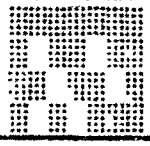
CHAPTER - II



MATERIAL AND METHODS

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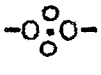

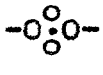

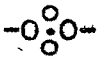

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1. MATERIAL :

As the reasons outlined in the plan of proposed research in the previous chapter for the present dissertation two species of fish were selected. One Rasbora-daniconius (Ham.), commonly called as 'Dandai' and second Cirrhina-fulungee (Sykes) commonly called as 'Mulicha gana'. Both the species were easily available in the river and tank of the Southern Maharashtra. As stated and reviewed in the introductory chapter, neither breeding cycle nor the lipids in the gonads of the above two species of fish have been carried out.

Because of the breeding cycle of R.daniconius and C. fulungee is not known, so the one of the positive point of the present dissertation is to describe the breeding cycles of the above two species. The breeding cycle of the above two species was worked out using histological studies of the gonads, for complete one year. Accordingly, the breeding activity the cycle is divided into four periods, as follows -

i) Pre-breeding/Pre-spawning period - From March to May

ii) Active breeding/Active spawning period - From June to August

iii) Post breeding/Post spawning period - From September to November

iv) Quiescent period - From December to February

i) Pre-breeding/Pre-spawning period ranging from March to May. During this period the testes exhibited cysts full of developing stages of spermatocytes, especially the secondary spermatocytes and spermatids. The interstitium was slightly reduced. In the pre-spawning females Oocytes were observed in various stages of development. Each Oocyte was encircled with a layer of follicular cells.

ii) Active breeding/Active spawning period commence on beginning of the monsoon i.e. from June and comes to end in August. The testes in the active breeding period exhibited cysts with lumina full of sperms and reduced interstitium. Ovaries in the active spawning period contained numerous spericle follicles each with mature ovum full of yolk and follicular cell layer.

iii) Post-breeding/Post-spawning period begun at the end of active breeding period i.e. from September extend to November. In post-breeding fishes, the sperms were found to have been evaculated from all the tubules, which hence appeared empty. In few tubules sperms and residual bodies were seen. In the post-spawning females, the ovaries were in regressed condition with few atretic follicles, germinal epithelium and enlarged stroma with connective tissue separated by variable gaps.

iv) Quiescent period onset from December to February. During the sexual quiescence, the testes were atrophied and reduced

to minimal size. The cysts (ampullae) were found empty except for the germinal epithelium and one or two layers of spermatogonia. The interstitial tissue between cysts was prominant. In sexually quiescent females, the ovaries contained few atretic follicles germinal epithelium and enlarged stroma.

As already reviewed in the introductory chapter no detail histochemical or biochemical studies on lipids in the gonads of these seasonally breeding fishes have been carried out.

Both the species of fish R.daniconius and C.fulungee, were collected from the Rajaram tank, near the Kolhapur city (Maharashtra, India). Fishes were brought on 15th day ± 2 days of every month since August 1989 to July 1990.

At a time 20 males and 20 females were collected and brought to the laboratory. They were kept in laboratory in glass containers for about two to three hours for acclimatisation. The average length of adult males and females ranged upto 8 to 11 cms. R.daniconius is brilliantly coloured when living with purple and green irridations, and has a black band running the whole length of the body, from the eye to the end of the tail, while C.fulungee is an brilliantly silvery coloured fish with smaller scales on the body and snout overhangs the mouth, lips are smooth.

The live fishes were dissected out from ventral side to expose the gonads. Both testes and ovaries were taken out,

blotted with blotting paper, weighed accurately and utilised for lipid study, some pieces of testes and ovaries were also fixed for histological details, to confirmed the breeding cycle.

2. CHEMICALS :

Each and every solvent used in the present research work were Analar (AR) Grade and were obtained from E.Merck and B.D.H. England. Solvent included Chloroform, Methanol, Hexane, Diethyl-ether etc. All solvents were redistilled in the laboratory under the ideal conditions before use.

a) Reference lipids :

During the present investigation following reference lipids were used -

Phosphatidyl choline, Phosphatidyl inositol, Spingomyelin, Phosphatidyl serine, and Phosphatidyl ethanolamine. The cholesterol, cholesterol ester, dipalmitin, oleaic acid, triolein, palmitic acid, n-tetracosane were obtained from Sigma chemical Co.,U.S.A. Other various chemicals used in detection techniques of lipids and assay methods were also analar grade, obtained from B.D.H. England.

b) Methods :

The extraction of lipids for the biochemical studies clearly depends upon a advanced methods, the lipid extraction from their natural source is carried out without any marked alterations. Entenman (1960), put forth various recommendations

in his article to carry out maximum extraction, of lipids with negligible alterations in its chemical structure. In last thirty years remarkable rapid advances occurred in chromatographic techniques of lipid analysis. Macek et al. (1968,1972) studied the thin layer chromatography and survey of applicants which is available. Marinetti (1967) has also discussed critically the developments in chromatographic analysis of lipids. Stahl (1969) put forth the quantification techniques of lipids. Skipski and Barclay (1969) have reviewed the details in various methods employed for quantitative analysis of neutral lipids and Skipski et al. (1964) have advanced the techniques of phospholipid determination.

3. BIOCHEMICAL TECHNIQUES :

i) Extraction of lipids :

The extraction and purification of lipids were carried out by using Floch's improved method (Floch et al.1957). Though some of the workers have tried to select a reliable method for complete extraction and purification of lipid moieties from the tissue by studying comparatively the existing methods (De. Longh and Pelt,1962), to our experience the method of lipid extraction by Floch et al.(1957) was found to be rapid, convenient and reproducible and also resulted in complete extraction of the lipids without degradation.

The fishes were dissected out ventrally and the reproductive organs as mentioned earlier were taken out. The tissues

were homogenised with 2 volumes of chloroform - 1 volume methanol (2:1 v/v) at room temperature the homogenate were allowed to stand for 2 to 3 hrs at 4°C and then filtered through the sintered funnel into a glass stoppered container, the precipitate was rehomogenised with 10 ml of chloroform - methanol mixture (2: v/v) and then filtered through the sintered funnel. Both the filtrates were pooled together and the resultant mixture was shaken well with 0.2 volume of glass-distilled water. Extracts were allowed to partition in to two distinct phases. The upper phase which generally contained the major part of the non-lipid contaminants was removed as completely as possible with a fine tipped pipette. The lower phase which mainly contained lipid fraction was transferred qualitatively through sodium sulphate to remove water completely from the lipid sample, then more chloroform was added to remove any lipid fraction from the sodium sulphate. Then it is transferred quantitatively into a glass stoppered container and evaporated under vaccum at 40°C. The lipid sample thus obtained was weighed and preserved in desication under vacuo at - 20°C for further use.

ii) Thin layer chromatography of neutral lipids :

a) Preparation of plates :

20 grams of silica gel G (about 200 mesh. containing $CaSO_4$ as a binder, E. Merck, Germany) was slurried with 40 ml of distilled water. The slurry was transferred to the applicator immediately and applied to the plates - (20 x 20 cm)

setting of the adjustable applicator was done at 0.25 mm, the plates were activated by heating in an oven at 110-115°C for 60 minutes. Plates are coiled and preserved in a desicator for further use.

b) Application of sample :

The edges of the plates were trimmed of excess silica gel. To each chromatoplate, lipid extracted from tissues and mixture of reference lipids of known quantity dissolved in chloroform were applied with Hamilton micro syringe (No.8206-B) 2.5 to 3.0 cm. from the bottom edge of the plates. The amount of standared compounds ranged from 5 to 25 µg, whereas, the total lipids of the tissues extracts applied ranged from 200 - 500 µg.

c) Development of chromatogram :

The chromatographic chambers (length 25 cm hight 30 cm and width 10 cm) were prepared 20 minutes before the insertion of the plates. The chambers were lined on three sides with Whatmann No.3 filter paper wetted with developing solvent. One step development system was followed, the plates were developed in haxane (b.p. 65-70°C) diethyl-ether, acetic acid (85:15:2 v/v) solvent system, as recommended by Gloster and Fletcher (1966). The solvent system was allowed to move approximately 13.50 cm from the bottom of the plate (approximately 40-45 minutes). The plates were pooled out, dried at room temperature.

d) Detection and identification of spots :

40% H_2SO_4 spray (Privett and Blank, 1962, Skipski et al. 1963) Dichromate sulphuric acid spray (Blank et al.1964), Iodine vapour (Sims and Larose,1962), 2.7-dichlorofluorscein spray (0.2% in ethanol) Mangold and Malins, 1960, Mangold, 1961) were used for general detection of all lipids. Spots on chromatogram of tissue extracts were identified according to their positions with respect to reference lipids. Identification of individual neutral lipid components was carried out by using specific chemical tests directly on the plates. The detection of cholesterol and cholesterol-esters was further confirmed by employing antimony trichloride spray (Weicker,1959) whereas, that of estrified fatty acids was confirmed by hydroxyamine ferric chloride spray (Weicker,1959).

e) Quantitative analysis of neutral lipids :

(i) Elution of lipids

The elution of lipids from silica gel scraping was performed separately for different classes of lipids. To test tubes containing silica gel with free fatty acids, triglycerides and diglycerides 5 ml of diethyl-ether were added. The tubes were vigorously shaken for ten minutes at room temperature, and were centrifused for 15 minutes (3000 g), diethyl-ether was removed with pipettes and the elution step was repeated with another 5 ml portion of diethyl-ether in a similar manner. Both the diethyl-ether elutes were pooled and filtered through a sintered glass

funnel. To the silica gel, 3 ml of diethyl-ether were added and again the tubes were vigorously shaken. The whole suspension was then transferred to a funnel with fine sintered glass and filtered.

Cholesterol esters, cholesterol and monoacylglycerides were eluted by the addition of 5 ml of chloroform-methanol (4:1 v/v) to the test tubes containing silica gel with adhered lipids. The tubes were shaken vigorously for 10 minutes. The elutes were removed with pipettes. The elution procedure was repeated twice in the same manner and three elutes were combined. The elutes of mono-acylglycerides were filtered through a fine sintered glass funnel to remove any traces of silica gel.

(ii) Analysis of lipids in the elutes

Lipid elutes were chemically analysed. The amount of triacylglycerides, diacylglycerides and monoacylglycerides in the elutes were estimated according to the method of Antonis (1960) modified by Viogue and Holman (1962). Each test tube with elutes was treated as follows :

Each tube is evaporated at 65 to 70°C till dryness. To the dried elutes add 3 ml di-iso-propyl-ether. One ml hydroxylamine-hydrochloride, wait for 20 to 25 minutes and add 6 ml of ferric perchlorate solution then colour obtained was measured after 5 minutes at 520 µm in Spectronic 20 - Bausch and Lomb Spectrophotometer against the solutions containing graded known amounts of Methyl palmitate which were treated as standards for colour development.

Cholesterol esters and free cholesterol were analysed by the method of Abell et al. (1952). The elutes from the cholesterol-esters and cholesterol scrappings were evaporated to dryness. Before that the cholesterol-esters were converted into the free cholesterol. After cooling to room temperature the tubes were stoppered with clear dry corks and were taken for colour development with the modified Liebermann-Burchard, reagent. The standard cholesterol solutions were also treated simultaneously in a manner identical to that of samples. The tubes containing dry residues from the samples and the standards and a clean empty tube to receive blank were arranged in a series, 6 ml of modified Liebermann-Burchard reagent were added first to the empty tube and then at regular interval to the other samples care was taken to wash down the entire inner surface of the tube with the reagent. The tubes were tightly corked, shaken and returned to the bath. The optical density of each sample was read against the blank in Spectronic 20 at 620 mμ. 30-35 minutes after the reagent was added.

The amount of free fatty acids were determined by the method of Itaya (1977). The clutes of free fatty acids were evaporated to dryness in tube at 65 to 70°C. The containt of test tube was redissolved in 10 ml chloroform. After proper shaking the tubes, the tubes, the containt is divided into two stoppered conical flask (5 ml in each flask) 2 ml copper TEA reagent was added in each flask, shake well and wait for 30 to 60 minutes. Take out 2 ml of lower chloroform layer in a clean

dry test tube (there should not be any Cu^{++} contamination). Add 1.5 ml diphenyl carbazide and diphenyl carbozone reagent in each test tube, shake the test tubes properly. The optical density of each sample was read against the blank in Spectronic 20 at 550 mμ.

So as to carry out proper confirmation of the results the assay of individual neutral lipid components was carried out in three different sets of tissue.

4. THIN LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS :

The basic procedure of thin layer chromatography of phospholipids originated from the work of Schlemmer (1961) and Wagner et al. (1961). These two laboratories introduced the use of silica gel G and the solvent system of Chloroform : Methanol : Water with the neutral chromatographic plates many a time one of the prominent phospholipid component of animal tissue, Phosphatidyl-serine is not properly separated. Under such circumstances the use of basic plates has been recommended by Skipski et al (1962), So to avoid trouble with phosphatidyl-serine, in the present investigation separation of phospholipids has been carried out on the neutral plates using silica gel H (about 200 mesh. without binder BDH, England). Rest of the conditions for preparation of plates were maintained as described earlier.

A) Development of chromatograms :

One dimensional one step thin layer chromatography was followed for the separation of phospholipids in the reproductive organs. The chromatographic chambers (length 28.5 cm, height 34 cm and width 11 cm) were lined on three sides with Whatmann paper No.3, wetted with developing solvent. The selection of suitable solvent system was made by taking several trails with the solvent systems recommended by various workers (Schlemmer 1961, Wogner et al. 1961, Dittmer and Loster 1964, Peterwood et al. 1964, Ways and Nanham, 1964, Skipski et al. 1964, Wuthier 1966 and Skipski et al.1967).

During the present investigation solvent system recommended by Skipski et al. (1963), for the separation of phospholipids was slightly modified i.e. the concentration of methanol was lessened and the volume of methanol was maintained nearly more than half of that of chloroform. So chloroform-Methanol-Glacial acetic acid-water in proportion, 50:26:10:5 v/v, were found to form a suitable solvent system for the reproductive organs. All the phospholipid components from the tissue samples were found to ressolve by this solvent system and all the components were found to be quite distinct from each other without tailing.

B) Detection and identification of spots :

The detection of phospholipids on dried plates was made by exposing the plates to the iodine vapour (Sims and Larose, 1962). The phospholipid spots were further identified by employing

the following sprays : Rhodamin 6G - (Witter et al. 1957) molybdanum blue spray (Dittmer and Lester,1964), Vasakovasky's modified spray - (Vasakovasky and Kostesky,1968) all for general phospholipids. Ninhydrin (0.2% in acetone lutidine, 9:1 v/v, Skipski et al. 1962). Dragendroff reagent (Wagner et al.1961). For the choline phospholipids, P-benzoquinone for phosphatdyl-ethanolamine, ammonium silver nitrate spray and mercuric oxide barium acetate spray for inositol. Colox-benzidine spray for sphingomyelin (Bischel and Austin, 1963), Skipski et al. (1967). Details of these sprays and their diagnostic importance in the thin layer chromatography are critically described by Marinetti (1962),Krebs et al. (1969) and Skipski and Barclay (1969).

C) Quantitative analysis of phospholipids :

a) Elution of phospholipids :

Each phospholipid was eluted from the silica gel by suspending the powder in the eluting solvent by vigorously shaking the tube. The first and second elutions were performed with the solvent mixture, chloroform-methanol-acetic acid-water (100:50:10:4 v/v) by using 3 and 2 ml portion for each elution. To facilitate the elution process the silica gel suspension in elution solvent was shaken for 10 minutes. The samples were then directly transferred to the digestion tube. All the sample elutes prior to digestion were evaporated to approximately 2 ml.

b) Phosphorus determination :

Bartlett's method (Bartlett,1959) modified by Marinetti (1962) was followed for the estimation of lipid phosphorus. Samples eluted from thin layer chromatograms were digested with 0.9 ml of 70% perchloric acid. Digestion was carried out for 15 minutes on a medium gas flame. The tubes were cooled, 7 ml of distilled water, 1.5 ml of 2.5% ammonium molybdate and 0.2 ml of the amino-napthol reagent described by Bartlett (1959) were added. The tubes were placed in boiling water for exactly seven (7) minutes, removed and allowed to cool for 20 minutes. The optical densities were determined at 830 mμ employing Bausch and Lomb Spectrophotometer. Approximate blank and standards were also run simultaneously.

For the determination of lipid phosphorous from total lipids, Marinetti's semimicrophosphorus method was followed (Marinetti,1962). Digestion was carried out with 0.9 ml of 70% perchloric acid and two drops of nitric acid for 15 minutes. After the flasks were cooled, 7 ml distilled water were added. To each flask were then added 1.0 ml of 2.5% ammonium molybdate and 1 ml of elon reagent. The solutions were mixed and left at room temperature for 30 minutes. The optical density of each solution was determined at 820 mμ by using Spectronic 20. The technique using elon as suggested by Marinetti (1962) was found to be more suitable than the technique using amino-naphthol-sulphonic acid in the semimicro analysis of the total lipid

phosphorus. This confirmed the earlier observations on the rat alimentary tract lipids (Varute and Sawant,1972) and on seasonally breeding vertebrates gonads (Pawar,1978). The values of phospholipids were calculated in terms of mg/g wet weight of the tissues by multiplying the phosphorus values obtained by a factor of 25.

For the confirmation of the results the thin layer chromatographic separations and the assays of the total and individual phospholipids were carried out in three different sets of the same tissue, and the average values were taken for consideration of the problem of alterations in the reproductive organs during the seasonal breeding cycles.