The present investigation has been carried out on cold water salmon (rainbow trout) inhibiting the lakes and rivers of Kashmir valley.

The Salmonidae is a teleost fish family of an immense economic value. Salmonids include most of the economical fishes, such as Pacific and Atlantic salmon, trout, charrs, and whitefish. The rainbow trout is known to be the most important fish species of the world. It is a salmoniform fish and it spawns only once in a year under natural conditions. In India trout (rainbow trout) was introduced by Mitchell in 1900 from Scotland in Kashmir valley. It was formerly known as *Salmo gairdneri*, but later on recognized as a member of Pacific Salmonidae, and named as *Oncorhynchus mykiss* (Kendall, 1988).

**Systematic Classification**

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Chordata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Actinopterygii</td>
</tr>
<tr>
<td>Order</td>
<td>Salmoniformes</td>
</tr>
<tr>
<td>Family</td>
<td>Salmonidae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Oncorhynchus</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>mykiss</em></td>
</tr>
</tbody>
</table>

### 3.1 COLLECTION OF MATERIALS (FISH SAMPLE)

In the present study, samples of mature rainbow trout weighing approximately 500 gm. were procured from Verinag (33.55°N and 75.25°E) and Kokernag (33.69°N and 75.22°E) hatcheries situated in Jammu and Kashmir. The fish samples were collected from June 2013 to March 2017. The collected fish were measured, weighed and dissected and the gonads and liver were taken out. Soon after dissection the fresh samples were taken to Department of Zoology, Kurukshetra University, Kurukshetra analyzed for various assays. The Gonadosomatic index as well as Hepatosomatic index was also determined.
Photograph showing Rainbow trout fish, ovary and different developmental stages of testis respectively.
3.2 MORPHOMETRIC ANALYSIS

Length-weight relationship (LWR): The relationship between length and weight of fish was analyzed by measuring length and weight of fish specimens collected from study area. The statistical relationship between these parameters of fishes were established by using the parabolic equation by Froese (2006) $W = aL^b$

Total length (TL) of each fish (n=70) was taken from the tip of the snout to the extended tip of the caudal fin nearest to 1mm by digital caliber and weighed to the nearest gram (g) by digital weighing machine.

The relationship ($W=aL^b$) when converted into the logarithmic form gives a straight line relationship graphically

$$\log W = \log a + b \log L$$

Where $b$ represents the slope of the line, $\log a$ is constant.

The relationship between length and weight for mean samples were used to calculate Fulton’s Condition Factor (Ricker, 1958) is estimated using the following equation:

$$CF = \{W/L^3\} \times 100$$

Where, $L$ is the length in centimeters (cm) and $W$ is the weight in grams (gm).

3.3 HISTOLOGICAL STUDIES (PEARSE, 1968)

For the histological studies rainbow trout gonads were fixed in Bouin’s fixative for 24 hrs and then were brought to laboratory (Kurukshetra University, Kurukshetra). The samples were thereafter dehydrated in various grades of alcohol. After proper dehydration specimens were embedded in paraffin wax at (58°-60° C). The ovaries and testis were sectioned serially at 5μm and stained with different histochemical stains. Slides were examined under light microscopy.

**Haematoxyylene and Eosin stain**

The paraffin sections were dewaxed in xylene and passed through decreasing grades of ethanol. Thereafter the sections were stained with haemotoxylene for 5 minutes and the section was allowed to develop for 5 minutes under tap water. After dehydration up to 70% of ethanol, the sections were stained with eosin for 2 minutes. After this sections were dehydrated further up to 100% ethanol and then were cleared in xylene. Finally the sections were mounted in DPX.
3.4 ULTRASTRUCTURAL STUDIES (ZAMBONI, 1976)

Transverse sections of fish gonads were fixed for 24 hours in 2.5 percent glutaraldehyde in 0.2M phosphate buffers saline at 7.2 pH. Tissue were trimmed to appropriate size (1mm), and post-fixed in 1.3 percent osmium tetraoxide. Samples were dehydrated in graded ethanol series and embedded in EPON-812 (Luft, 1961). 1μm thick sections were stained with toluidene blue and examined under light microscope to determine the section orientation. Thin sections were prepared with the help of glass knives in a porter-blum MT II ultracutE, mount on 100 mesh grids and stain with uranyl acetate and lead citrate (Reynolds, 1963). Sections were photographed under M-10 Phillips at All India Institute of Medical Sciences, New Delhi.

3.5 BIOCHEMICAL STUDIES

3.5.1 Protein Analysis

(a) Protein extraction

Gonad protein was extracted from both sexes by blending 0.5 gram of gonad with 5ml 0.1M Tris-HCL, pH 8.6 followed by mechanical homogenization. Samples of gonad protein were centrifuged for ten minutes at 4°C at 6,000 rpm. The supernatant liquid was pipetted into vials and stored at -20% until assayed.

(b) Protein estimation by Lowry’s method

Principle

Peptide bonds CO-NH in polypeptide chain reacts with copper sulphate in alkaline medium and gives a colored blue complex. The amino acids tryptophan and tyrosine of proteins causes reduction in phosphomolybdate and phosphotungstate in Folin to give blue color (Lowry et al., 1951).

Reagents:

<table>
<thead>
<tr>
<th>Solution A:</th>
<th>Sodium carbonate: 5gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide: 1gm</td>
<td>Distilled water: 250 ml</td>
</tr>
<tr>
<td>Solution B:</td>
<td>1% Copper sulphate solution</td>
</tr>
<tr>
<td>Solution C:</td>
<td>3% Na-K-tartarate (3gm in 100 ml of distilled water</td>
</tr>
<tr>
<td>Solution D:</td>
<td>Mixing of solutions</td>
</tr>
<tr>
<td>Solution A:</td>
<td>48ml</td>
</tr>
<tr>
<td>Solution B:</td>
<td>1ml</td>
</tr>
<tr>
<td>Solution C:</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>
Procedure

1. 10 test tubes were taken, out of these 3 were marked as S₁, S₂ and S₃ (Standard), while one was marked as blank, 6 test tubes were marked from T₁ to T₆.
2. 0.1, 0.2 and 0.4 ml of BSA was added in S₁, S₂ and S₃ respectively.
3. 1 ml of distilled water was added in blank.
4. 0.1 ml of sample was added in each test tube from T₁ to T₆.
5. Final volume of 1 ml was made in each test tube by distilled water.
6. After this, 5 ml of solution D was added in each test tube, and were kept for 30 minutes.
7. 0.5 ml of 1N Folin’s reagent was added in each test tube.
8. After 30 minutes, the absorbance was taken at 640 nm against reagent blank.

3.5.2 Lipid Analysis

(a) Extraction of Lipids

For the extraction of total lipids, Folch et al., (1957) method was followed. 5 gram of tissue (fish ovary and testis) was crushed in the presence of 10 gram of anhydrous sodium sulphate and a homogenous paste was obtained. Total lipids were extracted from the paste with chloroform: methanol (2:1 v/v) in the ratio of 1:15 (w/v) by intermittent shaking for at least 12 hours. The extract was filtered through G-3 sintered funnel. The filtrate was taken in a separating tunnel and was given a Folch washing with 0.9 percent normal saline (5:1 v/v) and funnel was left overnight. The total lipids were obtained in a crucible of known weight and evaporation of chloroform: methanol was facilitated. The difference in initial and final weight of crucible gave the quantity of total lipid content. The total lipids were then dissolved in 5 ml of chloroform, and was stored at 4°C and used for quantitative analysis.

(b) Estimations of different lipid classes

(i) Estimation of total cholesterol (Stadman, 1957)

Reagents

Acetic anhydride- cooled
Concentrated sulphuric acid- cooled
Chloroform
**Procedure**

Solution of lipid with amount of 0.7 ml was taken in a test tube and then 4.3 ml of chloroform was added to it to make the final volume of 5ml. After this 0.2 ml of pre cooled concentrated sulphuric acid and 1.2 ml of precooled acetic anhydride was added. Lastly the test tubes were kept in water bath at the temperature of 17-19°C and were allowed to develop color for 20 minutes. Then absorbance was taken at 625 nm against reagent blank.

A standard curve was prepared by taking cholesterol in the range of 10-80µg/ml.

**(ii) Estimation of glycolipids (Roughan and Batt, 1968)**

**Reagents**

1. 2% phenol solution
2. Concentrated sulphuric acid

**Procedure**

Lipid sample of (gonads) weighing 0.5 ml was taken in a test tube and then kept in oven at 50°C to dry the lipid sample. After this 1.0 ml of 2% phenol solution and 4ml of concentrated sulphuric acid was added to the test tube. The sulphuric acid increases the temperature of the solution to about 70°C. The test tubes were kept at room temperature for 15 minutes in order to lower the temperature of the solution. The absorbance was read at 480 nm on digital spectrophotometer against reagent blank.

The standard curve was prepared by taking 10-100µg/ml galactose.

**(iii) Estimation of free fatty acids (Lowry and Tinsley, 1976)**

Cupric acetate-Pyridine reagent

5g cupric acetate was dissolved in 100 ml of distilled water. The solution was adjusted at pH of 6.5 with pyridine added before making final volume.

**Procedure**

Lipid solution was heated at 50°C in order to evaporate the chloroform, after evaporating the sample 5ml of benzene was added. After this 1.0 ml of cupric acetate – pyridine reagent was added to the solution. The diphasic system obtained then was shaken for 5 minutes at 3000 rpm. From the two layers obtained, the upper layer was taken and the absorbance was read at 715 nm against reagent blank.

The standard curve was prepared by taking palmitic acid in the range between 1-14 µg/ml.
(iv) Estimation of Phospholipids (Ames, 1966)

**Reagents**

(1) 10% magnesium nitrate \([\text{Mg (NO}_3\text{)}_2]\)

(2) 0.5 N hydrochloric acid

(3) Color developing reagent: 10% ascorbic acid was dissolved with 6 parts of 0.42% ammonium molybdate in sulphuric acid

**Procedure**

Lipid solution of 0.5 ml was taken in test tube and then lipid solution was allowed to dry in oven at 60°C. After this 0.18ml of 10% magnesium nitrate in 95% ethanol was added to the solution and then was again kept at oven to dry the contents at 100°C. Thereafter 0.3ml of 0.5N hydrochloric acid was added in the test tube. Then test tube was kept in a boiling water bath for 15 minutes. After this the solution was kept in room temperature. After cooling the contents present in a solution, 0.7 ml of color developing reagent was added. At last test tubes were kept in water bath at 45°C for 20 minutes and the total volume was made 5ml by adding water. The absorbance was read at 820nm against reagent blank.

The standard curve was prepared by taking 2-6µg/ml \(\text{KH}_2\text{PO}_4\).

(v) Estimation of triglycerides (Vanhandel and Zilversmit, 1957)

**Reagents**

(1) 0.4% KOH in 95% alcohol

(2) 0.05 M sodium metaperiodate

(3) 0.2 N hydrosulphuric acid \((\text{H}_2\text{SO}_4)\)

(4) Sodium arsenite reagent- 2.2.5 g NaOH + 5g arsenic trioxide in 100 ml water.

(5) Chromotropic acid (224g) dissolved in 200ml water.

\(600\text{ml H}_2\text{SO}_4\) and was mixed with 300 ml of water and cooled thereafter.

Diluted \(\text{H}_2\text{SO}_4\) was mixed with chromotropic acid.

**Procedure**

Lipid solution of 1 ml was taken in a test tube containing 0.5 ml alcoholic KOH. Thereafter the test tube was kept in water bath at 40°C so that solution became warm. After this 0.5 ml of 0.2 N \(\text{H}_2\text{SO}_4\) was added to the solution and the mixture was again warmed for 15 minutes in water bath at 40°C. 0.1ml sodium metaperiodate was added to solution present in test tube and waited for 10 minutes. At last 0.1 ml of sodium arsenite solution was added to
stop the oxidation followed by 5 ml of chromotrophic acid solution. The test tubes were kept at 100°C in dark for half an hour. The absorbance was read at 570 nm.

(c) GC-MS Analysis

The GC MS was analyzed by Gas Chromatography coupled to a mass detector. 0.5 ml of gonadal tissue (Ovary and Testis) extracts were evaporated separately to dryness and final volume was made up to 5μL. The extracts were then subjected to GC MS. Inlet temperature at 280 °C, quadruple temperature at 150°C and Helium flow rate as 1.5 ml/min and ion source temperature at 230 °C. Injection was performed in the split less mode with a volume of 1 µL. The instrument was set to an initial temperature of 90 °C, and was maintained at this temperature for 5 min. After this the temperature was increased with the rate of 10 °C/min. The mass spectra of compounds in tissue samples were obtained by electron ionization at 70 eV, and the detector operated in scan mode from 60 – 800 atomic mass unit (amu). Identifications were based on the molecular structure and mass spectral matching with standard compounds in NIST library.

3.6 ENZYME ANALYSIS

3.6.1 Estimation of Acid Phosphatase (EC 3.1.3.2) and Alkaline phosphatase (EC 3.1.3.2)

Estimation of the acid as well as phosphatase was done by the method of Linhardt and Walter (1974).

Extraction:

5 grams of ovarian and testicular sample were homogenised in a chilled mortar 20 ml of chilled 50 mM Tris – HCl buffer (pH 7.6) containing 1 mM EDTA. After this the homogenate was filtered through double layers of cheesecloth and centrifuged at 20,000 g for 20 min. The supernatant was used for the enzyme assay.

Enzyme assay included 0.2 ml homogenate and 0.8 ml of citrate buffer (pH 4.0) containing 5.5 x 10-3 M p- nitrophenyl phosphate. Mixture was incubated at 40°C for 30 min. Reaction was stopped by addition of 4 ml of 0.1 N NaOH after incubation. The absorbance was measured at 405 nm on spectrophotometer.

Reagents

(1) 0.1N NaOH

(2) Substrate solution with 0.41g citric acid, 1.125g sodium citric acid and 180Mg Na-p-nitrophenyl phosphate dissolved in 100ml dissolved water
3.7 HORMONAL ANALYSIS

The sex steroid hormones (Testosterone, Progesterone and Estradiol) were analyzed using enzyme linked immunosorbent assay (ELISA). This was done by using commercially available enzyme linked immunosorbent assay kits from Cayman Chemical Company, USA. The sex steroids were determined following the assay kit procedures and methods described by Cuisset et al. (1991) and Nash et al. (2000).

Procedure:
50 µl of serum sample was added per well.
Thereafter 50 µl of progesterone/testosterone/estrogen ELISA antiserum was added in each well.
After this the wells were washed with PBS. Thereafter, 200 µl of Ellman’s reagent was added in each well.
The plate was then covered with plastic film for 90 minutes.
The bottom of the plate was cleaned to remove the fingerprints, dirt etc.
The plate was read at 412 nm.

3.8 STATISTICAL ANALYSIS

The results of analysis were subjected to one-way ANOVA followed by t-test student for comparison mean. Statistical analyses were performed using the SPSS 9.0 statistical package SPSS. The 5% level was taken as the significance level.