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Some Biological Aspects of *Anabas testudineus* (Bloch 1792) from Rudrasagar Lake (Ramsar site), India with Special Reference to Reproductive Biology

Vidyabhooshan, Pampa Bhattacharjee, Diamond Rajakumar Tenali

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ABSTRACT

The present study has been carried out from the Rudrasagar lake of Tripura. For which, a total of 350 fish specimens were collected during July 2020 to July 2021. The highest and lowest value of GSI was observed during month of May and January respectively. The mean HSI value started to decrease gradually from April to June and started to increase from July. The percentages of male fishes were observed highest in the month of January and lowest during the month of March. The percentages of female fishes were observed highest in the month of March (78%) and lowest during the month of January (18%). The egg diameter was found to vary from 0.13 mm to 0.54 mm. The highest value of ova diameter observed in the month of May and lowest during month of January. Macroscopic examination of ovaries revealed that ripe ovaries were pale yellow in color, almost mature ovaries were brownish yellow in color and immature ovaries were yellowish white in color. Histological study revealed that the mature oocytes were relatively prevalent from April to June, with a slight decline in June, when females may have spawned, as indicated a decrease in female GSI. Similarly, spermatozoa were abundant throughout the study period. These results indicate that *A. testudineus* gonads develop simultaneously, allowing for an extended breeding period. The findings of this study would be useful in the fields of culture, rearing, larval control and fisheries management.

Keywords Reproductive biology, *Anabas testudineus*, Rudrasagar lake, Ramsar site.

INTRODUCTION

In order to assess a population's reproductive potential, it is necessary to determine spawning and reproduction frequency during the season and throughout the life cycle of the fish. It is essential to evaluate the annual breeding cycle of culturable fishes in order to have success in fish farming. Fish spawn during a specific stage of their reproductive cycle ; some breed once a year, while others do so at regular intervals throughout the year (Praveen *et al.* 2017). Studying gonadal development of a species and spawning season allows for future research into the population's spawning frequency, which is crucial for management. The study of sex-ratio, length at first sexual maturity, maturation cycle and spawning periodicity, among other things, is an important element of fish reproductive biology research. For the sustainable use of fish populations and their long-term production, understanding fish reproductive biology is essential (Temesgen 2017). Understanding fish reproduction is also essential in providing reliable

Vidyabhooshan[1], Pampa Bhattacharjee[2], Diamond Rajakumar Tenali[3]*
[2]Assistant Professor
Department of Fisheries Resource and Management, College of Fisheries, CAU(I), College of Fisheries, Central Agricultural University (I), Tripura, West Tripura 799210, India
Email : diamondraj.t@gmail.com
*Corresponding author

scientific recommendations on fisheries management (Hossain *et al.* 2017, Khatun *et al.* 2019). *Anabas testudineus* is a high-priced air-breathing freshwater food fish belonging to the Anabantidae family and order Perciformes. Low-lying swamps, marsh regions, lakes, canals, pools, shallow pits and puddles are the most common habitats. This species with auxiliary respiratory organs may be cultured at high stocking density and in low-quality water, exhibit obligatory air-gulping behavior, omnivorus in habit and movements through ephemeral inflowing waterways and thus often ends up on the land when the rain ceases. It mostly feeds on diatoms, green algae, blue green algae, cladocerans. Since no work has been done on biology of *A. testudineus* from Rudrasagar lake of Tripura, hence conservation and culture strategies can be formed for its future management. Therefore, the present study is undertaken to study the biology of species *A. testudineus* under family Anabantidae from Rudrasagar lake of Tripura.

MATERIALS AND METHODS

Sample collection

Fishes were collected randomly during July 2020 to July 2021 in morning hours from Rudrasagar lake. The collected fish samples were immediately preserved in 5% formalin and transported to laboratory for further study.

Morphometry

The morphometric measurements were recorded as per the criteria. The fishes were identified up to species level with the help of timely updated different authentic keys.

Gonado-somatic index

The GSI was determined according to equation given as below :

$$GSI = \frac{\text{Gonad weight}}{\text{Body weight}} \times 100$$

Hepato-somatic index

The HSI was calculated using the formula as given below :

$$HSI = \frac{\text{Weight of liver}}{\text{Weight of fish}} \times 100$$

Fecundity

Fecundity of fish was calculated using the formula given as below :

$$N = \frac{\text{Total weight of ovary} \times \text{No. of oocytes in the subsample}}{\text{Weight of subsample}}$$

Histological study

In every sampling, four to five male and four to five female fishes were studied for histology of the gonads. After morphological examination, the gonads were cut into 3 pieces as anterior, middle and posterior portion. The middle portion is preferred for the further study. Both the middle parts were taken for histological study.

For histological study, the microscopic slides were prepared and for development stages of germ cells in the testes and the change of the oocytes in ovary were studied following methods.

Collection and fixation of tissue

For histological study, the middle parts of the gonadal tissues (testes and ovary) of *Anabas testudineus* were collected as stated earlier. The tissues were trimmed into 5 to 6 mm size for better penetration of fixatives into it. The tissues were put into Bouin's solution for 24 to 48 hrs as per size of tissues.

Washing

The tissue (testes and ovary) was removed from the fixatives and subjected to overnight washing with flowing clear tap water until the formaldehyde odor were vanished.

Dehydration

The tissues were dehydrated perfectly with graded

alcohols, starting from 30%, 50%, 70%, 90% and absolute alcohol (100%) to avoid the brittleness of the tissues.

De-alcoholization

Two changes of xylene (1 h each) were made to clean the tissues from alcohol. For better impregnation of wax into the tissue, the xylene penetrates into the tissue to become transparent and the material comes up to float on the top.

Infiltration

Paraffin wax (melting point 58-60°C) was used for infiltration of tissue. Three changes of wax (45 min each) were made to make the tissue xylene free.

Embedding

The embedding process was caried out using an automated tissue embedder (Shadon Histocenter 3, USA). For the preparation of blocks, pure paraffin wax was melted in water bath in between 58–60°C. Metal 'L' moulds were adjusted according to the size of blocking materials. The melted paraffin was taken from water bath and the blocking disc was filled. After permitting a layer of wax to be solidifying on the bottom disc, the completely infiltrated tissues were carefully taken from the paraffin wax and put inside the different blocking disc according to their size. Care was taken so that the wax on the top of the disc did not solidify during keeping material in the blocking disc. For this reason, a heated forceps was put only the upper portion or inside the wax of the disc. After the proper positioning of the tissues, the wax inside the disc was allowed to solidify. After few minutes, the 'L' moulds were removed from the wax block and prepared blocks were kept separately inside the labelled polythene packets.

Trimming and sectioning

The paraffin blocks were trimmed carefully to 6 to 7 mm sharp blades. The trimmed blocks were fixed to the wooden holder (peg) with the material facing away from it. Molten wax was poured on the holder and the block was kept on it. The block was padded with more wax at the base to make it strong. After being confirm, the blocks were firmly fixed with holder, the sectioning was done using rotary microtome (Leica RM 2245, Germany). On the microtome, each section was cut into 5µ thickness. The ribbons containing tissues were collected on clear glass slide (already a smear of egg-albumin was kept on that slide) with the help of a fine brush.

Spreading and fixing

Glass slides were cleaned properly Chromic-acid solution then soap and finally with tap water. After cleaning, the slides were air-dried and a thin layer of Glycerine. Then the ribbons with materials (about 10 to 15 sections depending on the size) were spread over the clean glass slides. Thin tissues were made wrinkle free and allowed to fix on slides keeping them on hot plates (30°C) for 2 to 5 minutes.

De-waxing and staining

Tissues fixed on slides were dewaxed with descending order of alcohols (100%, 90%, 70%, 50% and 30%) and stained the double staining method with Hematoxylin and Eosin using standard techniques.

Mounting

One or two drops of DPX (mountant) were put on the dried slide which one was ready for mounting. Then, a cover slip or slide was slowly lowered when the mountant will flow ahead of the descending glass without trapping air bubble between the cover slip and slide. The excess of mountant on the slides was removed with xylene soaked in cotton. After mounting, the slides were allowed for drying.

Labeling and storing

Labeling was done on the slide glass marking pen to avoid future confusion. The slides were stored in slide box to protect them from dust and dirt.

Microscopic observation

The histological sections on the prepared slides were thoroughly observed under light microscope (Leica,

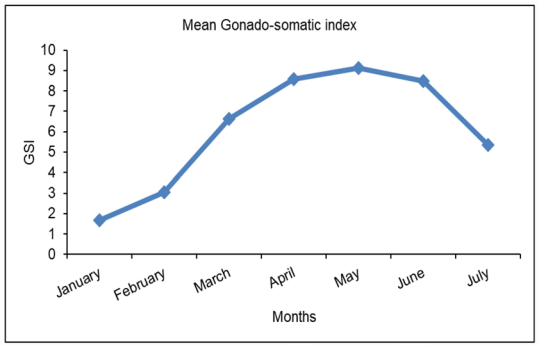

Fig. 1. Month wise GSI of *A. testudineus.*

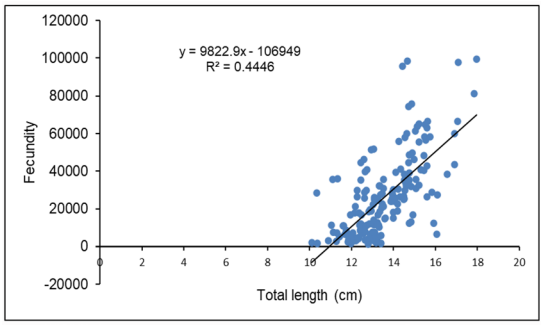

Fig. 3. Relationship between fecundity and total length of *A. testudineus.*

MODEL DM750) at different magnifications. The developmental stages of germ cells in the testes and changes of the oocytes of ovary were noticed carefully. Different analytical techniques of statistics like mean, standard deviations, standard error, correlation coefficient (r), coefficient of determination (R^2), coefficient of regression (b) were used to analyze and interpreted the results using Microsoft excel.

Data availability statement

All data generated or analyzed during this study are included in this published article.

RESULTS

Gonadosomatic index (GSI) and hepatosomatic index (HSI)

The GSI measures gonadal maturity of fish and development. It increases with the development of the fish and then decreases. The maximum mean GSI values were observed in the month of May (9.12) and the lowest value (1.67) in the month of January. The mean GSI value started to increase gradually from January and reached a maximum value in May. So, we can say that peak spawning season of this species is May.

The maximum mean HSI values were recorded in the month of January (4.10) and the lowest value (1.47) in the month of May. The mean HSI value started to decrease gradually from January to April and started to increase from May and June (Fig. 1). In this study, the GSI and HSI values (Fig. 2) were found to be negatively associated, indicating that energy is liberated from the liver and transferred to the ovary. The reduction in liver weight remained until the spawning was completed, at which point vitellogenin began to be deposited in preparation for the next spawning.

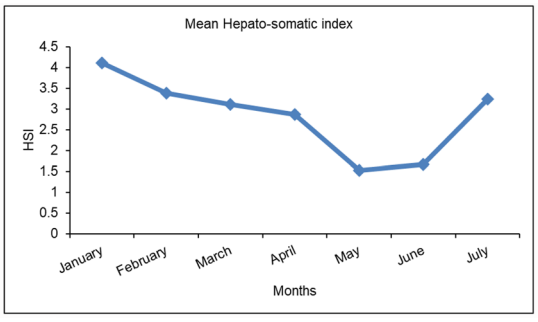

Fig. 2. Month wise HSI of *A. testudineus.*

Fecundity estimation

Fecundity in females differs from species to spe-

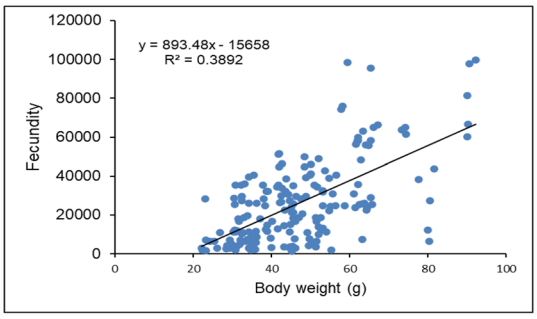

Fig. 4. Relationship between fecundity and body weight of *A. testudineus*.

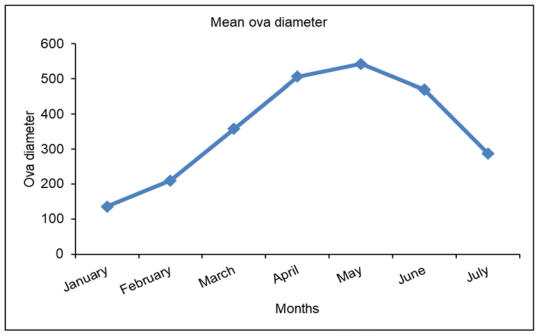

Fig. 5. Mean ova diameter of *A. testudineus*.

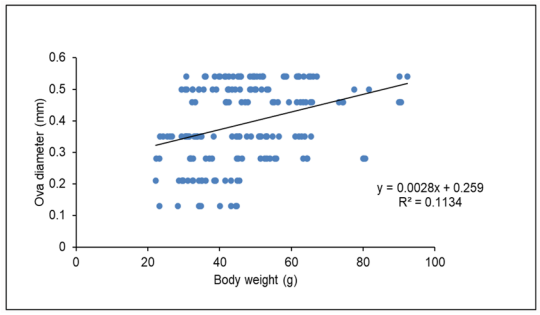

Fig. 7. Relationship between body weight and ova diameter of *A. testudineus*.

cies, age, body length, weight, and environmental conditions. The total 189 female fish was observed for determination of fecundity and it was found that the individual fecundity of fish varied from 1404 to 99,580, with mean fecundity of 27,593 for the fishes with 10.3 – 17.9 cm in total length (Fig. 3) with a mean of 13.67cm and the body weight ranged from 22.17 – 92.25 g with a mean of 45.11g.

The relationship between total fecundity and body length is stated the equation Y= a+bx, where Y= fecundity (TF), x = total length (TL), intercept (a), regression co-efficient (b) and correlation co-efficient (r) are shown in respective figures. The regression equation was calculated as TF= - 106949+9822.9TL. Fish fecundity and total body length were found to have a linear relationship. Coefficient of correlation is positive, as the value of 'r' is 0.683. When the body length reaches 10.3 cm, the number of eggs is 1404. The maximum number of eggs in the ovary is calculated as 99,580 with the body length of 17.9 cm. The relationship between fecundity (TF) and total body weight of fish (BW) was computed and expressed the equation F= – 15658 + 893.48 BW. A positive and linear correlation was observed and the 'r' value is 0.632 (Fig. 4).

Ova diameter

The egg diameter was found to vary from 0.13 mm to 0.54 mm (Fig. 5). The relationship between the total length and ova diameter is also linear and expressed equation OD = -0.094 + 0.035TL. The coefficient of correlation between the total length of fish and ova diameter is found to be positive as the value of 'r' is 0.415 (Fig. 6). The link between diameter of a fish ova and its body weight observed and expressed the equation OD = 0.259 + 0.0358 BW (Fig. 7).

A positive correlation was observed in the present study and the r value is 0.336. In the present study the smallest gravid fish, with total length =10.36 cm and

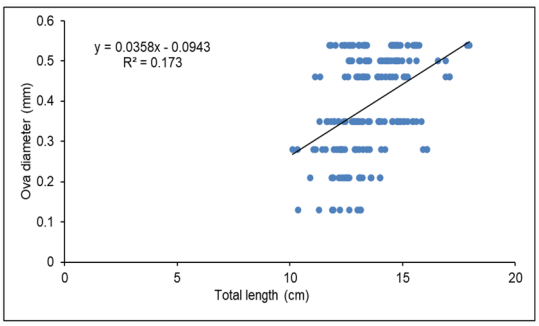

Fig. 6. Relationship between total length and ova diameter of *A. testudineus*.

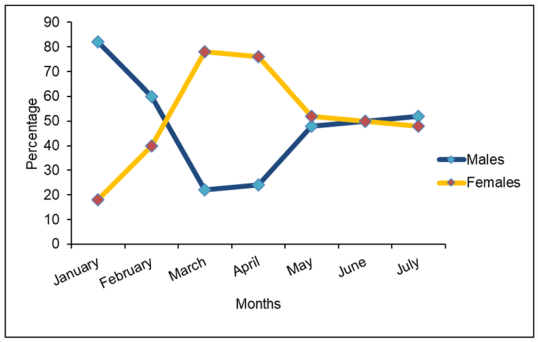

Fig. 8. Monthly variations of sex ratio of *A. testudineus*.

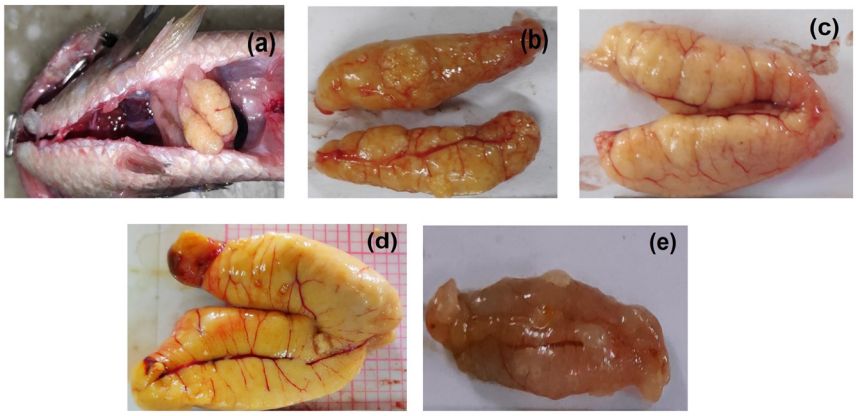

Fig. 9. Maturity stages of ovary of *A. testudineus* (a) Immature stage, (b) Maturing stage, (c) Mature stage, (d) Ripe stage, (e) Spent stage.

body weight 23.17 g had eggs with a mean diameter of 0.13 mm, whereas the biggest fish, with total length= 17.96 cm and body weight = 92.25 g, had a mean egg diameter of 0.54 mm.

Sex ratio

A total of 350 specimens of *A. testudineus*, were experimented, in which 169 (48.28%) were males and 181 (51.71%) were females. The sex ratio was recorded as 1:1.09. The number of females were higher than males (Fig. 8).

Maturity stages of the ovary

The ovaries were classified as immature (stage I), maturing (stage II), mature (stage III) and ripe (stage IV) based on gross appearance, such as ovary color and size, relative space occupied in the body cavity with ovary, and microscopic observations, such as yolk content and ova diameter measurements.

Stage I (Immature)

The ovaries were small, transparent, and taking up about a quarter of the body cavity. They were yellowish white in appearance and defining the ova without a microscope was difficult. The ovary was irregularly formed, with translucent eggs that are roughly spherical in shape and have a central nucleus (Fig. 9a).

Stage II (Maturing)

Ovaries expanded in size, taking up nearly a third of the body cavity and turning a brownish yellow color. The ovary had become slightly opaque due to yolk deposition at this point, and the ovary wall was still uneven. The nucleus could not be seen clearly (Fig. 9b).

Stage III (Mature)

The ovary grew in size, taking up nearly two-thirds of the body cavity. The ovarian wall was slightly thin, and the ova could be seen with wide eyes, but the nucleus was invisible due to yolk deposition, and the color of the ovary turned yellowish (Fig. 9c).

Stage IV (Ripe)

The ovary turned a pale yellowish color and expanded in size, with a very thin ovarian wall. Due to pushing the abdomen, the eggs came out readily at this stage (Fig. 9d).

Stage V (Spent)

Ovaries were flaccid, with a brownish color and a reduced volume and size. The ova were of various sizes, with a few maturing ova and ripe ones visible through the ovary wall (Fig. 9e).

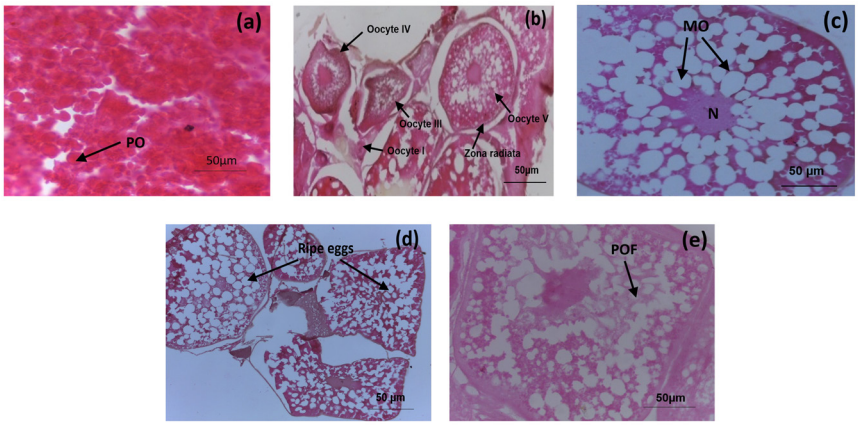

Fig. 10. Stages of oocyte development in the *A. testudineus* (H and E stain, 40X). a. PO: Primary oocytes, b. MO : Mature oocytes, c N : Nucleus, d POF: Post ovulatory follicle. e POFR: Post ovulatory follicle rapture.

Ovarian cyclicity

Ovarian histology revealed the many stages of ovarian development during oogenesis. The oogenesis process of *A. testudineus* has been divided into five stages (Fig. 10).

Stage I (Immature)

Oogania of this period were spherical cells that were quite tiny. The cytoplasm of each cell was thin and unclear, with a big nucleus. The primary oocyte was typically oval or spherical in form, and the size ranged from small to large (Fig. 10a).

Stage II (Maturing)

Various oocyte stages such as oocyte I, III, IV and V were observed at this stage. Between the ooplasm and the follicular layer, there is a vitelline membrane with zona radiata. There are also a few oocytes in the primary and early secondary growth phases (Fig. 10b).

Stage III (Mature)

At this stage, the ovary contained a considerable number of developed oocytes. Oocytes developed quickly and swelled in size, displaying follicular epithelium. In addition, the size and number of nuclei grew (Fig. 10c).

Stage IV (Ripe)

The oocytes grew in size during this time, and the ovaries were full with ripe eggs (Fig. 10d).

Stage V (Spent)

The ovigerous folds were uneven, convoluted, and included a considerable number of ruptured, post-ovulatory follicles on histological sections (Fig. 10e).

Testicular cyclicity

The process of spermatogenesis in *A. testudineus* has been categorized into five phases based on histological studies in the testes (I–V). In this investigation, it was discovered that *A. testudineus* matured only during the breeding season, which runs from March to June.

Stage I (Early immature)

Seminiferous tubules were tiny and interlobular gaps were densely packed with stroma on histological examination. Proliferation of spermatogonia characterizes this phase (Fig. 11a).

Stage II (Late immature)

During this time, histological observations revealed a significant level of spermatogenic activity. Testes had

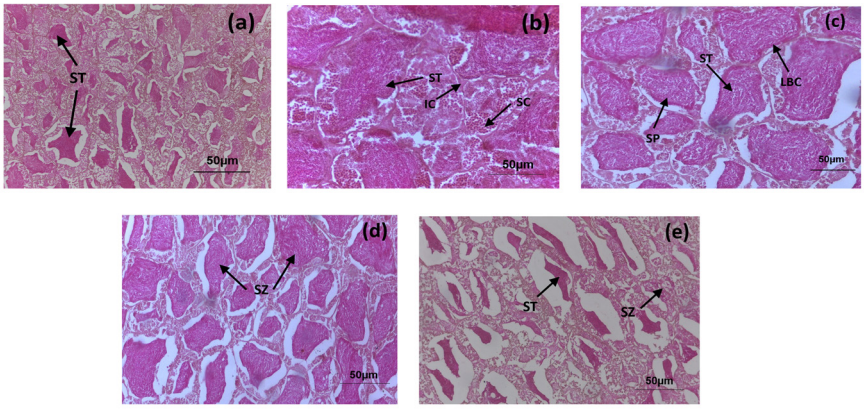

Fig. 11. Spermatogenic cell stages of development in the *A. testudineus* (H and E stain, 40X). ST : Seminiferous tubules, IC : Interstitial cells, SC : Spermatocyte, SP : Spermatids, LBC : Lobule boundary walls, SZ : Spermatozoa.

a plentiful supply of blood. The size of the lobules gradually grows larger, while the interlobular space shrinks, revealing conspicuous blood veins (Fig. 11b).

Stage III (Maturing)

During the latter portion of this period, strong spermatogenesis (the change of spermatocytes into spermatids) can be noticed histologically (Fig. 11c).

Stage IV (Mature)

The peak spermatogenic and spermiogenesis (transformation of spermatids into spermatozoa) processes were observed histologically in testes during this time. The spermatozoa were abundant in the seminiferous tubules, which were big in size. When the abdomen is lightly pushed during this period, milt seeps out (Fig. 11d).

Stage V (Spent)

The tunica albuginea thickens and the interlobular gap expands histologically. Seminiferous tubules are shown to be empty and collapsing, with some containing remnant or un expelled sperm. There were also some interstitial cells that emerge. Because of the diminished blood supply, the color shifts from pinkish red to light brown (Fig. 11e).

DISCUSSION

In this investigation, GSI and HSI values were found to be inversely associated, which indicated that energy is released from the liver into the ovary. The decrease in liver weight continued until the spawning was completed, at which point vitellogenin started to be stored in preparation for the next breeding. Similar findings were observed (Uddin *et al.* 2017) in *A. testudineus*. The peak spawning season might be predicted based on the high association between GSI and the number of adult fish.

The GSI, which reflects gonad growth and maturation, was higher from April to June in this study with a peak in May for both males and females. Similar results were observed (Mian *et al.* 2017) in *Channa punctatus*. The similar results were shown (Narejo *et al.* 2015) in *C. punctatus* and *C. striatus.*

Even among females of equal length and weight, there was a variation in fecundity. In this study, a fish with a total length of 15.63 cm, a body weight of 67.10 g and an ovary weight of 12.76 g produced 66352 eggs, while another fish with the same total length produced 63024 eggs. The fluctuation in *A. testudineus* fecundity could be linked to the lake's environmental factors. The linear relationship between fecundity and fish length and fecundity and body weight in the selected species was found using statistical analysis. In the present study it was revealed that as total length, total weight, and gonadal weight increase, fecundity increases linearly. The correlations revealed in the present study agreed with

previous findings for this species.

Due to the highest percentage of ripe ova available in the gonad during the spawning month, mean monthly ova reached its highest value, indicating April to June as the spawning month for *A. testudineus*. The frequency of occurrence of various size-classes of intra-ovarian oocytes correlated with the changing trend of mean monthly ova-diameter, indicating a synchronous development of oocytes in *A. testudineus*. Ripe eggs were found in the gonads from April to June, indicating that this was the breeding season of *A. testudineus*. Similar results were observed (Gupta and Banerjee 2013) in *Mystus tengara*.

In the case of *A. testudineus*, a preponderance of females over males was more noticeable, especially around the start of the breeding season in April and May. Female dominance could be linked to migration patterns and schooling behavior. This can be attributed to easy vulnerability of the migratory schools of spawners to the increased fishing pressure during the start of the breeding season. Similar observations also reported (Bindu *et al.* 2012) in *Horabagrus brachysoma.*

Macroscopic examination of ovaries revealed that ripe ovaries were pale yellow in color, almost mature ovaries were brownish yellow in color, and immature ovaries were yellowish white in color. The ovary was seen through naked eyes when the ovaries were higher in larger fish. (Amzad *et al.* 2015) found a similar statement in *C. punctatus*.

Throughout the study period, gonadal development stages were seen in the gonads of *A. testudineus,* whereas the abundance of different oocyte stages altered based on the season. The ovary contained both mature and atretic oocytes, which was unusual. However, mature oocytes were relatively prevalent from April to June, with a slight decline in June, when females may have spawned, as indicated a decrease in female GSI. Similarly, spermatozoa were abundant throughout the study period. These results indicate that *A. testudineus* gonads develop simultaneously, allowing for an extended breeding period. Similar findings reported (Bernal *et al.* 2015) for this species. This finding is consistent with the findings of (Behera *et al.* 2015) for this species.

The ovarian cycle is regulated the distinct phases of ova, which can be seen histologically in the ovaries. It has an ovarian wall that surrounds an ovocoel cavity. The tunica albuginea became thinner during the spawning time, but remained thicker during the rest period. The germinal epithelium is made up of a single layer of cuboidal cells with minimal cytoplasm. A substantial number of developed oocytes were found in the ovary at the peak breeding phase. In the spent phase, the ovary became flaccid and there were fewer unspawned ova. The ovary grew thin and the ovarian wall thickened during the resting period. Male *A. testudineus* had a normal histological structure, according to histological study of the testes. Immature phase, maturing phase, mature phase, ripe phase, and spent phase were identified as the five phases of maturity. All phases of spermatogenesis show germinal cysts in the lobules. A layer of connective tissue was present around the seminiferous tubules. Similar results were observed (Behera *et al*. 2011) in *C. bleheri*. This study may potentially aid efforts to breed *A. testudineus* in captivity for trade and conservation purpose. The investigation will provide baseline information on the feeding and reproductive biology of *A. testudineus* which would be useful in the management and conservation of this species in the natural environment.

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