CHAPTER – 3

Materials and methods

Present investigation has been carried out during the period from March 2011 to Feb 2013. To investigate the Helminthes parasites three different host species of fishes namely, *Anabas testudineus* (Bloch), *Trichogaster fasciata* (Bloch and Schneider, 1801), and *Trichogaster lalius* (Hamilton, 1822) were examined monthly during the two years study periods. Host fishes were collected from three wetlands viz. Hasila Beel, Kumri Beel and Urpad Beel of Goalpara District, Assam.

The seasonal variation of parasites were studied by dividing the seasons as Pre-monsoon (PRM, March-May), Monsoon (MON, June-August), Retreating monsoon (RTM, September-November) and winter (W, December-February) on the basis of regional climatological changes. The topographic survey for determining the locations of wetlands were done with the help of a Portable Global Positioning System (GPS) as cited in the map (Figure-1).

The collected parasite species were photographed with the computerized “Charged Coupled Device” (CCD) camera (Labomed LX-300) and digitized. Histological sections were photographed with Leica DM 3000 Fluorescent microscope.
**Determination of Physico-chemical parameters of water:**

The physicochemical parameters determined during the study periods were water temperature, Hydrogen ion concentration (pH), Dissolved Oxygen (DO), Total alkalinity (TA) after following APHA (2005) and Chattopadhay (1998).

For the analysis of dissolved oxygen, alkaline iodide- azide reagent & manganese sulphate were added to the sample water immediately after the collection to fix the sample to examine the dissolved oxygen. Modified Winkler’s method was followed to estimate the dissolved oxygen expressed as mg.l⁻¹. The mean pH of sample water was calculated by using digital pH meter in each month of the study period. Alkalinity of a fresh water body is mainly caused by the presence of hydroxide (OH⁻¹), carbonate (CO₃²⁻) and bicarbonate (HCO₃⁻¹) ions. Collected water samples were analyzed by titrimetric method to find the Total Alkalinity where phenolphthalein and methyl orange were used as indicator solution.

Water temperature was recorded at the respective study sites with the help of 0°C to 100°C mercury laboratory thermometer in the present study. The monthly average of water temperature during the study periods in the studied wetlands were recorded in Celsius scales (°C).

**Collection and identification of Host species:**

To investigate the parasite load, host species were collected from natural water bodies. Following Jhingran (1991) and Jayaram (2010) host fishes were identified.

The three host species examined for parasite infestation are belonging to the order Perciformes and two families namely Anabantidae and Osphronemidae. They are chiefly carnivorous in feeding habit and in the aquarium the blood worms are given as supplementary food.

Kingdom- Animalia

Phylum- Chordata

Class-Actinopterygii

Order-Perciformes
Family- Percinidae

Genus-Anabas

Species- 1. Anabas testudineus (Bloch)

Family-Osphronemidae

Genus - Trichogaster

Species - 2. Trichogaster fasciata (Bloch and Schneider, 1801)

3. Trichogaster lalius (Hamilton, 1822)
Photographs of Host Fish Species
Post Mortem (PM) and Microscopic Examinations

By inserting a pointed needle into brain through the upper part of the eye fishes were killed. Oesophagus, stomach, liver, gall bladder, air bladder, kidney, eye, brain, muscle were observed under a compound microscope.

Clinical alternation indicating disease conditions were examined using magnifying lens for detection of macroscopic parasites and other abnormalities.

Size Measurement of Host Fish Species and Parasites

By graduated meter scale the total body length of the examined host fishes were measured and weight on the electronic balance (ANAMED-MX-7240DA). The measurement procedures of the parasites were followed according to Schmidt (1998). Parasites were measured by using Oculu-stage micrometer.

Freshly killed host fish species were dissected to examine the helminthes parasites in different organs. Collected parasites were examined under a dissecting microscope (Labomed) at 20x to 40x magnification (connected with CCD camera) and a light binocular microscope (Labomed LX 300) at 40x to 400x magnification.

Observation of Organs

Eyes

Eyes were dissected out by cutting the muscle and optic nerve and observed for parasites.

Gills

Gills were taken out with the help of scissors, placed in a Petri-dish and gill filaments were dissected using needle and forceps and examined under the microscope.

Abdominal cavity

Host fishes were dissected through the anus and abdominal wall with a scissor and the next incision was made perpendicular to the fish directly behind the bronchial cavity. The third cut run from the anus to the head parallel to the middle line to make the internal organs clearly visible. The amount and nature of the abdominal fluids and presence of free parasites and cysts were marked.
Digestive system

Digestive organs were separated from the mesentery. Air bladder, heart, kidneys, liver and gall bladder were separated from the stomach and examined its colour, size, borders, consistency and the presence of parasites or nodules. Spleen was noticed for size, colour, consistency and presence of nodules or cysts.

Stomach and intestine

With the help of needle and forceps the stomach and intestine were opened for parasitic examinations. Colour of the mucus, oedema, haemorrhages and inflammations were marked. After thorough examinations the stomach and intestine were made into several pieces and immersed in 2% formalin solution overnight for unnoticed parasites. Physiological solution (7.5%) was also used for two to three hours for collection of some parasites before pieces were immersed in formalin.

Muscular tissue

Several cuts were made in different parts of the body surface. Search for parasites or their cysts as well as plerocercoids, presence of hemorrhagic foci or abscesses were done. Pieces of suspected muscles were kept in 2% formalin in normal saline (0.75%) solution to find out muscle living nematodes.

Air bladder

The structure of the inner and outer surface of the wall of air bladder, its content were noticed and examined for parasites.

Kidneys

The kidneys were searched for parasite by considering shape, size, colour, consistency and parasite nodule.

Processing of parasites

Processing of parasites including preservation, staining was done by following Kennedy (1979).
Mount preparation

Scrapings from stomach and gills were mounted on a slide in water or saline and examined for Helminthes. Gills were examined thoroughly under the compound microscope. Eyes were cut under the water and lens, humour and retina were examined.

Stomach, intestine, gall bladder and swim bladder were examined with microscope for detection of parasite infection including cysts in the muscles. Organs were cut into small pieces and thin sizes of pieces. Scrapings were compressed between two glass slides and examined under microscope. For parasitic cysts, squash preparations were made from liver, kidneys and spleen and examined under microscope.

Fixation and clearing of parasites

After collection of parasites from host species, Lectophenol was used to clear the parasites from debris and unwanted tissues. Moreover use of Lectophenol increased the transparency of the parasites which enhanced the visibility of the organ of the gastrointestinal cavity.

Fixation of fresh samples of nematode was carried by hot 70% ethyl alcohol (Boiling point 78.3°C, Meyer and Olsen 1975) for straitened and preserved in 80% alcohol with a few drops of pure glycerine or in 4% formalin.

As suggested by Kennedy (1979), for permanent slide preparation, the worms were taken on the slide and fixed by glycerine alcohol in a ratio of 1:4 for at least 15-45 minutes. Air bubble, under the slide were drive out by addition of small drop of pure glycerine; the upper slide or the cover slip carefully cleaned and was framed with dense Canada balsam and kept in horizontal position.

Trematodes were pressed between two slides in a proper position and Glacial Acetic Acid (GAA) was poured between two slides with the help of a syringe and exerted gentle pressure which prevent them from shrinking. All specimens were preserved in 70% alcohol after individual treatments.

Larger sized acanthocephalans were kept in air tight glass vial containing distilled water for adequate period of time in order to complete release of proboscis to the outside of the body. Mild pressure of a needle was applied to the worm where necessary. Worms were taken in between slides and cover slips and were observed for proboscis and other parts of the body.
Required pressure was given for complete release of proboscis over the cover slip and AFA solution (85% alcohol- 85ml, Formalin commercial- 10ml, Glacial acetic acid- 5ml.) was added in order to kill and fix the specimen instantly. All parasites were preserved in 70% alcohol after individual treatments.

**Staining and permanent mount preparation of the parasites**

**Trematodes**

Alum carmine was used to stain the Trematode worms. The fixed Trematodes were transferred from mount preparation into the dye for 10 minutes up to 1 hour depending on their size, then to remove excess stain. Fixed specimen were transferred from the dye into distilled water where they were washed for several times, then exchanged with 70% alcohol in Petri-dish in which the worms were again pressed between two slides and left for several hours. Finally the worms were transferred through an alcoholic series 50%, 70%, 90% and 100%; cleared in methyl benzoate and mounted in Canada balsam.

**Nematodes**

Staining and preparation of nematode for whole mount is followed by Krustalev *et al.*, (1996). The process along with reagents is as follows

**Reagent required**

1. Absolute methanol  
2. 70% ethanol  
3. Propionic acid (concentrated)  
4. Glacial acetic carmine

**Stain preparation (Carmine – Propionic acid stain)** 3 g of Carmine + 50 ml Propionic acid + 50 ml distilled water. Preparation is known as 3% carmine solution in 50% P.acid.

For staining *acanthocephalan* species same procedure was applied that applied to stain nematode species.

**Procedure**

1. Nematodes were picked up from fixatives and placed in the stock solution of staining for 4, 12 or 24 hrs depending upon the size of the nematode.
2. After staining, specimens are distained in a solution of 1: 1 =50% acetic acid: 70% ethanol.
3. Terminate distaining by transferring specimen to non acidified 70% ethanol.
4. Specimens were mounted by standard media including lacto phenol, glycerine, and phenol alcohol.

**Ecological terms are studied as per Margolis et al. (1982)**

(i) Prevalence = \[
\frac{\text{Total no of host infected}}{\text{Total no of host examined}} \times 100
\]

(ii) Mean Intensity = \[
\frac{\text{Total no. of parasite}}{\text{Total no. of infected host}}
\]

(iii) Relative Density or Abundance = \[
\frac{\text{Total no.of parasite}}{\text{Total no.of host examined}}
\]

**Taxonomic keys for identification of parasites**

Isolated parasites from the three host fishes were identified by following the taxonomic keys of Gibson et al. (2008), Anderson et al. (2000) and Soota (1983). Identified helminth species were confirmed for their taxonomic status at the helminthological laboratory of Zoological Survey of India, Kolkata, India.

**Histopathological techniques used for permanent slide preparation**

Normal as well as infected tissue from the freshly killed host species were immediately removed from the host and fixed in 10% buffered formalin, embedded in paraffin, cut at 5-\(\mu\)m section, and stained with haematoxylin and eosin. The stained tissue were washed off in tap water and the over stained ones were de-stained and finally mounted using DPX and dried (Raymond M. Cable, 1976). Later the slides were observed under microscope and photographed.

**Estimation of total protein content of Muscle tissue in infected and uninfected host**

The amount of total protein in a given sample of fish tissue is estimated by Lowry’s Method (1951).
Reagents Required:
1. **Reagent A**: 2% sodium carbonate in 0.1 N sodium hydroxide.
2. **Reagent B**: 0.5% copper sulphate (CuSO4.5H2O) in 1% potassium sodium tartarate. Prepare fresh by mixing stock solutions.
3. **Alkaline copper solution (Reagent C)**: Mix 50mL of reagent A and 1 mL of reagent B prior to use.
4. **Diluted Folin’s reagent (Reagent D)**: Dilute Folin-Ciocalteau reagent with an equal volume of 0.1 N NaOH
5. Cold 10% & 5% TCA
6. Alcoholic ether (3: 1= ethanol: diethyl ether)
7. **Standard**: Dissolve 50mg BSA in 50mL of distilled water in a volumetric flask. Take 10mL of this stock standard and dilute to 50 ml in another flask for working standard solution. One ml of this solution contains 200 μg protein.

**Apparatus and Glass wares required**: Test tubes, Pipettes, Colorimeter, etc.,

**Preparation of tissue sample**
1. Scrape muscle tissue of infected and uninfected host and weight immediately.
2. Prepare 5% homogenate (5g tissue in 100 mL or 1g tissue in 20 mL H2O) in a homogenizer.
3. Add 1 ml of 10% TCA to 5 ml of homogenate and allow standing on ice for 10 min. Protein reacts with diluted acid and precipitate out.
4. Discard the supernatant after centrifugation at 4000 rpm for 10 min and add 2.5 mL of 5% TCA to the precipitate.
5. After centrifugation at 1000 rpm for 5 min again discard the supernatant and wash the precipitate with absolute alcohol by centrifuging at 1000rpm for 5 min.
6. Mix the precipitate with 2-3 ml of alcoholic ether and centrifuge twice each with 5 min duration at 3000-4000 rpm.
7. Discard the supernatant and invert the tube containing pallate on a tissue paper for 30 min.
8. Add 3 ml of 0.1 N NaOH and place the tube in water bath a 37°C for 3-5 hrs, till a clear solution i.e. the unknown sample obtained.

**Preparation of standard and sample tube for estimation**
1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labelled test
tubes.
2. Pipette out 1 ml of the sample in another test tube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
4. Now add 5 ml of reagent C to all the test tubes including the test tubes labelled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and allow standing for 10 min.
6. Then add 0.5 ml of reagent D rapidly with immediate mixing well and incubate at room temperature in the dark for 30 min.
7. Now record the absorbance at 660 nm against blank.
8. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 660 nm along Y-axis.
9. Then from this standard curve calculate the concentration of protein in the given sample.

Result: The given unknown sample contains ----μg protein/ml.

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**Estimation of total activity of vital tegumental enzyme in some helminth species**

The total activity of alkaline phosphatase (AlkPase) and acid phosphatase (AcPase) are estimated by following the method of Plummer (1988).

For both the enzyme estimation, principle and techniques are same except the buffer used to prepare the sample homogenate.

**Principle**

Alkaline and acid phosphatase act on phosphoric esters with the liberation of inorganic phosphate. P-Nitro phenyl phosphate is used as substrate and the p- nitro phenol released by enzymic hydrolysis is measured colorimetrically. P-nitro phenol absorbs at 405 nm. The substrate p- nitro phenyl phosphate does not absorb at this wave length so the progress of the enzyme-catalyzed reaction can be readily followed by measuring the change in extinction at 405 nm.
Materials

1. Sodium acetate buffer (pH 6.2)
2. Glycine buffer (pH 10)
3. Sodium carbonate-bicarbonate buffer (0.1 mol/litre, pH 10)
4. P-Nitrophenyl phosphate substrate solution. (5 mol/litre in the alkaline buffer)
5. p-Nitrophenol standard (50 µ mol/litre)
   This is prepared by dissolving 69.9 mg. p-Nitrophenol in 100 ml of alkaline buffer, then diluting this solution one in a hundred with the buffer. Solution 4 and 5 should be freshly prepared.
6. 0.02 N NaOH
7. Spectrophotometer
8. Cuvetts.
9. Test tubes
10. Water bath at 37°C
11. Timer
12. Pipettes and micropipettes
13. Freshly prepared sample

Sample preparation for estimation of acid phosphatase activity (AcPase)

0.5 g wet weight of helminthes is homogenized in 5ml of sodium acetate buffer (10% w/v) and centrifuged at 5000rpm at 4°C for 20 minute in a cooling centrifuge (REMI C4, India). The supernatant obtained is used as the enzyme source.

Sample preparation for estimation of alkaline phosphatase activity (AlkPase)

0.5 g wet weight of helminthes was homogenized in 5ml of Glycine buffer (10% w/v) and centrifuged at 5000rpm at 4°C for 20 minute in a cooling centrifuge (REMI C4, India). The supernatant obtained is used as the enzyme source.

Method

Test: 0.8 ml of the carbonate-bicarbonate buffer was added to 2ml of the substrate solution and mix thoroughly. At zero time, 0.2 ml sample was added. The reaction was stopped by adding 0.02N NaOH & after 15 minutes incubation at 37°C the change was followed in extinction at 405 nm
Blank: This is the same as the test except that 0.2 ml of buffer replaces the sample.

Standard: A range of p-Nitrophenol solution is prepared and a graph of extinction at 405 nm against concentration is plotted.

Calculation

The extinction of the blank is subtracted from the test and the amount of p-Nitrophenol released is calculated from the standard graph of p-Nitrophenol. One unit of AcPase or AlkPase activity is defined as that amount which catalysed the formation of 1mM of p-nitrophenol/hour at 37°C.

Effect of Helminth Infestation on Gonado-Somatic Index (GSI) and Fecundity:

To find the effect of parasitism on GSI and Fecundity, gonad weight of both uninfested and infested fish of same group (Total length and body weight) were recorded monthly during 2012-13. Gonads fill with ova or sub samples of gonads were kept in Gilson’s fluid for 3-4 weeks and then counted for fecundity.

Fecundity:

Fecundity is estimated by the following formula:

\[ F = n \frac{G}{g} \]

where ‘F’ is Fecundity, ‘n’ is the average no. of eggs, ‘G’ is the weight of Gonad and ‘g’ is the weight of sub samples of Gonad. According to the size of eggs, three sub samples from the two lobes of each ovary is taken and the eggs from each were counted under magnifying glass and mean value of eggs were computed.

Gonado-Somatic Index (GSI):

The development of the gonad can be estimated by determining its weight relative to body weight. This is expressed as follows:

\[ G = \frac{\text{Weight of gonad}}{\text{total weight of fish}} \times 100 \]
Scanning Electron Micrographs (SEM) of Some Helminthes:

Samples were processed in the following way

1. Primary fixation was done with 2.5-3% Glutaraldehyde or Karnovsky’s fixative for 24hrs
2. Helminthes were then washed in 0.1 M Sodium Cacodylate Buffer in three changes of 15 min at 4°C
3. Dehydration:
   All steps were carried at 4°C
   - 30% acetone 15min x 2changes
   - 50% acetone 15min x 2changes
   - 70% acetone 15min x 2changes
   - 80% acetone 15 min x 2 changes
   - 90% acetone 15 min x 2 changes
   - 100% acetone 15 min x 2 changes
   (Dry acetone)
4. Drying:
   - Critical point drying: Samples were tube dried with liquid carbon di oxide at its critical point i.e. 31.5°C at 1100 p.s.i.
   - TMS method: the specimens were immersed in Tetra-Methyl-Silane for 5-10 mins for 2 changes at 4°C then they are brought to room temperature (25-26°C) to dry.
5. Mounting:
   - The specimen were mounted on Brass or aluminium stubs
6. Sputter coating:
   - Coating was carried out using silver or gold, the coating should be about 35nm thick.

After processing of the helminthes SEM was carried out under JSM – 6360, Jeol scanning electron microscope.
Statistical Analysis

The effects of five main factors i.e.- seasonal variation of parasites, number of fishes examined for parasites, fish length, fish weight, number of parasites per fish were analyzed by Microsoft EXCEL tools.

Standard statistical computations (Intensity of infection, standard deviation, prevalence, abundance, density and dominance) were carried out using PAST. Correlations between different parameters were calculated with the help of SPSS (9.1 versions).