4 to 6 µm thickness, which was double stained with haematoxylin and eosin, and finally mounted in DPX mountant (Roberts and Smail 2001). Triplicate slides of gonadal tissues were examined under the microscope and photographed. Briefly, each slide of ovarian and testicular sections was examined under a light microscope at 40 or 400X magnifications depending on the stages of gonadal development. The morphometric analysis of oocyte stages was performed using a canon shot camera fitted to the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope, and the diameter of the oocyte was presented in µm. Similarly, the spermatogenic cells were analysed and photographed using Research Microscope, and the measurement was made using ImageJ software (version 1.52a) (National Institutes of Health, USA), and the values are expressed in µm. However, the stages of oocyte and spermatogenic cell composition were shown in percentage (Bernal *et al.* 2015).

2.4 Serum hormone analysis

Blood collected from the caudal vein of male and female fish were transferred into non-heparinised tubes and centrifuged at 1700 g for 10 min at 4°C, and the serum collected was stored at –80°C for the analysis of testosterone and estradiol hormones using the commercial ELISA kits purchased from Cusabio Biotech Co., Ltd. (China) according to the prescribed protocols.

2.5 Data analysis

The statistical package SPSS V-21.0 was used as the statistical tool to measure various morphometric and hormonal parameters. Levene's test was conducted to ensure the equality and homogeneity of variance. One-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range as post hoc test was used to set the significance within and between variations in GSI, HSI, serum hormones, and difference in diameter of gametes at different stages of reproduction of *A. testudineus*. The data were presented as mean ± standard deviation (SD) in graphs setting significance at $p < 0.05$, denoted by different superscripts against each data point. Correlation between the fecundity and fish weight, length, and weight of ovary were performed using Pearson's correlation coefficient.

3 | RESULTS

3.1 Identification of gonadal stages

The present study was initiated in February, which showed no remarkable morphological distinction in the sex of the animal. Fish were randomly sampled from the stock, and the abdomen is carefully dissected for identifying the different developmental stages of gonads. Thus preparatory and pre-spawning stages were identified by dissecting the abdomen of the fish sampled during February to April, and May to June respectively. However, in the spawning stage (July to August), sexual dimorphism was distinct by the morphological characters as bulging of abdomen and modification of vent while post-spawning stage was more prominent during September to January characterised by regressed gonads. Also, milt and oocytes were easily extruded from the vent by applying gentle pressure on the abdomen during the spawning stage.

3.2 Growth performance

The average length and body weight of the fish, based on the gonadal maturity stage were shown in Figure 1. There was a significant ($p < 0.05$) and progressive increase in the body weight and length of the fish from the preparatory to post-spawning phases in both sexes.

3.3 Absolute weights of tissues

On the month-wise evaluation, the weight of liver tissues in female and male fish showed a remarkable increase from December to April while in the rest of the months no significant variations were noted (Table 1). The weight of liver tissues during the different stages of reproduction showed significant ($p < 0.05$) and progressive increases during the post-spawning and preparatory stages in both sexes (Table 2). However, the month-wise estimation of gonadal weights showed a notable increase in May to August, and similarly during pre-spawning and spawning stages of reproduction (Tables 1 and 2).

3.4 Relative weights of tissues

3.4.1 Gonadosomatic index (GSI)

The monthly evaluation of the GSI showed the peak values from May to August, which was the pre-spawning and spawning stage of female reproduction (Figure 2A and 2C). Similarly, in males, the GSI increased significantly ($p < 0.05$) during the May to August months, where the stages were identified as pre-spawning and spawning phases of reproduction (Figures 2B and 2C). There was a progressive

increase in the GSI values from February to April, and a sudden decline was observed from September to January months in both male and female fish (Figures 2A and 2B).

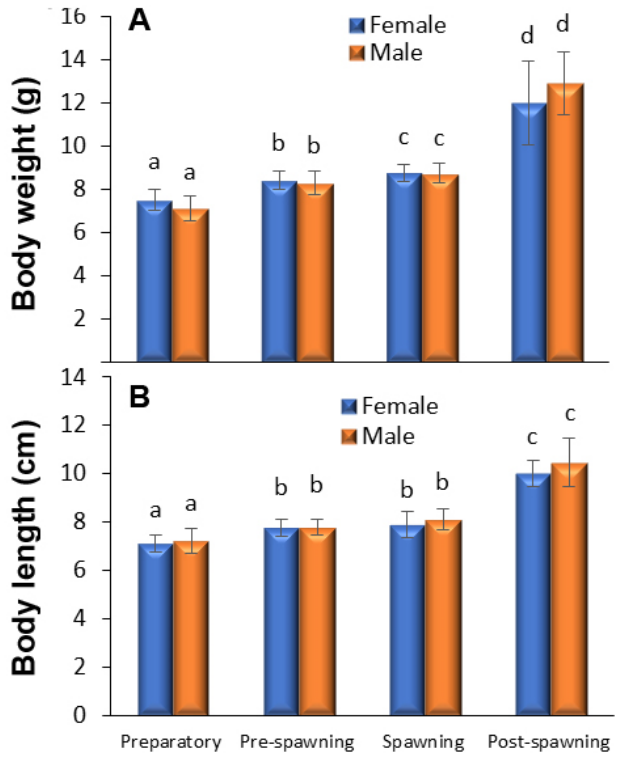

FIGURE 1 Body weight (A) and length (B) of the *Anabas testudineus* during different reproductive stages (Mean ± SD; n = 10 individuals group^{-1}; significant differences among means are indicated by different superscripts letters, $p < 0.05$).

TABLE 1 Monthly variation in the weight of tissues of male and female *Anabas testudineus* (mean ± SD; n = 10 individuals per group).

Month	Weight of liver (mg)		Ovary	Testis
	Female	Male	weight (mg)	weight (mg)
Feb	128.6±6.47	139.0±4.72	128.04±6.76	26.87±1.45
Mar	95.15±4.86	99.89±8.23	354.62±9.54	39.00±3.45
Apr	78.53±5.76	75.16±6.06	636.39±6.24	52.67±0.16
May	65.85±7.26	68.62±2.66	668.13±9.35	77.10±4.69
Jun	64.68±5.51	63.45±3.87	988.99±8.25	96.45±5.45
Jul	63.80±4.63	64.37±7.58	1051.2±9.10	113.0±5.49
Aug	60.38±4.22	62.03±7.27	1366.4±10.6	125.7±1.98
Sep	65.05±3.76	69.71±5.43	328.0±8.53	86.12±3.27
Oct	66.74±2.78	83.19±4.72	164.6±6.50	26.32±4.43
Nov	78.53±5.76	75.16±6.06	35.71±1.95	15.26±2.78
Dec	112.5±6.68	119.9±9.41	28.74±8.70	14.26±1.62
Jan	127.6±5.83	120.2±8.33	48.57±3.92	20.82±2.79

3.4.2 Hepatosomatic index (HSI)

The HSI of both male and female fish showed a negative correlation with GSI and the HSI begin to increase from December to April whereas no significant changes were observed in the remaining months (Figures 2A and 2B). The data when expressed based on reproductive stages showed a significant ($p < 0.05$) progressive increase in the percentage of HSI during post-spawning and preparatory stages than pre-spawning and spawning phases in both sexes (Figure 2D).

TABLE 2 Variations in the tissue weight (mean ± SD; $n = 10$ individuals group^{-1}) of *Anabas testudineus*. Significant ($p < 0.05$) differences among means are indicated by different superscript letters.

Tissue	Tissue weight (mg)			
	Pre-paratory	Pre-spawning	Spawning	Post-spawning
Liver - Female	118.61 ± 0.05^a	83.36 ± 0.74^b	73.36 ± 0.64^c	74.19 ± 0.15^d
Liver - Male	97.07 ± 0.05^a	87.50 ± 0.14^b	81.50 ± 0.23^c	83.12 ± 0.05^d
Ovary	438.4 ± 12.9^a	926.9 ± 11.8^b	1205 ± 13.9^c	86.53 ± 2.21^d
Testis	45.50 ± 0.94^a	90.39 ± 3.39^b	120.7 ± 3.17^c	22.05 ± 0.96^d

3.5 Fecundity

The relationship of fecundity to body weight (Figure 3A), ovary weight (Figure 3B), and fish length (Figure 3C) showed a very high degree of positive correlation. The number of eggs produced ranged between 6500 and 11000 in a female parent weighing 7.5 to 9 g with the regression equation as y = 1050.5x; R^2 = 0.8928 and r = 0.9528 (Figure 3A) possessing the ovary weight between 900 and 1700 mg that occur in the spawning period of reproductive phase showing the regression equation of y = 6.2803x; R^2 = 0.6557 and r = 0.9353 (Figure 3B), and the fish size of 7.5 and 9 cm range with the regression equation of y = 1093x; R^2 = 0.8848 and r = 0.9024 (Figure 3C).

3.6 Histomorphological analysis of ovary

Microscopic examination of the ovary revealed different developmental stages throughout the reproductive cycle. Both chromatin nucleolus and perinucleolus stages have occurred from February to April. Chromatin nucleolus consisted of a single, large nucleus, while perinucleolus contained a thin band of dense, compact, and basophilic cytoplasm surrounding the nucleus having an irregular nuclear membrane and 6 – 12 peripheral nucleoli (Figures 4A and 4B), represented the preparatory stage of ovary (Figure 5A). The chromatin nucleolus of 35 – 37 µm diameter contributed 41.3%, and the perinucleolus stage of 73 – 76 µm diameter occupied 35.4% of the preparatory stage ovary, and the remaining 23.3% were presented by vitellogenic, vitellogenic, mature, and atretic stages (Figures 7A and 7C). From May and June, in the pre-spawning stage of the ovary, the pre-vitellogenic oocytes (166 – 174 µm diameter) began to develop into vitellogenic follicles filled with yolk granules and cortical alveoli pos-

sessing a centrally located germinal vesicle (Figures 4C and 5B). The pre-spawning ovary consisted of 28.7% of the pre-vitellogenic and 35.3% of the vitellogenic stage oocytes while 36% of the ovary was formed with chromatin nucleolus, perinucleolus, mature and atretic oocytes (Figures 7A and 7C).

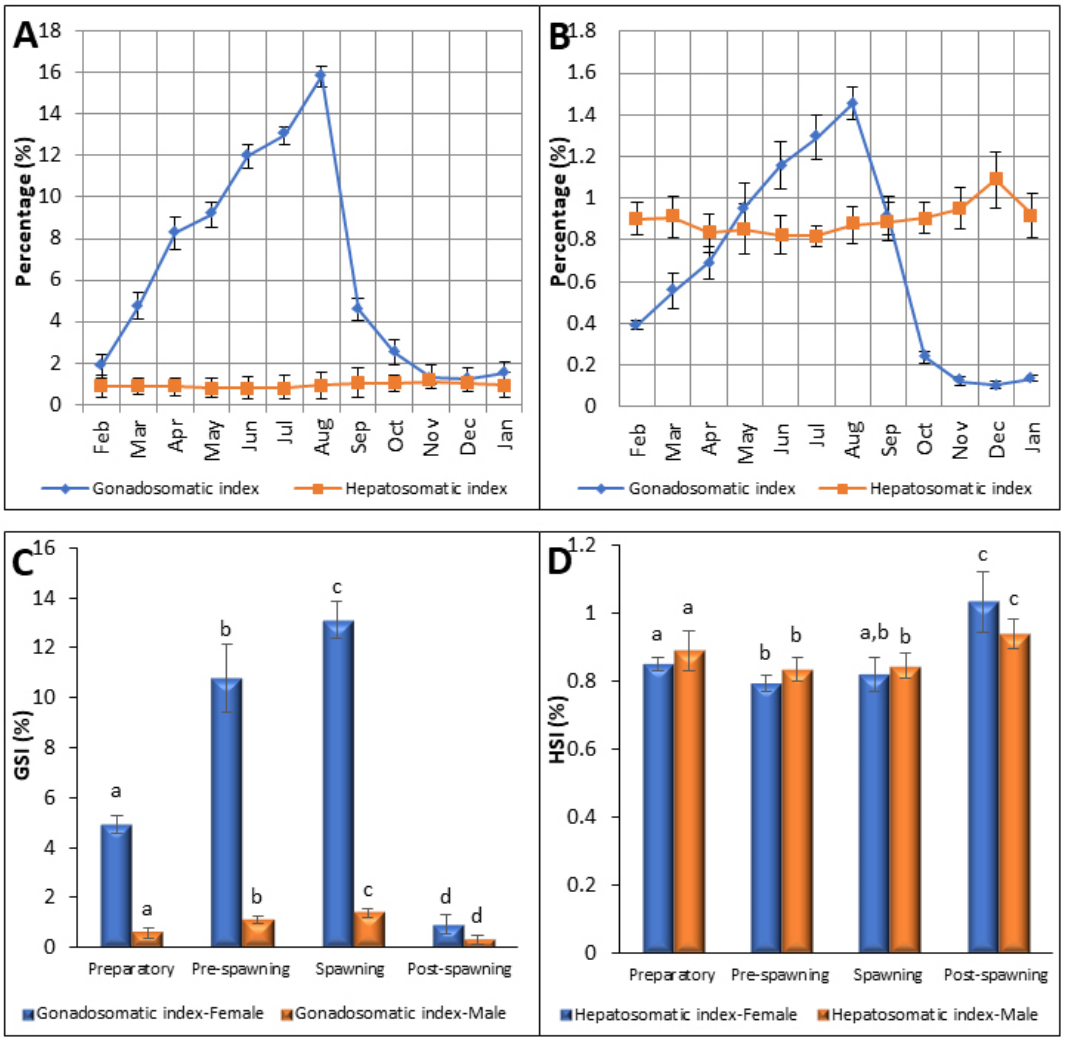

FIGURE 2 Monthly variations in gonadosomatic index (GSI) and hepatosomatic index (HSI) in the females (A) and males (B) and variations in GSI (C) and HSI (D) during different stages of reproduction of *Anabas testudineus* (mean ± SD; *n* = 10 individuals per group; significant differences among means are indicated by different letter superscripts).

From July to early September, the ovary occupied by the loosely arranged spherical matured eggs of pale yellow colour with centrally located large oil vacuole (Figures 4D and 4E), and were recognised as the spawning stage (Figure 5C). In the spawning stage, 35.9% of the vitellogenic (447 – 469 µm diameter) and 44.4% of the mature (599 – 625 µm diameter) oocytes have occupied the ovary followed by pre-vitellogenic, perinucleolus, atretic and chromatin nucleolus (Figures 7A and 7C). The post-spawning stage of the reproductive cycle occurred from the end of September till January that were identified by a mixture of atretic, spent as well as some pre-vitellogenic and immature oocytes (Figures 4F, 4G and 5D). In this stage, chromatin nucleolus (43.2%) predominated, followed by perinucleolus (29.3%) and atretic (20.7%) oocytes (Figure 7A).

3.7 Histomorphological analysis of testis

All spermatogenic stages of the testis were present throughout the annual reproductive cycle (Figure 6A). However, certain stages were more common in specific months; accordingly, the stages were identified as preparatory stages from February to April months having abundant spermatogonia, spermatocytes, spermatid and relatively fewer spermatozoa (Figure 6B). The pre-spawning stage occurred between May and June consisted of all stages of spermatogenic cells enclosed in large seminiferous tubules (Figure 6C). In July to early September, the spawning stage testis was formed of abundant spermatozoa that dilated the seminiferous tubule [meaning is not clear] (Figure 6D), and milt was easily extruded upon exerting slight pressure on the belly.

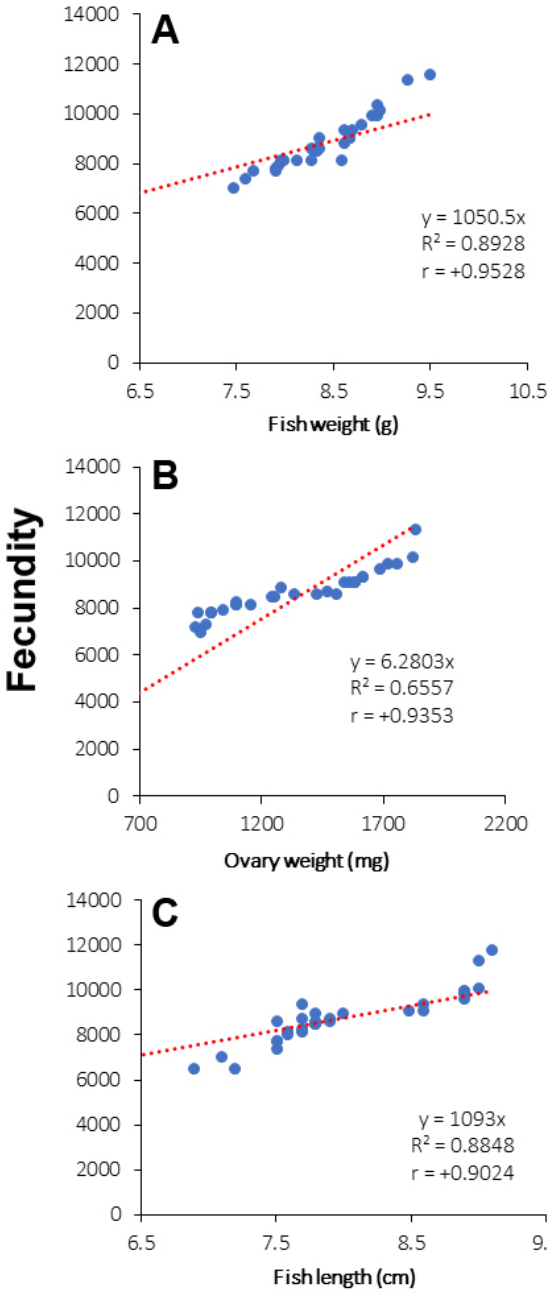

FIGURE 3 Correlation between the fecundity and fish weight (A), ovary weight (B) and fish length (C) in *Anabas testudineus*.

The post-spawning stage began in the late September and continued until January which was characterised by loosely packed spermatozoa while some part of the seminiferous tubule remains empty. This was also the time when other spermatogenic cells begin to appear (Figure 6E). The composition and size of different stages of spermatogenic cells were also estimated in male. It was found that the percentage of spermatozoa was 9.6 in the preparatory stage, and increased to 82.05% and 93.9% in pre-spawning and spawning stages respectively. However, it decreased to 41.1% in the post-spawning stage (Figure 7B). The mean sizes of spermatogenic cells were 1.56 ± 0.06 µm, 0.80 ± 0.04 µm, 0.61 ± 0.06 µm and 0.37 ± 0.09 µm for spermatogonia, spermatocyte, spermatid and spermatozoa respectively (Figure 7D).

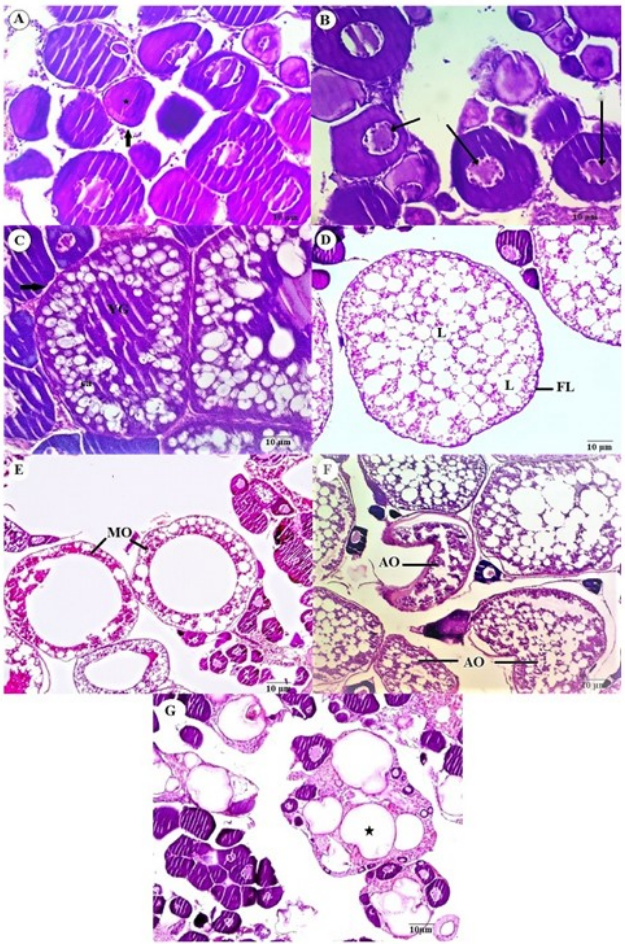

FIGURE 4 Photomicrographs of ovaries of *Anabas testudineus* during different developmental stages of gametes. A, chromatin nucleolus stage (arrow); B, perinucleolus stage (arrow); C, previtellogenic stage; follicular layer is represented by solid arrow; ca, cortical alveolus; YG, yolk granule; D, vitellogenic stage; FL, follicular layer; L, lipid droplet; E, mature stage; MO, mature oocyte; F, atretic stage; AO, atretic oocyte; G, spent stage; spent oocyte are represented by asterisks.

3.8 Serum sex hormones

In female fish, the level of estradiol in the blood serum showed a significant increase ($p < 0.05$) during pre-spawning and spawning stages of ovarian development along with the slight increase in the level of testosterone when compared with preparatory and post-spawning stages (Figure 7E). In male, a progressive and significant increase ($p < 0.05$) in the level of testosterone and estradiol from preparatory to pre-spawning stages were observed. However, it showed a significant reduction ($p < 0.05$) in the post-spawning stage (Figure 7F).

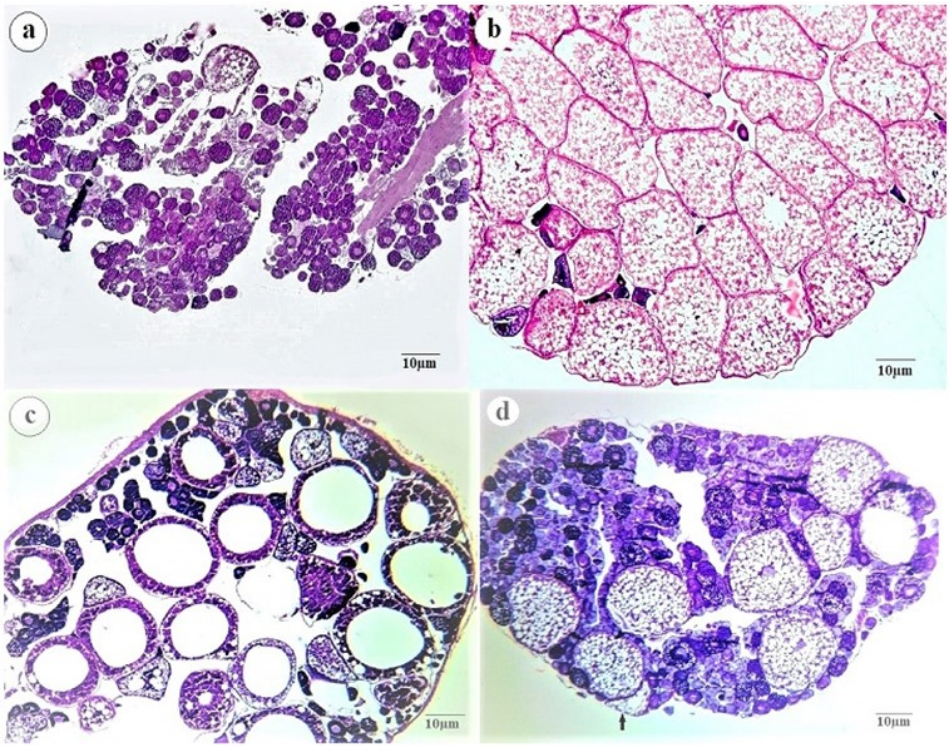

FIGURE 5 Photomicrographs of ovaries of *Anabas testudineus* showing different stages of reproduction. a, preparatory ovary; b, prespawning ovary; c, spawning ovary; d, postspawning ovary.

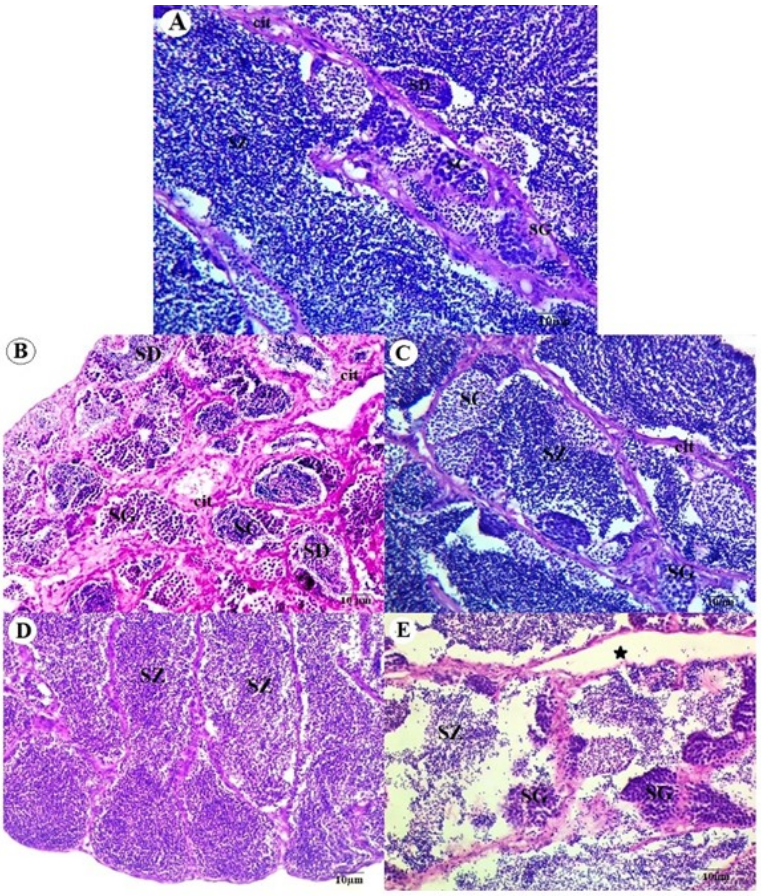

FIGURE 6 Photomicrographs of testis of *Anabas testudineus* showing different stages of reproduction. A, testis of all stages; B, preparatory stage; C, prespawning stage; D, spawning stage; E, postspawning stage. Spent testis with loosely packed spermatozoa inside the seminiferous tubule. SG, spermatogonia; SC, spermatocytes; SD, spermatids; SZ, spermatozoa; cit, connective interstitial tissue; Empty seminiferous tubule are represented by asterisks.

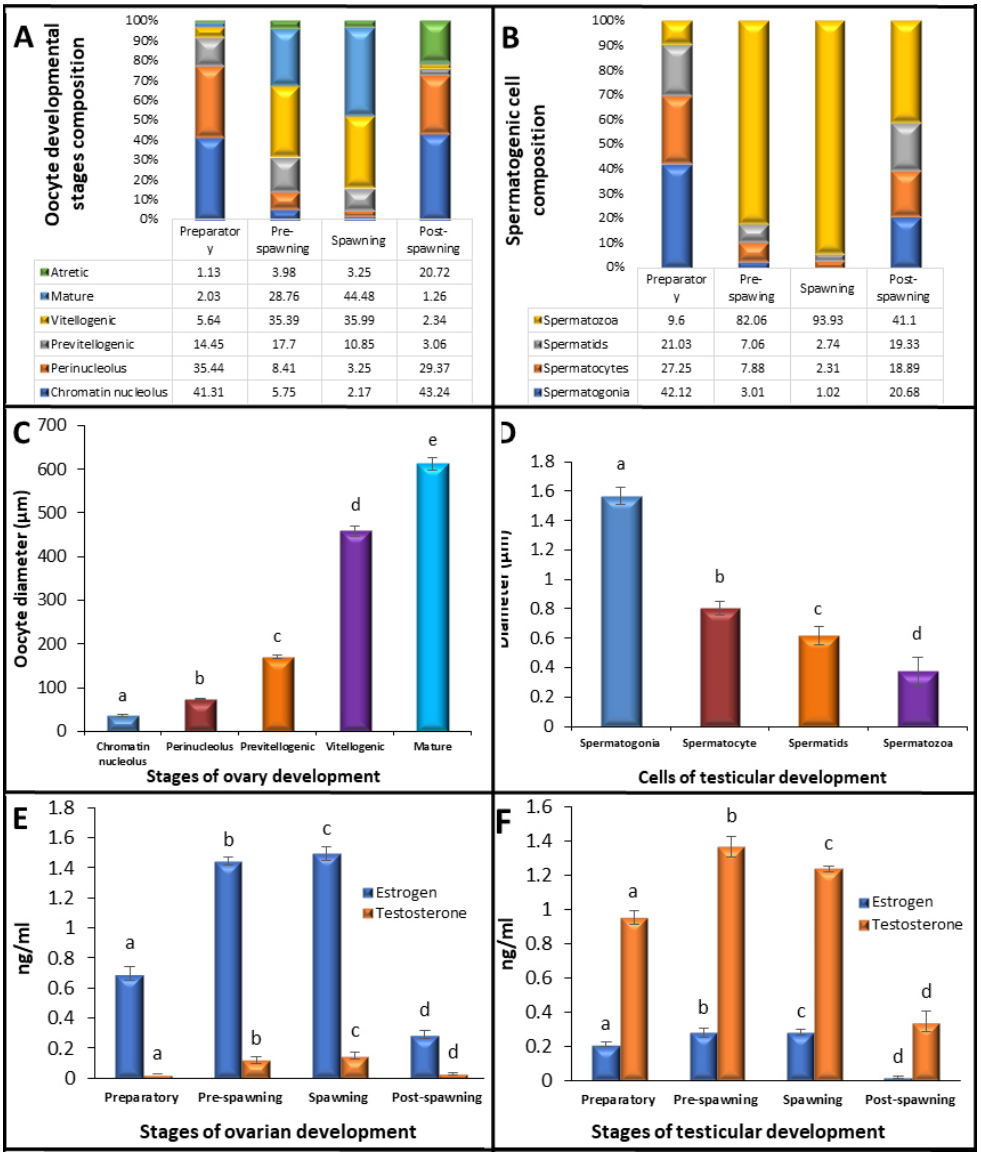

FIGURE 7 Percentage of oocyte stage composition (A), spermatogenic cell composition (B), oocytes diameter (C), diameter of spermatogenic cells (D), levels of sex hormones in females (E) and males (F) of climbing perch *Anabas testudineus*, during different reproductive stages (mean ± SD; n = 10 individuals per group; significant differences among means are indicated by different letter superscripts, $p < 0.05$)

4 | DISCUSSION

Sexual dimorphism in *A. testudineus* was remarkable only when it approaches the breeding season, and no differences were noticed during the non-breeding season in the present study. Thus, the body colour, belly structure, and vent characteristics were used as the main secondary sexual characters to distinguish the sex during the breeding season (Lagler *et al.* 1977). The appearance of the reddish-yellow colour on the ventral surface differentiated the mature fish, irrespective of its sex (Dehadrai *et al.* 1973). Bulginess in the belly of female fish than male was recorded during the breeding season (May – August) as it contained developing ovary with vitellogenic, matured, and ripe oocytes. Whereas, the belly of both sexes appeared the same outside the breeding season. Similar features of sexual dimorphism in *A. testudineus* have been observed by Behara *et al.* (2015) during the breeding season in the natural environment. During the preparatory phase of reproduction (February – April), there was a slight bulging in the belly of both sexes which made impossible to differentiate the sex. These observations are in agreement with the previous studies (e.g. Behera *et al.* 2015). The vent is an opening found between the anus and anal fin on the ventral side, often referred to as the genital papilla or ovipositor in females. During the breeding season, the vent of the female individual developed with red colour which was not the case for males. These remarkable morphological changes served as a basis for differentiation of fish sexes during the study; however, based on the stages of gonadal maturity such as preparatory, pre-spawning, spawning and post-spawning , the growth performance of the fish were estimated.

In this study, the growth performance of *A. testudineus* was evaluated in the laboratory by maintaining optimal water quality and physico-chemical conditions. The average weight and length of the fish for both sexes in-

creased gradually from preparatory to post-spawning period in contrast to an abrupt increase in the weight of the fish due to developing gonads, thus the present study evidenced only the progressive growth index. This could be due to the energy reallocation from the body growth to gametogenesis and other reproductive behaviours for maintaining normal reproduction (Araujo *et al.* 2019). Generally, several fish species are known to suppress their growth to maintain their reproductive capacity (Coward and Bromage 1999).

The gonadal and liver weight for both sexes were estimated monthly, and also during the different stages of reproduction. The month-wise evaluation showed a remarkable increase in the weight of liver tissues during December to April in female and male fishes. The weight of liver tissues showed a progressive increase during the preparatory and post-spawning stages in both sexes. The monthly mean values of liver tissues showed regular fluctuations with a remarkable decrease during May to September months, corresponding to the pre-spawning and spawning stages, and this could be due to the energy trade between the liver to the gonads. Thus liver plays a major role in gonad maturation as reflected by the change in the weight of the tissue (Larson 1973). The observation was further substantiated by the month-wise estimation of gonadal weight, which showed a prominent increase in the weight during May to August, parallel to the pre-spawning and spawning stages of reproduction. Hence it was clear that there occurred an inverse relationship between the weight of the liver and gonads, which in turn coincided with the reproductive activity of the fish.

The GSI or the relative weight of gonads is widely used as an indicator to determine the gonadal development in fish, particularly used to identify the period and season of spawning. Monthly estimation of the GSI showed the peak values from May to August, which was the pre-spawning and spawning stage of the female reproduction. The deposition of large amounts of lipids and proteins into the developing oocyte was responsible for the rise in the GSI during gonad maturation (Patino and Sullivan 2002). The highest peak in July and August indicated the spawning period of female fish, which was followed by a sudden decrease in GSI from September to February suggesting the post-spawning or spent stage of the female fish. The GSI of male fish also varied from minimum in the months of October-February, which seems to be the post-spawning stage and attained maximum during May-August during the spawning stage. Hence, the GSI decreased during the non-reproductive season and increased during reproductive seasons in both sexes. The observations from the present study also revealed that the female fish exhibited a high percentage of GSI than the males, due to the size of ovaries. Similar results have been reported after analysing the correlation among relative gonad weights in *Cyprinus carpio* and *Ctenopharyngodon idella* under a composite culture system with special reference to the pond fertilization (Mahboob and Sheri 2002). Another study on snow trout, *Schizothorax plagiostomus* also showed comparable fluctuations in GSI indicating the variation in gonad maturation during different reproductive phases (Jan and Ahmed 2016).

The HSI is associated with energy reserves and metabolic activity of liver tissues and is often used as a biomarker to indicate metabolism in fish. The HSI has estimated month-wise and different stages of gonadal development showed fluctuations in both sexes. The percentage of HSI increased from December to April at the maximum during the post-spawning and preparatory stages, while decreased during pre-spawning and spawning period in both sexes. The liver is the major site where the synthesis of vitellogenin, the major precursor protein of egg-yolk occurs mainly during the post-spawning and preparatory phase of reproduction, which can be correlated to the increase in the HSI percentage. The synthesised yolk proteins are then released from the liver into the circulatory blood for transportation, and get deposited as yolk in the developing oocytes. The deposition of yolk protein usually occurs during the pre-spawning and spawning period of the reproductive cycle, which is associated with an increase in GSI and a decrease in HSI. Thus the fluctuations in the HSI along with GSI could be considered a good indicator for the reproductive fitness of the species (Pandit and Gupta 2019). Similar fluctuations in HSI with the highest peak during post-spawning and preparatory and minimum range during pre-spawning and spawning phases have been reported in the catfish *Heteropneustes fossilis* (Singh and Srivastava 2015).

Fecundity is the physiological process used to measure the reproductive potential of the fish with the number of ripe eggs produced during the spawning season (Bromage *et al.* 1992). Fecundity is an important factor in fish stock management where it quantifies the reproductive capacity of individual fish that likely shows variations even within the stock. However, fecundity depends on several factors including body weight, age, length, nutritional status, environmental conditions, water quality, and food availability (Lawson 2011). In the present study, fecundity was correlated to body weight, ovary weight, and fish length, which showed a very high degree of positive correlation. The current study observed the fecundity of *A. testudineus* ranged between 6500 and 11000 for the individuals weighing 7.5 to 9 g. The coefficient of determination (R^2) obtained was 0.8928 with a high degree of positive correlation (r) at 0.9528 indicating that more than 89% of the variability of the fecundity was explained by the body mass. In another study, the fecundity of *A. testudineus* varied between 12355 and 41820 eggs for individuals of 30.24 – 66.62 g and 12.3 – 15.5 cm (Banu *et al.* 1985). Female climbing perch of the mean body mass

of 61.10 ± 17.32 g showed mean fecundity of 24120.5 ± 3328.24 (Amornsakun *et al.* 2005) while individuals of 32.2 – 50 g body weight had the fecundity from 5979 to 13565 (Ziauddin *et al.* 2016). In our observation, the fecundity showed a high degree of positive correlation. Similarly, a high degree of positive correlation (r = 0.8642) has been reported in *A. testudineus* on the fecundity of the female fish possessing the ovary weight from 80.5 mg to 586 mg, indicated that fecundity was directly depending on the ovary weight (Ziauddin *et al.* 2016). The relationship between the fecundity and the fish size of female *A. testudineus* showed more than 88% of the variability of the fecundity was explained by the size of the fish. Similarly, another study reported a linear and positive relationship between the fecundity (3120 – 84690) and body weight (35.54 – 94.70 g), body length (12.7 – 17.24 cm), and ovary weight (0.60 – 16.70 g) in *A. testudineus* (Marimuthu *et al.* 2009). Thus, the statistically significant coefficient of correlation values obtained between the fecundity and other body parameters were sufficient to enumerate the spawning period and rate of egg production without sacrificing the animals.

In most teleost fish, the ovary is a hollow sac-like organ, whereas in *A. testudineus* it is a slightly unequal cylindrical, paired lobe attached to the dorsal coelomic wall by mesovarium, and opens through a common oviduct leading to the vent (Jacob 2005). The ovarian wall consists of an outer thin, transparent peritoneal layer called serosa, and inner thick, elastic tunica albuginea containing connective tissues, smooth muscles, and blood vessels. Ovigerous folds the finger-like folding of ovarian wall projects into the ovocoel, the lumen of the ovary, in which different developmental stages of oocytes were loosely packed (Selman and Wallace 1989; Nishimura *et al.* 2018).

Based on the shape, size, colour, texture, and microscopic observations, the developing oocytes were classified into five arbitrary stages namely immature, maturing, mature, ripe, and spent, represented as stages I to V, respectively (Newton and Kilambi 1969). The GSI and ova diameter are widely used parameters to predict the maturity and periodicity of spawning or breeding season of the fish. Accordingly, the reproductive cycle of female *A. testudineus* was further classified into four stages namely preparatory, pre-spawning, spawning, and post-spawning (Pal *et al.* 2018). In the present study, microscopic analysis of the ovary revealed the four developmental stages, which were used to determine the degree of maturity of *A. testudineus*. The first and immature stage was the preparatory stage that occurred from February to April, which enclosed both chromatin nucleolus and perinucleolus oocytes. The gonadal differentiation and development vary among different species, and it has been reported that the gonadal development in two-year-old starlet, *Acipenser ruthenus* were asynchronous, predominantly consisting of chromatin nucleolus and perinucleolus stages (Wang *et al.* 2017). The histological analysis performed during May and June was identified as the pre-spawning stage with pre-vitellogenic and vitellogenic oocytes. In this stage, the cortical alveolus began to develop on the periphery of the ooplasm with abundant yolk granules and a similar observation was reported in the vitellogenic oocytes of zebrafish, *Danio rerio* (Koc *et al.* 2008). During the spawning stage, the matured eggs with centrally located large oil vacuoles observed in the ovary were loosely arranged. The last stage of oocyte development in *A. testudineus* includes the post-spawning stage, which was identified at the end of September till January with the mixtures of atretic, spent as well as some pre-vitellogenic and immature oocytes. However, atresia is a normal physiological event characterised by the disintegration of the nucleus, breakdown of the vitelline envelope, and degeneration of yolk granules and follicular cells, influenced by several factors such as alterations in the levels of hormones, environmental conditions, temperature, photoperiod, nutritional supply, and water quality (Miranda *et al.* 1999). In the reproductive season, the abundance of mature oocytes along with some immature oocytes in the spawning stage ovary indicated a group-synchronous type of ovarian development in *A. testudineus*. In the present study, the diameter of mature ova of *A. testudineus* was ranged from 599 – 625 µm, while another study reported the ova diameter of *A. testudineus* from the water bodies of Malaysia has been found to vary between 0.54 and 0.80 mm for the fish weighing 36.84 to 105.26 g and 12.4-19.2 cm length (Marimuthu *et al.* 2009). The present results were also in agreement with another study that reported that *A. testudineus* possessing four distinct stages of reproduction with the same range of oocyte size based on the body mass (Pal *et al.* 2018).

In *A. testudineus*, testes are paired and elongated organs attached to the dorsal coelomic cavity by a thin membranous layer, mesorchium that joins caudally and converges into spermatoduct, which finally opens the exterior through the urogenital pore (Uribe *et al.* 2015). In the present study, histological analysis revealed the lobular type of testis in *A. testudineus* formed of many cysts-like grouped seminiferous tubules as identified in the Denison barb, *Sahyadria denisonii* (Sajan and Mercy 2016). During development, the growth of testis is accompanied by the gamete formation and production of viable sperm. Spermatogenesis in fish is a highly organised and coordinated process that is characterized by morphologically distinct germ cell stages such as spermatogonia, spermatocytes, spermatocytes, spermatids, and spermatozoa (Schulz *et al.* 2010).

Four spermatogenic stages of testis in *A. testudineus* were identified in this study which has been observed throughout the annual reproductive cycle and the composition of these stages determined the testicular maturity (Rutaisire *et al.* 2003; Montchowui *et al.* 2012). In the

preparatory stage, a thin, delicate, and transparent testis consists primarily of spermatogonia was noticeable. However, the composition of spermatozoa increased in pre-spawning and spawning stages as the testis became thick, flattened, appeared creamy white in colour consisting of all other stages at a relatively low amount and the seminiferous tubules were greatly dilated. The post-spawning was characterised by loosely packed spermatozoa or empty lumen of the constricted seminiferous tubules. A similar pattern of testicular development has been reported in *A. testudineus* collected from the water bodies of the Philippines (Bernal *et al.* 2015), and in *Sahyadria denisonii* from Western Ghats of India (Sajan and Mercy 2016). The largest cell of spermatogenesis was spermatogonia of 1.56 µm in size and the smallest one was spermatozoa of 0.37 µm in diameter. All the four cells of spermatogenesis have been identified throughout the annual cycle, but the spermatozoa were dominant during the pre-spawning and spawning stages, and these observations suggest a group-synchronous development in *A. testudineus*.

Hypothalamic-pituitary gonadal axis regulate the reproduction of fish by the release of gonadotropin-releasing factor from the hypothalamus in a pulsatile fashion and stimulate pituitary for the secretion of luteinizing hormone and follicle-stimulating hormone, which in turn act on the gonads and releases estradiol and testosterone (Meethal and Atwood 2005). They were involved in the development, maintenance, and functioning of reproduction. The HSI axis is controlled by either positive or negative feedback mechanism acting either on the hypothalamus, pituitary, or gonad tissues. The circulating level of gonadotropins increases during oocyte development and binds with the receptors of thecal cells to synthesize aromatizable androgen for the formation of estradiol (Redding and Patino 1993). In the present study, the level of estradiol in the blood serum of female fish increased during pre-spawning and spawning stages of ovarian development along with the slight increase in the level of testosterone when compared with preparatory and post-spawning stages. The rise in the level of estradiol during the spawning period is responsible to trigger the liver for the production of vitellogenin, which was later incorporated into the developing oocytes (Pelissero *et al.* 1993).

The present results revealed that the male fish showed an increase in the level of testosterone in pre-spawning and spawning stages of testicular development along with the decreased level of estradiol in post-spawning stages stating the role of testosterone in spermatogenesis during the spawning period. The level of testosterone also coincided with the increased GSI during pre-spawning and spawning period as reported in the male golden rabbitfish *Siganus guttatus* in captivity (Pham and Le 2020). Another study also reported a rise in the levels of testosterone during the pre-spawning period in the testis and seminal vesicles of the catfish *Heteropneustes fossilis* (Chaube *et al.* 2018). The evaluation of sex steroid hormones provides key information about certain reproductive parameters such as gonadal differentiation, gametogenesis, steroidogenesis, and fecundity to predict the reproductive success of the species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

PCV accomplished data collection, data analysis, acquired funding and drafted the manuscript. **KCC** assisted in the planning and supervision of the study, critically reviewed the manuscript and edited photographs.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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