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[^{125}I] gonadotropin binding to the ovary of an Indian major carp, *Catla catla,* at different stages of reproductive cycle

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Abstract. At different stages of the annual reproductive cycle of *Catla catla,* a major Indian carp, specific binding of gonadotropic hormone to the plasma membrane receptors was demonstrated. Maximum specific binding of [^{125}I] *Catla* gonadotropic hormone was obtained at 30°C and pH 7·5 during 2 h of incubation. *Catla* gonadotropic hormone binding was saturable with high affinity. Competitive inhibition experiment showed that binding site was specifically occupied by piscine gonadotropic hormone, *Catla* gonadotropic hormone and murrel gonadotropic hormone, human chorionic gonadotropin was a weak competitor while bovine thyroid stimulating hormone, bovine prolactin and ovine follicle stimulating hormone had no effect. Scatchard analysis of *Catla* gonadotropic hormone binding to the plasma membrane preparation from the carp oocytes of different reproductive stages showed that the range of dissociation constant (K_d) varied from 0·78 to 0·97 × 10^{-10} M. However, maximum binding capacity (B-max) varied remarkably between the different stages of reproductive cycle, it was 6·11 ± 0·36 fmol/mg protein in the preparatory stage which increased to about three-fold in prespawning stage of reproductive cycle (17·0 ± 0·29 fmol/mg protein) and spawning (18·7 ± 0·17 fmol/mg protein) and lowest in postspawning stage of reproductive cycle (5·28 ± 0·28 fmol/mg protein). Fluctuation in the number of gonadotropic hormone binding site at different stages of annual reproductive cycle was found to be coincided well with the pattern of ovarian steroidogenesis in response to *Catla* gonadotropic hormone as determined by the formation of progesterone from pregnenolone.

Keywords. Gonadotropin receptor; gonadotropin; ovary; fish reproduction; steroidogenesis.

1. Introduction

Action of gonadotropin on the target tissue depends on its specific binding to the receptors located in the plasma membrane of gonadal cells. In teleostean fish, as in other higher vertebrates, gonadotropin (GTH) stimulates ovarian steroidogenesis following its binding to the ovary (Nagahama *et al* 1985; Jamaluddin and Bhattacharya 1986; Kanamori and Nagahama 1988). Specific binding of radioiodinated fish or mammalian GTH to GTH-binding sites have been demonstrated in the ovaries of Chum salmon, *Oncorhynchus keta* (Van der Kraak and Donaldson 1982; Van der Kraak 1983), amago salmon, *Oncorhynchus rhodurus* (Kanamori *et al* 1987) murrel, *Channa punctatus* (Jamaluddin and Bhattacharya 1986) and in the testis of rainbow trout, *Salmo gairdneri* (Schlaghecke 1983) and goby, *Glossogobius olivaceus* (Ishii and Aida 1982; Aida and Ishii 1985). It is known that majority of the teleostean fish are seasonal breeders and they breed at specific

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Abbreviations used: GTH, Gonadotropic hormone; cGTH, *Catla* gonadotropic hormone; hCG, human chorionic gonadotropin; bTSH, bovine thyroid stimulating hormone; bPRL, bovine prolactin; oFSH, ovine follicle stimulating hormone; PEG, polyethylene glycol.

time of a year. To spawn at a certain time of year fishes have to use various environmental cues to complete gonadal recrudescence so that gametes are matured in time for spawning (Peter and Hontela 1978). In mammals it is now an established fact that the quantity of gonadotropin receptors modifies the sensitivity of gonad to gonadotropins (Richards 1979; Catt *et al* 1979, 1980; Adashi and Hsueh 1984). The annual cycle of activity of teleostean gonad is well documented and divided in a number of semiarbitrary phases (Yamazaki 1965; Vlaming 1972; Khoo 1975; Sundararaj and Vasal 1976; Malhotra *et al* 1989). It is expected that in tune with the variation of gonadal cyclic activity, the content of gonadotropin receptor in the gonad will also vary. Practically nothing is known about it except a recent report by Kanamori and Nagahama (1988) where they have shown that during oogenesis there are developmental changes of gonadotropin receptor properties in the ovarian follicles of amago salmon.

Majority of the Indian teleosts breed once a year and that is only during monsoon (rainy season) and to perform spawning in monsoon, maturation of gonad takes place in pre-monsoon phase. The reproductive cycle of Indian teleost may be divided into four stages, preparatory, prespawning, spawning and postspawning (Sundararaj and Vasal 1976; Chakraborty and Bhattacharya 1984) and at each stage gonads show a discrete change but there is no direct evidence of GTH involvement in these changes.

The present work is the first attempt to observe the binding pattern of homologous GTH to the ovarian tissue of an Indian major carp, *Catla catla,* at different stages of its reproductive cycle. Functional relevance of GTH occupation of the receptor at various stages of ovarian cycle of this carp has also been examined.

2. Materials and methods

2.1 *Preparation of oocyte plasma membranes*

Reproductive cycles of Indian teleosts can be divided into four stages and in the eastern India these four stages covered the following months of a year (Chakraborty and Bhattacharya 1984)—(i) preparatory (February to April), (ii) prespawning (May to middle of June), (iii) spawning (middle of June to middle of September) and (iv) postspawning (middle of September to January). For the present study ovary was collected from C. *catla,* during the middle portion of each phase.

Ovaries from *C. catla* were dissected out and immediately placed in a sterile petridish (placed on ice) containing ice-cold fish oocyte culture medium as described earlier by Mukherjee and Bhattacharya (1982). The mesovarium covering was cut with a scissor and a long incision made from anterior to posterior region to remove the tunica albuginea and germinal epithelial layer. Oocytes were isolated and washed several times with the medium. The same procedure was followed for fish belonging to different stages of reproductive cycle. Oocytes were then homogenized (1:5, w/v) very gently with sodium phosphate buffer (0·01 M, pH 7·5) in a Potter-Elvehjem homogenizer under ice for 30 s. The homogenate was passed through a single layer cheese cloth to remove fat. Oocyte plasma membrane was then

prepared using the method of Birnbaumer and Swartz (1982). Protein content of the membrane preparation was measured according to the method of Lowry *et al* (1951) using bovine serum albumin (BSA; fraction V; Sigma Chemical Co., St. Louis, Mo, USA) as the standard.

2.2 *Hormones*

Purification of *Catla* GTH (cGTH), with an estimated molecular weight of about 40,000 Da, was carried out with the acetone dried pituitary powder by following the method of Banerjee *et al* (1989). Purified human chorionic.gonadotropin (hCG) (CR-121; biological potency 13450 IU/mg) was obtained as a gift from Dr R E Canfield, Columbia University, New York, USA. Bovine thyroid stimulating hormone (bTSH, NIADDK-bTSH-11), bovine prolactin (bPRL, NIAMDD-P-B_4) and ovine follicle stimulating hormone (oFSH, NIAMDD-oFSH-S13) were obtained as generous gifts from NIADDK, Bethesda, Maryland, USA.

2.3 *Preparation of* [^{125}*I*] *labelled cGTH*

[^{125}I] cGTH of specific activity 30-35 μCi/μg was prepared by following the same procedure as previously described by Jamaluddin and Bhattacharya (1986). Briefly, cGTH was iodinated using chloramin T (Sigma Chemical Co., USA), following the procedure of Greenwood *et al* (1963). Separation of radiolabelled cGTH from non-reactive radioiodine was performed on a Sephadex G-75 column equilibrated with 0·01 M sodium phosphate buffer, pH 7·5 containing 0·15 M NaCl, 0·5 % (w/v) BSA and 0·1 % (w/v) KI.

2.4 *Binding assay*

Binding assay was performed using the procedure described by Jamaluddin and Bhattacharya (1986). Incubation medium used for binding assay contained 5 mM $MgCl_2$, 100 mM sucrose and 0·1 % (w/v) BSA in phosphate buffer, 0·01 M, pH 7·5. Oocyte membrane preparation (2mg) was incubated with 20 μl of [^{125}I] cGTH solution (approximately 1×10^5 cpm corresponding to 4ng cGTH), The specific binding was calculated from the difference between total binding and nonspecific binding which was calculated from the incubation in the presence of a 800-fold excess of unlabelled cGTH. The reactants were added in the following order : incubation medium, excess unlabelled cGTH, labelled cGTH and membrane preparation from oocytes in a final volume of 1·0 ml at pH 7·5. After mixing the contents of the tubes by vortexing they were incubated at 30°C for 1 h in a shaking waterbath. The reaction was terminated by placing the tubes under ice and by the addition of ice-cold mixture of 0·1 % (w/v) bovine y-globulin and 0·1 M NaCl. Ice-cold polyethylene glycol (PEG, 20% w/v) was then added to each tube under ice and the contents of the tubes were vortexed and centrifuged at 1500 *g* for 20 min. The supernatant was discarded by careful aspiration and the pellet thus obtained was washed three times with ice-cold mixture of 0·1 M NaCl and 20% PEG. The radioactivity of the pellet was determined in a gamma-ray spectrometer.

2.5 *Ovarian steroidogenesis in response to cGTH*

To observe cGTH sensitivity in terms of ovarian steroidogenesis at different stages of reproductive cycle, hundred oocytes of each stage were incubated in the presence or absence of 500 ng cGTH. Total incubation time was 6 h. Oocytes were allowed to incubate for 2h and at 2h cGTH was added. A 2h preincubation time was necessary for the recovery of oocytes. Incubation medium and method for checking the viability of oocytes were same as described earlier from this laboratory (Jamaluddin *et al* 1989).

To observe ovarian steroidogenesis, oocytes were homogenized in a medium containing 250 mM sucrose, 1 mM tetrasodium EDTA and 1 nM potassium phosphate buffer, pH 7·5. Homogenization was carried out by gentle movement of teflon pestle along a loose fitting tube. Homogenate was then centrifuged at 2,500 *g* in a refrigerated centrifuge (IEC, India). The pellet was rejected and supernatant was subjected to ultracentrifugation at 10,000 *g* (Beckman, USA, model L7-55) to obtain mitochondrial pellet. Supernatant of this fraction was further fractionated at 150,000 *g*. The supernatant of 150,000 *g* was saved from both control and cGTH treated oocytes. Mitochondrial steroidogenesis was assayed by a short term *in vitro* incubation of oocyte mitochondria and the procedure adopted was described earlier from this laboratory (Deb and Bhattacharya 1988) which was a modification from Dimino (1977). Briefly, 2·0 mg of mitochondrial protein was incubated with 10 nmol of Δ^5 [4, 7–^{3}H]-pregnenolone (obtained from Amersham, England) in a medium containing 200 mM sucrose, 25 mM Tris-HCl buffer (pH 7·5), 10 mM KCl, 5 mM $MgCl_2$, 5mM NADPH, 5 mM Na-succinate, 0·2 mM tetrasodium EDTA and 1% (w/v) recrystallised fatty acid-free BSA. Incubation was started by adding mitochondria and final volume was fixed at 1·0 ml. It was shown from this laboratory earlier that to observe GTH effect on mitochondrial steroidogenesis there is a need to add 150,000 *g* supernatant (volume 200 μl, protein 800 μg/incubation) of both control and GTH treated oocytes to the mitochondrial incubation (Deb and Bhattacharya 1988). Incubation was carried out for 1 h at 30 ± 1°C in a metabolic shaker bath and terminated by the addition of 1·0 ml of methanol containing 4 μg of pregnenolone and 2 μg of progesterone. Steroids were extracted from mitochondria and subjected to thin-layer chromatography on a pre-coated silica gel (Kiesel gel 60, E Merck, Darmstadt, Germany) against a solvent system composed of diisopropyl ether : petroleum spirit : acetic acid (70:30:2; v/v) Progesterone was clearly separated from the pregnenolone which was then scraped out, eluted and mixed thoroughly with toluene based PPO and POPOP and counted in a liquid scintillation spectrometer (ECIL, LSS20).

2.6 *Data analysis*

All data were statistically analysed by Student's *'t'* test for difference between replicates. Since no significant differences between the replicates were observed, replicate data were combined. Data in connection with the response of different stages of oocytes to cGTH were subjected to one way analysis of variance (ANOVA), where *F* values indicated significance, means were compared by Schefe's test (Snedecor and Cochran 1971).

3. Results

3.1 [^{125}I] cGTH binding to carp oocyte plasma membrane preparation

Optimum binding conditions of [^{125}I] cGTH to oocyte membrane preparation of carp belonging to prespawning stage was determined at different temperatures, time intervals, pHs and PEG concentrations. Optimum binding occurred at 30° C, pH 7·5 and 20% PEG concentration, and it was found that specific binding of [^{125}I] cGTH increased till 1 h of incubation and at 2 h it reached to saturation (data not shown). To observe the effect of radiolabelled cGTH concentrations on binding, oocyte membrane preparation (collected from fish belonging to prespawning stage) was incubated with varied concentrations of [^{125}I] cGTH (0·5 ng to 10·0 ng) under optimal binding conditions. [^{125}I] cGTH binding increased steadily with the increase in radiolabelled hormone concentrations and reached saturation at 4·0 ng (figure 1). The data from saturation experiment were used for Scatchard plot analysis. It could be seen from the inset of figure 1 that the maximum binding capacity (B-max) of oocyte membrane preparation was 17·0 ± 0·29 fmol/mg protein and K_d was 0·86 × 10^{-10} M.

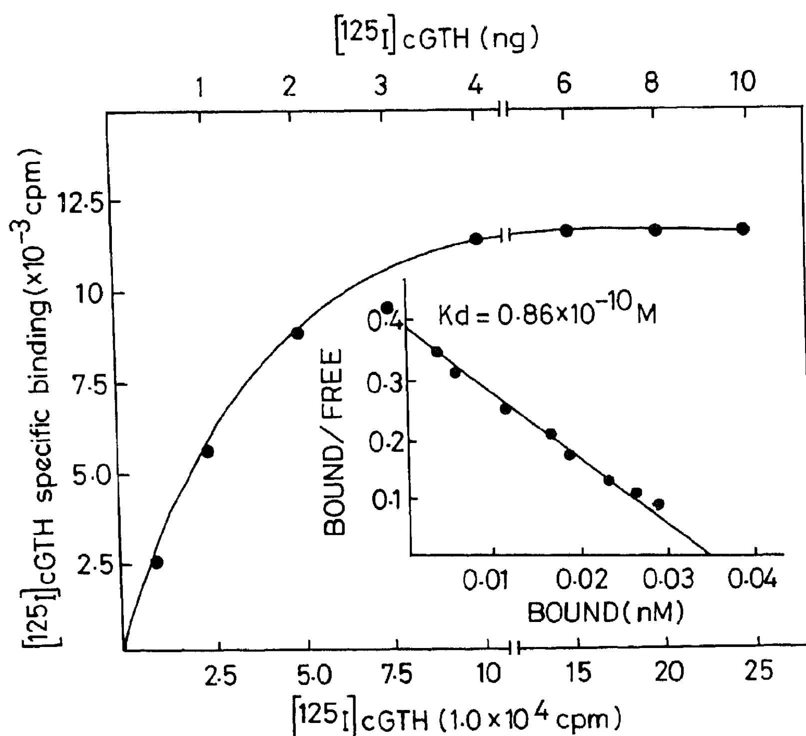

Figure 1. Effect of increasing concentrations of [^{125}I] cGTH on its binding to oocyte membrane preparation. Two mg of oocyte membrane preparation was incubated separately with varying amounts of [^{125}I] cGTH with fixed amount of cGTH (800-fold) to determine nonspecific binding. *Inset:* Scatchard plot of [^{125}I] cGTH binding to *Catla* oocyte membrane preparation.

3.2 *Competitive inhibition of* [^{125}*I*] *cGTH binding*

The specificity of the binding of [^{125}I] cGTH to oocyte plasma membrane was investigated by measuring the ability of number of unlabelled hormones to compete for the cGTH receptor sites. Figure 2 demonstrates that unlabelled cGTH is the most effective competitor of [^{125}I] c G T H binding. Fifty per cent binding inhibition was reached with 1·76 μg. Another teleostean GTH, purified from the pituitaries of a murrel (Banerjee *et al* 1989), *Channa punctatus* (murrel GTH), was an active competitor for the cGTH binding sites because it displaced [^{125}I] cGTH in a dose dependent manner and 50% inhibition was reached with 3·08 μg. hCG was found to be a weak competitor while bTSH, bPRL and oFSH did not affect the binding Tissue specificity of [^{125}I] cGTH binding was evaluated by incubating the plasma membrane preparations from ovary, kidney, skeletal muscle and heart of carp and it could be seen from figure 3 that except ovarian tissue cGTH does not bind to other tissues.

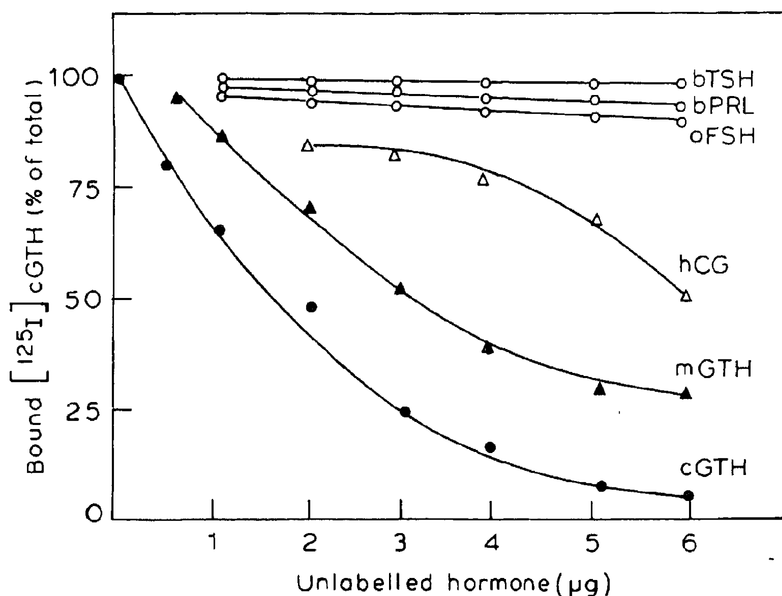

Figure 2. Specificity of the binding of [^{125}I] cGTH to carp oocyte plasma membrane preparation. Competitive inhibition is expressed as a percentage of [^{125}I] cGTH binding when incubation mixtures contained varied concentrations of unlabelled hormones. Each point is the mean value of four observations.

3.3 *Determination of* K_d *and B-max at different reproductive stages*

Oocytes from *Catla* were collected from each stage of reproductive cycle and plasma membrane preparations from them were subjected to binding assay with increasing concentrations of [^{125}I] cGTH (0·5 ng to 6·0 ng) in the presence of 800-fold excess of cGTH. Results obtained from Scatchard analysis of four reproductive cycles have been shown in table 1. There was a little alteration of K_d value between

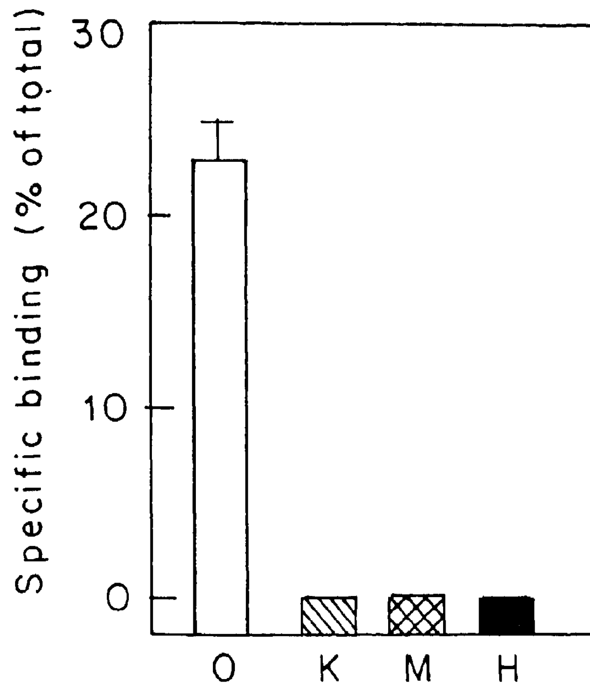

Figure 3. Tissue specificity of [^{125}I] cGTH binding. Binding incubations were carried out in the same manner as described in §2 except the addition of 2 mg of different tissues collected from *C. catla*. Data represent the mean ± SEM of five determinations.
O, Ovary; K, kidney; M, muscle; H, heart.

the four different stages whereas receptor occupancy varied significantly. B-max was highest in spawning stage oocytes which was comparable to prespawning oocyte while it was three times less in preparatory stage oocytes as compared to spawning stage and it was lowest in postspawning oocytes.

Table 1. Determination of cGTH affinity and B-max in the ovary of *Catla* belonging to different stages of reproductive cycle.

Stage of fish	K_d ($\times 10^{-10}$ M)	Maximum binding capacity (fmol/mg protein)
Preparatory	0·92	6·11 ± 0·36
Prespawning	0·86	17·0 ± 0·29
Spawning	0·78	18·7 ± 0·17
Postspawning	0·97	5·28 ± 0·28

[^{125}I] cGTH binding profile to oocyte membrane preparation was obtained from the saturation experiments. Determination of *Kd* and B-max was made from the Scatchard analysis of binding experiments. Data presented are the mean of four independent determinations.

3.4 *Functional response of different stages oocyte to cGTH*

To observe cGTH sensitiveness to oocytes of different reproductive stages, oocytes from each stage was incubated with 500 ng of cGTH and then processed to obtain steroidogenic profile. Effect of varied concentrations (100 ng to 2 μg) of cGTH on the ovarian steroidogenesis was tested and since 500 ng had half-maximal stimulation (data not given), its effect on different stages of oocytes is shown in figure 4. Formation of progesterone from pregnenolone was highest in the oocytes of prespawning and spawning stage and lowest in post spawning stage oocyte.

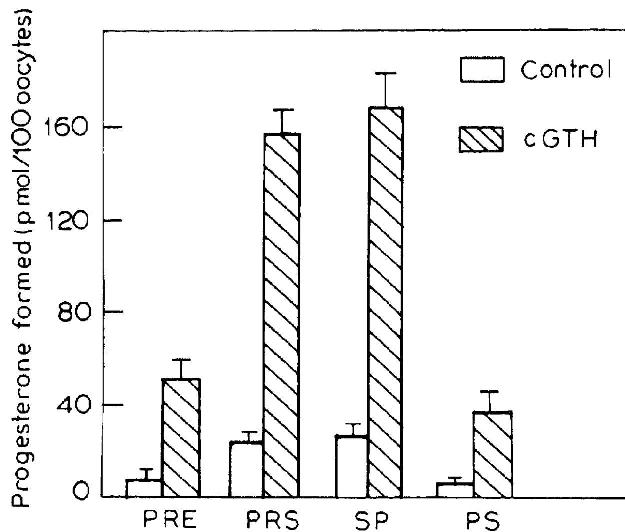

Figure 4. Response of different stages oocytes to cGTH. Oocytes were collected from the carp belonging to four different reproductive stages and incubated with 500 ng of cGTH and then processed for determining the amount of radiolabelled progesterone formed from radiolabelled pregnenolone. Each value represents the mean ± SEM of five separate determinations.
PRE, Preparatory; PRS, prespawning; SP, spawning; PS, postspawning.

4. Discussion

In the present study we have used homologous radiolabelled piscine GTH [^{125}I] cGTH for receptor binding assay with fairly pure plasma membrane preparation from *Catla* oocytes. Standardization of cGTH receptor binding assay clearly showed maximum binding at 30°C with pH 7·5. These two characteristics are coinciding well with the natural environment when GTH activity is in peak and spawning occurs. Annual reproductive cycle of Indian teleosts can be divided into four distinct phases, preparatory, prespawning, spawning and postspawning (Sundararaj and Vasal 1976; Chakraborty and Bhattacharya 1984). Indian teleosts spawn during monsoon, which is the rainy season and precise timing of monsoon differs in different parts of this country. Monsoon comes earlier (middle of June) to eastern India *(i.e.* the site of present investigation) than that of western or northern

India (middle of July) while south India has two monsoon seasons. To breed during monsoon, gonadal development and maturation of Indian teleosts must have to be preceded earlier. Prespawning is a vitellogenic phase when growth and maturation of oocyte is nearly completed except the germinal vesicle breakdown (Mukherjee *et al* 1989). Water temperature during May to July (covering prespawning and spawning phases) varied between 30°C to 35°C and pH of the water is weakly alkaline (7·3 to 7·6). Shift of pH towards acidity (6·6 to 6·8) worstly affects maturation and breeding, which often occurs due to the environmental pollution. However, our standardization of [^{125}I] cGTH specific binding to carp oocyte membrane preparation at 30°C and pH 7·5, corresponds to natural environment and indicate physiological binding. To observe the specificity of cGTH binding, identically processed plasma membrane preparation from muscle, kidney, heart and ovary were subjected to $^{[125I]}$ cGTH binding assay and results showed that specific binding of cGTH was strictly restricted to ovarian tissue only. Similar specificity of gonadotropin binding to only gonads where no other tissues *i.e.* kidney, muscle, brain and liver showed specific binding was also demonstrated in amago salmon (Salmon *et al* 1984). Specificity of cGTH binding sites to oocyte plasma membranes was checked by inhibition study. Only mGTH was found to be a competitor of cGTH but about 1·8-fold excess of mGTH was required for 50% displacement of [^{125}I] cGTH as compared to unlabelled cGTH.

One of the interesting aspects of the present investigation is the use of homologous radiolabelled cGTH for receptor binding assay with carp oocyte plasma membrane preparation. Scatchard plot analysis showed that the affinity of receptor binding (oocytes collected from carp belonging to prespawning stage) is appreciably high *i.e.* K_d = 0·86×10^{-10} M. Binding affinity of GTH with the oocyte plasma membrane preparation of another Indian teleost, a freshwater murrel *Channa punctatus* was comparatively lower. Jamaluddin and Bhattacharya (1986) from this laboratory used oocyte plasma membrane preparation from spawning stage murrel and found *Kd* value of 1·27 × 10^{-10} M with silver carp GTH and 2·35 ×10^{-10}M with hCG, while this was 0·78 × 10^{-10} M in the case of carp belonging to spawning stage. However primary reason for such a difference between Indian carp and murrel may be the use of homologous GTH in carp and heterologous GTH in murrel. The K_d values of all four reproductive stages range between 0·78 to 0·97 × 10^{-10} M, affinity here appears to be higher than that of the value obtained with preovulatory ovarian follicles of amago salmon, *O. rhodurus* using chum salmon GTH, CSG-S II (0·2–0·8 nM; Kanamori and Nagahama 1988), rainbow trout testis using hCG (0·4 nM; Schlaghecke 1983), rainbow trout ovary using hCG (0·3-0·6 nM; Schulz *et al* 1985), chum salmon gonad using salmon gonadotropin (0·3-0·7 nM; Van der Kraak and Donaldson 1982), ovary of brown trout using salmon gonadotropin (0·3-0·6 nM; Breton *et al* 1986) and goby testis using salmon gonadotropin (2·5–200 nM; Ishii and Aida 1982; Aida and Ishii 1985). Our dissociation constant value is very close to the value obtained with European carp gonadotropin binding to eel ovary (about 0·1 nM; Salmon *et al* 1987).

Although reproduction in majority of teleostean fish is seasonal, there is very little information about the pattern of gonadotropin receptor in different stages of annual reproductive cycle. Kanamori and Nagahama (1988) made Scatchard plot analysis of CSG-S II binding to the membrane preparation from ovarian follicle of amago salmon during oogenesis and observed high affinity sites in the intact follicles, isolated thecal layers and isolated granulosa cells at all stages of

development. The values of K_d in amago salmon varied at all stages of development which is very similar to our observation. Vitellogenesis in Indian carp starts in February (preparatory stage) and completes by the second week of June (prespawning stage). The number of GTH binding site in amago salmon increased for the membrane preparations from intact follicles during follicular development but the same is not true when membranes were prepared from granulosa cells or thecal layers (Kanamori and Nagahama 1988). The reason for such differences between intact follicle and isolated cells is not known, it possibly indicate an alteration in the membrane property imposed upon by the separation of cells from the follicles. However, we have used plasma membrane from intact follicles and found a conspicuous increase in the number of cGTH binding site (B-max) from preparatory to prespawning and spawning stages of carp (from 6·11± 0·36 fmol/mg protein to 18·7 ±0·17 fmol/mg protein) which sharply declined in the oocytes of post-spawning stage fish (5·28 ±0·28 fmol/mg protein). There was practically no binding with the ovarian tissue collected from the spent fish *i. e.* which already spawned. This clearly suggests a significant increase in the quantity of GTH receptor with the developmental progress of oocytes and their absence after spawning. There is very little difference between prespawning and spawning stages oocytes when GTH receptor amount was determined on the basis of per mg protein. B-max was 17·0± 0·29 fmol/mg protein in the oocytes of prespawning stage and 18·7±0·17fmol/mg protein in the oocytes of spawning stage but when we determined the total number of receptors per oocyte, it was about 1,00,000 per oocyte with prespawning stage of fish and 1,30,000 per oocyte with spawning stage fish. In fact, there was only 15 days difference between these two determinations, about 1,00,000 receptor per oocyte was found at the end of May (prespawning) and 1,30,000 receptor per oocyte was observed in the middle of June after the first shower of monsoon (spawning) which is the beginning of spawning time. To observe the physiological relevance of varied amount of cGTH receptor at different stages of reproductive cycle, oocytes of different stages were incubated with fixed amount of cGTH and conversion of radiolabelled pregnenolone to radiolabelled progesterone was assessed. Response to cGTH of different stages of oocytes varied considerably. There was a significant increase in progesterone formation during preparatory to prespawning ($P < 0.005$), highest was obtained with spawning stage oocytes and lowest with postspawning stage oocytes, a profile which clearly matches receptor number at different stages of reproductive cycle. In conclusion it may be stated that GTH receptor number fluctuates in *Catla* ovary at different stages of reproductive cycle and its profile follows the functional status of ovary schedule at each stage of annual reproductive cycle.

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