MATERIALS AND METHODS

3.1. Collection of the fish

A total number of 464 specimens of *Badis badis* belonging to all available size classes were taken for this present study. They were collected mostly from Dattafuliya of North 24 Parganas, situated at West Bengal and Bangladesh border from March, 2017 to February, 2018 and necessary measurements were recorded. After collecting the fishes were packed in oxygenated packet with methylene blue and the packets were arranged in thermocol box for further transportation. Then the fishes were safely transferred to "Ornamental Fish ResearchCentre" of ICAR-Central Institute of Fisheries Education, Kolkata Centre.

3.2. Acclimatisation of procured fish

After procuring, fishes were disinfected by giving bath treatment with 5 ppm KMnO4 until fishes show the symptoms of stress. After discarding dead and weak fishes the bath treated fishes were transferred to fiber tanks of 1000 litre capacity with continuous aeration. Fishes were acclimated for 15 days in laboratory conditions before starting of the further experiments.

3.3. Experimental set up for captive rearing

The fish were stocked in 02 different systems after acclimatization. i.e., RAS (flow through system) with the dimension of 160 cm length × 60 cm height × 90 cm width (water volume – 50 lit) and Cement (confined system) with the dimension of 75 cm

length × 30 cm height × 30 cm width (water volume – 450 lit). The average size of stocking was about 1.5 cm with the body weight of around 250 mg. The habitat for fish were made with the help of sandy bottom, gravels, stones along with plantation of some of the ornamental plants like Amazon, *Vallisnaria*, *Hydrilla* in RAS system while apart from above the *Lemna*, *Cabomba*, *Hydrila*, *Cryptocorine*, *Ceratophyllum* and *Salvinia* were planted in the cement tanks (**Fig. 1, 2 and 3**).The depth of water was maintained about 1 ft. in the cement tank. The fish were fed with Plankton, *Artemia* naupli, Tubifex and chlorella twice a day.

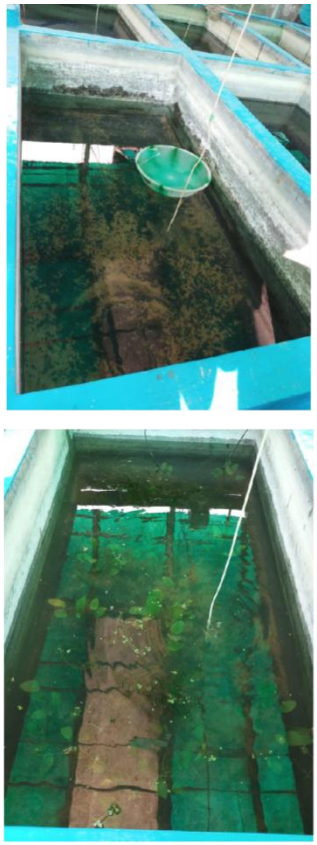

Fig. 1 Habitat preparation for captive maturation of *Badis badis* under semi-natural condition (See colour photo in Plate 2)

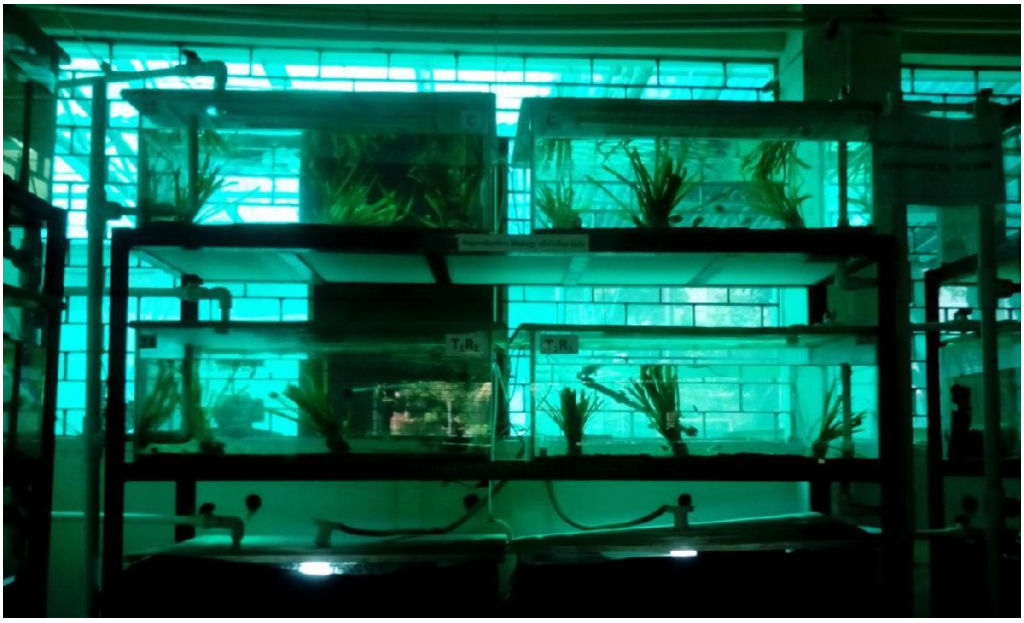

Fig. 2 Rearing of *Badis badis* in RAS (Flow through system)
(See colour photo in Plate 3)

Fig. 3 Rearing of *Badis badis* in cement tank
(See colour photo in Plate 3)

3.4. Captive rearing

Maintenance of fish- Regular unconsumed feed was removed by siphoning after 2 hours offeed given. The filter medium was cleaned before feeding once in 3 days. Only 25-30% water was exchanged thrice a week to remove the accumulated faces. The fish were kept under natural photoperiod throughout the experiments. Bottom substrate and aquarium plant wash once in a week and treated with 5ppm $KMnO_4$ solution.

Physico-chemical parameters analysis-

The water temperature recorded daily twice at 9:30AM and 2:30PM. The water samples were tested weekly to study the various physico-chemical parameters of habitat for the selected species. DO, Alkalinity, Total Hardness and Free CO_2 werestudied following standard procedures (APHA, 2005). Ammonia and Nitrates were tasted by using test kits developed by Seachem Laboratories, USA.

Water temperature: The water temperature was recorded with the help of a mercury centigrade thermometer with 0.5 ^oC accuracy.

pH: pH was analyzed by using of digital pH meter.

Dissolved Oxygen: Winkler's method was used to estimate the dissolved oxygen level of water. The value was expressed in ppm.

Free CO_2: Phenolphthalein indicator with N/80 Sodium Hydroxide method was used to detect the free Carbon di-oxide level of water. The value was expressed in ppm.

Alkalinity: Alkalinity of the water was estimated by titration with N/50 Sulphuric acid using Methyl orange and Phenolphthalein indicator. The value was expressed in ppm.

Total Hardness: Total hardness i.e. total multivalent cations' concentrations were measured by 0.01M EDTA using EBT indicator. The value was expressed in ppm.

Feeding of the experimental fishes–

Live food was used for feeding. After one day of procuring the fishes were fed on tubifex @ 2% of total body mass twice in a day at 10:00AM and 5.30 PM. Based on regular requirements constant supply of live feed, live food like Tubifex, Mosquito larvae and Daphnia are cultured in the laboratory. Artemia nauplii also hatched from dry cyst in laboratory, are also used as food.

Tubifex culture: The culture method followed as per Marian and Pandian, 1984. In cemented channel with continuous running water flow Tubifex was cultured (**Fig. 4.**). 75% raw cow dung and 25% soft sand were used as substratum. The tubifex worms were incorporated @ 10g/cm^2 after 2 days of substrate preparation. Fresh raw cow dung was added @ 25g/m^2 once in 4 days. Harvesting was done in every 15 days @ 50mg/cm^2.

Mosquito larvae culture: For mosquito larvae culture milk solution was added with rain water in a fibre tank of approx 500Lt. capacity and left undisturbed. Medium mash sized

net used as cover to avoid the accumulation of unwanted leaf and dirt. After 2-3 days mosquito larvae were harvested @ 20-30 Nos./tank.

Daphnia culture: Daphnia was cultured by the process as mentioned by Mahapatra, 2017. At first 4 cm deep soft soil base was added in 500Lt. cemented tank (**Fig. 5.**). Then $CaCl_2$ and raw cow dung were added @ $4g/m^2$ and $1kg/m^2$ respectively. Filtered water addedafter 2 days and after 1 week Daphnia inoculated @ 10 Nos./Lt. Phytoplankton was added @ 1mg/Lt. as food for Daphnia at every alternative day. After 1 month daphnia was harvested by plankton net.

Artemia nauplii hatching: Filtered NaCl solution (@ 12-15g/Lt) was added in a cylindrical jar for hatching of Artemia cyst. In room temperature (26-30^oC) pH maintained at 7.5-8.2 and continuous vigorous aeration was given. Artemia cysts were added @ 0.5-0.75g/Lt. and electric bulb light was added for faster the hatching process. After approx 26 hours hatched Nauplii were harvested.

Studies carried out-

During the captive rearing phase at ICAR-CIFE, Kolkata, study of the general biology of *Badis badis* was carried out from June 2018 to June 2019.

Fig. 4 Tubifex worm culture system
(See colour photo in Plate 4)

Fig. 5 Mixed zooplankton culture unit
(See colour photo in Plate 4)

3.5. General biology

3.5.1. Sample collection and preservation

For biological study fish samples were collected from the experimental setup on monthly basis. After collection serial photography was made by Sony DSC W800 camera and fishes were preserved in 5% formaldehyde solution. Total length (mm) of individual fish specimen was taken from the tip of the snout to tip of the caudal fin using Mitutoyo Digital Slide Calipers to the nearest 0.01 mm. Body weight (g) of each fish specimen was taken to the nearest gram using a top Mark Electronic Balance after blot-drying excess water from the body. Before dissecting out the guts, the length and weight as well as colouration of individual specimen were documented. After dissection the weight of guts were recorded to nearest 0.01 g. Different aspects of morphometric, meristic, food, feeding and reproductive biology of selected fish species were as follows:

3.5.2. Morphological study

Study of morphology was consisting of: i) morphometric and ii) morphomeristics. Morphometric and Morphomeristic studies were made following Jayaram (1999). Morphometric measurements were made by using vernier caliper. Morphomeristic characters were taken using magnifying glass and microscope. All morphometric measurements were taken in nearest millimeter.

A total of 50 samples of the fish species were studied for morphological analysis. The total lengths of the fishes to the nearest centimetre were recorded point to point with

binocular stereo microscope and Digital slide calliper by following the literature of Kullander and Britz (2002).

Morphometric Measurement: Morphometric measurements were recorded to the nearest 0.1cm. from left side of the specimen followed by Kullander and Britz (2002) and expressed as percentages of Standard length (SL). Various morphometric subunits were examined such as total body length, standard length, pre dorsal, pre pelvic, pre pectoral, pre anal length, head length, eye diameter, snout length, body depth at pelvic point (**Fig. 6**).

Total length (TL): Total body length represents the maximum elongation of the body. It is distance in between tip of the pre-maxilla and the tip of the tail.

Standard length (SL): It is measured between the tip of snout and the caudal fin base.

Head length (HL): Distance between the tip of snout to the posterior most edge of opercular bone.

Eye diameter (ED): Distance between anterior and posterior rims of the eye.

Snout length (SnL): From tip of snout to the anterior end of orbit.

Body depth (BD): It was measured from the dorsal and ventral surface at pelvic point.

Pre-dorsal length (PDL): Distance between anterior most part of the body and thefirst dorsal fin ray.

Pre-anal length (PAL): Distance between anterior most part of the body and thestarting point of anal fin.

Pre-pelvic length (PVL): Distance between anterior most part of the body and thebase of the pelvic fin.

Pre-pectoral length (PPL): Distance between anterior most part of the body and thebase of the pectoral fin.

3.5.3. Morpho-meristic count

A sample size of 50 fish was taken, and the following meristic characters are counted, using a hand magnifying glass and needle:

Dorsal Spines (DS) – The hard spines present at the anterior portion of dorsal fin, are counted.

Dorsal Soft Rays (DSR) – The soft rays present behind the dorsal spines are counted.

Anal spines (AS) - The hard spines present at the anterior portion of anal fin, are counted.

Anal Soft Rays (ASR) - The soft rays present behind the anal spines are counted.

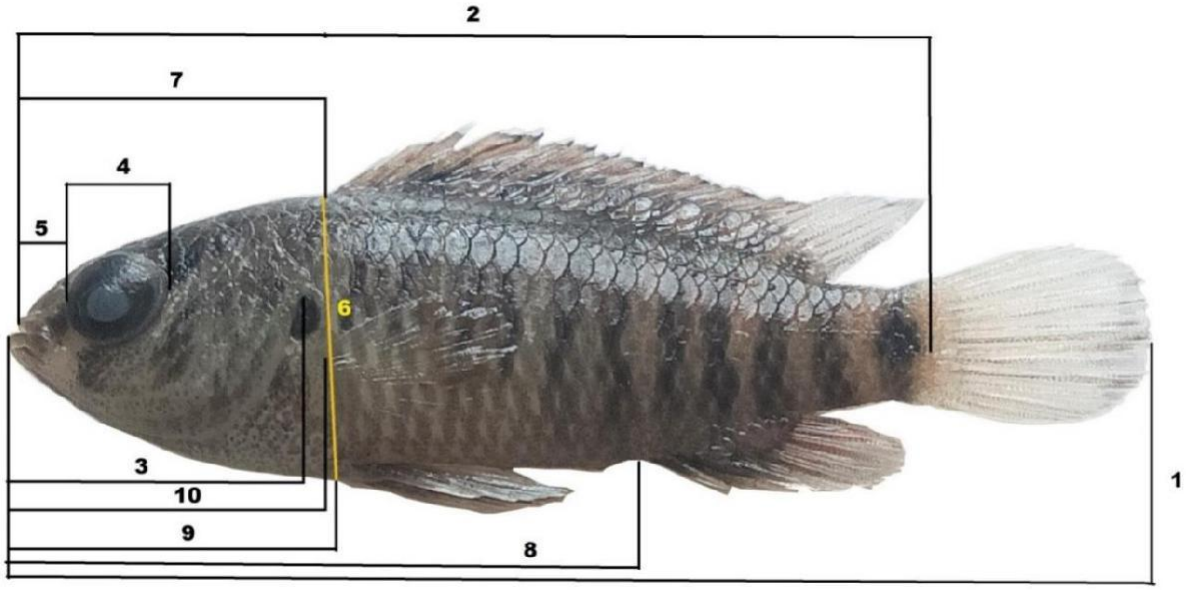

Fig. 6 Different morphometric measurements of *Badis badis*
(See colour photo in Plate 5)

1. Total length
2. Standard length
3. Head length
4. Eye diameter
5. Snout length
6. Body depth
7. Pre-dorsal length
8. Pre-anal length
9. Pre-pelvic length
10. Pre-pectoral length

3.5.4. Statistical analysis for morpho-meristics and Length-weight relationship

The mean, standard deviation, minimum range, maximum range and range difference were calculated of all the ten morphometric characters. Correlation Coefficient, R2 value and Regression Equation were worked out in comparison to total length (TL). Relationships were analysed using a standard linear regression expression Y = a + b X, where dependent variable is 'Y', independent variable is 'X' the, 'a' is constant (Y intercept) and 'b' (slope) is the regression coefficient, were fitted for all the variables for different localities.

All 464 specimens of different size group were taken to study of length-weight relationship and condition factor. The total length and standard length was recorded to the nearest millimetre. The weights were taken to the nearest gram. The length-weight data were analysed according to the method given Le Cren (1951). The equation of the parabolic relationship of the form $W=aL^b$ was used where W represents weight of the fish in gram. L the total length in millimetre and 'a' the constant and 'b' an exponent to which L can be raised. The equation expressed in logarithmic form becomes: Log W = Log a + b Log L. In the present study the length-weight data were analysed after classifying the fishes in the following 2 major groups: i) combined group and ii) different size length. Condition Factor was calculated by using the following formula $K=100W/L^3$ as given by Bagenal and Tesch, 1978. Statistical relations were measured with the aid of computer using SPSS-16.0 and MS Excel.

3.6. Feeding biology

For determination of the food and feeding habits of the fish species GaSI, gut content, mouth size and shape were studied as per standard methods.

3.6.1. Relative Length of Gut (RLG)

The characterization of fish as carnivore, herbivore or omnivore is done by using the Relative gut length (RLG) as the main morphological variable. A sample size of 10 fish were taken every month and dissected. Before dissection the total length of the fish was taken, and then later the gut length was taken (**Fig. 7**). Relative gut length (RGL) was calculated by the following formula given by Al-Hussaini, 1949.

RLG= (Total length of gut (mm))/(Total body length (mm))

3.6.2. Gastro-somatic index (GaSI)

Feeding intensity of fish was determined as the Gastro-somatic Index, a numerical value. A sample size of 10 fish was taken every month and dissected. The body weights and gut weight were measured by using an electronic balance. Gastro-somatic index (GaSI) was calculated by the following formula.

GaSI= (Weight of gut (gm))/(Total weight of fish (gm)) x100

3.6.3 Gut content analysis

After measuring the total length, mouth to anus length and weight of specimen, the alimentary canal was dissect out and preserved in 10% formalin for microscopic examination of food items.

Fig. 7 Intestine of *Badis badis*
(See colour photo in Plate 6)

3.7. Reproductive biology

To study the reproductive biology about 225 live fish specimens covering all the size ranges were measured, weighed, aged and sexed for studying the different maturity stages, sex ratio and size at first maturity. The gonads were removed by dissection, and weighed to the nearest milligram. The morphological characters of the gonads will then preserve in Gilson's fluid for further microscopic examination. Some female were

selected for ova diameter studies. As recommended by Clark (1934) and Qasim and Qayyum (1961) 100 ova from both the lobes will collect randomly and will measure with the aid of an ocular micrometer. The sex ratio was calculated and the Chi-square formula

$X^2= (O-E)^2/E$ was used to test the homogeneity in the distribution of males and females where O denotes the observed and E the expected values.

3.7.1. Sexual dimorphism

Randomly 200 adult fishes were collected from the stock and kept in a transparent aquarium and their size, colouration, fins shape and body shape was observed and recorded. The observations were carried out for the entire captive rearing period, so as to find the differences in the characters in breeding and non-breeding period. A couple of fishes were dissected for sex confirmation.

3.7.2.Gonadosomatic index (GSI)

Monthly samplings of 50 fish were carried out from the captive culture stock from July, 2018 to June, 2109. The gonadosomatic index, abbreviated as GSI is a tool for measuring the sexual maturity of animals in correlation to ovary development and testes development. It is the calculation of the gonad mass as a proportion of the total body mass. It is represented by the formula

$$GSI= (\text{Weight of gonads})/(\text{Total weight of fish}) \times 100$$

3.7.3. Fecundity

Absolute fecundity is the total number of eggs that are likely to be spawned in one spawning period. Matured female fishes were dissected, and then ovaries were carefully removed from the body, and sub samples were collected from the anterior, posterior and middle portions of right and left ovaries put into 10% neutral buffered formalin (NBF) for fixation. Then the eggs were loosened from the ovary by shaking the tube and counted under microscope to find out fecundity (Grimes and Huntsman, 1980).

3.7.4. Breeding behaviour

Male and female fishes were segregated for fifteen days. They were given Tubifex as feed during these fifteen days. Then breeding setups were arranged by taking one adult male and one adult female in each aquarium. The breeding setup was left undisturbed and observations were made and recorded without disturbing the breeding pair.

3.7.5. Captive breeding, seed production and rearing

Artificial structure e.g. hideouts, floating weed cover, flow through systems etc. were tried to simulate the natural habitat of the fish in captivity. Ecological parameters (e.g. pH, dissolved oxygen, ammonia, hardness, alkalinity of water etc.) in captive condition were maintained by water exchange, aeration, thermostat, proper feeding and creation of suitable environment as mentioned above. Natural feeding was done during breeding.