MATERIALS AND METHODS
3. MATERIALS AND METHODS

With a view to fulfilling the objectives of the study a research was executed during the period 2012 to 2015 both in the Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal and Fishery Laboratory in Uttar Banga Krishi Viswavidyalaya, Cooch Behar, as per the Experimental Design given in Fig.2.

3.1. Experimental Design

![Experimental design diagram]

Fig.: 2. Experimental design of the research work done in the Field (Kaljani river) and in Captivity (Laboratory)
3.2. Study area and sampling sites

The present study was executed in the Cooch Behar district situated on the foothills of Eastern Himalaya, lying between 25° 57’ to 26° 36’ North latitude and between 89° 54’ to 88° 47’ East longitude. The district having a total water area of approximately 6121 ha includes hill stream rivers, ponds and beels. The aquaculture resource of the district is very rich with fishes being the major living components of these water bodies. The Eastern Himalaya with rich biodiversity is under immediate threat of species extinction and habitat destruction due to tremendous pressure from demotechnic growth and natural environmental changes. With a view to rearing and breeding of loaches in captivity, which are Vulnerable and Endangered, their conservation and ichthyofaunal diversity of river Kaljani, Cooch Behar district a study was executed during the period August 2012 to July 2015.

River Kaljani situated in Cooch Behar district covers a stretch of about 9 Km upto the lower reaches of the river, that is, from Amlaguri in the north to Chhat Bhelakopa in the south. The Kaljani river, has a total length of about 96 km., and runs down through the districts of Alipurduar and Cooch Behar, originating from Gabaur Bachhra forest lying in the borders of Bhutan and West Bengal and outfalls into Shiltorsa in Cooch Behar. The sampling areas which were divided into four sites and having a distance of 3 km between them included Amlaguri (26° 34’ N latitude and 89° 58’ E longitude), Chhatoa (26° 32’ N latitude and 89° 58’ E longitude), Jaigir Chilakhana (26° 31’ N latitude and 89° 58’ E longitude), and Chhat Bhelakopa (26° 29’ N latitude and 89° 58’ E longitude).
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Fig. 3. Physical map showing the sampling sites of river Kaljani (Source: Google map)

Fig. 3a: Site 1                                                   Fig. 3b: Site 2

Fig. 3c: Site 3                                                 Fig. 3d: Site 4

Fig. 3a -3d: Photographs showing four different sampling sites (Amlaguri, Chhatoa, Jaigir Chilakhana and Chhat Bhelakopa) of River Kaljani
3.3. Collection Procedure

3.3.1. Water (Wild and Captive)

Water samples were collected from both natural water system (Kaljani river) and glass aquariums (Dimensions: $3' \times 1.5' \times 1.5'$) with aeration in captive system (Laboratory) during the entire period of study in the early morning (6am to 8am). For analysis, water samples were transported to the Aquaculture and Limnology Research Unit, Department of Zoology, University of North Bengal, taking all precautions.

![Water samples](image)

Fig. 3e and 3f: Glass aquariums showing live fishes in captive condition

3.3.2. Fish (Wild)

Fishes were collected from different sites of the river Kaljani during the months of September, October, April and May of respective years with the help of fishermen using different types of gears namely, gill nets, cast nets, dip nets, drag nets and other locally designed fishing gears like Katal fishing gear. In Katal fishing technique, some area of the river was temporarily fenced off by bamboo and Eichhornia or Pistia sp. After a few days, these areas were covered by nets and the fishes caught by cast net. This method was applied throughout the year except monsoon.
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Botia species are less abundant, Endangered and Vulnerable whereas other genus of loach is abundant and Least Concern. Therefore, Botia species are selected as experiment fish of the present study. These loaches are high demanding species having both ornamental and food value. The fishes are very colourful with bright bands, barbles, peaceful nature, and small cycloid scales are usually immersed in mucous. Botia loaches consume different types of snails and therefore control naturally of the freshwater snail population. Four Botia loaches namely Botia almorhae, Botia dario, Botia lohachata and Botia rostrata were selected for the research study. Live fishes of Botia (Botia almorhae, Botia dario, Botia lohachata and Botia rostrata) were collected from different sampling sites of Kaljani river and immediately oxygen packed in sterile polythene bags and transported in cartons to the laboratory in the University.

3.4. Examination Procedures

3.4.1. Water Quality

Water samples for physico-chemical analysis of water were collected from the experimental areas at monthly interval during the study period. All standard procedures described in APHA (2012); Trivedy and Goel (1984) and Chattopadhyay (1998) were followed. Water samples were intensively studied during the breeding periods of selected fishes Botia almorhae, Botia dario, Botia lohachata and Botia lohachata in captivity.

3.4.1.1 Temperature

Temperature is an important parameter for growth of fish. Air and water temperatures were recorded at all sampling sites by standard mercury Celsius (°C) thermometer. Air temperature was also taken at the sampling sites avoiding direct sunlight. Water temperature was recorded immediately after collection of water.
3.4.1.2. pH (Hydrogen Ion Concentration)

pH, the concentration of hydrogen ions (H+) present is a measure of the acidic or basic property. pH of the water was measured by portable pH meter (Eutech). For measuring pH, water sample was taken in a beaker and pH meter was immersed in the beaker to record the pH.

3.4.1.3. Specific Conductivity

Specific conductivity, expressed in μS, is a measure of the ability of a solution to carry an electric current. Specific conductivity was determined by using portable Specific Conductivity meter (Eutech). Water sample was kept in a glass-beaker and conductivity meter was immersed in beaker to record the reading.

3.4. 1.4. Total Dissolved Solids (TDS)

Total dissolved solids (TDS) is the total amount of mobile charged ions, including minerals, salts or metal dissolved in water. TDS of the natural water body mainly depends upon the nature of the bedrocks. Solids are composed mainly of carbonates, bicarbonates, chlorides, phosphates and nitrates of calcium, magnesium, sodium, potassium and manganese, organic matter, salt and other particles. Total dissolved solid was determined by using portable meter (Eutech). Water sample was kept in a glass-beaker and TDS meter was immersed in the beaker to record the readings.

3.4. 1.5. Dissolved Oxygen

Dissolved Oxygen is the most important water quality parameter for respiration of fish and other aquatic organisms. Dissolved oxygen either diffuses from air into water or enters water with the help of photosynthesis by algae and plants. Dissolved oxygen was
measured by modified Winkler’s Iodometric method. Water samples were collected in BOD bottles 300ml capacity avoiding air bubbles. 2ml of Winkler’s A (Manganous sulphate) and Winkler’s B (Alkaline potassium iodide) reagents were added to immediately fix the samples. A brown coloured precipitate appeared which was later dissolved by 2 ml concentrated Sulphuric acid. The sample was then titrated against N/40 Sodium thiosulphate (Na$_2$S$_2$O$_3$) solution using starch (1%) as an indicator. The concentration of dissolved oxygen (mg l$^{-1}$) was calculated using the following formula,

$$Dissolved \text{ Oxygen} = \frac{\text{Volume of Na}_2\text{S}_2\text{O}_3 (\text{ml}) \text{ consumed} \times 0.025 \times 8 \times 100}{\text{Volume of the sample titrated}} \text{ mg l}^{-1}$$

Where,

- Molecular weight of Oxygen=8,
- Volume of the sample to be taken for titration =101.35 ml (Calculated as shown below),
- Volume = \(\frac{100\times300}{(300-4)}\) ml

Where, Original sample to be taken as per procedure = 100ml, Volume of the BOD bottle = 300ml, Total volume of Winkler’s A and Winkler’s B = 4ml

3.4. 1.6. Free Carbon Dioxide

Carbon dioxide is the end product of organic carbon degradation in almost all aquatic environments. Carbon dioxide remains in water in three closely related forms, namely (a) Free CO$_2$ (b) HCO$_3$ and (c) CO$_3$. Each remains in the water based on pH. Natural waters contain low concentration of free CO$_2$ (< 6.0mg l$^{-1}$). Carbon dioxide can be determined by titrating the sample using a strong alkali N/44 NaOH to pH 8.3. At this pH all the free CO$_2$ is converted into bicarbonates. The concentration of free CO$_2$ (mg l$^{-1}$) in 100ml sample water was analyzed using 5 drops of 50% Alcoholic Phenolphthalein
indicator and N/44 NaOH as titrant as described by Trivedy and Goel (1984). The concentration was calculated using the following formula,

$$\text{Free Carbon dioxide} = \frac{\text{Volume of N/44 NaOH solution consumed} \times 1000}{\text{Volume of the sample titrated}} \text{mg l}^{-1}$$

### 3.4. 1.7. Total Alkalinity

Alkalinity is a measure of the buffering capacity of the water which considerably maintains the pH by absorbing excess H+ ions and protects the water body from pH fluctuation. Alkalinity of water is due to the presence of hydroxides, carbonates, phosphate, nitrates and bicarbonates. Total alkalinity, that is, carbonate and bicarbonates can be estimated by titrating the sample with strong acid (HCl or H$_2$SO$_4$), first to pH 8.3 with alcoholic Phenolphthalein as an indicator and then further using Methyl Orange as an indicator (pH maintained at 4.2 and 5.4). Total alkalinity is the value of the Phenolphthalein alkalinity (PA) and Bicarbonate alkalinity (BA). Low water alkalinity, that is, less than 20mg l$^{-1}$ of CaCO$_3$ is very vulnerable to fluctuation of pH due to low buffering capacity.

Total alkalinity present in the 100 ml water samples were estimated as mg l$^{-1}$ using 5 drops alcoholic phenolphthalein and 2 drops of Methyl Orange as indicator and then titrating against $\frac{N}{50}$ H$_2$SO$_4$ (Golterman et al., 1978) and Phenolphthalein alkalinity was estimated only when free carbon-dioxide was found to be absent (Trivedy and Goel, 1984). The concentration (mg l$^{-1}$) of each alkalinity (carbonate and bicarbonate) was calculated by using the following formula,

**Concentration of Total Alkalinity**

$$= \frac{\text{Amount of titrant solution (ml) consumed} \times 1000}{\text{Volume of sample used}} \text{ mg l}^{-1}$$
3.4.1.8. Total Hardness

Total Hardness is caused by the Calcium and Magnesium ions of a water body. This is because to Ca$^{2+}$ and Mg$^{2+}$ are bound to the alkalinity bases bicarbonate and carbonate. Usually the concentrations of the total alkalinity and total hardness are similar because the calcium magnesium, bicarbonate and carbonate are derived from the solution of limestone. However, greater fish production occurs from the higher concentration of phosphorus and other essential elements that increase along with alkalinity and hardness. Calcium and magnesium form a complex wine red colour with Eriochrome Black T indicator at pH of 10.0 ± 0.1. The EDTA has got a stronger affinity towards Ca$^{++}$ and Mg$^{++}$ ions and therefore by addition of EDTA the former complex is broken down and a new complex is formed.

Ethylene diamine tetra acetic acid (EDTA) method (APHA, 2012) was followed to estimate total hardness of 100ml water samples and expressed as mg $^{-1}$. It was estimated by titrating the water sample against EDTA after adding 0.5 ml ammonium buffer and 6 drops Eriochrome Black T as indicator. The end point was indicated by blue colour. The total hardness was calculated by using the following formula,

\[
\text{Total Hardness} = \frac{\text{Amount of EDTA (ml) consumed} \times 1000}{\text{Volume of sample taken}} \text{ mg $l^{-1}$}
\]

3.4.1.9. Ammonium-N

Ammonium is the second most important water quality parameter for fish production. The source of ammonia in water is organic, inorganic and through air deposition. The most important ammonia contamination in water is excessive use of ammonia rich fertilizer, excretion of nitrogenous wastes from animals and sewage contamination in aquatic environments. Ammonia is required for life, but it is toxic to
aquatic organisms when the normal level exceeds. Ammonium concentration in water exists in two forms, (a) Unionized ammonium (NH₃) and (b) Ionized ammonium (NH₄). NH₃ is more toxic than NH₄ to fish. The amount of total NH₃ depends on pH and temperature. Recommended safe level of NH₃ –N is 0.025mg l⁻¹ and 1.0 mg l⁻¹ for total ammonium.

Ammonium-nitrogen (NH₄-N) of 25ml filtered water sample was estimated by Phenol-Hypochlorite Method (APHA, 2005). 1ml of Alcoholic phenol solution, Sodium Nitropruside solution and oxidizing solution (10ml alkaline citrate solution with 2.5 ml hypochlorite) were added to the sample. The sample was then kept for one hour at room temperature and wrapped with aluminium foil. A blue colour appeared which was stable for 24 hours. The blue compound, indophenol, is formed by the reaction of ammonium, sodium oxidising solution and alcoholic phenol catalysed by Sodium Nitropruside. The NH₄-N concentration of the sample was directly estimated through the double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 640nm wavelength. A standard curve was prepared by using stock ammonium chloride solution (0.3819g anhydrous NH₄Cl in 100ml DW) to estimate the Ammonium-N concentration of the water sample.

3.4. 1.10. Nitrite-N

Nitrite is the intermediate product of nitrification. Nitrite-nitrogen is an unstable product, formed during nitrogen cycle (nitrification and denitrification process). High amount of nitrite present in water bodies will indicate pollution. Nitrite is present in low concentration in water and culture systems. A safe level of nitrite toxicity to fish is 0.02 to 1.0 mg l⁻¹. Nitrite-N of 50 ml filtered sample was estimated by α-Naphthalamine and Sulphanilic Acid Method (APHA, 2012). 1ml of EDTA, Sulphanilic acid and α-Napthalamine hydrochloride were added to the sample. A pink colour appeared after 10
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minutes. The Nitrite-N concentration of the sample was directly estimated through the double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 520 nm wavelength. A standard curve was prepared by using NaNO₂ to estimate the Nitrite-N concentration of water sample.

3.4. 1.11. Nitrate–N

Inorganic nitrogen present in water is Nitrate-N. It is the main nutrient that accelerates the growth of hydrophytes and algae (Lodh et al., 2014). Human activities like food production, agriculture and manure disposal of domestic and industrial sewage contribute to nitrate-N. High level of nitrate is found in rural areas because of extensive application of nitrogenous fertilizers in agriculture. In urban areas, sewage water rich in nitrate contaminates surface water and thus increasing the nitrate amount (Tank, 2013; Gopalkrushna, 2011).

To estimate the concentration of Nitrate-N, Brucine Method (Trivedy and Goel, 1984) was followed. Filtered sample was taken to remove residual chloride from the sample by adding one drop of Sodium Arsenite solution. 10 ml of sample was placed in a cool water bath and 2ml of 30% NaCl solution was added. 10ml H₂SO₄ solution was added after mixing the contents thoroughly swirled by hand. 0.5 ml Brucine reagent was added and the sample was placed in hot water bath for 20 minutes. After cooling, the concentration of Nitrate-N was estimated by double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 410 nm wavelength. A standard curve was prepared by using KNO₃ to estimate the Nitrate-N concentration of water sample.
3.4. 1.12. Phosphate-P

Phosphate is in a limited source in nature and important factor for productivity of water body. Phosphate may occur in lake as a result of domestic waste, detergent and agricultural runoff containing fertilizer (Gopalkrusna, 2011). The Phosphate concentration of water sample was determined by Stannous Chloride method (APHA, 2005). 2ml Ammonium Molybdate solution (25.0 g Ammonium Molybdate + 280ml concentrate H$_2$SO$_4$ + 1000ml distilled water) and 5 drops Stannous Chloride in glycerol were subsequently added to the properly filtered 50 ml water samples. A blue colour appeared in the sample. The readings were recorded within 10-12 minutes. The Phosphate-P concentration of the samples was directly estimated through the double beam UV-Visible spectrophotometer (Ray Leigh UV-2601) at 690 nm wavelength. A standard curve was prepared by using known concentration of Phosphate-P solution (10mg P/1ml) to estimate the Phosphate-P concentration of water sample.

3.4.2. Study of the Fish Biology Parameters in Captivity (Glass Aquarium)

The Botia loaches are high demanding ornamental fishes which have very beautiful bright bands, colour and barbles. Four Botia loaches namely Botia almorhae, Botia dario, Botia lohachata and Botia rostrata were studied for the biology in captivity. To study the fish biology of Botia species in aquarium, juvenile fish (length 3 to 4 cm; weight 2 gm) were collected from the river sites and reared for 7 to 9 months to produce broodstock fish. In the rearing tanks, temperature of $27^0$ to $32^0$ C was maintained with the help of regulated water heaters (Thermostat). This temperature was good for optimum growth of loach and also gonad maturation. Broodstock can be managed in aquarium to promote gonad development. In the present study, the broodstock were fed with blood
worm, tubifex and commercially available fish food at 5 to 10% of the total body weight per day. Excess feeds were removed regularly twice in a day to prevent water contamination. The fishes were monitored regularly for morphological indicators of maturation.

3.4.2.1. Study of fish growth parameters

For this study Gonado-somatic index (GSI), gonad length with body length, body length versus body weight of the fish were determined.

3.4.2.1.1. Gonado-Somatic Index (GSI)

Gonad weight gives an easy measured quantitative record of changes in gonad condition. Thus, GSI is an indirect method of estimating spawning season of a fish species. The Gonado-somatic Index values of fishes indicate cyclical change during growth, maturation, spawning, post-spawning and resting phases of the gonads. The season change in gonad weight is more profound in female than male. To determine the relation between gonad weight and body weight it is calculated by Gonado-somatic index (GSI). Gonado-somatic Index was expressed according to the method of Vlaming (1982). This Index is calculated as

\[
\text{Gonado-somatic Index (GSI)} = \frac{\text{Gonad weight} \times 100}{\text{Total body weight}}
\]

3.4.2.1.2. Condition Factor

Condition of fish means robustness (fitness) of a cultivated fish with respect to the same species taken from other water bodies or to other species of fish taken from the same water body. The robustness is calculated by Condition Factor or “K- factor” or “Ponderal Index”. According to Mir et al., (2012), the Condition Factor is used for comparing the condition, fatting or well-being of fish, based on the assumption that
heavier fish of a given length are in better condition. Difference in the Condition Factor have been interpreted as a measure of histological events such as fat reservation, adaptation to the environment and gonadal development (Le Cren., 1951). The Coefficient of Condition (K) was calculated using Fulton (1904) expression.

\[
\text{Condition Factor or Coefficient of condition (K)} = \frac{W \times 100}{L^3}
\]

where, W= Weight in gram, L=length in cm, and 100 is a factor to bring the value of K near unity (Froese, 2006).

3.4.2.1.3. Length-Weight Relationship

The Length-Weight relationship is a standard method providing authentic biological information. It helps to calculate weight from length of fish; it is direct method of converting logarithmic growth rat into weight and provides taxonomic differences and events in the life history. To determine the Length-Weight relationship the Method of Least Squares is applied, that is, the relation \( W = aL^b \) is considered where, \( W = \) fish weight in g, \( L = \) fish length in cm; ‘a’ is a constant being initial growth and ‘b’ is the growth coefficient which is equal to or greater than 3 (Le Cren, 1951). The relationship \( W = aL^b \) or \( W = aL^3 \) (Cube Law) when converted into the logarithmic form gives the expression for the straight line \( Y = a + bX \) (Linear Regression). The Correlation of Coefficient \( (r) \) that is the degree of association between the length and weight was computed from the linear regression analysis:

\[
\log W = \log a + b \log L
\]

where, \( W \) is the weight, ‘b’ represents the slope of the line and ‘a’ is a constant.
3.4.2.2. Study of fecundity and fertilization rate of the selected fishes

3.4.2.2.1. Fecundity

The fecundity of a fish is defined, as the number of ova found in the ovary of a female fish prior to spawning. There are different methods for the estimation of fecundity. In this study, eggs were collected from three regions of the gonad like anterior, middle and posterior as the distribution pattern was not uniform. The Absolute Fecundity was calculated according to the method of Hartman and Conkle (1960) using the expressions

\[
\text{Fecundity (F)} = \frac{n \times G}{g}
\]

where, F is Fecundity; n is mean numbers of eggs in the sub-samples, G is weight of ovaries and g is weight of sub-samples.

3.4.2.2.2. Fertilization rate

After 1 hour of spawning 2 litres of water and eggs were collected from the hatchery and continued for 4 hours. Counting of the fertilized and unfertilized eggs were done. Fertilization rate was estimated by using the following formula (Udit et al., 2014).

\[
\text{Fertilization rate (\%)} = \frac{\text{Fertilized eggs x 100}}{\text{Total number of eggs in sample}}
\]

3.4.2.3. Standardization of breeding protocol of Botia species

For induced breeding of loaches, initially, 2- phenoxy ethanol @ 2ml in 20 lit. of water was used to anesthetize the fishes (Fig.3g) for easy handling prior to injection of the fish. This also prevented the fish from getting stressed. Thirty two (32) pairs of each group of matured fish were injected (Fig.3h) with different doses of synthetic hormone WOVA-FH (Biostadt India limited, Mumbai). The breeding trail was done twice. A total
of 64 pairs of fish were taken (8 pairs of *Botia almorhae*, 8 pairs of *Botia dario*, 8 pairs of *Botia lohachata* and 8 pairs of *Botia rostrata*) for the breeding experiment.

![Fig. 3g. Anesthetized fishes](image)

![Fig. 3h. Hormone being injected at pectoral fin site](image)

**Tab. 3:** Summary of the protocols of experimental Set-up and design for induced breeding of Genus *Botia* in 100 litre tanks with running water system.

<table>
<thead>
<tr>
<th>No. of Set-up</th>
<th>Species name</th>
<th>Sex ratio</th>
<th>Number of Fish</th>
<th>Dose of hormone (WOVA-FH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set-up-01</td>
<td><em>B. almorhae</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.5ml/kg</td>
</tr>
<tr>
<td>Set-up-02</td>
<td><em>B. almorhae</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.25ml/kg</td>
</tr>
<tr>
<td>Set-up-03</td>
<td><em>B. almorhae</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.025ml/kg</td>
</tr>
<tr>
<td>Set-up-04</td>
<td><em>B. almorhae</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.0125ml/kg</td>
</tr>
<tr>
<td>Set-up-05</td>
<td><em>B. dario</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.5ml/kg</td>
</tr>
<tr>
<td>Set-up-06</td>
<td><em>B. dario</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.25ml/kg</td>
</tr>
<tr>
<td>Set-up-07</td>
<td><em>B. dario</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.025ml/kg</td>
</tr>
<tr>
<td>Set-up-08</td>
<td><em>B. dario</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.0125ml/kg</td>
</tr>
<tr>
<td>Set-up-09</td>
<td><em>B. lohachata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.5ml/kg</td>
</tr>
<tr>
<td>Set-up-10</td>
<td><em>B. lohachata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.25ml/kg</td>
</tr>
<tr>
<td>Set-up-11</td>
<td><em>B. lohachata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.025ml/kg</td>
</tr>
<tr>
<td>Set-up-12</td>
<td><em>B. lohachata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.0125ml/kg</td>
</tr>
<tr>
<td>Set-up-13</td>
<td><em>B. rostrata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.5ml/kg</td>
</tr>
<tr>
<td>Set-up-14</td>
<td><em>B. rostrata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.25ml/kg</td>
</tr>
<tr>
<td>Set-up-15</td>
<td><em>B. rostrata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.025ml/kg</td>
</tr>
<tr>
<td>Set-up-16</td>
<td><em>B. rostrata</em></td>
<td>1:1</td>
<td>2 pairs</td>
<td>0.0125ml/kg</td>
</tr>
</tbody>
</table>
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Insulin syringe of 1 ml, normally divided into 40 parts, that is, 1 part is 1/40 = 0.025 ml, was used. The fish were injected at the base of the pectoral or pelvic fin and then released within an hour’s in running water of breeding tanks of 100 litres capacity. After injection of hormone WOVA-FH to the fish and the fish were transferred in the tanks. The detailed of the different Set-up of experimental protocols followed are given in Tab.3.

3.4.2.4. Pattern of behaviour of Botia species prior to breeding

Behaviour study is difficult in loach species because, Botia loaches are bottom feeders. In aquarium or tank they always try to escape to the sand or stone bottom. Botia species prefer to take rest in covered areas where sun or light does not reach. Behaviour was monitored continuously from morning to night. During breeding, in an aquarium male and female fishes were allowed to stay together and photographs were taken very quickly as they move fast to escape. At the breeding time, Botia species feel uneasy to spawn with interference of sound and light and are afraid to see moveable objects.

3.4.2.5. Embryonic development

Egg samples on hatching were examined hourly and the developing stages were documented through microphotograph. Eggs were collected as the fish were spawning. Initially photographs were continuously taken for the first two hours to capture the egg getting fertilized and the zygote stage undergoing cell division in the different stages and time frame. The standardization of rearing and culture of larvae and spawn to get healthy fingerlings were aquranched in natural habitat. The yolk sac absorbed fry were harvested and stocked in well prepared cemented tanks for further rearing. Feed was given twice daily. The monthly total length, body depth and body weight were taken.
Before breeding season the F1 generation of captive bred adult fishes were aquaranched in different rivers of Terai, West Bengal, India for conservation of loach species.

**3.4.2.6. Supplementary feed for larval rearing of *Botia* species**

Four glass aquariums, (Tank A, Tank B, Tank c and Tank D) containing 50 litres water capacity was used for feeding experimental trials (Tab. 4). Juveniles from the same parents were stocked in different experimental tanks with same stocking density ( 20 fishes per tank). Among the 20 fishes, 5 numbers of healthy juveniles of *Botia dario*, *Botia almorhae*, *Botia lohachata* and *Botia rostrata* were taken in four different aquariums for feeding experiment. In aquarium marked **Tank-A** fish were fed only commercial fish feed (Fig.4a); **Tank-B** with different types of live zooplanktons (Fig.4b); **Tank-C** only boiled minced meat (Fig.4c) and **Tank-D** only minced snail or bivalve flesh (Fig.4d) which were available in the natural water bodies. The fish were fed with experimental diet thrice a day for 45 days. Excess feed were removed regularly twice in a day to prevent water contamination. Biological filter was used in all the experimental tanks. In the rearing tanks temperature of 27\(^0\)-32\(^0\) C was maintained with the help of regulated water heaters (Thermostat). Absolute growth rate of fish were calculated by using the formula given by Rao and Kumar (2014).

\[
\text{Absolute Growth Rate} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Days of Experiment}}
\]
**Materials and Methods**

Tab. 4: Summary of the protocols of Experimental Set-up of larval rearing of *Botia* spp.

<table>
<thead>
<tr>
<th>Tank No.</th>
<th>Water capacity</th>
<th>No. of fish</th>
<th>Feeding time</th>
<th>Types of fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tank-A</td>
<td>50 litres</td>
<td>20 fish</td>
<td>Thrice a day</td>
<td>Commercial fish feed</td>
</tr>
<tr>
<td>Tank-B</td>
<td>50 litres</td>
<td>20 fish</td>
<td>Thrice a day</td>
<td>Zooplanktons</td>
</tr>
<tr>
<td>Tank-C</td>
<td>50 litres</td>
<td>20 fish</td>
<td>Thrice a day</td>
<td>Boiled minced meat</td>
</tr>
<tr>
<td>Tank-D</td>
<td>50 litres</td>
<td>20 fish</td>
<td>Thrice a day</td>
<td>Minced snail or bivalve flesh</td>
</tr>
</tbody>
</table>

Fig. 4a-4d: Plate shows different types of supplementary feeds given to *Botia* spp.
3.4.3. Histological study of the gonads of selected fish

Fishes exhibit periodic or cyclic reproductive behaviour. A thorough knowledge of maturation cycle and depletion of gonad help to understand and predict the annual changes that population undergoes. This, therefore, involves morphological and histological examination of the gonads of the fishes to be studied. For histological study, the development stages of germ cells of the testes and the oocytes in ovary were studied by the following methods (Agarwal, 1996).

3.4.3.1. Collection and fixation of tissue

For histological study, a live fish was dissected and gonad removed from the fish (Fig. 5a and 5b). The tissues were trimmed into small size for better penetration of fixatives into it. The tissues were kept in Formaldehyde Saline (Baker, 1944) for 24 to 48 hours as per size of tissues for fixation.

![Fig. 5a. Collection of ripe testes from live matured male Botia spp.](image)

![Fig. 5b. Collection of ripe ovary from live matured female Botia spp.](image)

3.4.3.2. Post fixation treatment

3.4.3.2.1. Washing and Dehydration

The tissue (testes and ovary) were removed from the fixatives and subjected to overnight washing with flowing clear tap water until the formaldehyde odour had disappeared. Water was removed from the tissue through ascending series of alcohols, starting from 30%, 50%, 70%, 90% and Absolute alcohol (100%) for 15 minutes each.
3.4.3.2.2. De-alcoholization and Infiltration

After dehydration the tissue were transferred to xylene for one hour to clear tissue from alcohol. For better impregnation of wax into the tissue, the xylene penetrated into the tissue to make it transparent and make the material to float to the top (Behera et al., 2015). In the main time, Paraffin wax kept at 60 °C and allowed to melt for infiltration of the tissue. Three changes of wax (45 min each) were made to make the tissue xylene free and then kept in the incubator overnight at 60°C.

3.4.3.2.3. Embedding

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 the water bath and the block disc filled with it. A layer of wax was allowed to be solidified at the bottom of the disc. The completely infiltrated tissues were then 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 the material in the block disc. For this reason, a heated needle or forcep was put at the upper portion or inside the wax of the disc. After proper positioning of the tissues, the wax inside the disc was allowed to solidify. The 'L' moulds were removed from the wax block after a few minutes (Behera et al., 2015)

3.4.3.2.4. Trimming and sectioning

The paraffin blocks were trimmed carefully by sharp blades. The trimmed blocks were firmly fixed to a holder and sectioning was done using a microtome. The sections
were cut at 5μm thickness. The ribbons were placed on clear glass slide smeared with egg albumin with the help of a fine brush.

3.4.3.2.5. Spreading and fixing

As the Behera et al., (2015), Glass slides were cleaned properly by chromic-acid solution, soap and finally tap water. After cleaning, the slides were air-dried and a thin layer of glycerin egg albumin was smeared over it. The ribbons were then spread over the clean glass slides with a drop of water, the thin tissues were made wrinkle free and allowed to fix on slides by keeping them on a hot plate (30 °C) for 2 to 5 minutes.

3.4.3.2.6. De-waxing and staining

Dried slides were dipped in xylene to remove the wax and rehydrated in descending order of alcohol (100%, 90%, 70%, 50% and 30%) for 5 minutes at each step. The sections were stained with aqueous Haematoxylin for 1 minute and the slides then washed with tap water to remove excess stain. The slides were dehydrated through ascending order of alcohol 30%, 50% and 70% (each step for 5 minutes). The slides were then dipped in Eosin for one minute and again dipped in 70% alcohol. The slides were then transferred to 90% and 100% alcohols for 5 minutes. At the end, slides were cleared in xylene.

3.4.3.2.7. Mounting

One or two drops of DPX (mountant) were put on the dried slide which was ready for mounting. Then, a cover slip or slide was slowly lowered over it when the mountant flowed ahead of the descending glass without trapping any air bubble between the cover slip and slide. The excess mountant on the slides was removed with xylene soaked in cotton. After mounting, the slides were allowed to dry. The excess mountant on the slides
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were removed with xylene soaked in cotton wool. Dried slides were observed under optical microscope at 10X and 40X magnifications. Photographs were taken by camera (Nikon, Coolpix L24).

3.4.4. Molecular characterization of DNA barcoding and Evolutionary relationship among *Botia* species

For taxonomic identification of *Botia* species, some fish samples were fixed in ethanol and some live fish were oxygen packed in sterile polythene bags and kept in cartons for transport to the laboratory of Molecular Biology and Biotechnology Division, National Bureau of Fish Genetic Resources (Indian Council of Agriculture Research), Canal Ring Road, Dilkusha, Lucknow. Firstly all samples were tagged with the help of tagging gun (Fig. 6a). Tagged fish image were displayed as in Fig. 6b. The fins and muscles were then collected from the sample (Fig. 6c) without skin as the skin of the fish had chance of microbial contamination.

![Fig.6a. Tagging gun](image.png)  ![Fig. 6b. Tagging fish](image.png)  ![Fig. 6c. Collection of tissue](image.png)

3.4.4.1. DNA extraction

Total genomic DNA was isolated from approximately 50 mg of pectoral or pelvic fins and muscle tissue following standard phenol/chloroform method (*Sambrook et al.*, 1989), which removed proteins and other cellular components from the nucleic acids and pure genomic DNA was obtained. Precipitated DNA was resuspended in TE buffer
(10mM tris –HCl, 0.1 mM EDTA, pH 8) with a final concentration of 100 ng/µl using Nanodrop 2000 (Thermo Scientific, USA), for all samples.

3.4.4.2. Determination of quality and quantity of isolated DNA

Estimation of the DNA concentration was done on 0.7% agarose gels in submarine gel casting units (BIO-RAD). The qualitative and quantitative estimation was done by observing the bands in ultraviolet light on UV transilluminator with UV shield and UV protective goggles. The DNA was diluted to get a final concentration of 50 ng/µl.
3.4.4.3. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is known as revolution method developed by Kary Mullis (1990). It is a powerful tool precisely because it can be done using as little as a single or few copies of template DNA (Hofreiter et al., 2001). The universal set of primers

FishF1 – TCAACCAACCACAAAGACATTGGCAC

FishR1 - TAGACTTCTGGGTGGCCAAA GAATCA.

were used to amplify the mitochondrial gene cytochrome C oxidase I (COI) (Ward et al., 2005).

Fig. 6j. COI gene PCR amplified products of Botia species

A 20 μl PCR amplification of mitochondrial gene COI was performed with 2 μl of each template DNA. The reagents procured from Bangalore Genei (Bangalore) were used in PCR reactions containing 2 μl of 10X Taq polymerase buffer, 0.8 μl of MgCl₂ (25 mM), 0.8 μl of dNTP (2.5 mM each), 0.4 μl of each primer (10 mM) and 0.4μl of Taq polymerase (3U/μl). Veriti Thermal Cycler (Applied Biosystems) was used for PCR amplification. The PCR products were visualized on 1.2% agarose gels documented using Gel Documentation system (UVP, GelDoc-It™310 Imaging System). Products with concentration between 50 to 100 ng per μl were selected for sequencing.
3.4.4.4 Sequencing

Once target sequences were selected and successfully amplified, sequence reactions were performed. Sequencing was performed following the dideoxynucleotide chain termination method (Sanger et al., 1977), using automated techniques in 3500 Genetic Analyzer (Thermo Fisher Scientific) sequencer.

3.4.4.4.1. Sequencing PCR

Products were labeled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc). Reagent quantity for one sequencing PCR reaction cocktail was Terminator Ready Reaction Mix (2.5X) 8.0 µl, BigDye Sequencing buffer (5X) 4.0 µl, PCR product (50ng/µl) 1 µl, Primer (3 µM) 1.0 µl (forward in one PCR tube and reverse in other tube), deionized water 6.0 µl, to make the total volume to 20 µl. Cycle sequencing PCR conditions was 96°C for 1 min and 25 cycles of 96°C for 10s, 50°C for 5s, 60°C for 4 min.

Fig. 6k. 3500 Genetic Analyser used to analyse DNA structure

Fig. 6l. Working mode of sequencer, sequencing the DNA
3.4.4.4.2. Sequence Editing

Both Forward (light) and Reverse (heavy) strand sequences were obtained for the Cytochrome oxidase I gene. Forward strand sequences were generally better than reverse strand. Reverse strand sequences were inverted (reversed and complimented) and aligned with the forward strand sequence. Ambiguities were referenced against the sequencing chromatograms and corrected as per necessity. Full length sequences were made from
forward and reverse strands for all samples of species and aligned using CLUSTALW
(Thompson et al., 1994). The edited sequences were blasted in NCBI Genbank for the
nearest similar sequence matches and submitted to Genbank.

3.4.4.5. Genetic distances and Phylogenetic or Evolutionary analysis

To analyze the evolutionary isolation of six species and the level of divergence
within species, K2P distance was calculated by averaging pair wise comparisons of
sequence difference across all individuals by the Kimura 2-Parameter method (Saitou
and Nei, 1987) under Gama distribution estimated in MEGA 5.1(Molecular Evolutionary
Genetics Analysis) software (Tamura et al.,2011).

The first phylogenetic tree was constructed by Neighbor-Joining method (Saitou
and Nei, 1987) based on bottom-up clustering and distance between each pair of taxa
was also calculated. The second phylogenetic tree was inferred by Maximum-Likelihood
Tamura-Nei model (Tamura and Nei, 2011) using the Maximum Composition
Likelihood approach.

3.4.4.6. DNA Barcoding of four Botia loaches

Barcode of Life Database Systems was created and is maintained by University of
Guelph, Ontario, Canada. It offers researchers with a way to collect, manage and analyze
DNA barcode data. DNA barcoding involves a four step uploading process through
BOLD systems (Barcode of Life Data Systems) Version 3. A steps involve 1) to upload
specimen data, 2) to upload image of the specimen, 3) to upload primers name and traces
of the DNA sequence and 4) to uploaded FASTA sequence of the Botia species in BOLD
systems. All the barcode sequences are deposited in the BOLD. The aim of this database
is to establish a large scale reference sequence database against which new or unknown sample sequence can be queried for species identification.

The International Nucleotide Sequence Database Collaborative (INSDC) is a partnership among the Genbank of NCBI in USA, the Nucleotide Sequence Data (NSD) of European Molecular Biology Lab (EMBL) in Germany and DNA Data Bank of Japan (DDBJ). They have recognized “CBOL” data standards for DNA Barcode records (Singh et al., 2014).

3.4.5. Survey the Ichthyofauna diversity of river Kaljani

Besides selected loaches (Botia sp.), other fishes were also harvested and preserved in 10 % solution of formaldehyde for identification to study the fish biodiversity of river Kaljani. Fish photographs were taken from fresh samples by camera (Nikon, Coolpix L24) and were identified following their general body form, morphometric and meristic characteristics according to Talwar and Jhingran (1991), Jayaram (1999) and Vishwanath et al. (2011), Conservation status of fish is given as per Conservation Assessment and Management Plan (CAMP, 1998) and International Union for Conservation of Nature (IUCN, 2010).

3.5. Statistical analysis

3.5.1. Study of correlation between body weight, gonad weight, body length, gonad length, fecundity and Gonado-somatic index

To establish the mathematical relationship between body weight, gonad weight, body length, gonad length, fecundity and Gonado-somatic Index the values of Correlation Coefficient (r) were established by using the statistical formula using MS Excel. The Scatter Diagram of different growth parameters will showed a linear relationship by using the linear regression equation Y = a+ bx, where, ‘a’ and ‘b’ are
constants and X and Y are the variables. In order to determine the strength of obtained data between length – weight relationship, Correlation analysis was performed. To show the linearity between Gonado-somatic Index among male and female, gonad length and body length, fecundity and body weight and body weight and gonad weight linear regression analysis was executed. All significant differences studied at 0.05 and 0.01 levels. Mean and Standard deviation were performed for Physico-chemical parameters to show the average and variation among the data. For the computation, software package using Microsoft Office Excel.

Fig.6o and 6p: Length and depth of fishes being measured using calipers

Fig.6q and 6r: Total weight and gonad weight of the fishes being measured