SEASONAL VARIATIONS OF PROTEINS IN THE PITUITARY GLAND IN RELATION WITH TESTICULAR CYCLE OF THE INDIAN MAJOR CARP, *LABEO ROHITA* (HAM.)

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Abstract:

The hormones of anterior pituitary are proteins or large polypeptides, while hormones of posterior pituitary are derivatives of proteins or amino acids. These hormones activate the genes of the cells which causes the formation of intracellular proteins that initiate specific cellular functions. Reproduction in fishes depends upon the coordinated actions of various proteins associated with brain-hypothalamus-pituitary gonad axis. In L. rohita reproductive cycle is divided into five phases. In the preparatory phase total protein content in the pituitary gland is moderate. It has been distinctly increase in the pre-spawning phase and reduce drastically in the spawning phase. In the post-spawning phase total protein content show negligible increase and resting stage show remarkable increase in the total protein content of the pituitary gland. These changes repeat with every reproductive cycle. This could be due to involvement of GTH-I in early gonadal development and also due to stimulatory role of GTH-II in spermiation. Seasonal changes in the expression of specific proteins coinciding with testicular cycle suggest the involvement of these proteins in reproduction.

Introduction:

Reproduction in fishes depends upon the coordinated actions of various hormones, associated with brain-hypothalamus-pituitary gonad axis. At each level of this axis, a limited number of target cells are under the influence of many factors. The final cellular response results from integrated effects of these regulatory factors on the intracellular signal transduction (Evans, 1998).

The pituitary gland cells that underwent correlative seasonal cyclic changes are identified as gonadotropin-secreting cells (Prasada Rao, 1969). Ng and Idler (Idler 1982, Idler and Ng 1983), succeeded in isolating a carbohydrate-poor gonadotropin from the pituitaries of American Plaice (*Hippoglossoides platessoides*), the Winter Flounder (*Pseudopleuronectes americans*) and the Chum Salmon (*Oncorhynchus keta*). This hormone stimulates the production of a steroid binding protein in the male and vitellogenesis in the female.

Number of studies are carried out in the reproductive cycle of seasonally spawning teleosts, mainly on salmonid and cyprinid fishes (Peter, 1981, Dodd and Sumpter, 1983). Hormonal changes during the seasonal reproductive cycle have been studied in number of teleosts such as the rainbow trout, *Salmo gairdneri* (Billard *et al*., 1978; Lou *et al*., 1984), goldfish-*Carassius auratus* (Kobayashi *et al*., 1986), and *Cyprinus carpio* (Billard *et al*., 1978).

Indian major carp, *Labeo rohita*, is economically important but there is no information available on its pituitary gland proteins and its role in testicular cycle. As pituitary gland is known to play an important role in reproduction, attempts are made to study the seasonal changes in pituitary proteins with respect to testicular cycle.

Material and methods :

Fishes were collected from natural habitat all around the Nagpur City. They were brought to the laboratory in tin containers and acclimatized in small ponds. Sexually matured males were selected with body weight ranging between 1.5 kg to 2 kg and length 30 to 40 cms.

Gonadosomatic index (GSI):

Six males of *Labeo rohita* were used regularly from January to December fortnightly for continuous two complete reproductive cycles, after acclimatization. Fish were anesthetized, weighed and length was measured. The gonads were removed, weighed and Gonado-somatic index was calculated by the following formula to ascertain the gonadal activity throughout the year.

$$GSI = \frac{\text{Weight of gonads}}{\text{Weight of fish}} \times 100$$

Methods For Calculation Of Seasonal Changes In Testis:

On the basis of histomorphological changes, stages of spermatogenesis were identified. Percentage of different stages of spermatogenesis was calculated in number of sections chosen at random. On the basis of GSI and the dominance of particular type of stages, annual reproductive cycle is reported.

Histological Methods:

Fixation : The fishes were anesthetized pituitary gland and testes were dissected out and immediately fixed in Bouin's fixative for 18 to 24hrs.

Embedding And Sectioning:

Tissues were transferred to 70% alcohol and dehydrated in graded series of alcohol, cleared in xylene and embedded in paraffin wax.

The testes were cut at 8 to 10 μm thickness. The olfactory organ and brain with pituitary gland were cut at 10 μm thickness in transverse as well as saggital planes.

Haematoxylin Eosin:

The sections of testis were deparaffinized in xylene and were passed through descending grades of alcohol and brought to water. They were stained for 5 minutes in haematoxylin solution and kept in running water for 15 minutes. The sections were partially dehydrated by passing them through alcohol series up to 90% alcohol and were then stained with eosin for two minutes. They were differentiated in 90% alcohol, dehydrated in absolute alcohol, cleared in xylene and mounted in DPX.

Histochemical Methods For Proteins:

The fishes were anesthetized and necessary tissues were dissected out and fixed in Cornoy's fixative for 4 to 6 hrs, transported to rectified spirit and absolute alcohol, cleared in xylene and embedded in paraffin wax at 58^0-60^0C. The sections of olfactory organ were cut at

10 µm thickness. Sections were deparaffinised and hydrated in descending grades and stained in Bromophenol blue, rinsed in 0.5% acetic acid, then differentiated in terbutyl alcohol, cleared in xylene and mounted in DPX.

Biochemical Methods For Proteins:

Tissue Extract:

L. rohita were killed by decapitation. Olfactory organ, hypothalamus, pituitary and testis were dissected out in the ice cold Ringer's solution. The tissues were weighed and homogenized at 0^0C in ice cold Ringer's solution using pestle and mortar.

Estimation:

Tissue proteins were estimated by Lowry *et al.*, method (1951) with minor modifications. One ml of homogenate was mixed with 1 ml of 10% Trichloroacetic acid (TCA) and centrifuged for 15 mintues at 10,000 RPM. The precipitate was dissolved in 3 ml of 0.1 N NaOH, of which 1 ml of dissolved precipitate was taken in a clear test tube, which was diluted upto 4 ml by adding 3 ml distilled water, then 5.5 ml of alkaline copper sulphate reagent was added and kept for 30 minutes. Simultaneously, Bovine serum albumin (2 mg/1ml) as standard and distilled water (Blank) were taken in separate test tubes, 3 ml distilled water and 5.5 ml of alkaline reagent was added with vigorous shaking in each test tube. The test tubes were kept for 30 minutes and colour intensity was noted at 650 nm in the calorimeter. The standard graph was drawn with five standard BSA solutions and from this, the unknown amount of proteins was determined from the extracted samples. Values are expressed in mg proteins/gm of tissue weight.

Statistical Methods :

All statistics presented in this study were mean standard error. Students "t" test was made use of testing the significance of difference between the mean of readings of experimental and control groups in this study, using 5% level of significance. The relevant test statistics is given below

$$t = \frac{\bar{X} - \bar{Y}}{\sqrt{SE(X)^2 + SE(Y)^2}}$$

Where

t = Student's test

$\bar{X}$ = Mean of Experimental readings

$\bar{Y}$ = Mean of control reading (Resting phase)

$SE(X)^2$ = Square of standard error of experimental reading

$SE(Y)^2$ = Square of the standard error of control reading.

Result:

Biochemical changes of protein in pituitary in relation to testicular cycle of the Indian major carp *Labeo rohita* have been studied. The reproductive cycle of *L. rohita* is divided into five phases on the basis of gonadosomatic index and histological details viz. Preparatory (February to March), Prespawning (April to June), Spawning (July to August), Post-spawning (September to October) and Resting phase (November to January).

Preparatory Phase (Feb-Mar):

Photoperiod increases gradually from the month of February and at the same time (Fig.4), temperature also increases sequentially (Fig. 3). Rainfall is scanty in this season (Fig. 2). The gonadosomatic index (GSI) starts increasing gradually in preparatory phase (Fig. 1).

In Preparatory phase gametogenesis commences and testis shows different stages of spermatogenesis (Fig. 5). In the early Preparatory phase, in the month of February, primary spermatogonia are 96.7% which is the highest and remaining 3.3% are secondary spermatogonia but in late Preparatory phase in the month of March, primary spermatogonia are 24.1% which get converted in secondary spermatogonia. With 45.2%, these dominate the gamete development. The primary spermatocytes are 15.2%, secondary spermatocytes 10.13%, and spermatids are 5.37%.

In Preparatory phase, total protein content in the pituitary gland is 14.71±0.181 mg/gm of tissue weight in the first cycle of the year and 16.45±0.102 mg/gm of tissue weight in the second cycle of the year (Fig.6). Histochemically proteins are demonstrated in Proximal pars distalis (PPD) of pituitary gland. In the PPD gonadotroph cells show moderate staining of Bromophenol blue (Fig.7A).

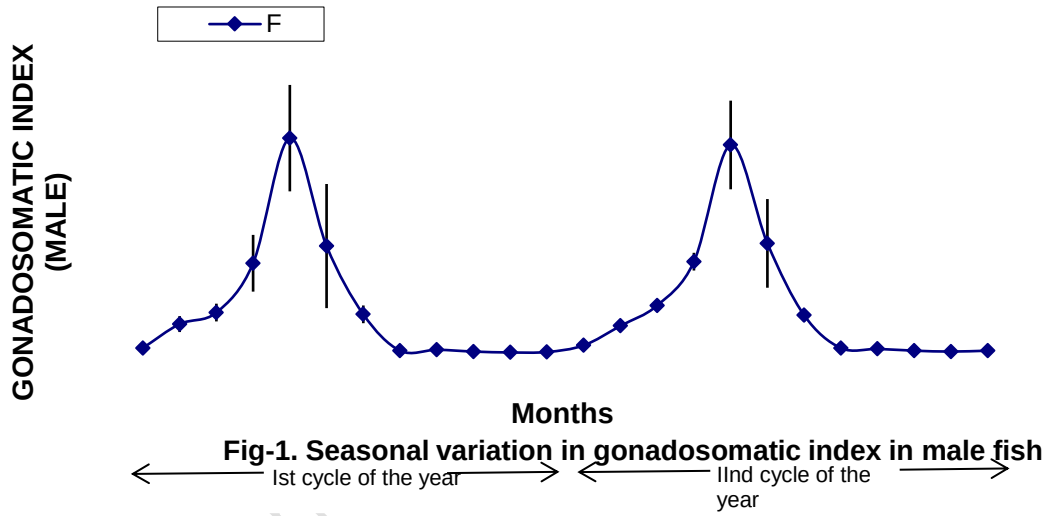


Fig-1. Seasonal variation in gonadosomatic index in male fish

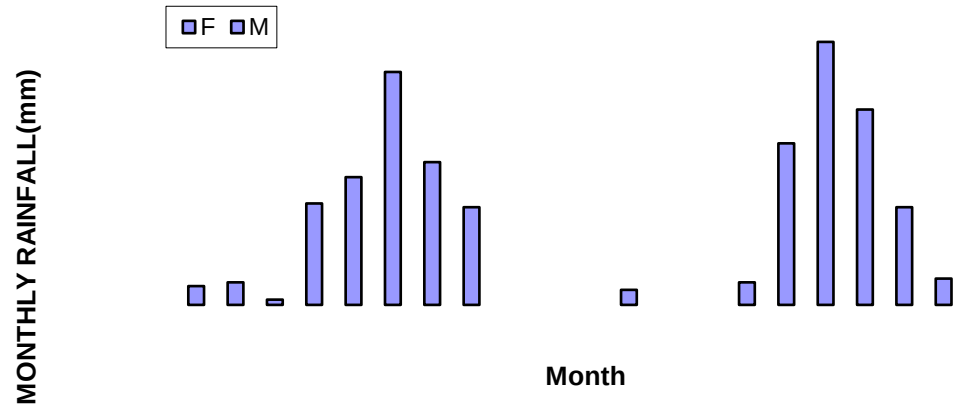
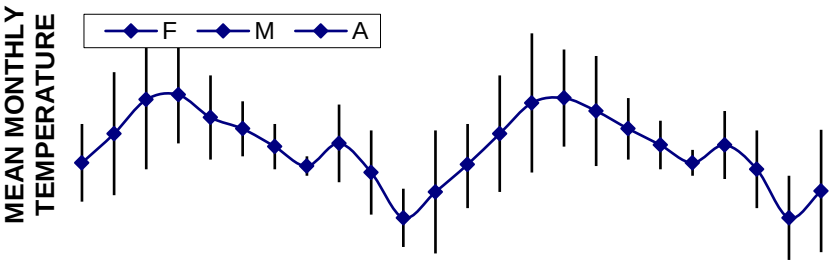


Fig-2. Seasonal varation in annnul rainfall

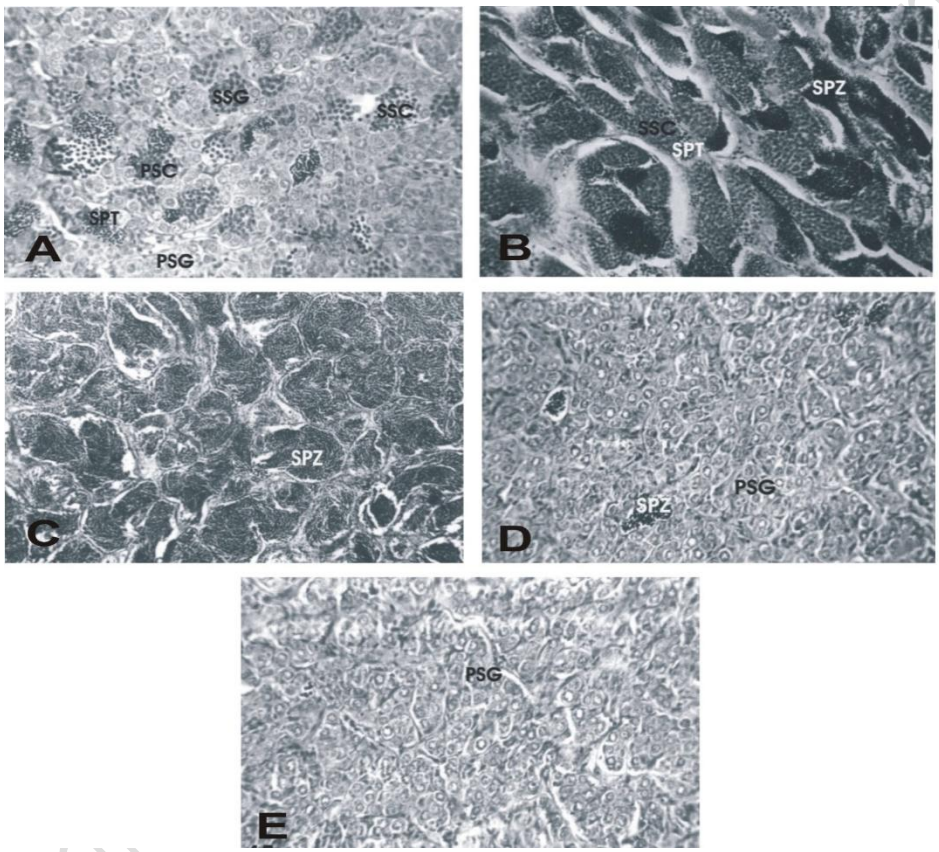


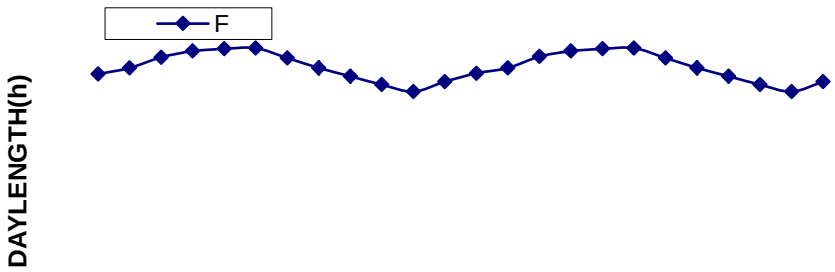
Months

Ist year of cycle

IInd year of cycle

Fig- 3. Seasonal changes in mean temperature





Month

Fig- 4. Seasonal Variation of Daylength

Fig-5. Transverse section of testis in the (A) Preparatory phase showing primary spermatogonia (PSG), secondary spermatogonia (SSG), primary spermatocytes (PSC), secondary spermatids

(SPT). (B) Prespawning phase showing secondary spermatocytes (SSC), spermatids (SPT) and spermatozoa (SPZ). (C) Spawning phase showing spermatozoa (SPZ) with broken wall of seminiferous lobules. (D) Postspawning phase showing primary spermatogonia (PSG) and unspent spermatozoa (SPZ). (E) Resting phase showing primary spermatogonia (PSG). 210 X.

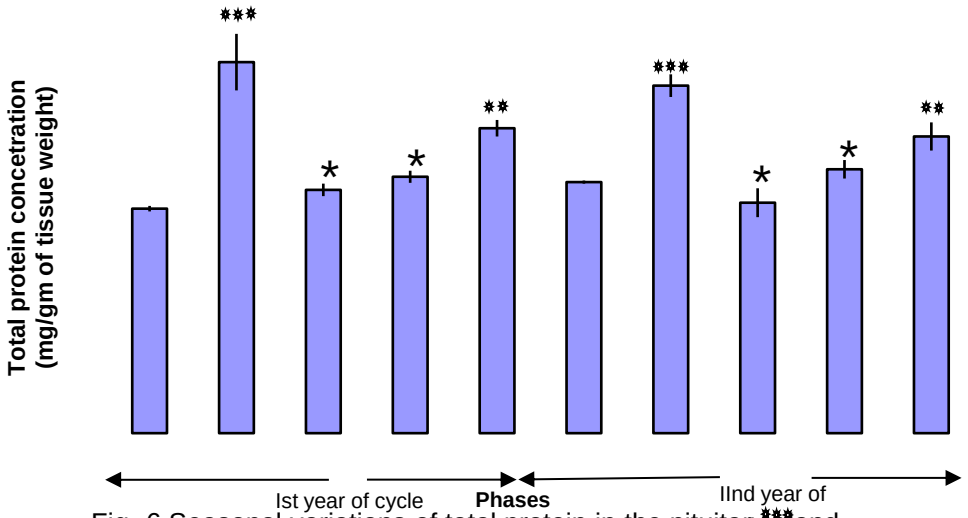


Fig- 6.Seasonal variations of total protein in the pituitary gland.
P<0.0001; * P<0.005 ;

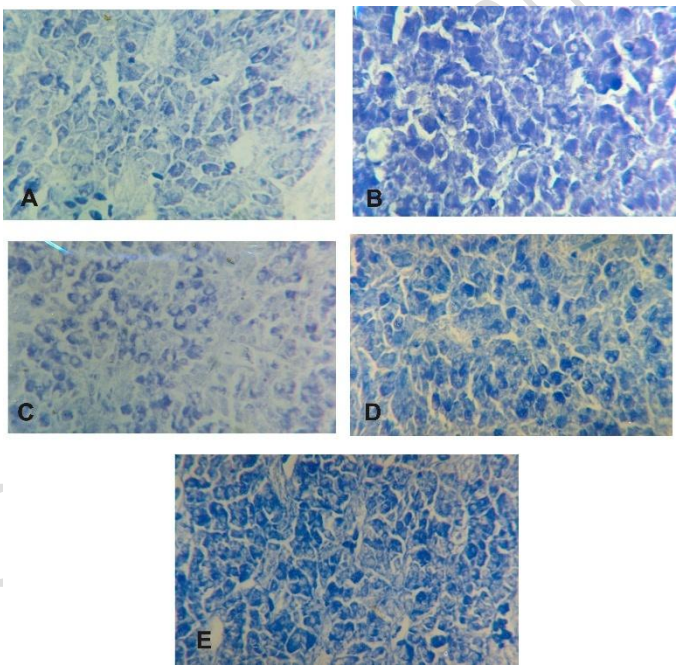


Fig. 7 Transverse section of pituitary gland A) in preparatory phase showing weak staining of bromophenol blue, B) in pre-spawning phase showing intense staining of bromophenol blue, (C) in spawning phase showing light staining of bromophenol blue, (D) in post-spawning phase showing weak staining of bromophenol blue and (E) in resting phase showing slightly intense staining of bromophenol blue. 400X.

Prespawning Phase (APR- JUN):

In this phase which spans approximately from April to June, maximum temperature and day length were noticed (Fig.3). Monsoon rainfall starts in late pre-spawning phase and

temperature goes down (Fig.2, 3). In this phase, testes enlarge in size and volume with maximum GSI in late pre-spawning phase in the month of June (Fig.1).

In the early pre-spawning, in the month of April when temperature starts soaring, recrudescence of different types of gametes starts. Primary spermatogonia or sperm mother cells are about 6.4%, secondary spermatogonia are 10.1% and the primary spermatocytes dominate with 40.9%; followed by secondary spermatocytes with about 27.2%. These are converted into spermatids by meiotic division which are 10.4% and further into spermatozoa which are 5% of the total gametes. In the month of May, when maximum temperature is recorded, conversion of secondary spermatocytes in spermatids hastens, which constitute about 44%, which is the highest of the total count.

Spermatozoa also register a little rise in percentage, which is 8.12%. Primary spermatocytes record a drastic fall in percentage from initial 40.9% to 8.43% from April to May.

Pituitary gland shows peak in the total protein content in Prespawning phase (Fig.6). In this phase total protein content in the pituitary is 22.81 ± 0.7858 mg/gm of tissue weight in the first cycle of the year and 22.812 ± 0.7422 mg/gm of tissue weight in the second cycle of the year (Fig.6). In the PPD of Pituitary gland, proteins are demonstrated histochemically. These cells show intense staining of Bromophenol blue (Fig.7B).

Spawning Phase (JUL – AUG) :

In the early spawning phase in the month of July, day length is maximum but the temperature is reduced substantially due to rainfall (Fig. 2). In the month of August, day length is reduced and the temperature is decreased with heavy rainfall (Fig.2, 3. 4). In this phase, there is fall in the GSI level (Fig.1) as milt is released from the testes and ruptured seminiferous lobules are observed. In this phase testes are filled up with spermatozoa only.

In the pituitary gland, the total protein content decreases remarkably in the Spawning phase (Fig. 6). In this phase, content in the pituitary gland is 15.94±0.410 mg/gm of tissue weight in the first cycle of the year and 15.10±0.947 mg/gm of tissue weight in the second cycle of the year. Histochemically, gonadotrophs in the PPD show light staining of bromophenol blue (Fig.7C).

Postspawning Phase (OCT-NOV):

Temperature in Postspawning phase, in the month of September, is slightly decreased as the rainfall continues and reduction in the temperature continues in the month of October with scanty rainfall (Fig.2, 3). In these months, day length gradually decreases (Fig. 4). GSI suddenly falls down with initiation of Postspawning phase (Fig.1).In this phase some primary spermatogonia are seen along with few unspent spermatozoa in the seminiferous lobules.

In the pituitary gland there is remarkable increase in the total protein content in the Postspawning phase (Fig.6). In the Postspawning phase total protein content in pituitary is 16.8 ± 0.397 mg/gm of tissue weight in the first cycle of the year and 17.29 ± 0.605 mg/gm of tissue weight in the second cycle of the year (Fig.6). Histochemically, proteins in the pituitary gland are localized by the Bromophenol blue and it shows moderate staining in gonadotroph cells of PPD of pituitary gland (Fig.7D).

Resting Phase (NOV-JAN):

In resting phase, due to onset of winter, temperature reduces and day length also is much reduced (Fig. 3, 4). Rainfall is not noticed in these months (Fig.2). GSI is minimum in

the resting phase (Fig.1). In this phase, in the testis only primary spermatogonia are seen (Fig. 5).

In the pituitary gland increase in the total protein content in the resting phase is seen (Fig.6). In this phase total protein content in the pituitary gland is 19.18 ± 0.542 mg/ gm of tissue weight in the first cycle of the year and 19.44 ± 0.923 mg/ gm of tissue weight in the second cycle of the year (Fig. 6). Histochemically, protein shows strong staining of Bromophenol blue in the gonadotroph cells in pituitary gland (Fig.7E).

Discussion:

Reproductive cycle of the annual breeder in the male of *Labeo rohita* is divided in five phases Preparatory (February-March), Prespawning (April-June), Spawning (July-August), Postspawning (September-October) and Resting (November-January). Maturation in fishes is reported to follow a cyclic pattern in such a way that Spawning is adjusted to the most propitious time of the year, where maximum survival and faster growth of the young ones is ensured (Malhotra *et al.*, 1989).Spawning in *Labeo rohita* takes place during monsoon months when conditions are most suitable. An essential pre-requisite of periodicity is an interaction between successive gonadal stages and the exteroreceptive factors (Marshall, 1942; Amorosso and Marshall, 1960). These exteroreceptive factors are temperature, light, rainfall, water level of the habitat and food supply influencing breeding periodicity. These environmental cues influence on target organs including gonads through nervous and reflex stimulation which further culminate in secretions of gonadotropic hormones from the pituitary (Malhotra *et al.*, 1989).

In present study maximum day length is observed in the month of June which is slowly reduced in July and august. Rainfall is highest in the month of August in the first year of the cycle and in the month of July in the second year of the cycle. Along with the rainfall, temperature also lowers down successively from June towards July and there is substantial fall in the month of August. These environmental factors along with the intrinsic factors viz., condition of gonads and their secretions, general conditions of the body appear to influence the physiology and psychology of the breeders so that the target organs respond to external influences. A long photoperiod and warm temperature regime stimulates testicular maturation in *Channa punctatus* (Srivastava & Singh, 1993), *C. reba* (Verghese, 1967,1970,1975), *Mystus tengara* (Guraya *et al*, 1976)and *H. fossilis* (Sundararaj & Vasal, 1976).

In case of *L. rohita* highest rainfall, lowering of temperature and lowering of day length seems to favor breeding in bullhead catfish *Ictaluras nebulosus* rainfall plays an important role in reproductive cycle, either as a direct environmental cue or through effects on water conditions (Salinity, PH) or food availability (Rosemblum, *et al*, 1987). However Malhotra *et al.*, (1989) reported that same species at different latitudes and altitudes may breed at different times in response to different ecological conditions.

Thus it is necessary to wart out the reproductive cycle separately in region where the studies are carried out.

In the present study even after maintaining the time scale, histological procedures were implicated to ensure that the groups are homogenous enough regarding testes development and it was further substantiated by calculating gonadosomatic indices.

In the males of *L. rohita*, highest GSI values are obtained in the month of June in both the years. This period corresponds to late Prespawning period and the testes are in the final phases of maturation. Maximum value of GSI in final stages of spermatogenesis and spermiation in the males is also reported for pink salmon *Onchorhynchus gorguscha* (Dye *et al.*, 1986). In *Fundulus heteroclitus* (Mathews, 1938), also maximum weight is attained by testes just before Spawning and rapidly decreases immediately after Spawning. Lower GSI values are observed in *L. rohita* from September to January which corresponds to Postspawning and Resting phases of the life cycle.

This is due to the fact that milt passes out of the body during this period. Histologically also the lumen of seminiferous lobules have a few unspent spermatozoa in the middle and spermatogonia are visible at the lobular wall seasonal testicular changes are worked out in Pacific salmon, *Onchorhynchus nerka* (Weisel, 1943); Bluengill, *Lepomis macrochirus* and large mouth bass, *Huro salomoides* (James, 1946), in *Heteropneustes fossilis* (Sunderaraj, 1960; Hunge, 2002) and in the Cyprinid fish *Natropis bifrenatus* (Harrington,1957). Seasonal cycle is influenced not only by environmental factors but a variety of hormones associated with brain-hypothalamus-pituitary-gonad axis work in coordination to bring about reproduction in teleosts (Evans, 1998). Ovarian and testicular function in teleosts and other fishes are controlled not only by the pituitary gonadotropins (GTH I &GTH II), but also by multiple hormones and growth factors which act in an endocrine, autocrine and or paracrine manner (Evans, 1998). To have the insight along this axis, proteins are histochemically located during the entire testicular cycle, in the olfactory organ, hypothalamus, hypophysis and testes and they are estimated biochemically in all these tissues. An attempt is made to establish the correlation of fluctuation in protein content pituitary gland and annual testicular cycle; because it is well known that both the energetic and nutritional requirements of maturing fish increase during the process of gametogenesis (Mommsen and Walsh, 1988; Dickhoff *et al*; 1989; Billard, 1992) and many energy consuming reactions are involved in sexual maturation (Ng *et al*, 1986).

All these changes involve an increased energy demand which has been demonstrated in several fish species (Diana and Mackay, 1979; Montecchia *et al*, 1990).

GTH I and GTH II are the two chemically different glycoproteins which are characterized in four orders of teleosts (Kawauchi et al., 1989; Swonson, 1991; Querat, 1995 ;Elizur et al., 1996). In PPD, intensely stained cells are seen and highest amount of proteins in pituitary obtained in pre Spawning phase in the males of *L. rohita* ($P < 0.005$) can be as a result of GTH I content because GTH I in the males is primarily important in early gonadal development during spermatogenesis and GTH II, which stimulates spermiation (Swonson, 1991; Parat et al., 1996).

Intensity of staining is reduced and there is a drop in protein content in pituitary during Spawning. Reduction of protein in Spawning in pituitary might be due to the action of Dopamine (DA), another neuropeptide, which inhibits the GnRH induced GTH II response via D_2 receptors on gonadotrophs in a wide array of teleosts including cyprinids, salmonids, tilapia, cat fish, eel, and Chinese loaches (Peter et al., 1991a, b; Yu et al., 1997). In some of these species DA also reduces basal GTH II secretion directly at pituitary cell level.

Protein content slowly increases in Postspawning ($P<0.005$). Cells are darkly stained and a second surge is noted in Resting period which can be due to non utilization of proteins

by target organs during this phase.

It is well known that neurohypophysial peptides may act as chemical messengers delivered to target cells as hormone, neurotransmitter, or locals paracrine factor (Hazon and Baltment, 1998).

Neruosecretory fibers are shown to directly invade the pars distalis of pituitary in teleosts (Holmes and Ball, 1974; Peter and Crim, 1979). It is established that gonadotropic activity of teleost pituitary is regulated by gonadal hormones and by neurohormones mainly of hypothalamic origin (VanOordt and Pelte, 1983). In *L. rohita*, cells in proximal pars distalis are intensely stained for proteins in Prespawning phase and the amount is also highest during this phase ($P<0.0001$ in 2001 and $P<0.005$ in 2002) and in the Resting phase ($P<0.005$), also, cells are intensely stained and the quantity of protein is high ($P < 0.005$). In rest of the phases, the staining intensity is moderate to light which matches with the quantification of the proteins.

The proximal pars distalis, where GTH cells are located, receive innervations from neurosecretory fibers in number of fish species, in Goby, *Gillichthyes mirabilis* (Zambrano, 1970 a, b, 1971), Roach , *Leucisus rutilus* (Ekengren, 1975; Bage *et al*., 1974; Ekengren *et al*., 1978), Black molly, *Poecila latipinna* (Peute *et al*., 1976), Atlantic salmon and Rainbow trout (Terlou and Ekengren, 1979). GnRH neurons are known to innervate the pituitary gland and direct connection with pituitary gland were demonstrated in the electric fish (Johnston and Maller, 1992 and in the goldfish (Anglade et al., 1993). Innervation of pituitary by the fibers from the brain is also observed in *L. rohita.* Thus, involvement of GnRH in regulation of GTH secretion cannot be ruled out in this fish.

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