Introduction

Urbanization, industrialization and hi-tech agricultural processes are indicators of the advancement of civilization. However, this process can unfortunately lead to unhealthy ecosystems and also cause biodiversity degradation. In fact, modernization results often in the release of various toxic chemicals into the environment and it represents a growing global concern today. Heavy metals, especially mercury, are common pollutants released by the advanced civilization process and mercury is the most studied pollutant since 1950. However, most of the studies have concentrated on the biomagnification or bioaccumulation of mercury and its impact on human health. Any pollutant exerts its action on organisms or populations by affecting their normal endocrine function as well as reproduction. Scanty information is available (Kirubagaran and Joy, 1988; Mukherjee *et al*., 1994) regarding the role of mercury as endocrine disrupting compounds (EDCs) in teleost fish. Thus, to find out the role of mercury on reproductive dysfunction of teleost fish is essential.

Mercury exists in the environment as inorganic form by making complexes with other substances such as chloride, hydroxide, and sulfide (Morel *et al*., 1998; Wang *et al*., 2010). Among them, mercuric chloride ($HgCl_2$) is the dominant pollutant as it is used as an ingredient in antiseptics, disinfectants and preservatives, insecticides, batteries and also in metallurgical and photographic operations (Goldberg, 1996; Carpi and Lindberg, 1997; Freeman *et al*., 2003). These sources contaminate natural aquatic ecosystems through surface run off. In India, mercury pollution is becoming a new environmental threat as the range of mercury in industrial effluents (0.058-0.268 mg/L) is much higher than the limit decided by the World Health Organization (0.001 mg/L) (Srivastava and Srivastava, 2002.). Atmospheric mercury releases from coal combustion contributed to further contaminate the aquatic ecosystems (Glover *et al*., 2010).

Being an important part of the aquatic food web, fishes are more susceptible to mercury toxicity. However, only a limited number of reports are available on mercury toxicity in fish. In killfish, *Fundulus heteroclitus,* mercury pollution induces teratogenecity (Weis and Weis, 1995) and chromosomal abnormality (Perry *et al*., 1988). An adverse effect of mercury on fish health was reported by several studies (Kitamura, 1968; Lockhart *et al*., 1972). Mercury is able to induce reproductive impairment by inhibiting gonadotropin secretion, 3β-HSD activity, spermatogenesis and by decreasing the gonadosomatic index (GSI) of catfish and guppy

(Kirubagaran and Joy, 1988; Kirubagaran and Joy, 1992; Wester and Canton, 1992). Thus, to assess how mercury induces reproductive impairment in fish, both *in vivo* and *in vitro* experiments are needed.

Fully grown oocytes from most species are arrested at prophase of meiosis- I. In echinoderms and lower vertebrates this meiotic arrest is released upon exposure to species-specific maturation-inducing hormone (MIH). The oocytes then undergo a process of meiotic maturation termed oocyte maturation occurs prior to ovulation and is a prerequisite for successful fertilization. The first visible event of the onset of oocyte maturation is the migration of germinal vesicle towards the animal pole following which breakdown of germinal vesicle, condensation of chromosome, formation of spindle and extrusion of first polar body takes place to produce an egg that can be fertilized (Masui and Clark, 1979). Oocyte maturation has been studied in variety of vertebrates and invertebrates, including mammals, amphibians, fishes and starfish (Masui and Clark, 1979; Nagahama, 1987; Kishimoto 1988; Nagahama *et al.,* 1995), but the endocrine regulation of oocyte maturation has been investigated most extensively in amphibian and fishes (Nagahama *et al.,* 1995).

In teleosts, meiotic resumption is initiated by the production of an ovarian maturation-inducing hormone (MIH). Numerous studies have been conducted to test the effects of various steroids on the induction of oocyte maturation *in vitro*. It is now suggested that among the C_{18}, C_{19} and C_{21} steroids tested so far, the C_{21} steroids have always been reported to have more potent maturation-inducing activity than the other two groups of steroids (Goetz, 1983; Nagahama *et al*., 1983; Greeley *et al*., 1986; Nagahama, 1987; Canario and Scott, 1988; Trant and Thomas, 1988). The compound 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) has been recognized as the most effective and potent MIH of several fish species such as *Salmo gairdneri* (Nagahama *et al*., 1983), Northern pike, *Esox lucius* (Jalabert, 1976); goldfish, *Carassius auratus* (Nagahama *et al*., 1983); brook trout, *Salvelinus fontinalis* (Duffey and Goetz, 1980); amago salmon, *Oncorhynchus rhodurus* (Nagahama *et al*., 1983; Young *et al*., 1983); common carp, *Cyprinus carpio* (Sen *et al*., 2002); Indian catfish, *Mystus vittatus* (Upadhyaya and haider, 1986); Indian perch, *Anabas testudineus* (Bhattacharyya *et al*., 2000). In perciform fishes, another C_{21} steroid, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), has been identified as MIS (Trant *et al*., 1986; Thomas, 1994; Garcia-Alonso *et al*., 2004). MIH activity was also demonstrated for corticosteroids (deoxycorticosterone and cortisol) in several teleosts including catfish (Goswami and Sundararaj, 1974; Sundararaj and Goswami, 1977; Goetz, 1983; Upadhyaya and Haider, 1986; Rao and Haider, 1992; Haider, 1997). Thus, there seems to exit species specificity in the nature of MIH in teleosts. Since a report on the nature of MIH of *T. fasciata* is unknown, we considered it necessary to identify the nature of MIH in *T. fasciata*, one of the most important wetland fish in this subcontinent. For this, in the present study we examined the relative ability of several physiological steroids, including estrogen, progestogene and corticosteroids to induce maturation in *in vitro* of intact follicles from *T. fasciata* considering GVBD as the criterion of oocyte maturation. We also tried to elucidate the involvement of specific types of gonadotropins in the regulation of oocyte maturation in this fish.

Materials and methods

Animals

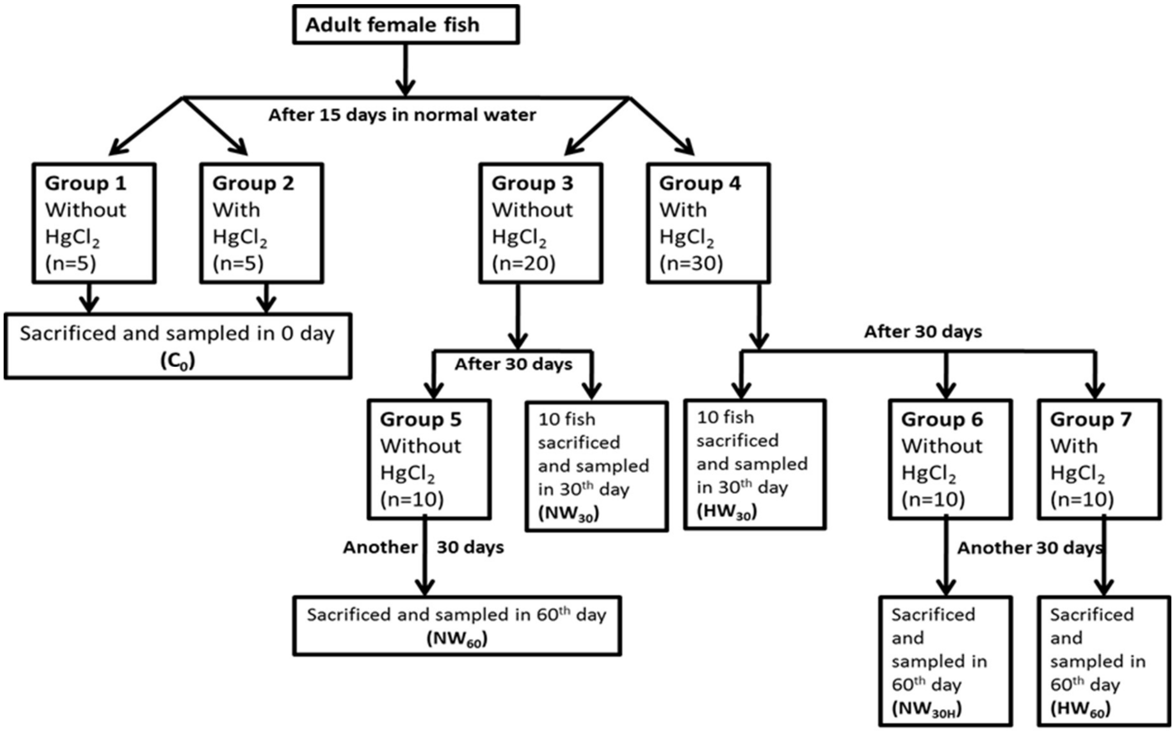
9–10 cm length young adult female *T. fasciata* collected from a local fisherman. According to Banu and Bhakta (1985), *T. fasciata* attains the first maturity at the age of 10–12 months and at this age, the length is around 10 cm. To avoid mixed age group, approximately 10 cm length fishes were selected for experiments. Fishes were kept in laboratory aquaria (50L capacity: Size – 30″×18″×15″) at 23±1 °C at least for 15 days prior to the experiments to acclimatize them to the laboratory environment. They were fed with commercial fish food (Shalimar fish food, Bird and Fish food manufacturer, Mumbai) twice a day. During the month of May and June, the ovaries of female *T. fasciata* are comprised mostly of pre-vitellogenic (0.2–0.3 mm diameter) and vitellogenic follicles (0.3–0.4 mm diameter), respectively.

Chemicals

$HgCl_2$ was purchased from Sigma Chemicals. Human chorionic gonadotropin (HCG) was obtained as a gift from A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases). Labeled steroids, $[^3H]$ estradiol-17β (specific activity 75.0 Ci/mmol) and $[^3H]$ testosterone (specific activity 95.0 Ci/mmol) were purchased from Amersham Biosciences. E_2 antibody was a generous gift from Gordon Niswender (Colorado State University, Fort Collins, Colorado, USA). Cocktail T (for scintillation) was purchased from Spectrochem Pvt. Ltd. All other chemicals were used of analytical grade.

In vivo experiment

For *in vivo* experiment, initially, we divided all fish into four (4) different groups (for summary of the *in vivo* experiment, please see the flow chart). Group 1 and 2 reared (5 fish for each group) in normal water and $HgCl_2$ treated water for 0 days i.e. the fish were sacrificed at the starting day of experiment. Both these group served as 0 day control (C_0). Group 3 and group 4 (20 and 30 fishes respectively) reared in normal water and ecological relevant dose of $HgCl_2$ treated water (50µg/L) respectively for 30 days. After 30 days, this group divided into two group (10 fish each), – 10 fishes were sacrificed to collect sample and rest of the fish (group 5: n=10) reared again in the normal water for another 30 days. The sample collected from the sacrificed fish at 30th day served as 30 days control (NW_{30}) and sample collected from 60th day after second time rearing served as 60 days control (NW_{60}). 10 fish from group 4 sacrificed after 30 days (HW_{30}) and other 10 fishes were reared in normal water again for another 30 days (group 6) to assess whether fish has the ability to regain its reproductive potentiality or not ($NW_{30}H$). Rest of the group 4 fishes (n=10) were reared again in $HgCl_2$ treated water (group 7) and sacrificed after 30 days to collect the sample (HW_{60}). Both the control and the test solutions were renewed every 7th day. During the experiment, 13.5 h:11.5 h light and dark phase were maintained. After the experiment, fish were deeply anaesthetized with MS 222 (150 mg/L), sacrificed and ovary and blood samples were collected for further analysis. A portion of ovarian samples were fixed in paraformaldehyde for normal histology and immunohistochemistry. Furthermore, 200 µl of blood from each fish were also processed for extraction and estimation of T and E_2 levels by specific radioimmunoassay (RIA).



Adult female fish
After 15 days in normal water
Group 1 Without HgCl2 (n=5)
Group 2 With HgCl2 (n=5)
Group 3 Without HgCl2 (n=20)
Group 4 With HgCl2 (n=30)
Sacrificed and sampled in 0 day (C0)
After 30 days
After 30 days
Group 5 Without HgCl2 (n=10)
10 fish sacrificed and sampled in 30th day (NW30)
10 fish sacrificed and sampled in 30th day (HW30)
Group 6 Without HgCl2 (n=10)
Group 7 With HgCl2 (n=10)
Another 30 days
Another 30 days
Sacrificed and sampled in 60th day (NW60)
Sacrificed and sampled in 60th day (NW30H)
Sacrificed and sampled in 60th day (HW60)

Chart – I: Flowchart for *in vivo* experiments.

In vitro Experiments

Two different *in vitro* studies were conducted for assessing hormones-stimulated oocyte maturation. The first one with steroids like E_2, 17α-OH-P, T, 17,20β-P, P and in the second one with gonadotropins like FSH, LH and HCG. For each experiment only one fish was used to avoid the individual variation of responsiveness towards the steroids and gonadotropins.

Under laboratory condition the whole ovary was transferred into a sterile petridish containing about 50 ml of ice-cooled incubation medium. With the help of watch-maker forceps and scissor ovary was cut into small pieces which were further teased with watch-maker forceps for separation of individual follicles. Approximately 35 mg were transferred to sterile 5 ml glass-beaker containing 1 ml of medium and the respective steroids or gonadotropins. Steroid in appropriate concentrations 0, 0.05, 0.1, 0.2, 0.5,1,5 and 10 μg/ml were added in 5 μl ethanol. Controls contained medium and ethanol alone. Unless otherwise stated in the text, incubation was continued for 24 h. The entire experiment was conducted in an air-conditioned laboratory where temperature was maintained at 23±1° C. Ovarian follicles were pre-incubated for 1 h with inhibitors prior to the addition of inducers. At the end of incubation the follicles were separated from the medium and subjected for GVBD analyses. The rate of GVBD was expressed as the mean percentage GVBD of three replicate incubations for each fish.

Extraction and assay of steroids

This has been described in Chapter II.

Histology

Ovarian samples were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). Fixed tissues were dehydrated in ascending concentrations (50, 70, 90 and 100%) of ethanol series, cleared in xylene and embedded in paraffin blocks at 60-62ºC. The blocks were sectioned with a rotary microtome at 8 µm thickness. Tissues were stained with Hematoxylin and Eosin following regular protocols and observed under bright field microscope.

Statistical analysis

All *in vitro* experiments were repeated in five different fish. As three replicates from a single donor fish showed similar tendency, the mean of these replicates was considered as a single data point. All data were expressed as mean±SEM of five separate experiments, each with a different fish. Statistical differences were calculated by the non-parametric Kruskal–Wallis test followed by Mann–Whitney *U* test for the determination of differences among groups, using StatView 5.0 (SAS Institute). The level of significance was chosen at $p<0.05$.

Results

Effect of mercuric chloride ($HgCl_2$) on follicular development/ folliculogenesis

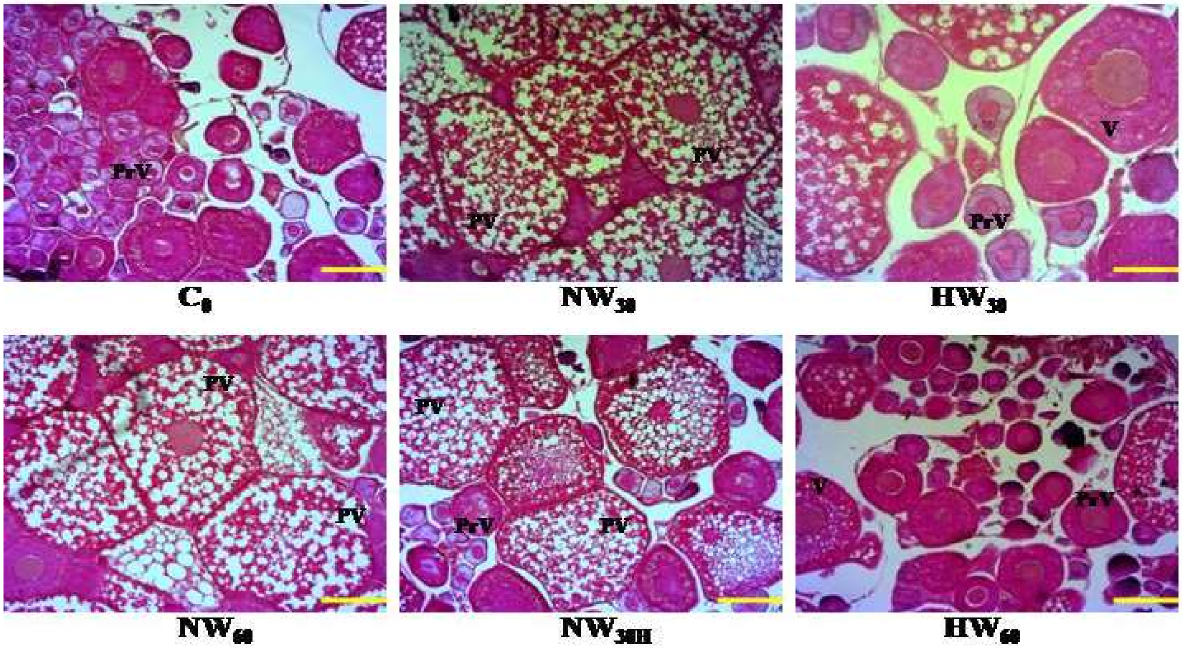
Histological analysis showed that most of the oocytes of $HgCl_2$-treated fish (HW_{30}, HW_{60}) were arrested in either the previtellogenic or vitellogenic stage like the C_0 fish. However, oocytes of non-treated fish (NW_{30}, NW_{60}) progressed through the postvitellogenic stages. Histological section of $NW_{30}H$ clearly showed that fish has the ability to recover their normal folliculogenic potentiality as oocytes progressed through postvitellogenic stages similar to the NW_{30} and NW_{60} group (Fig. 1A). It is also notable from the figure that the distance between two oocytes are more in Hg-exposed fish (HW_{30}, HW_{60}) in comparison to control group (NW_{30}, NW_{60}). Ovary unable to recovered this compactness, when it reared in normal water ($NW_{30}H$).

Fig. 1B shows that the diameter of oocytes in the NW_{30}, and NW_{60} (0.52±0.087 and 0.62±0.073 mm respectively) is significantly higher than the C_0 (0.21±0.027 mm) and other exposed groups (HW_{30}, HW_{60}: 0.29±0.17 and 0.19±0.032 mm respectively). Oocyte diameter increased (0.56±0.22 mm) after a recovery period of four weeks ($NW_{30}H$ group).

GSI data corroborates the histological analysis since the GSI increased normally in the control group (NW_{30}, and NW_{60}: 2.97±0.19 and 3.28±0.31 respectively) whereas fish reared in

$HgCl_2$-contaminated water (HW_{30}, HW_{60}) showed significantly lower GSI values (1.62±0.17 and 1.52±0.34) (Fig. 1C). $HgCl_2$-exposed fish, when reared in normal water ($NW_{30}H$ group), showed potentiality to recover their normal GSI value (3.16±0.54).

A.


PrV
FV
V
PrV
C_0
NW_{30}
HW_{30}
FV
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$NW_{30}H$
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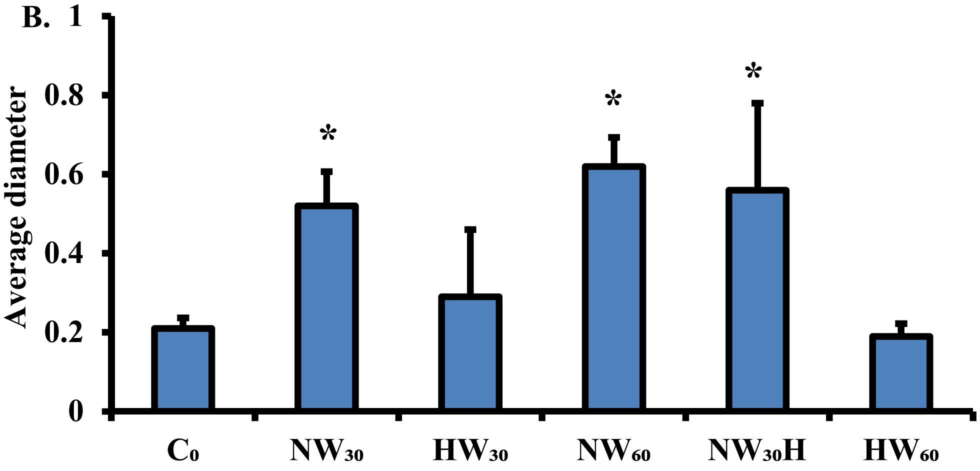


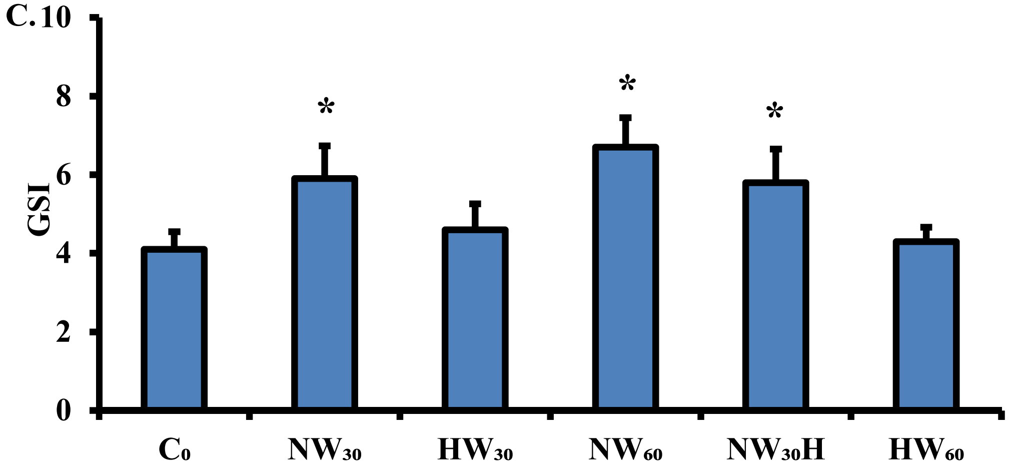


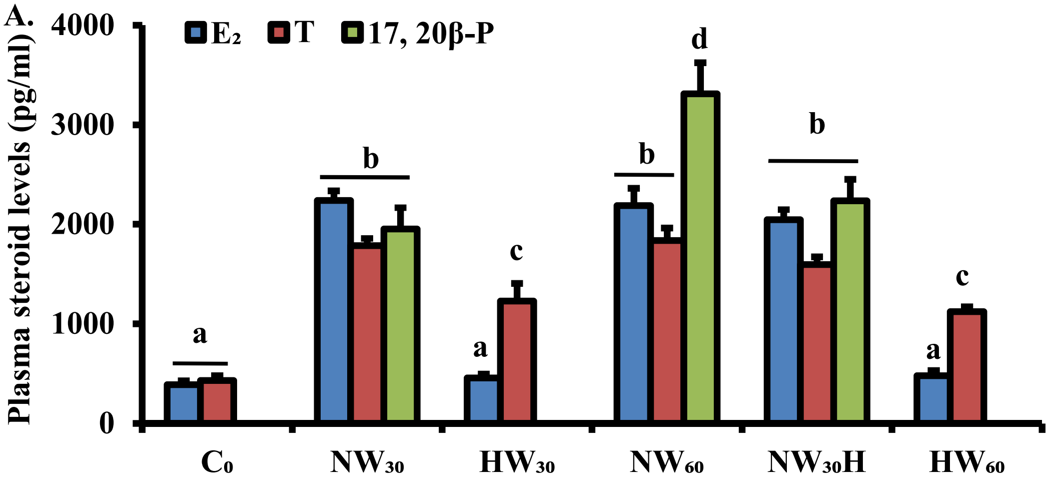
Fig. 1 Effect of mercuric chloride ($HgCl_2$) on follicular development of *Trichogaster fasciata.* Hematoxylin-Eosin staining of the ovary of fish reared in normal water and $HgCl_2$ –treated water for 0 days (C_0), normal water for 30 days, $HgCl_2$ -treated water for 30 days (HW_{30}), normal water for 60 days, normal water of HW30 fish for another 30 days (NW30H) and $HgCl_2$ -treated water for 60 days (HW_{60}) (A). NW_{30}, NW_{60} and NW_{30H} ovaries contain mainly postvitellogenic stage oocytes, whereas ovaries of C_0, HW_{30} and HW_{60} contain mostly previtellogenic and vitellogenic stage oocytes. Scale bar in (A) is 200 µm. Diameter of oocytes (B) and GSI percentage (C) of fish. Data are expressed as mean ± SEM of five experiments. * Denotes significant difference ($p \leq 0.05$) from other groups.

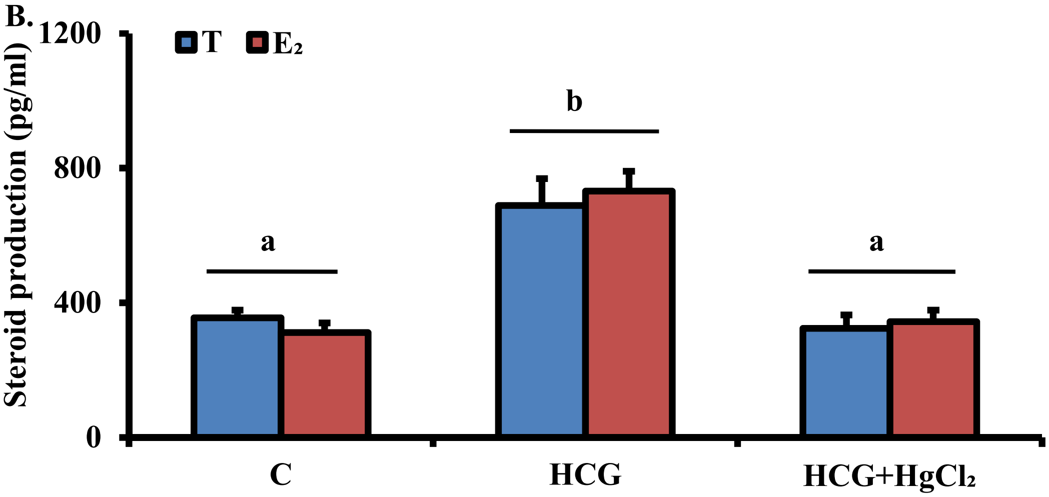
Effect of $HgCl_2$ on in vivo and in vitro steroid production

Starting from the 0 day of experiment (C_0) (E_2 −389.45±32.34, T – 432.62±58.57 pg/ml and 17, 20β-P – not detected), female banded gourami showed significantly increased level of both E_2 (NW_{30} and NW_{60} 2438.46±285.43 and 2189.76±327.67 pg/ml respectively), T (NW_{30} and NW_{60} 1786.24±216.73 and 1837.25±210.34 pg/ml respectively) and 17, 20β-P (NW_{30} and NW_{60} 1954.25±256.12 and 3312.35±312.54 pg/ml respectively) production when reared in normal water. After exposure to 50µg/L of $HgCl_2$ for 30 (HW_{30}) and 60 (HW_{60}) days, result shows a significantly decrease level of plasma E_2 (458.27±64.46 and 478.45±77.73 pg/ml respectively) and T (1228.49±137.89 and 1023.67±94.78 pg/ml respectively) and 17, 20β-P was not detected (Fig. 2A). However, when $HgCl_2$-exposed fish was reared in normal water ($NW_{30}H$) for another 30 days, our results showed that fish successfully recovered their normal steroidogenic potentiality as plasma E_2, T and 17, 20β-P levels increased to 2046.89±189.76 pg/ml, 1594.56±170.45 pg/ml and 2236.12±255.21 pg/ml respectively.

To investigate the direct effects of $HgCl_2$, isolated vitellogenic oocytes were incubated in the absence of $HgCl_2$ or in combination with 100 ng/ml of HCG. Our results showed that HCG alone significantly increased E_2 (730.7±58.69 pg/ml) and T (689.7±79.87 pg/ml) production and that addition of $HgCl_2$ significantly attenuated steroid production *in vitro* (Fig. 2B).

In post vitellogenic oocytes were incubated in same way the absence of $HgCl_2$ or in combination with 100 ng/ml of HCG *in vivo* experiment. The results revealed that HCG significantly increased 17, 20β-P production and $HgCl_2$ significantly decreased steroid production (2C).





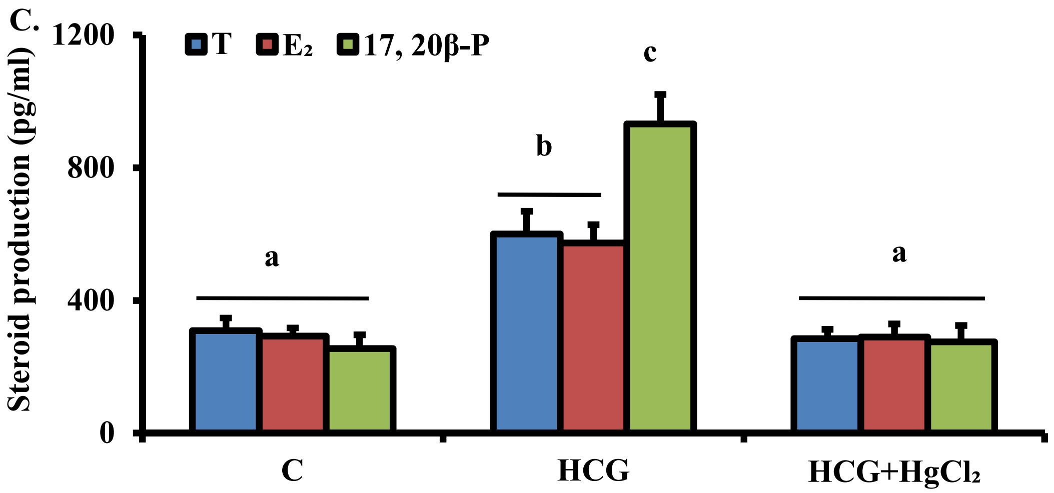


Fig. 2 *In vivo* (A) and *in vitro* (B & C) effect of mercuric chloride ($HgCl_2$) on 17β-estradiol (E_2) and Testosterone (T) production in female *T. fasciata*. (A) Plasma levels of E_2 and T were assayed from fish reared in normal water and $HgCl_2$ –treated water for 0days (C_0), normal water for 30 days (NW_{30}), $HgCl_2$ -treated water for 30 days (HW_{30}), normal water for 60 days, normal water of HW_{30} fish for another 30days ($NW_{30}H$) and $HgCl_2$ -treated water for 60 days (HW_{60}) by standard RIA methods. Each point represents the mean±S.E.M. Different letter represents the significant results from each other. (B and C) For *in vitro* study, follicles (100 mg) were incubated in 1 ml incubation medium containing HCG (100 ng/ml) in the presence or absence of $HgCl_2$ for 20 h in vitellogenic and post vitellogenic oocytes respectively. Each point represents the mean±S.E.M. of five separate experiments, each comprised of three replicates from each individual fish. Different letters denote values significantly different from others ($p<0.05$).

Oocyte maturation study

In *T. fasciata*, the yolk of postvitellogenic (pre-maturational) follicles is somewhat opaque, rather than translucent as in many other fish, and the nucleus or GV- which migrates from the central position of the follicle to periphery prior to resumption of maturation is visible with the necessity of chemical clearing.

The first visual indication of the onset of *in vitro* follicle maturation *T. fasciata* includes an accelerated clarification of the yolk, and an increase in the size of the follicle due to hydration. Both these events occurred approximately at six hours following exposure to stimulatory steroids (Fig. 3). Between six to twelve hours nuclear envelope begins to break down (GVBD), a process normally completed by sixteen hours. Subsequent to GVBD, the follicle undergoes additional size increase due to hydration and lipid droplets continue to coalesce. We considered the occurrence of GVBD to be in this species, the most consistent and reliable indicator of oocyte maturation *in vitro*.

Initial screening of steroids

In *T. fasciata,* ovary is very cyclic and oocyte mature at regular interval prior to spawning. Ovarian follicle also matured under laboratory conditions. As expected, the responsiveness of *T. fasciata* post vitellogenic oocyte to steroid stimulation of meiotic maturation *in vitro* also was cyclic in a similar fashion. Therefore, in an attempt to standardized results, all initial experiments were performed utilizing only post vitellogenic or pre-maturational follicles with centrally located GV.

We have used progestogens, androgens, and estrogens for our initial steroid screening test. Three progestins were 17α-OH-P, 17,20β-P and P. Androgens was T and estrogen was only 17β-estrodial. A dose kinetic study with all these steroids reveals that among the three progestins, the most effective and potent progestins were 17,20β-P in inducing oocyte maturation (Table 1). Nearly 47.21% oocytes underwent maturation at the dose of 0.05 μg/ml after 24 h of incubation. With increasing doses, GVBD increased gradually and significantly and nearly 97% oocytes underwent GVBD at 0.5 μg/ml. P and 17α-OH-P were not effective at the dose of 0.05 μg/ml in initiating maturation. Nearly, 12-16% oocytes underwent maturation at the dose of 0.1 μg/ml which gradually increased with increasing doses with a maximum of 46-53% at dose of 5 μg/ml. T was equipotent and they were being capable of initiating GVBD at concentrations as low as 0.1 μg/ml. 17β-estrodial, the only estrogen we have used for screening test, showed impotent and ineffective maturation inducing property (Table-I).

Postvitellogenic follicles were incubated with 1 μg/ml of all the steroids used in dose–kinetic study and GVBD was scored at 0, 3, 6, 9, 12, 16, 20 and 24 h of steroid addition. It appears (Fig. 3) that in all steroids induction of GVBD started at 3 h and GVBD induced by 17,20β-P and significantly with increasing time and reached maximum almost 90% at 16 h of incubation. Other steroids although stimulated GVBD with increasing time, but only 38% oocytes underwent GVBD at 16 h with no further increase thereafter.

Steroids	**Concentrations (µg/ml)**						
	0.05	**0.1**	**0.2**	**0.5**	**1**	**5**	**10**
P		12.05±3.23	19.65±4.84	43.61±4.45	46.58±5.50	52.12±4.5	53.56±4.56
17α-OH-P		15.58±2.45	22.12±3.45	30.05±2.87	36.12±4.56	46.23±5.47	45.23±3.32
17,20β-P	47.21±6.39	67.05±7.21	88.56±5.75	96.3±7.54	97±6.5	98±1.75	98.5±1
T		10±3.24	12.75±3.3	23.37±3.76	34.81±2.76	37.55±6.55	39.09±3.79
E_2		9.1±2.45	12.6±2.5	19.6±3.34	23±3.25	24±4.15	26.4±3.23
AD		7.5±2.34	9.75±2.57	17.56±5.76	31.5±7.45	33.78±6.75	33.9±9.25

Table 1: Effects of various steroids at increasing concentrations on the percentage of GVBD in intact postvitellogenic follicles of *Trichogaster fasciata* after 24 h of incubation. Each value represents the mean ±SEM of five observations obtained from five donor fish.

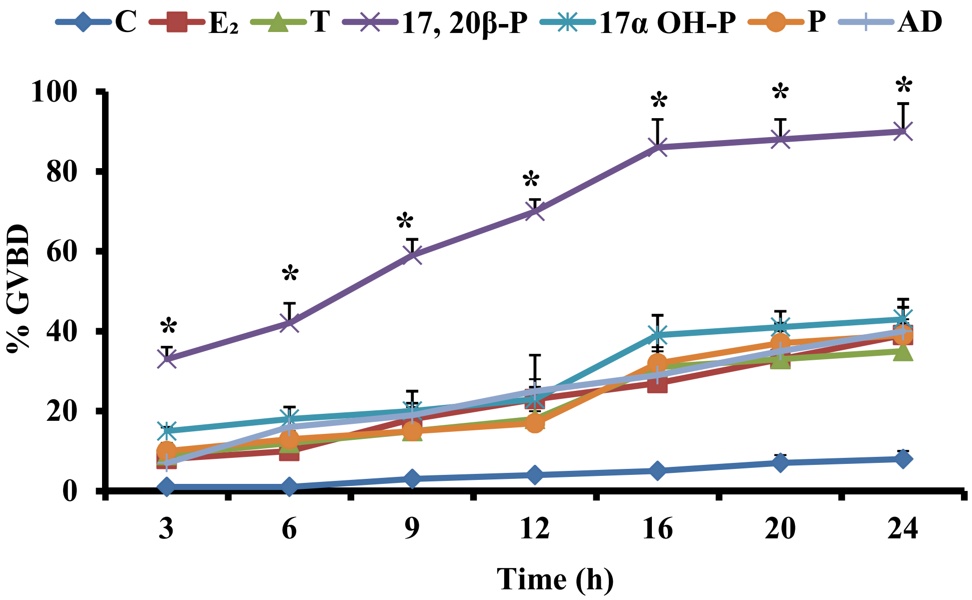


Fig. 3 Effects of time on exogenously added different steroids on oocyte maturation of *T. fasciata*. Intact follicles were incubated with 1 µg/ml of all steroids (E_2, T, 17,20β-P, 17α-OH-P, P and AD). GVBD was scored after 0, 3, 6, 9, 12, 16, 20 and 24 h of steroid addition. Each point represents the mean (±SEM) of four replicates obtained from four donor fish. Asterisks indicates significant ($p<0.05$) differences of 17,20β-P from other steroids at each period of incubation.

Follicle sensitivity

The relative effectiveness of 17,20β-P in stimulating GVBD in prematurational follicle were further examined in follicles from ovaries in two different reproductive stages – i) ovaries containing fully grown postvitellogenic follicles and ii) ovaries containing vitellogenic follicles. Postvitellogenic follicles were found to be much more sensitive to steroid stimulated oocyte maturation *in vitro* than vitellogenic follicles (Fig. 4) Although 70% of the postvitellogenic follicles underwent maturation *in vitro* with 17,20β-P at doses as low as 0.1µg/ml, follicles at vitellogenic stage failed to respond *in vitro* even at relatively high steroid concentration (0.5-5 µg/ml).

Effects of FSH, LH and HCG on in vitro oocyte maturation

Effects of various concentrations of FSH, LH and HCG on GVBD induction is shown in Figure 5 A and B. LH or HCG at 0.05 µg/ml dose were able to stimulate GVBD significantly after 24 h of incubation and maximum GVBD (almost 90%) was achieved at the dose of 0.1 µg/ml to 1 µg/ml of both the gonadotropins. FSH-stimulated oocyte maturation was much less at all concentration tested compared to LH and HCG (Fig. 5A). The maximum GVBD (47%) were achieved with 0.5 µg/ml FSH.

A time course study on the effects of FSH, LH and HCG (each at the dose of 0.1 µg/ml) on oocyte maturation in post vitellogenic follicles of *T. fasciata* is shown in figure 5B. It appears that both HCG- and LH-induced GVBD started from 3 h and completed by 16 h after incubation, commencement of same by FSH was noticed at 6 h with a maximum at 20 h.

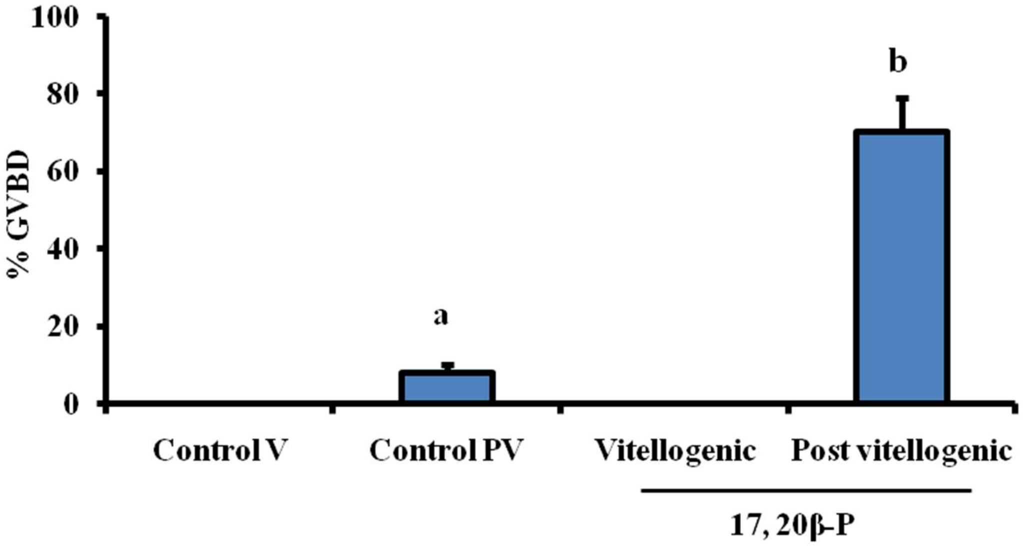


Fig. 4 Oocyte maturation in vitellogenic and postvitellogenic oocytes exposed to 17,20β-P (0.1 µg/ml) for 24h. Each point represents the mean ± SEM of five incubations taking follicles from five donor fish. Alphabets denote values significantly different from either those treated without hormone or low concentration of hormone ($p<0.05$).

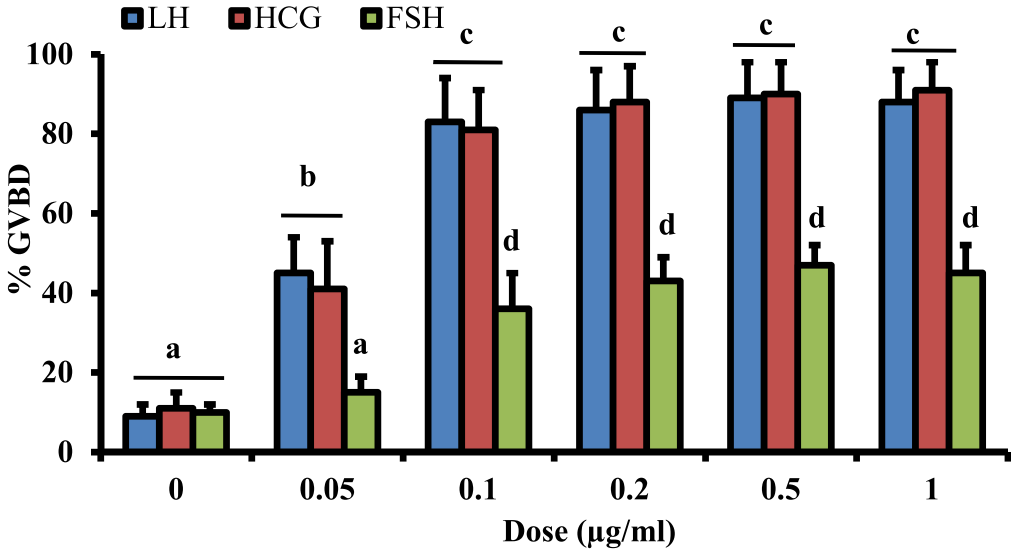


Fig. 5A *In vitro* effects of various concentrations of FSH, LH and HCG on the oocyte maturation in *T. fasciata* postvitellogenic ovarian follicles after 24 h of incubation. Each bar indicates the mean (±SEM) values of five observations from five donor fish. Different letter represents the significant results from each other.

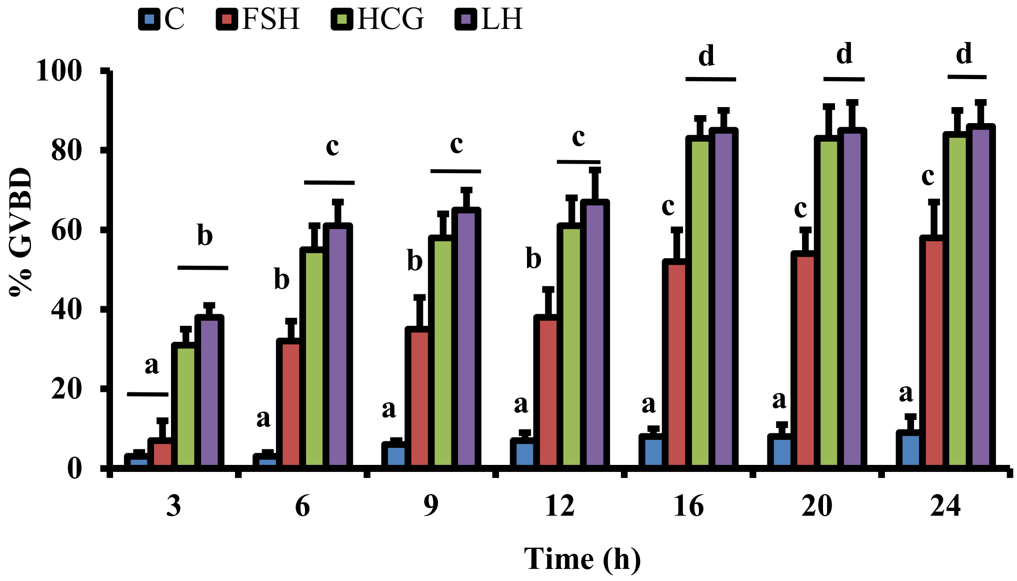


Fig. 5B Time course study of oocyte maturation (GVBD) in untreated and FSH-, LH- and HCG-treated (0.1 µg/ml) postvitellogenic follicles of *Trichogaster fasciata*. Each bar represents the mean (±SEM) values of five replicates from five donor fish. Different letter represents the significant results from each other.

Effects of inhibitors of steroidogenic enzyme and protein synthesis on HCG-induced oocyte maturation

Since LH and HCG showed equal potency in inducing GVBD, this experiment was conducted only with HCG. To observe the effects of trilostane an inhibitor of 3β-HSD on oocyte maturation, postvitellogenic follicle of *T. fasciata* were incubated with HCG (0.1 μg/ml) and various concentration of trilostane (each at 0.1, 0.5, 1 and 1.5 μg/ml) for 24 h. Cultures were pre-incubated with trilostane for one hour prior to the addition of HCG. After termination of incubation, GVBD were scored. Fig. 6A shows that trilostane at increasing concentration significantly inhibited HCG induced oocyte maturation almost in a dose dependent manner.

Follicle when exposed to graded doses of protein synthesis inhibitor cycloheximide or actinomycine-D (each at 0.1, 0.5, 1 and 1.5 μg/ml) show a significant reduction in HCG-induced GVBD (Fig. 6B and C). Both the inhibitors at concentration 0.5 μg/ml significantly reduced the percentage of oocyte maturation.

Effects of gap junction uncouplers on HCG-induced GVBD

Postvitellogenic follicles were pre-incubated with graded dose of n-alkanol gap-junction uncouplers, namely 1-octanol and 1-heptanol for one hour followed by incubations with HCG for 24 hours. Ethanol control was maintained in each case. It appears from Fig. 7 that 1-octanol and 1-heptanol inhibited HCG induced GVBD significantly with increasing concentration. Ethanol alone had no effect on GVBD.

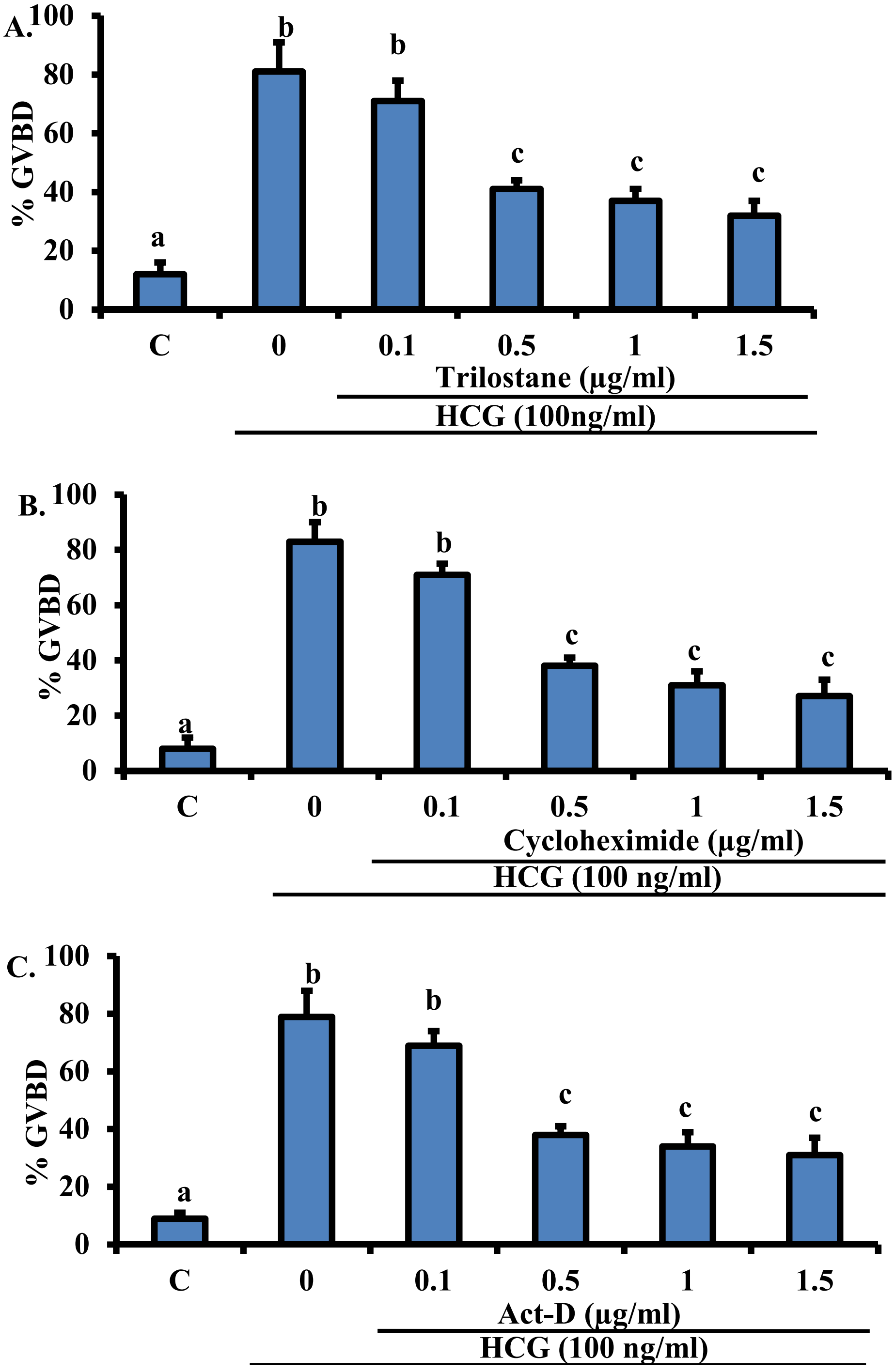


Fig. 6 Effects of trilostane, cycloheximide and actinomycine-D of HCG (100 ng/ml)-induced GVBD after 24 h of incubation in postvitellogenic ovarian follicles of *T. fasciata*. Follicles were pre-incubated with the inhibitors 1 h prior to the addition of HCG. Each point represents the mean (±SEM) value of four replicates obtained from four donor fish. Different letter represents the significant results from each other ($p<0.05$).

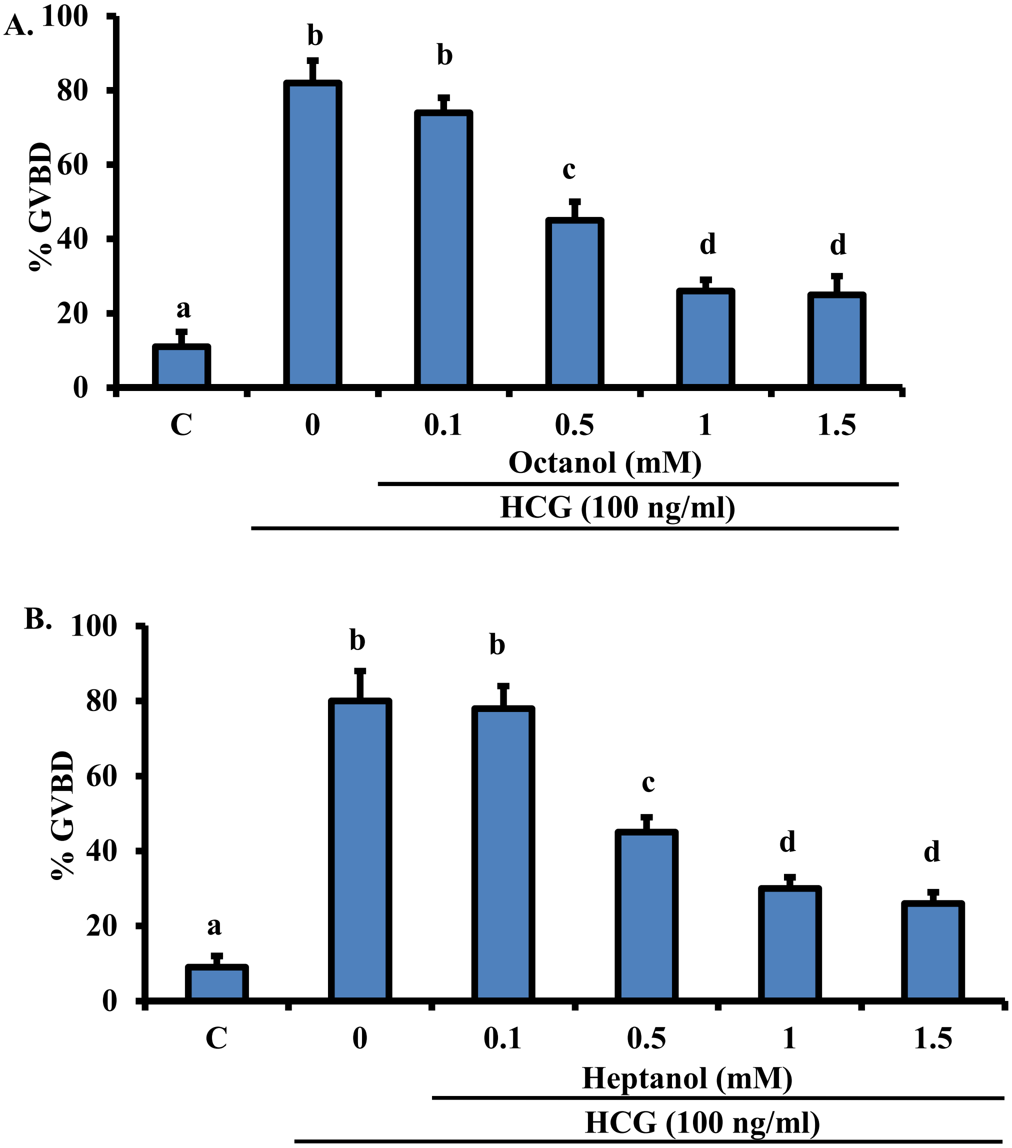


Fig. 7 Percent of GVBD in postvitellogenic ovarian follicles of *T. fasciata* exposed to HCG in the presence or absence of gap junction uncouplers for 24 h. Follicles were pre-incubated for 1 h in uncouplers, 1-octanol and 1-heptanol. Each point represents the mean (±SEM) of five incubations taking follicles from five donor fish. Different letter represents the significant results from each other (p<0.05).

Effects of $HgCl_2$ on HCG induced in vitro oocyte maturation

In postvitellogenic oocytes of *T. fasciata* were incubated with HCG (0.1 µg/ml) and various concentration of $HgCl_2$ (1, 2.5, 5, 10, 20 and 40 ng/ml) for 24 h. Cultures were pre-incubated with $HgCl_2$ for one hour prior to the addition of HCG. After termination of incubation GVBD were scored. Fig. 8A shows that $HgCl_2$ at increasing concentration significantly inhibited HCG induced oocyte maturation almost in a dose dependent manner. The GVBD (14.7%) were achieved without killing oocyte at 5 ng/ml of $HgCl_2$.

A time course study on the effects of $HgCl_2$ (5 ng/ml) on oocyte maturation in post vitellogenic follicles of *T. fasciata* is shown in Fig. 8B. The result shows that $HgCl_2$ was efficiently block GVBD at 16 h.

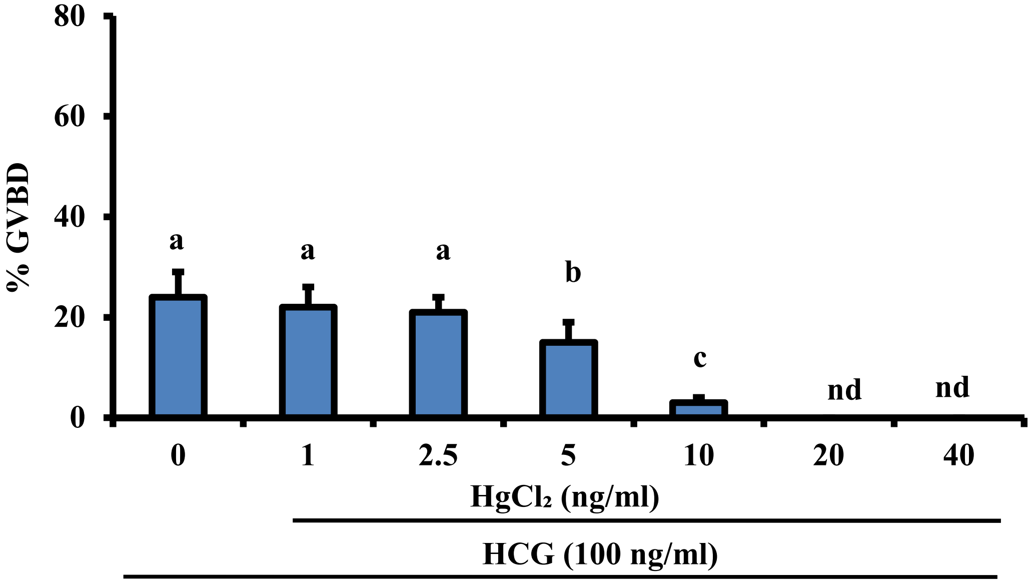


Fig. 8A *In vitro* effects of various concentrations of $HgCl_2$ on HCG (0.1 µg/ml)-induced oocyte maturation after 24 h of incubation in post vitellogenic ovarian follicles of *T. fasciata*. Follicles were pre-incubated with the inhibitors 1 h prior to the addition of HCG. Each point represents the mean (±SEM) value of four replicates obtained from four donor fish. Different letter represents the significant results from each other (p<0.05).

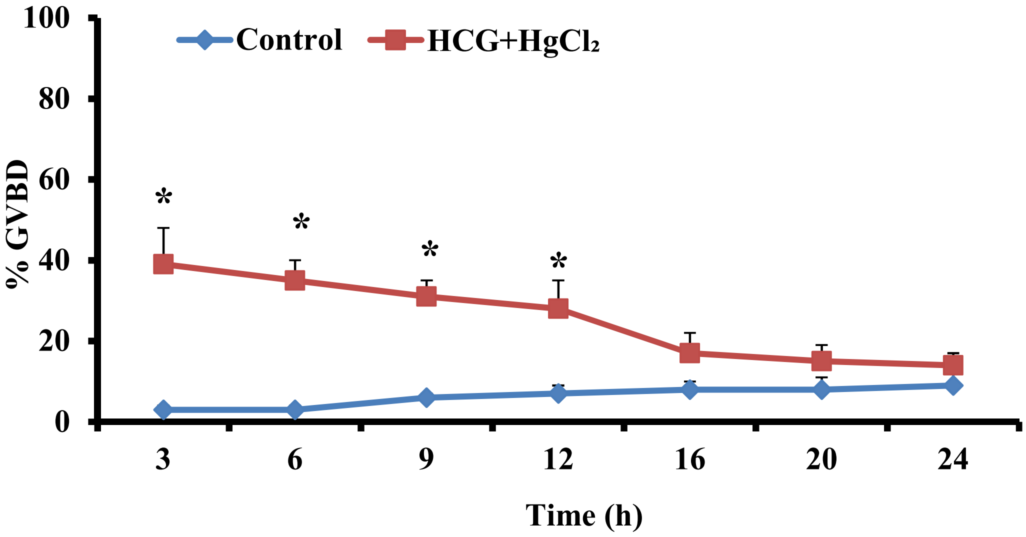


Fig. 8B Time course study of oocyte maturation (GVBD) in untreated and $HgCl_2$+HCG treated postvitellogenic follicles of *T. fasciata*. Each bar represents the mean (±SEM) values of five replicates from five donor fish. Different letter represents the significant results from each other.

Effects of $HgCl_2$ and other inhibitors on HCG-induced oocyte maturation

Since LH and HCG showed equal potency in inducing GVBD, this experiment was conducted only with HCG. To observe the effects of $HgCl_2$ and other inhibitors on oocyte maturation, post vitellogenic follicle of *T. fasciata* were incubated with HCG (0.1 µg/ml) and various concentrations of inhibitors ($HgCl_2$- 5 ng/ml, Act-D- 500 ng/ml, Chx- 500 ng/ml, Oct - 0.5 mM and Hept – 0.5 mM). Cultures were pre-incubated with inhibitors for one hour prior to the addition of HCG. After termination of incubation GVBD were scored. Fig. 9 shows that all inhibitors significantly inhibited HCG induced oocyte maturation.

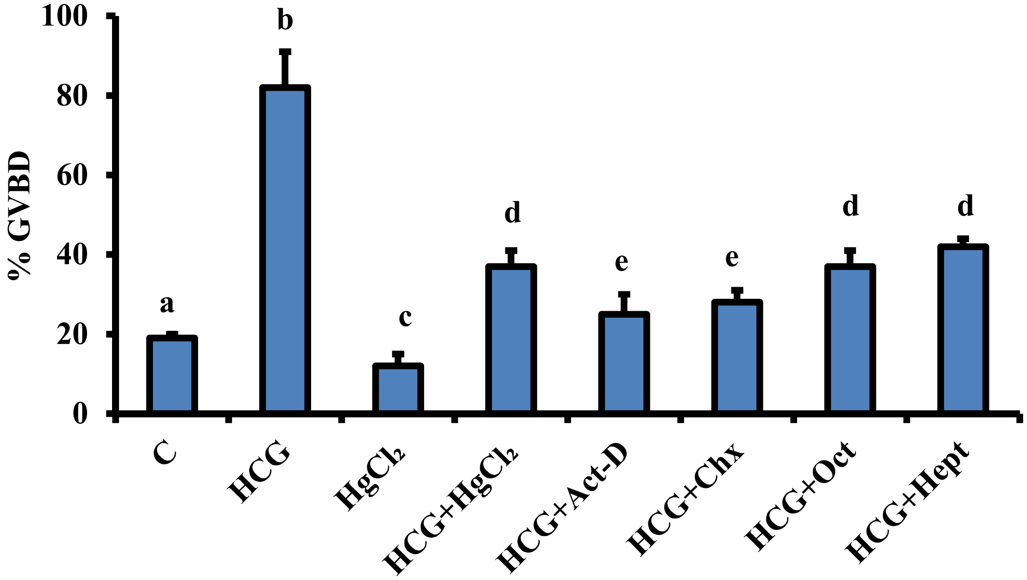


Fig. 9 Effects of only $HgCl_2$ and $HgCl_2$, cycloheximide, actinomycine-D, octanol and heptanol of HCG (0.1 µg/ml)-induced GVBD after 24 h of incubation in postvitellogenic ovarian follicles of *T. fasciata*. Follicles were pre-incubated with the inhibitors 1 h prior to the addition of HCG. Each point represents the mean (±SEM) value of four replicates obtained from four donor fish. Different letter represents the significant results from each other ($p<0.05$).

Discussion

In this study, $HgCl_2$ was used as a source of Hg to determine the effect of Hg contamination on fish reproduction by assessing its impacts on follicular development, steroid biosynthesis and aromatase activity in the banded gourami, *Trichogaster fasciata*. The results indicated that environmentally relevant concentrations of $HgCl_2$ (50 µg/L) act as endocrine disrupting chemicals (EDC) by inhibiting folliculogenesis, oocyte maturation and normal steroidogenic production. This study also attempted to determine whether Hg-exposed fish have any potentiality to recover their normal reproductive functions if they have been re-cultured in a pollution-free environment. The results obtained from this study suggest that this fish species has the potential to recover their normal reproductive function if it is cultured in pollution-free water.

Available reports showed an inhibitory role of Hg on GnRH secretion, gonadal development, steroidogenesis and sex ratio in teleosts (Kirubagaran and Joy, 1988; Mukherjee *et al*., 1994; Dey and Bhattacharya, 1989; Mulvey *et al*., 1995; Friedmann *et al*., 1996). However, contradictory reports are also available. Specifically, unaltered GSI values were observed by Friedmann *et al*., after exposure of fish in Hg (Friedmann *et al*., 2002) and increased steroidogenic enzyme activity in the fish ovary was observed by Mondal *et al* (1997). In the present study, we observed that both gonadal development and GSI values were inhibited by $HgCl_2$ exposure and this inhibitory effect is a temporary phenomenon because if $HgCl_2$-treated fish are cultured in normal water, they recover their normal reproductive potentiality immediately. Results obtained from the *in vivo* study revealed that ovarian steroid secretion is inhibited in fish that are cultured in Hg-contaminated water. Pre-vitellogenic stage fish exposed to ecologically relevant doses of $HgCl_2$ had reduced levels of testosterone and 17β-estradiol production by ovarian follicles and this inhibition might be due to lower gonadotropin secretion from the pituitary or to inhibitory effects on ovarian enzyme responsible for steroid production. Similar reports are available from various fish species exposed to different EDCs (Mukherjee *et al*., 1994; Das *et al*., 2016), providing support for our results.

Overviewing the pattern of plasma and ovarian steroids in vitellogenic and postvitellogenic stage wild caught *T. fasciata,* (Chapter I and II) it is evident that in an around oocyte maturation there was a sharp increase in plasma 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) levels. Interestingly, unlike other fishes, plasma and ovarian 17β-estradiol levels remained high at postvitellogenic stage. As nature of the MIH in *T. fasciata*, has never been elucidated, we thought for an initial screening test for different steroids, including progestogens, estrogen, androgens and corticosteroids etc.

The result of the present study demonstrated that a number of different steroids at relative high dose (1 μg/ml) including progestogens, estrogens, androgens and corticosteroids – can initiate oocyte maturation in *T. faasciata* postvitellogenic follicles *in vitro*. Such a ubiquitous responses of follicles to various steroids at high doses is not unusual among the teleosts examined to date (Goetz, 1980; Canario and Scott, 1988), and is probably due, at least in part, to the metabolism of many steroids to more active forms by some components of the follicular investements. However, in *T. fasciata* like many other teleosts, 17,20β-P appears to be far more effective in inducing oocyte maturation in intact follicle than other steroids especially at lower doses.

Among androgens, testosterone (T) treatment induces oocyte maturation in *O. latipes* (Hirose, 1972; Iwamatsu, 1978) and *O. rhodurus* (Young *et al*., 1982). In *M. vittatus*, androstenedione (AD) is sufficiently effective to evoke good percentage of GVBD events at its lowest concentration (Iwamatsu, 1978). However, in the present study both T and AD appears to be a poor inducer for oocyte maturation in *T. fasciata* postvitellogenic follicles *in vitro*. Both T and AD at their highest concentrations tested (5 µg/ml) evoked 39.09% and 43.5% GVBD respectively.

Among C21 steroids, relative *in vitro* effectiveness of progestogens was examined and the result shows that 17,20β-P was the most potent and effective MIH for *T. fasciata* oocytes as it has the ability to initiate GVBD at a very low concentration (0.05 µg/ml, 47.21% GVBD). The optimal effective dose for 17,20β-P was 0.5 µg/ml. On the other hand, remaining C21 steroids namely, progesterone and 17α-OH-P failed to exhibit any such property at such a low concentration. These findings thus corroborate the earlier reports for various species namely, *S. gairdneri* (Jalabert, 1976; Nagahama *et al*., 1983), *S. fontianalis* and *P. flavescens* (Goetz and Bergman, 1978), *O. latipes* (Iwamatsu, 1980), *O. kisutch* (Sower and Schreck, 1982), *O. rhodurus* (Young *et al*., 1983; Nagahama *et al*., 1983), *F. heteroclitus* (Petrino *et al*., 1993) and *A. testudineus* (Bhattacharyya *et al*., 2000).

From the present study it appears that the effectiveness of 17α-OH-P, which posses considerably high percentage of oocyte maturation property in some teleosts, (Trant *et al*., 1986; Thomas, 1994; Garcia-Alonso *et al*., 2004) was far less than 17,20β-P both at low and high concentrations tested. Maturation inducing potency of 20β-S was also much lower than 17,20β-P in the present study. 20β-S, thus may not be the MIH of this fish; although 20β-S has been identified as MIH in many teleosts such as atlantic croacker, *M. undulates* and spotted seatrout, *C. nebulosus* (Trant and Thomas, 1989a; Thomas, 1994).

In teleosts, follicles undergo oocyte maturation before ovulation. This final maturation has been linked to gonadotropic promotion of maturation involving steroid production. It is now known that fish gonadotropins, GTH-I and GTH-II are chemically and biologically similar to tetrapod FSH and LH respectively (Kawauchi *et al.,* 1989; Swanson, 1991). Like FSH, GTH-I is secreted during the period of gonadal growth (vitellogenesis) and function to stimulate gonadal growth and steroidogenesis at this stage. Like LH, plasma levels of GTH-II increase during the period of final oocyte maturation to alter steroidogenesis and to promote oocyte maturation (Swanson, 1991). In the present study FSH, LH and HCG-induced oocyte maturation in postvitellogenic follicles of *T. fasciata* clearly indicates the involvement of GTH-II i.e. LH or HCG in this physiological process. Dose and time were required for minimum and maximal induction of GVBD was comparable with those applied in earlier studies with fish oocytes.

Present study demonstrates that vitellogenic follicles of *T. fasciata* were incapable to undergo final oocyte maturation (FOM) in response to FSH, LH and HCG at their highest concentrations tested. This indicates that sensitivity of *T. fasciata* oocyte to MIS at this stage has not developed. Current information on other teleosts indicate that LH regulates oocyte maturation in two stages and that "oocyte maturation" can be appropriately described within the broader context of ovarian follicle maturation. During the first stage of maturation the follicle cells acquire the ability to produce MIS and the oocyte to respond to MIS (i.e., oocyte maturational competence, OMC) where as in the second stage the follicle cells produce MIS and consequently the oocyte is released from meiotic arrest (see Review Patino *et al.,* 2001).

LH has been well known to be involved in inducing final oocyte maturation and ovulation. A preovulatory LH surge has been reported in the rainbow trout (Breton *et al*., 1997), spotted seatrout (*Cynoscion nebulosus*) (Thomas *et al*., 2001), gilthead seabream (Gothilf *et al*., 1997), and goldfish (Stacey *et al*., 1979). However, according to the information available, FSH in teleosts has generally been considered to be the gonadotropin involved in promoting follicle growth or vitellogenesis, and it is less likely to play significant roles in oocyte maturation or ovulation. FSH is less potent than LH in stimulating maturation-inducing hormone (MIH) production from the postvitellogenic follicles in the amago salmon (Suzuki *et al*., 1988), and it is incapable of inducing maturational competence development and oocyte maturation in the red seabream (*Pagrus major*) *in vitro* (Kagawa *et al.,* 1998).

Induction of GVBD by HCG in postvitellogenic follicles of *T. fasciata* was inhibited by actinomycin D suggest that oocyte maturation induced by HCG required synthesis of mRNA. Some earlier studies with act-D in red seabrem, *Fandulus* and striped bass oocytes on this aspect have reported similar results (Kagawa *et al*., 1994; Nagatu *et al*., 1998; Weber and Sullivan, 2000; Mukherjee *et al*., 2006). Inhibition of GVBD induced by HCG in the presence of cycloheximide suggests the requirement of translation of mRNA to protein for mediating the actions of HCG. This translation blockage indicates the inhibition of induction of MPF or protein required for the activation of MPF (Kagawa *et al*., 1994; Nagatu *et al*., 1998; Mukherjee *et al*., 2006).

Results of the present study using n-alkanol gap junction uncouplers indicate the requirement of functional gap junctions for gonadotropin-induced GVBD in such fish. Gap junction communication play an important role in the growth and differentiation of fish ovarian follicles, but the precise function of this communication during the period of ovarian follicle maturation is controversial. One view holds that uncoupling of heterologous gap junction (granulosa –oocytes) causes the resumption of meiosis as found in ovarian follicles of *Fundulus* (Cerda *et al*., 1993). The other view is that uncoupling of heterologous gap junction impairs the ability of oocytes to resume meiosis in response to MIH. This has been observed in striped bass (Weber and Sullivan, 2000) and in atlantic croaker (Yoshizaki *et al*., 2001). Our results with HCG-induced GVBD in *T. fasciata* oocytes strengthen the second view that functional gap junctions are required for GTH-induced oocyte maturation.

Employing a specific inhibitor of 3β-HSD, in the incubation mixture it is evident that HCG-induced GVBD was blocked by this inhibitor. This observation clearly indicates that HCG induced 17,20β-P production through Δ5-Δ4 pathway. Likewise, this inhibitor also blocked gonadotropin-induced oocyte maturation in many teleosts (Young *et al*., 1982; Petrino *et al*., 1989b). We concluded from this observation of 17,20β-P synthesis that the enzymatic step catalysed by 3β-HSD is essential for the biochemical and physiological responses of *T. fasciata*.