Introduction

Gonadal development and reproduction in vertebrates is always depends on the two gonadotropins (GTHs), FSH and LH which are produced by the pituitary gland. In fish, ovarian steroids synthesis, like other vertebrates, is under the control of these two distinct gonadotropins, GTH-I and GTH-II, which are homologous to tetrapods FSH and LH respectively (Suzuki et al, 1988; Planas et al., 1993; Li and Ford, 1998; Yoshiura et al., 1999; Swanson et al., 2003). Plenty of reports are available regarding the steroidogenic potency of FSH and LH in both testes and ovary of teleost fishes (Suzuki et al., 1988; Swanson et al., 1989; Van Der Kraak et al., 1992; Tanaka et al., 1995; Kagawa et al., 1998; Planus et al., 2000; Kagawa et al., 2003; Gen et al., 2003; Ko et al., 2007; Pramanick et al., 2013). Generally, steroidogenesis begins with the secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus. GnRH stimulates FSH and LH which binds to the specific receptors on ovarian follicular cells to begin steroid secretion (Kumar and Trant, 2001; Bogerd et al., 2005; Mukherjee et al., 2017). Due to availability of purified FSH and LH from salmonid pituitaries, steroidogenic and maturational activity of salmon FSH and LH has been relatively well described in salmon and trout. In salmonids, FSH is elevated during vitellogenesis and regulates early phases of gametogenesis, whereas LH appears during final stage of oocyte maturation and ovulation in females (Prat et al., 1996; Gomez et al., 1999). In theca-interstial cell layer from all stages of coho salmon, both FSH and LH stimulated testosterone (T) and 17α -hydroxyprogesterone (17α -OH-P) production, but LH being more potent than FSH, whereas in granulosa layers from all stages, LH, not FSH, stimulated 17 β -estradiol (E₂) and 17 α ,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) production through conversion of T and 17a-OH-P respectively (Planas et al., 2000). This conversion of T to E_2 and 17 α -OH-P to 17,20 β -P is thought to be rate-limiting steps in E_2 and 17,20 β -P biosynthesis in salmon ovarian follicles. Moreover, it has also been suggested that in salmonids, FSH regulate E₂ production through stimulating P450arom gene expression and aromatase activity during vitellogenic period (Kagawa et al., 1983).

It has been shown that there are two types of GTH receptors in salmon ovary, one binds both FSH and LH (type-I receptor) and another which binds specifically LH (type-II receptor) (Yan *et al.*, 1992; Miwa *et al.*, 1994). They also reported that during oocyte growth type-I receptors are present in both theca and granulosa cells, while during final oocyte maturation in parallel with the rise in plasma LH level the type-II GTH receptor appears in the granulosa cells. In 2005, Ge have cloned and characterized zebra fish FSH and LH receptors (FSHR and LHR). During ovarian and follicular development, both FSHR and LHR have low expression in the follicles of primary growth stage. However, during vitellogenesis, there is a significant increase in FSHR but not LHR expression. The expression of LHR significantly increases in the midvitellogenic stage and show peak in the full grown stage. These results suggests that the two gonadotropin receptors have distinct functions in folliculogenesis (Kwok *et al.*, 2005), with FSHR being involved in follicle recruitment and vitellogenic growth and LHR being important in the later stages of follicle development including final oocyte maturation.

T. fasciata belongs to the order perciformes is one of the most ecologically important fish in the rural West Bengal, India. From available literature, it appears that although only scattered information is available regarding the biology and induced breeding of *T. fasciata;* (Abujam *et al.*, 2015; Islam *et al.*, 2017) no report has yet been available on steroidogenic potentiality of ovarian follicles and effects of gonadotropins and other hormones thereon of this fish.

The aim of the present study was to investigate the steroidogenic potentiality of ovarian follicles at different stages of reproductive cycle and effects of gonadotropins on *in vitro* production of steroids.

Materials and Methods

Chemicals

The materials and methods specifically used for conducting the experiments mentioned in this chapter are discussed below. All others have already been described under "Materials and Methods" of Chapter-1.

Human chorionic gonadotropin (HCG) and follicle stimulating hormone (FSH) was a gift from National Hormone and Pituitary program, Torrance, California. All cold steroids, cycloheximide, actinomycin D and trilostane were purchased from Sigma Chemical, St. Louis, MO, USA. 1-heptanol and 1-octanol were purchased from ICN Biochemicals Inc (USA). All other chemicals were used of analytical grade.

Dilution of hormones and other effectors

HCG was diluted in Idler's medium before use. All cold steroids were dissolved in ethanol before dilution in the medium. The amount of ethanol did not exceed 0.5% of the final volume and this concentration had no influence on basal or stimulated steroid production. Cycloheximide and actinomycin-D were dissolved directly in the incubation medium. Trilostane was dissolved in dimethylsulphoxide (DMSO).

Incubation Medium

For *in vitro* incubation of *T. fasciata* ovarian follicles we have selected Idler's medium containing streptomycin (100 μ g/ml) and penicillin (100 IU/ml) adjusted to pH 7.4 with little modification. The composition of Idler's medium was NaCl-1.080g, KCl-0.272g, Glucose-0.108g, CaCl₂-0.32g, MgCl₂.6H₂O- 0.404g, Tris-0.96g, NaH₂PO₄- 0.034g, NaHCO₃- 0.42g, in 1.0 litre triple distilled sterilized water.

In vitro incubation of ovarian follicles

T. fasciata was collected from local fisherman, reared in the laboratory condition 2 days, killed by decapitation and the ovaries were dissected out in aseptic condition. They were then placed in ice cooled Idler's medium containing streptomycin (100 µg/ml) and penicillin (100IU/ml) adjusting pH 7.4 (Bhattacharyya et al., 2000). Ovarian samples were then cut into small pieces and quickly transported to the laboratory. Oocytes with follicle layers were separated by repeated pipetting and collected in the fresh medium with stimulators and inhibitors. T. fasciata is a semelparous fish which exhibits synchronous follicle development i.e., most of the follicles in the ovary are at the same developmental stages in the same time. Ovarian pieces and follicles weighing approximately 100 mg were then placed in individual wells of a 24-well culture plate (Tarson, India) that contained 1.0 ml fresh medium with stimulators and inhibitors. Inhibitors were added 1 h prior to the addition of the test compounds. Cultures were placed in a metabolic shaker bath at 23±1°C under air. Viability of ovarian follicles was observed to be about 90% as detected using 0.1% trypan blue dye exclusion. At the end of incubation, medium samples were aspirated, centrifuged (5000×g) and stored at -20° C for steroid measurement by specific radioimmunoassay. All experiments were repeated using ovarian follicles collected from five different fishes.

Extraction and assay of steroids

This has been described in Chapter-II.

Statistical analysis

All experiments were repeated in ovarian follicles or theca and granulosa cells collected from five fishes. Data from each experiments were subjected to analysis of one way variance (ANOVA) followed by Bonferroni's multiple range test. Differences were considered to be significant at p<0.05.

Results

Effects of FSH, LH and HCG on T, E_2 and 17,20 β -P production by ovarian tissues at various developmental stages

Previtellogenic, vitellogenic, post vitellogenic and post GVBD stage follicles (100 mg each) of *T. fasciata* were incubated with various increasing concentrations of FSH, LH and HCG (each 0, 25, 50, 100, 200, 400 ng/ml) for 24 h and the steroid concentrations in the medium were analyzed. It appears from Fig 1 that, previtellogenic stage follicles showed very low response to FSH, LH and HCG in T (Fig. 1A) and E_2 (Fig. 1B) production *in vitro*. Among the three hormones tested, FSH showed a little higher stimulatory effect on T and E_2 production at this stage of development than LH and HCG. The optimum effective doses of all the three hormones showed effective response was 25 ng/ml (Fig. 1).

In vitellogenic follicles, FSH, LH and HCG at their increasing doses caused a gradual and significant stimulation in the production of T (Fig. 2A) and E_2 (Fig. 2B). Responses of such follicles to the hormones on steroid production were much higher than that of previtellogenic stage follicles (Fig. 1). The optimum effective dose of FSH, LH and HCG in stimulating both the steroid production was shown to be 100 ng/ml. The minimal tried concentration at which three hormones showed effective response was 25 ng/ml. (Fig. 2). Moreover, at this stage all the three hormones showed almost equal effects on steroid production. Interestingly, at this stage of follicular development, media 17,20 β -P content was non-detectable and even hormone treatment has no stimulatory effect on 17,20 β -P production.

Postvitellogenic stage follicles incubated for 24 h with LH and HCG produced T (Fig. 3A), E_2 (Fig. 3B), and 17,20 β -P (Fig. 3C) gradually and significantly with increasing doses. Increasing doses of FSH only could stimulate E_2 production (Fig. 3B). FSH-stimulated T and 17,20 β -P production was noticed only at 100 ng/ml with no further increase thereafter (Fig. 3A & 3C). Follicles at this stage responded more to LH and HCG than to FSH in producing E_2 . Optimum effective doses for LH and HCG were 100 ng/ml. The minimal tried concentration at which LH and HCG showed effective response for T and E_2 was 25 ng/ml but for 17, 20 β -P was 50 ng/ml.

In post GVBD follicles, FSH, LH and HCG at their increasing doses caused a significant production of T (Fig. 4A) and E_2 (Fig. 4B) in very low levels. Responses of such follicles to the hormones on steroid production were much lower than that of post vitellogenic stage follicles (Fig. 3). The optimum effective dose of FSH, LH and HCG in stimulating both the steroid production was shown to be 100 ng/ml. The minimal tried concentration at which three hormone showed effective response was 25 ng/ml (Fig. 4). Interestingly, at this stage of follicular development, media 17,20 β -P content was non-detectable and even hormone treatment has no stimulatory effect on 17,20 β -P production.



Fig. 1 Effect of graded doses of FSH, HCG and LH on steroid production by previtellogenic stage follicles of *T. fasciata* after 24 h of incubation. Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish.





Fig. 2 Effect of graded doses of FSH, HCG and LH on steroid production by vitellogenic stage follicles of *T. fasciata* after 24 h of incubation. Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish. T- Testosterone and E₂- 17β-estradiol.



Fig. 3 Effect of graded doses of FSH, HCG and LH on steroid production by postvitellogenic stage follicles of *T. fasciata* after 24 h of incubation. Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish. T- Testosterone, $E_{2^{-}}$ 17 β -estradiol and 17,20 β -P-17 α ,20 β -dihydroxy-4-pregnen-3-one.



Fig. 4 Effect of graded doses of FSH, HCG and LH on steroid production by post GVBD stage follicles of *T. fasciata* after 24 h of incubation. Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish. T- Testosterone and E₂- 17β-estradiol.

Time-course effect of FSH, LH and HCG on steroid production

Previtellogenic, vitellogenic and post vitellogenic follicles of *T. fasciata* were incubated with optimal effective doses of FSH, LH and HCG for various length of time up to 24 h on T, E_2 and 17,20 β -P production. It appears from Fig 5A and B that, in previtellogenic follicles, FSH, LH and HCG, (each at 200 ng/ml dose) caused significant stimulation of E_2 and T production with times. Responses of such follicles to FSH on steroid production was little higher than LH and HCG. When FSH stimulated T and E_2 production started from 6 h and completed by 20 h after incubation, stimulation of the same by LH and FSH was noticed at 12 h with maximum at 16 h (Fig. 5A and B).

Vitellogenic follicles were incubated with FSH, LH and HCG at the dose of 100 ng/ml (optimal effective dose) for 24 hr. Fig. 6A and B showed that all the three hormones caused significant stimulation in the production of T and E_2 after 6 h of incubation and increased gradually and significantly up to 20 h and then levels off. Responses of the follicles to all the three hormones in the production of T and E_2 at this stage of development were shown to be almost similar.

Post vitellogenic stage follicles were incubated with FSH, LH and HCG at the dose of 100 ng/ml (optimal effective dose) for 24 h. Fig. 7A, B and C reveals that LH and HCG only caused a gradual and significant increase in T, E_2 and 17,20 β -P production from 3 h onwards and attended maximum levels at 16 h of incubation and then levels goes fall down. Interestingly, at this stage of follicular development FSH showed a very low stimulation of T, E_2 and 17,20 β -P production with increasing time (Fig. 7A, B & C).

In post GVBD follicles were incubated with FSH, LH and HCG at the dose of 100 ng/ml for 24 hr. Fig. 8A and B show that all three hormones caused stimulation in the production of T and E_2 very low level than post vitellogenic stage after 16 h of incubation and increased gradually and significantly up to 20 h and then levels goes fall down. Responses of the follicles to all three hormones in the production of T and E_2 at this stage of development were shown to be almost similar.



Fig. 5 Time-course effect of FSH, HCG and LH on steroid production by intact previtellogenic follicles of *T. fasciata*. Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from 3, 6 and 9 h (*p<0.05). C- Saline control, T- Testosterone and E₂- 17 β -estradiol.



Fig. 6 Time-course effect of FSH, HCG and LH on steroid production by intact vitellogenic follicles of *T. fasciata.* Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from 3, 6, 9 and 12 h (*p<0.05). C- Saline control, T-Testosterone and E₂- 17 β -estradiol.



Fig. 7 Time-course effect of FSH, HCG and LH on steroid production by intact postvitellogenic follicles of *T. fasciata*. Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from 3, 6, 9 and 12 h (*p<0.05). C- Saline control, T- Testosterone, E₂- 17 β -estradiol and 17,20 β -P- 17 α ,20 β -dihydroxy-4-pregnen-3-one.



Fig. 8 Time-course effect of FSH, HCG and LH on steroid production by intact post GVBD follicles of *T. fasciata*. Each point represents the mean \pm S.E.M. of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from 3, 6, 9, 12 and 16 h (*p<0.05). C- Saline control, T- Testosterone and E₂- 17 β -estradiol.

Effects of actinomycin-D (Act-D) and cycloheximide (Chx) on HCG-induced steroid production

Since, steroidogenic effects of LH and HCG at postvitellogenic stage were almost similar and the effect of FSH on this aspect was very low, for our present experiment we selected only HCG as an inducer of steroidogenesis.

A dose kinetic study on the effects of protein synthesis inhibitors, actinomycin-D (Act-D) and cycloheximide (Chx) on HCG-induced T, E_2 and 17,20β-P production were conducted with postvitellogenic follicles. Initially, follicle were pre-incubated for 1 h with increasing concentrations of Act-D and Chx and incubated for 24 h with HCG (0.1 µg/ml) for each inhibitor. It appears from Fig. 9 that both Act-D and Chx inhibited HCG-stimulated T (Fig. 9A), E_2 (Fig. 9B) and 17,20β-P (Fig. 9C) production almost in a dose-dependent manner. Both the inhibitors reduced media levels of steroids significantly at the concentration of 0.1 µg/ml and maximum inhibitors on T and E_2 production by the previtellogenic and vitellogenic follicles and 0.2 µg/ml in vitellogenic follicles) for 16 h. Results showed that both the inhibitors caused significant inhibition of HCG-stimulated T and E_2 production at these doses tested (Fig. 10 and 11).

Effects of aminoglutethemide and trilostane on HCG-induced steroid production by the postvitellogenic follicles

Since LH and HCG at postvitellogenic follicles had almost similar effects on steroid production, this experiment was conducted only with HCG in presence of inhibitors. Ovarian follicles (100 mg each) were incubated with 0.1 μ g/ml of HCG in absence or presence of increasing doses of aminoglutethemide (inhibitor of cholesterol side-chain cleavage enzyme) and a potent 3 β -HSD inhibitor, trilostane for 24 h. Result showed that both aminoglutethemide and trilostane caused a gradual and significant inhibition of T (Fig. 12A), E₂ (Fig. 12B) and 17,20 β -P (Fig. 12C) production with increasing doses (0, 0.1, 0.5, 1 and 1.5 μ g/ml) of inhibitors (Fig. 12A, B and C). A maximal inhibitory effect of aminoglutethemide and trilostane was obtained at 0.5 μ g/ml and 1 μ g/ml dose respectively.



Fig. 9 Production of T (A), E_2 (B) and 17,20 β -P (C) in postvitellogenic ovarian follicles (100 mg) exposed to HCG (0.1 μ g/ml) for 24 h in the presence or absence of actinomycin-D (Act-D) and cycloheximide (Chx). Each point represents the ±SEM of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from those shown for tissues incubated with exogenous hormone alone (*p<0.05). C- Saline control, T- Testosterone, E_2 - 17 β -estradiol and 17,20 β -P- 17 α ,20 β -dihydroxy-4-pregnen-3-one.



Fig. 10 Production of T (A) and E_2 (B), in previtellogenic ovarian follicles (100 mg) exposed to HCG for 16 h in the presence or absence of Act-D and Chx. Each point represents the ±SEM of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from those shown for tissues incubated with exogenous hormone alone (*p<0.05). C- Saline control, T- Testosterone and E_2 -17 β -estradiol.



Fig. 11 Production of T (A) and E_2 (B) in vitellogenic ovarian follicles (100 mg) exposed to HCG for 16 h in the presence or absence of Act-D and Chx. Each point represents the ±SEM of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from those shown for tissues incubated with exogenous hormone alone (*p<0.05). C- Saline control, T- Testosterone and E_2 -17 β -estradiol.



Fig. 12 Production of T (A), E_2 (B) and 17,20 β -P (C) in postvitellogenic ovarian follicles (100 mg) exposed to HCG (0.1 μ g/ml) for 24 h in the presence or absence of aminoglutethemide (Ami) and trilostane (Tri). Each point represents the ±SEM of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from those shown for tissues incubated with exogenous hormone alone (*p<0.05). C- Saline control, T- Testosterone, E_2 - 17 β -estradiol and 17,20 β -P- 17 α ,20 β -dihydroxy-4-pregnen-3-one.

Effects of gap-junction uncouplers on HCG-induced steroid production by the postvitellogenic follicles

Postvitellogenic follicles were pre-incubated with gradually increased doses of n-alkanol gap-junction uncouplers, namely, 1-octanol and 1-heptanol, for 1 h followed by incubation with HCG for 16 hr. Ethanol control was maintained in each case. It appears from Fig. 13 that 1-octanol or 1-heptanol at their increasing concentrations were able to inhibit the HCG-stimulated T (Fig. 13A), E_2 (Fig. 13B) and 17,20 β -P (Fig. 13C) production. Ethanol alone had no effect on steroid production. Significant levels of inhibition were observed at the concentration of 0.1 mM 1-octanol or 1-heptanol. Further increase in concentration caused gradual decline in the media steroid levels.



Fig. 13 Production of T (A), E_2 (B) and 17,20 β -P (C) in postvitellogenic ovarian follicles (100 mg) exposed to HCG (0.1 μ g/ml) for 24 h in the presence or absence of 1-octanol (Oct) and 1-heptanol (Hept). Each point represents the ±SEM of five incubations taking follicles from five donor fish. Asterisks denote values significantly different from those shown for tissues incubated with exogenous hormone alone (*p<0.05). C- Saline control, T- Testosterone, E_2 - 17 β -estradiol and 17,20 β -P- 17 α ,20 β -dihydroxy-4-pregnen-3-one.

Discussion

Present study reports about the *in vitro* steroidogenic effects of heterologous gonadotropins: FSH, LH and HCG in the ovarian follicles of *T. fasciata* at different developmental stages. In previtellogenic stage ovarian follicles, although all three gonadotropins stimulated T and E₂ production, but the responses of the tissues at this stage to the hormones were very low. Moreover, out of these three gonadotropins, FSH was shown to be more effective in E₂ and T production than LH and HCG. Vitellogenic stage follicles were shown to be sufficiently responsive to all the gonadotropins tested and in producing T and E₂. All these three gonadotropins are almost equipotent in releasing both the steroids. Interestingly, all these hormones at this stage of development were not able to produce any detectable amount of 17,20β-P. During postvitellogenic stage, apart from basal T and E₂, production of 17,20β-P was also noticed and LH and HCG stimulated the production of all three steroids such as E₂, T and 17,20β-P with increasing doses and time. FSH practically had no or little stimulatory effect on the production of all these steroids at this stage of development.

In vitro basal production of T and E_2 by immature tissues and vitellogenic follicles and E₂, T and 17,20β-P by the postvitellogenic follicles was highly correlated with plasma levels of these steroids and GSI values (see results Chapter II). Steroidogenic responses of ovarian follicles to gonadotropins stimulation and endocrine mechanism underlining steroid production have been described in many teleosts (Zohar et al., 1982; Young et al., 1983a; Zhao and Wright, 1985; Nagahama, 1987; Petrino et al., 1989; Planas, 1997; Mukherjee et al., 2006). Most of these studies were restricted to either vitellogenic stage or during final oocyte maturation. Reports on responses of early stage ovarian tissues to gonadotropins in the production of steroids in teleosts are limited. Our results with immature stage tissues indicate that sensitivity of these tissues to gonadotropins in the production of steroids just commenced and the tissues at this stage were moderately responsive to gonadotropins. Almost similar results were obtained with C. carpio and A. testudeneous (Mukerjee et al., 2006; Bhattachriya et al., 2002). The more effectiveness of FSH compared to LH and HCG in the production of E₂ and T at this stage of development indicates that early stage of folliculogenesis and steroidogenesis of T. fasciata like other fresh water teleosts is regulated by GTH-I which is comparable to FSH. In our study we did not use gonadotropins of T. fasciata origin even we did not use fish gonadotropins but the trends of our results like other fish supports the two gonadotropin theory in regulating folliculogenesis and

steroidogenesis in female *T. fasciata* (Suzuki *et al.*, 1988; Planas *et al.*, 1993; Planas *et al.*, 2000; Ko *et al.*, 2007; Paul *et al.*, 2010).

In teleosts, increase in size of oocyte occurs mainly due to accumulation of vitellogenin synthesized by the liver which is stimulated by 17β -estradiol secreted by the follicles. Maximum production of E₂ was observed in vitellogenic stage follicles in response to FSH, LH and HCG. The responses of the follicles to all the three gonadotropins were almost in equal magnitude in producing E₂. Our data thus suggested that during vitellogenesis in *T. fasciata* both GTH-I and GTH-II are equipotent in the production of E₂. In most other teleosts the situation is different. In common carp and red seabream, it is LH, not FSH stimulated the production of E₂ during vitellogenesis (Kagawa *et al.*, 2003; Paul *et al.*, 2010). Our result also shows that all the three gonadotropin stimulated the production of E₂ in intact vitellogenic follicles by stimulating testosterone production.

In vitro production of $17,20\beta$ -P by postvitellogenic follicles and its stimulation by LH and HCG clearly indicates that GTH-induced synthesis and release of this steroid is acquired prior to final oocyte maturation. In postvitellogenic follicle significantly high production of 17,20 β -P in response to LH and HCG (not by the FSH) concomitant with less production of E₂ compared to vitellogenic follicle suggests that just immediately prior to final oocyte maturation, important changes occur in the mechanism controlling the steroid biosynthetic pathway in the follicle. A shift from estrogenic to progestational hormone production is evident in the fish. It has been reported that injection of GTH into ayu, Plecoglossus altivelis stimulated the activity or induced the formation of 20β-hydroxysteroid dehydrogenase (20β-HSD), the enzyme control the conversion of 17a-OH-P to 17,20β-P as assessed by conversion of precursor steroid to 17,20β-P by ovarian homogenate (Suzuki et al., 1981). Reports are also available that GTH can stimulate 20β-HSD in isolated granulose cell layer from amago salmon (Young et al., 1983). Postvitellogenic follicles of guppy, Poecila reticulate, were also shown to convert 17a-OH-P to large amount of 17,20β-P (Venkatesh et al., 1992). All these information indicate that during final oocyte maturation activity of 20β-HSD in the ovarian follicle is highly active which convert 17α -OH-P to $17,20\beta$ -P. Our results thus indicate that LH and HCG in intact postvitellogenic follicles stimulated the activity of 20β-HSD for higher production of 17,20β-P. Therefore, these results suggest that GTH-II may be an important regulator of steroidogenesis and final oocyte maturation in T. fasciata and changes in ovarian steroidogenesis that occur during vitellogenesis

and final oocyte maturation may be regulated in part by circulating FSH and LH and in GTH receptor type and distribution. The result also shows that FSH, HCG or LH, stimulated the conversion of T to E_2 during vitellogenesis in ovarian follicles of *T. fasciata*. Immediately prior to oocyte maturation a decrease in FSH activity concomitant with an increase in 17,20β-P production suggest a regulatory role of HCG or LH in steroidogenic shift from estrogenic to progestational steroid in this fish.

Cycloheximide and actinomycin-D were shown to block HCG-induced 17,20 β -P production in *T. fasciata* oocytes. It could be interesting to ascertain whether the novel protein for 17,20 β -P production is 20 β -HSD enzyme (Nagahama, 1987) or the MIH receptor (Patrino and Thomas, 1990) or a maturation promoting factor (MPF).

We observe that T, E_2 and 17,20 β -P production in response to HCG was inhibited by 1octanol and 1-heptanol indicating that homologous gap junction among granulosa cells is associated with the action of HCG to produce 17,20 β -P. The present findings thus suggest that, for GTH induction of steroidogenesis and oocyte maturation, both homologous and heterologous gap junction formation are required.

Results of the present study also indicate that stimulatory effect of HCG upon T, E_2 and 17,20β-P production in isolated ovarian follicles of postvitellogenic stage *T. fasciata* depends upon the activities of cholesterol side-chain cleavage enzymes. HCG-stimulated production of these steroids by the ovarian follicles was shown to be significantly inhibited by aminoglutethemide, an inhibitor of cholesterol side-chain cleavage enzyme (Hall, 1984). Petrino *et al.* (1989) also reported the inhibition of fish pituitary extract-stimulated steroid biosynthesis by aminoglutethemide in ovarian follicles of *F. heteroclistus*. The present work thus provides an evidence that enzymatic step catalyzed by cholesterol side-chain cleavage enzyme is essential for HCG-stimulated T, E_2 and 17,20β-P by the ovarian tissue of *T. fasciata*.

The importance of enzymatic step (3β -HSD) that transforms pregnenolone to progesterone has been studied in fish ovaries by different workers (Theofan and Goetz, 1983; Petrino *et al.*, 1989; Bhattacharyya *et al.*, 2000). Cyanoketone, an inhibitor of 3β -HSD, was found to prevent gonadotropin or forskolin-stimulated E₂ production by the ovaries of amago salmon (Young *et al.*, 1986) and gold fish (Tan *et al.*, 1986). Likewise pregnenolone-induced oocyte maturation in killifish was blocked by trilostane (Petrino *et al.*, 1989). Fish pituitary

extract-induced pregnenolone metabolism to progesterone and HCG-induced steroid production was blocked by trilostane (Bhattacharyya *et al.*, 2000; Set *et al.*, 2002). In the present study employing trilostane in the incubation of ovarian follicles, it is clear that HCG stimulated the synthesis of 17,20β-P through by Δ 5- Δ 4 pathway. We conclude from this observation that enzymatic step catalyzed by 3β-HSD is essential for the biochemical and physiological responses of *T. fasciata* postvitellogenic follicle to HCG.

In conclusion, the present study highlights the steroidogenic potency of female T. fasciata ovarian follicles from maturing to post-GVBD stage of ovarian development and gonadotropins have a discrete regulatory role over steroid production at each stage. Less amount of T and E₂ production by the maturing stage follicles and comparatively greater stimulatory action of FSH indicates that during early stage of folliculogenesis in T. fasciata, GTH-I or FSH have a regulatory role on steroidogenesis. The equipotent activity of FSH, LH and HCG at vitellogenic stage follicle in the production of T and E₂ indicate that during this stage of development both FSH and LH and equally active. Our results clearly indicate that steroidogenesis in postvitellogenic follicles is regulated by LH and HCG, not by FSH. In post-GVBD stage, the responsiveness of oocytes to any gonadotropin (FSH, LH and HCG) is lower down. Our result also indicates a shift in steroidogenesis from estrogenic to progestetional steroid from vitellogenic to postvitellogenic stage and high production of 17,20β-P during final oocyte maturation indicates its involvement in oocyte maturation. To get clear picture on the gonadotropin-regulated ovarian steroidogenesis in T. fasciata, further works are required specifically by using fish FSH and LH, and by demonstrating the activity of 20β-HSD and P450 aromatase.