3. MATERIALS AND METHODS

3.1 Animal

H. fossilis (common name - singhi) is a freshwater species of India, Burma, Pakistan, China and Sri Lanka. Body of the fish is elongated, dark leaden brown in colour (youngs are redish) and devoid of scales. The dorsal fin is small and commences in the anterior 1/3 of the body, the ventral fin reaches upto the third or fourth anal ray, pectoral fin with spine is serrated internally with a few serrations at its anterior end externally. It is 2/3 as long as the head. Anal and caudal fins are separated by a distinct notch. It frequent the surface of water to gulp in free air and is capable of outside water for several hours. The systematic position of H. fossilis according to Berg (1940) is as follows:

Phylum	Chordate
Sub-phylum.....	Vertebrata
Super-class.....	Gnathostomata
Series.....	Pisces
Class.....	Teleostomi
Sub-class.....	Actinopterygii
Order.....	Cypriniformes

Division ..... Siluri

Sub-order..... Siluridae

Family..... Heteropneustidae

Genus..... Heteropneustes

Species..... fossilis

3.2 Collection, acclimatization, stocking and maintaining of fish

Adult H. fossilis around 40 g body weight and 18 cm length were collected from the ponds in the vicinity of Jaunpur city, India. They were stocked in rectangular cemented aquaria (4' x 3' x 2') supplied with tap water. Before stocking, aquaria were washed with 2% solution of potassium permangnate to remove fungi and bacteria on the inner walls of the containers. After 24 hours male and female fishes were seperated on the basis of presence of anal papilla in male. Female fishes were used in the present study and were treated (by immersion) with 0.6% physiological saline to protect them from fungal and bacterial infections and then kept in separate aquaria. The saline treatment was repeated once a week during acclimatization for three weeks. For the experimental studies only acclimatized fishes were used and they were kept in groups in seperate aquaria. The details regarding the number of fish in a group, size of aquaria and the amount of water kept in them are mentioned seperately in each experiment described later. Fishes were allowed to feed on a compound ground mixture of goat liver, dry prawn and wheat flour ad libitum daily during acclimatization and through out the experiments unless otherwise mentioned. After feeding, remaining feed were taken out and water of the aquaria was replaced by fresh tap water.

3.3 Histological techniques

3.3.1. Evaluation of thyroid activity

Thyroid activity was measured according to method adopted by Fortune (1955) in some teleosts. We deeply realise that such histological method of evaluating thyroid activity is relatively old technique and has its own limitations for which severe criticism has come up these days regarding the validity of the results. However, because of the lack of necessary facilities for more recent methods of measuring thyroid activity like radioiodine uptake or radioimmunoassay techniques in our laboratory, I have followed the histological methods as adopted by Fortune (1955) for which we sincerely apologise. Nevertheless, it would be seen from this study that Fortune's method as adopted here is a sensitive one and reflects the variations in thyroid gland changes well under various natural and experimental conditions.

The lower jaw of fish, containing thyroid follicles were fixed in Bouin's fluid. Decalcification of thyroid region was achieved by changing the fixative twice a week for three weeks. After decalcification, paraffin sections were cut at 5 µm thickness by rotary microtome and stained with PAS-Haematoxylin and Heidenhan's Azan techniques. Camera lucida drawing outlines of 50 thyroid follicles of all sizes were drawn at 150 times magnification on a standard paper. They were then cut out and weighed. Epithelia outlines of weighed paper were also cut out and weighed. Finally, the ratio between the total epithelial weight and total follicular

weight of the paper cutting was taken as thyroid activity index (TAI).

3.3.2 Ova-diameter measurements

The diameter of oocytes was measured with the help of oculometer as adopted by Prabhu (1956) and Dixit (1960). The ovary of fishes were fixed in Bouin's fluid for 24 hours, parrafin sections were cut at 5 µm thickness and stained with Haematoxylin and Eosin. All the oocytes in the sections of the ovary were not symmetrical due to action of fixative and the collateral pressure exerted by the developing oocytes. Therefore, to minimise the error, only round and oval oocytes were considered in this study. Average diameter of 100 largest ova from each fish were recorded for ova-diameter measurements.

3.3.3 Evaluation of thyrotrops in the pituitary

The pituitary gland was exposed by cutting and lifting the parasphenoid bone from the posterior side and then immediately fixed in Bouin's fluid. After 12 hours the pituitary alongwith brain was carefully separated from the cranium and again fixed in fresh fixative for 24 hours. The paraffin sections were cut at 5 µm thickness and stained with Mallory's triple staining method and studied with compound light microscope. Prasada Rao (1972), Baker et al. (1974) and Haider (1978) made attempts to differentiate gonadotrops and thyrotrops in the pituitary of H. fossilis under various natural and experimental conditions at light microscope level. All of them agree that gonadotrops are large basophils and localize in most part of the proximal pars distalis (PPD) alongwith acidophils. However, regarding the topography of thyrotrops, there

is difference of opinion among these workers. Prasada Rao (1972) observed smaller basophils as thyrotrops localised in the ventral and lateroventral region of the PPD. Baker et al. (1973) reported these thyrotrops in the centrally situated neurohypophysis and in the pars intermedia (PI). Haider (1978) described that thyrotrops have a tendency to migrate in all parts of the pituitary if the fish is treated with radioiodine (^{131}I). In the present investigation smaller basophils were thus taken as thyrotrops while larger ones as gonadotrops. However, such thyrotrops were observed in small number as compared to gonadotrops and also thyrotrops were found to be present in all regions of the pituitary. The activity of thyrotrops were judged using degranulation as the criteria for high activity or stimulation.

3.4 Biochemical techniques

3.4.1 Protein estimation from fish tissue

Protein was estimated according to method of Lowry et al. (1951). A known weight of fresh tissue (50 mg) was homogenised with 1 ml of cold 10% TCA (Trichloroacetic acid) with electric homogenizer (Remi, India) for 10 minutes and centrifuged at top level in laboratory centrifuge (Remi, India) for 10 minutes. Supernatent was discarded and precipitate was washed with 1 ml of 5% TCA and centrifuged again for 10 minutes. Thereafter, residue was dissolved in required volume of alkaline solution (1 N NaOH). 0.5 ml of this solution was taken in a test tube and it was mixed with 5 ml of freshly prepared protein reagent (100 ml of 4% sodium carbonate + 1 ml of 2% copper sulphate + 1 ml

of 4% sodium tarterate). After few minutes, 0.5 ml of normal folin reagent was added in the test tube and allowed to stand for 10 minutes, optical density was then measured at 620 nm with spectrocolorimeter (Systronics, India). The concentration of the protein was calculated from the standard curve which was prepared by 100, 200, 300, 400 and 500 µg bovine serum albumen/ml alongwith the unknown sample and subjecting them to same procedure. The standard curve was linear.

3.4.2 Glycogen estimation from fish tissue

The colorimetric method of Kemp and Adrienne (1954) as modified by Srinivasan and Krishnaswamy (1961) was adopted for the estimation of total glycogen. A known weight (50 mg) of tissue was homogenised in 1 ml of cold 10% TCA and centrifuged at the top level in a laboratory centrifuge for 10 minutes. The supernatant was collected in the test tube and pellet was ground with 1 ml of 10% TCA and centrifuged as before. The pellet obtained was again ground with 1 ml of 10% TCA and centrifuged as before. The three supernatant fractions were pooled and the final volume was measured. 1 ml of supernatant was taken in a test tube and 6 ml of concentrated sulphuric acid was added to it. The tube was kept in boiling water bath for exactly 6.5 minutes. The optical density of the solution was measured with spectrocolorimeter at 530 nm. The concentration of the glycogen was determined from the standard curve which was prepared by 100, 200, 300, 400 and 500 µg/ml alongwith the unknown sample and subjecting them the same procedure. The standard curve was linear in this range.

3.4.3 Moisture estimation from fish tissue

For the determination of water content, preweighed tissues were dried in a hot air oven at 100°C. Weight of the dried tissues were taken at the interval of 12 hours until constant weight were obtained. Moisture content was then calculated by the taking out the difference of the weight of fresh and dried tissues.

3.5 Measurement of gonosomatic (GSI) and hepatosomatic indices (HSI)

GSI and HSI were calculated using the following formulae:

$$GSI = \frac{\text{Weight of gonad}}{\text{Weight of body}} \times 100$$

$$HSI = \frac{\text{Weight of liver}}{\text{Weight of body}} \times 100$$

3.6 Method of hypophysectomy

The pituitary of *H. fossilis* is situated at the level between first and second gill clefts in the floor of the cranium above the parasphenoid bone and attached with a short stalk. Hypophysectomy was performed using the method of Singh (1969). Fishes were anaesthetized with MS 222, Sandoz Switzerland, (0.07 g/litre). It was then placed on cotton wool which is fixed on a groove of a drawing board. The fish was kept belly upward in position with the help of rubber bands. During the operation, the fish was slightly tilted on one side, second gill cleft was opened with the help of stretching pins. A fine incision was made in buccal epithelium in mid longitudinal position and at that place buccal epithelium was stretched with the help of

forceps and the pituitary gland became clearly visible. The parasphenoid bone was then drilled smoothly at the place of pituitary by hypophysectomy drill and the pituitary was sucked out with a rubber tube having slightly large diameter than that of the pituitary. After the operation fishes were transferred to aquaria containing 0.6% physiological saline for two days to prevent fungal and bacterial infections. Thereafter, fishes were kept in tap water for six weeks to ensure complete ovarian regression. At autopsy pituitary was checked individually and cases of incomplete or partial hypophysectomy were eliminated from the present investigation.

3.7 Method of ovariectomy

Surgical method was adopted to remove the ovaries. Fishes were anaesthetized with MS 222 as before. They were placed on cotton wool in belly upward position using rubber bands. A ventrolateral incision of 1 to 1.5 cm, anterior to anal aperature was made with the help of fine scissor and abdomen was kept wide open with the help of stretching pins. The ovary was quickly removed with forceps and incision was sealed with silk thread. Following operation, fishes were transferred into 0.6% physiological saline and treated with penicillin powder to avoid bacterial infection. The infection was further checked by not providing the food for one week during the course of which wound healing occurred naturally. Complete removal of ovary was checked at the time of autopsy and any incomplete or partial cases were discarded from the present investigation.

3.8 Statistical analysis

The degree of relationship between two variables was measured by correlation coefficient (r) and significance of 'r' was checked by student t-test. The statistical significance between the means of treated and control groups was also calculated by student t-test and P value < 0.05 was taken to be significant.

3.9 Experimental studies

3.9.1 Annual changes in thyroid gland in relation to ovary and liver of H. fossilis

Twenty five adult specimens of H. fossilis (body weight, 38-42 g and length, 17.5-18.5 cm) were locally collected around 15th of every month for one complete year from ponds in and around Jaunpur city. They were brought alive to laboratory.

Ten fishes were rapidly sacrificed and their lower jaw containing thyroid region and ovary were fixed in Bouin's fluid for the study of thyroid activity index and ova-diameter measurement, respectively.

Another batch of 15 fishes were decapitated and their ovary and liver were taken out, quickly washed with cold 0.6% physiological saline, blotted to remove excess saline and blood, weighed separately and stored at -20°C until biochemical analysis were made. From these, tissues (both ovary and liver) of five fishes were analysed for the determination of protein, those of another five fishes for glycogen and of the remaining five fishes for moisture contents according to the methods described earlier. Total

weight of ovary and liver from these fishes were recorded separately for the measurements of GSI and HSI.

3.9.2. Effects of thyroid hormone on ovary and liver of H. fossilis

Hormones and drug

Thyroxine : L- thyroxine (T_4) was bought from Sigma Chemical Company USA. 20 mg T_4 was dissolved in 5 ml of 0.1 N NaOH and required volume was made up by adding 0.6% saline solution.

Thiourea : Thiourea was purchased from BDH, India. The required concentration of the drug was made in 0.6% physiological saline.

Gonadotropin : Salmon gonadotropin (SG-G100) was obtained as a gift from Dr. E.M. Donaldson, Vancouver, Canada. It was dissolved in 0.6% saline. Mixture of T_4 and SG-G100 was prepared by addition of equal volume of solution of same concentration of both hormones.

Prior to treatment, fishes were lightly anesthesised with MS 222 (0.07g/litre). The different hormones and drug (the stock solution of hormones and drug used in this investigation were kept at -20°C and were brought to room temperature and then diluted to the required concentration at the time of treatments) were administered through intramuscular injection in 0.1 ml carrier vehicle.

Following two experiments were performed during the months of December, January and February (late post-spawning to early preparatory phase).

Experiment - 1: Effects of thyroxine and thiourea on ovary and liver of intact fish

Sixty adult H. fossilis were divided into three groups of 20 each and placed in separate glass aquaria of 2' x 1' x 1' size containing 30 litres of tap water. They were subjected to following treatments on alternate days throughout the duration of the experiment.

Groups	Treatments
I	0.1 ml saline/fish (control)
II	0.05 μg T_4/g body weight
III	5.0 μg thiourea/g body weight

Experiment - 2: Effects of thyroxine, thiourea, salmon gonadotropin and thyroxine in combination with salmon gonadotropin on ovary and liver of hypophysectomised fish

Fishes were hypophysectomised according to the method described earlier. Six weeks after hypophysectomy fish were weighed. The fish which did show an increase in weight or no change were taken as incompletely hypophysectomised and rejected. The completeness of hypophysectomy was also checked occasionally by autopsy at different sampling intervals of the experiments. 100 fish were divided into 5 groups of 20 each and kept as described earlier in experiment-1. They were given following treatments on alternate days throughout the duration of the experiment.

Groups	Treatments
I	0.1 ml saline/fish
II	0.05 µg T_4/g body weight
III	5.0 µg thiourea/g body weight
IV	0.05 µg SG-G100/g body weight
V	0.05 µg SG-G100 + 0.05 µg T_4/g body weight

Twenty sham operated fish received comparable quantity of saline only which served as control.

The fish of each group of both the experiments were sampled after 2 and 4 weeks intervals. They were decapitated 24 hours after last injection. The lower jaw containing thyroid region and ovary of 5 fish of each group were fixed in Bouin's fluid for the measurements of thyroid activity index and ova-diameter measurements respectively using the methods described earlier. Ovary and liver of remaining 5 fish were quickly washed with physiological saline, blotted to remove excess saline and blood and stored in deep freeze at -20°C until sample were analysed for the determination of protein, glycogen, GSI and HSI.

3.9.3. Influence of sex steroids on thyroid gland, ovary and liver of H. fossilis

Steroids : The steroids like methyl testosterone (MT), testosterone propionate (TP), estradiol (E) and progesterone (P) were obtained from Sigma Chemical Company USA. The suspension of

these steroids were prepared in seasame oil and thoroughly mixed with the help of a Vortex Mixer.

Following three experiments were carried out.

Experiment-1: Effects of male sex steroids on thyroid gland ovary and liver of H. fossilis

Seventy adult fish were divided into 7 groups of 10 each and were placed in separate glass aquaria of 2' x 1' x 1' size containing 30 litres water. They were given following treatments on alternate days through out the duration of the experiment.

Groups	Treatments (µg/g body weight)
I	0.1 ml seasame oil (control)
II	1.25 µg Methyl testosterone
III	2.50 µg Methyl testosterone
IV	5.0 µg Methyl testosterone
V	1.25 µg Testosterone propionate
VI	2.50 µg Testosterone propionate
VII	5.0 µg Testosterone propionate

Experiment-2: Effects of female sex steroids on thyroid gland ovary and liver of H. fossilis

Seventy adult female specimens of H. fossilis were equally divided into seven groups and maintained as above. They were subjected to following treatments on alternate days through out the duration of the experiments.

Groups	Treatments (µg/g body weight)
I	0.1 ml seasame oil/fish (control)
II	0.60 µg Estradiol
III	1.25 µg Estradiol
IV	2.50 µg Estradiol
V	0.60 µg Progesterone
VI	1.25 µg Progesterone
VII	2.50 µg Progesterone

All the fishes of both the experiments were first lightly anesthesised as described before and intramuscular injections of freshly prepared suspension of the different steroids were given in 0.1 ml carrier vehicle (seasame oil). All injections were given on alternate days till each fish received a total of four injections. 48 hours after last injection, all the fishes of each group of both the experiments were killed by decapitation and their lower jaw and pituitary were fixed in Bouin's fluid for the evaluation of thyroid activity index and pituitary thyrotrops, while their ovaries and liver were taken out and washed with 0.6% physiological saline, blotted to remove excess saline and blood, weighed and stored at -20°C until samples were analysed for protein, glycogen, moisture, GSI and HSI values using the methods as described earlier.

Experiment-3: Effects of ovariectomy on thyroid gland of H. fossilis

Thirty five fishes were ovariectomised as reported earlier. Equal number of fishes were sham operated which were used as controls. Both ovariectomised and sham operated control fishes were separately maintained in glass aquaria of 2' x 1.5' x 1' size having 50 litres of water. 8, 12 and 20 weeks of post ovariectomy, groups of 10 fishes were sampled from experimental as well as control and sacrificed by decapitation. Their lower jaw and pituitaries were fixed in Bouin's fluid for the study of thyroid activity index and pituitary thyrotrops.