Journal of Environmental Biology
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September 2009, 30(5) 633-640 (2009)
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Testosterone and estradiol-17β dependent phospholipid biosynthesis in ovariectomized catfish, *Heteropneustes fossilis* (Bloch)

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(Received: April 15, 2008; Revised received:October10,2208 ; Accepted: November 03, 2008)

Abstract: *Effect of cumulative doses (7, 14 and 28 mgkg^{-1} body weight) of testosterone (T) and estradiol-17β (E2) on total phospholipids (TP), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) in tissues were investigated during the gonadal recrudescence, in prespawning phase of the annual reproductive cycle in intact and ovariectomized freshwater catfish, Heteropneustes fossilis. After ovariectomy, the hepatic levels of TP and PE were elevated and remained unaffected for PC, PS and PE when compared with control. In general, T and E2 were stimulatory for a specific class of phospholipid in tissues of intact and ovariectomized catfish. These effects were higher at 14 and 28 mg kg^{-1} body weight in ovariectomized catfish whereas 7 mgkg^{-1} body weight of T and E2 have pronounced effect in intact ovaries. In conclusion, the various phospholipid biosynthesis were under T and E2 dependent. Among the phospholipid, the PC was the main constituent and was sex steroid dependent biosynthesis during prespawning phase.*

Key words: *Sex steroids, Phospholipid, Ovariectomy, Reproductive growth, Fish*
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Introduction

The liver is the chief source of energy and biomembrane lipids during oocyte growth under the control of ovarian estradiol-17β (Singh and Singh, 1990). Vitellogenic gonadotropin (s) in turn influences the sequestration of lipids in circulation by the growing oocytes (Lal and Singh, 1987; Sundararaj *et al.*, 1982). Lipid is the component of vitellogenin (Vg) synthesized in the liver (Bradley and Grizzle, 1989; Burzawa-Gerard and Dumas-Vidal, 1991; Sehgal and Goswami, 1994, 2001, 2005; Phartyal Rajendra *et al.*, 2005). Vg, the egg-yolk precursor is an estrogen-inducible multi-component protein which is synthesized and post transcriptionally modified by hepatocytes and secreted into the bloodstream (Wiegand, 1996; Singh and Singh, 2007a). Vg is transported to ovaries and incorporated into oocytes by receptor mediated endocytosis. Specific enzymatic cleavage breaks it into lipovitellin and phosvitin which are deposited as yolk granules. Vg is present in the adult vitellogenic females, but is absent in males and in immature as well as in non-vitellogenic females (Ng and Idler, 1983). It can be induced in either sex at any time of the year by injection of estradiol-17β. Owing to its specificity as marker of estrogenic compounds, Vg has often been used as a biomarker of endocrine disruption in fishes (Chen *et al.*, 1986; Cheek *et al.*, 2004; Om Prakash *et al.*, 2007). Estrogen not only induces the synthesis of Vg, but also of a non-phosphorylated protein, choriogenin (Chg) which is the precursor of egg-envelope protein (Hamazaki and Murata, 1992; Murata *et al.*, 1994). During zonagenesis, Chg is transporated from the liver via blood into the developing oocytes and is accumulated in the chorion (Hamazaki and Murata, 1992; Sehgal and Goswami, 2005). Choriogenin can also be used as a biomarker of estrogenic compounds (Arukwe *et al.*, 1997; Folmar *et al.*, 2001; Berg *et al.*, 2004).

Estrogen affects free fatty acids, cholesterol and phospholipids (de Vlaming *et al.*, 1977, 1984; Wiegand and Peter, 1980 a,b; Van Bohemen and Lambert, 1981). Testosterone has been shown to increase plasma free fatty acids (Wiegand and Peter, 1980b) in *Carassius auratus.* Lipogenesis in ovary and its stimulation by gonadotropin(s) have also been reported in fish ovary (Wiegand and Idler, 1982, 1984). Singh and Singh (1990) have reported a circannual variation of plasma testosterone, estradiol-17β and 17β-hydroxyprogesterone produced from the ovary in which all three were elevated during the preparatory phase and reached their peak in the prespawning phase in *H. fossilis*. These authors also reported a positive correlation between sex steroid and hepatic lipid biosynthesis during gonadal recrudescence.

Lal and Singh (1987) have demonstrated the effect of three steroids- testosterone, estradiol-17β and progesterone hormone which were of ovarian origin and have recorded the suppressive, inducive and no effects respectively on plasma lipids in ovariectomized freshwater catfish, *Clarias batrachus* during prespawning phase without considering different phospholipids. These workers demonstrated that estradiol-17β was most potent in inducing plasma lipids. The phospholipid was the chief constituent of vitellogenin (Fremont and Riazi, 1988) and membrane lipids were the major parameters of interest in our study during gonadal recrudescence (prespawning phases). Since the phospholipids is

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the constituent of vitellogenin synthesized in the liver under the influence of estradiol-17β, it necessitated the observance of the effect of testosterone and estradiol-17β in ovariectomized catfish to get the clear picture of various phospholipid biosynthesis and its mobilization in intact and ovariectomized catfish, *H. fossilis* during gonadal recrudescence.

Most previous estimates of total lipids or some specific class of lipid have been made in one or two tissues in non-ovariectomized fish, which does not provide a full analysis of lipid metabolism in response to sex steroids. To date, no attempt has been made to study the effect of testosterone and estradiol-17β on the levels of phospholipids in liver, ovary and plasma of both intact and ovariectomized catfish, *H. fossilis*. Since lipid undergoes rapid breakdown, re-synthesis and inter-conversions in response to changes in the circulating levels of several hormones, it is essential that different classes of lipids in more than one tissue be taken into account simultaneously when considering the response of ovariectomized catfish to steroid hormones. This prompted us to examine the effect of testosterone and estradiol-17β control of phospholipid biosynthesis on total phospholipids (TP), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) in the intact and ovariectomized tropical freshwater catfish, *H. fossilis* during the reproductively active phase or gonadal recrudescence during its annual reproductive cycle when the maximum activity for lipid biosynthesis and steroidogenesis takes place (prespawning phase). Present study is at clarifying whether different phospholipid biosynthesis are estrogen (testosterone and estradiol-17β) dependent or not during gonadal recrudescence?

Materials and Methods

Experimental fish: The research reported herein was conducted under ethical guidelines for the treatment of animals in behavioral research and teaching (Animal behavior, 1998). The experimental fish, *H. fossilis* (Bloch) was selected because of its economic importance as well as its suitability in terms of laboratory maintenance. This fish is an annual breeder with reproductive cycle (Sundararaj and Vasal, 1976). Eighty female *H. fossilis* were purchased from the same ponds around district Jaunpur (Lat. 25.46 N : Long. 82.44 E) of an average weight of 45 g (ranged between 40-50 g) and length 17 cm (ranged between 16 -18 cm) during prespawning phase and kept under natural photoperiod (13.0 light : 11.0 dark) and temperature (31 ± 2°C) for one week of acclimation. Fish were maintained in earthen pots supplied with a constant flow of dechlorinated tap water. Experiments began after one week of acclimation in the earthen pots. On alternate days fish were fed *ad libitum* with chopped goat liver.

After acclimation, fourty fish were ovariectomized during the prespawning phase as described by McLean (1994) and kept further for a week before experimentation. There was 5% mortality in control and experimental fish after ovariectomy. The rest 40 fish were with intact ovaries. The experimental fish were divided into 16 batches of 5 each in an earthen pot containing 20 l water at room temperature.

Sex steroid treatments: Lipid standards and sex steroids-testosterone (T) and estradiol-17β (E2) were purchased from Sigma Chem. Co. (St. Louis, MO). Exogenous administration was given in intact and ovariectomized catfish as per protocol (Table 1).

Lipid extraction and separation: At the termination of the experiments on the 15th day, blood was collected by caudal puncture from each fish in heparinized syringes and centrifuged at 4,000 rpm for 15 min. The plasma was kept at -20°C for later analysis of phospholipids. Liver and ovaries were taken out, washed in 0.6% saline, blotted and kept at -20°C for analysis. Phospholipid extraction and their quantification were done as per method described by Singh and Singh (2007b).

Statistical analysis: Statistical errors were expressed as the standard error of the mean (mean ± SEM). The concentrations of phospholipids were expressed in mg g^{-1} of liver or mg ml^{-1} of plasma (mean ± SEM). Individual values for all parameters were compared by Students *t*-test followed by two way analysis of variance (TW ANOVA) by the Microsoft Tool pack (data analysis) at a level of 0.05 (Brunning and Kintz, 1977).

Results and Discussion

There was no significant difference in the lipid concentrations between vehicle injected fish and non-vehicle injected, so the two types of controls were pooled. Summary of results for TW ANOVA for the changes in phospholipids in tissues in response to different doses of testosterone (T) and estradiol-17β (E2) hormones during prespawning phase of reproductive cycle have been given in the Table 2.

Effect of T and E2 on total phospholipids (TP): After ovariectomy, the hepatic level of TP was elevated by T and E2 treatments during prespawning phase when compared with intact fish. The hepatic level of TP was elevated at 14 and 28 mg kg^{-1} body weight after T and E2 treatments but declined at 7 mg kg^{-1} body weight of E2 as compared to control. The level of TP in plasma was elevated at 14 mg kg^{-1} bw after T and E2 treatments. The ovarian level of TP was elevated at all tested doses of T and E2 during prespawning phase as compared to control. After ovariectomy, hepatic and plasma levels of TP was elevated at 14 and 28 mg kg^{-1} body weight of T and E2. The level of TP in liver was declined at 7 mg kg^{-1} body weight of T and E2 but elevated at 7 mg kg^{-1} body weight in plasma as compared with control (Fig. 1).

Effect of T and E2 on phosphatidylcholine (PC): After ovariectomy there was no effect on PC levels during prespawning phase when compared with intact fish ovaries. The levels of PC was elevated in liver at 14 and 28 mg kg^{-1} body weight but declined

its levels to 7 mg kg^{-1} body weight of T and E2 treatments as compared to control. In plasma, PC was elevated at 14 mg kg^{-1} body weight by E2. In ovary, PC was elevated at 7 and 14 mg kg^{-1} body weight by T whereas E2 elevated PC level at all tested doses as compared to control during prespawning phase. In ovariectomized fish, the PC level in liver was elevated at 14 mg kg^{-1} body weight by T and 14 and 28 mg kg^{-1} body weight by E2 as compared to control. The plasma levels of PC were elevated at all tested doses of T and E2 but declined at 7 mg kg^{-1} body weight when compared with control during prespawning phase (Fig. 2).

Effect of T and E2 on phosphatidylserine (PS): After ovariectomy there was no effect on PS during prespawning phase when compared with intact fish ovaries. The hepatic level of PS was elevated at 14 and 28 mg kg^{-1} body weight of E2 during prespawning phase. The plasma and ovarian level of PS was elevated at tested doses of T and E2 when compared with control during prespawning phase. After ovariectomy, the hepatic level of PS was elevated at all doses of T but only at 14 mg kg^{-1} body weight by E2. The plasma level of PS was elevated at 7 and 14 mg kg^{-1} body weight of E2 when compared with control during prespawning phase (Fig. 3).

Effect of T and E2 on phosphatidylinositol (PI): There was no effect on PI after ovariectomy when compared with intact fish during prespawning phase. The hepatic level of PI was elevated at all tested doses of T but by E2 at 14 and 28 mg kg^{-1} body weight. The plasma level of PI was reduced but elevated in ovary at all tested doses of T and E2 when compared with control. In ovariectomized fish, the T at dose 14 and 28 mg kg^{-1} body weight elevated the hepatic level of PI whereas E2 elevation was only at 14 mg kg^{-1} body weight. The plasma level of PI reduced at 7 and 14 mg kg^{-1} body weight of T but elevated at all tested doses of E2 as compared to control during prespawning phase (Fig. 4).

Effect of T and E2 on phosphatidylethanolamine (PE): After ovariectomy, the level of PE was elevated in liver but remained unaffected in plasma when compared with intact fish ovaries during prespawning phase. The hepatic level of PE was elevated at all tested doses of T whereas E2 at 14 and 28 mg kg^{-1} body weight could elevate PE level. The ovarian level of PE was elevated at all tested doses of T and E2 as compared to control. In ovariectomized fish, the level of PE was elevated by 14 mg kg^{-1} body weight but declined at 7 mg kg^{-1} body weight of T injections. After E2 treatments, the hepatic level of PE was elevated at 14 and 28 mg kg^{-1} body weight whereas its level was high at all doses in plasma when compared with control during prespawning phase (Fig. 5).

In the present investigation during prespawning phase in response to T treatments, there was an increase in hepatic and plasma levels of TP and its elevation in ovary which indicated that hepatic synthesis was promoted in intact fish. In ovariectomized fish,

Table - 1: Treatments of testosterone (T) and estradiol-17β (E2) sex steroid hormones during prespawning phase of intact and ovariectomized catfish, *H. fossilis*

Batches	Hormone treatments
1	Control + no vehicle
2	Control vehicle
3	Testosterone 1 μg g^{-1} body weight
4	Testosterone 2 μg g^{-1} body weight
5	Testosterone 4 μg g^{-1} body weight
6	Estradiol-17β 1 μg g^{-1} body weight
7	Estradiol-17β 2 μg g^{-1} body weight
8	Estradiol-17β 4 μg g^{-1} body weight

All the injections were given intraperitoneally in sesame oil to the fish on alternate days for 15 days. Changes in various lipids were measured against total amount received (7, 14 and 28 mg kg^{-1} body weight) of each hormone

Table - 2: F* and P* values for different variables and their interactions for phospholipids in different tissues in response to testosterone and estradiol-17β treatments in intact and ovariectomized catfish *H. fossilis* during prespawning phase

Variables phospholipids	Tissue		Concentration		Tissue x concentration	
Testosterone -	F	p	F	p	F	p
Total phospholipid (TP)	68.00	< 0.001	34.35	< 0.001	31.65	< 0.001
Phosphatidylcholine (PC)	10.39	< 0.025	1.86	NS	0.91	NS
Phosphatidylserine (PS)	11.00	< 0.025	4.25	NS	0.57	NS
Phosphatidylinositol (PI)	473.0	< 0.001	72	< 0.001	58.00	< 0.001
Phosphatidylethanolamine (PE)	264.00	< 0.001	21.00	< 0.005	72.00	< 0.001
Estradiol-17β -						
Total phospholipid (TD)	50.37	< 0.001	2.21	NS	1.25	NS
Phosphatidylcholine (PC)	15.44	< 0.01	1.00	NS	1.37	NS
Phosphatidylserine (PS)	12.2	< 0.025	19.8	< 0.005	1.4	NS
Phosphatidylinositol (PI)	6.4	< 0.05	1.8	NS	1.00	N
Phosphatidylethanolamine (PE)	0.59	NS	0.01	NS	S0.03	NS

* The test of significance (F) is the variance ratio which is obtained by dividing the 'between mean sum of squares' by the 'within mean sum of squares'. Probability level (p) is the level of significance. NS- not significant at the level of 0.05 ($p > 0.05$)

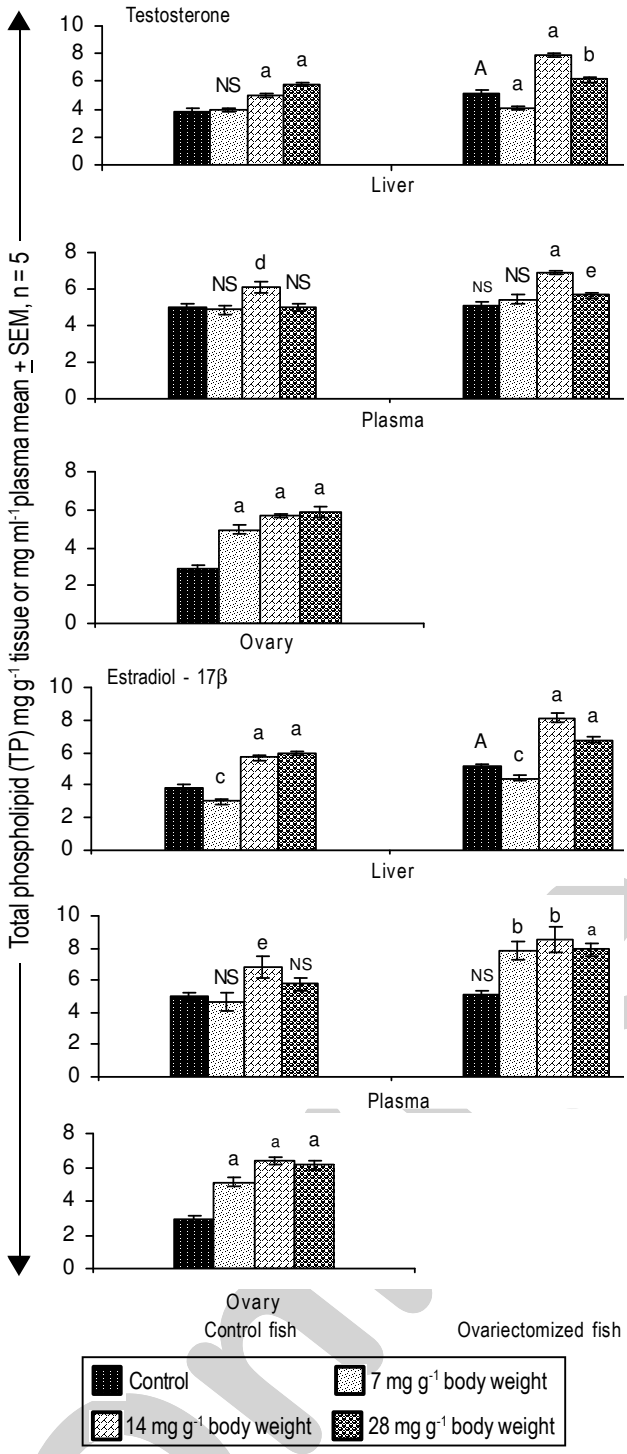

Fig. 1: Effect of testosterone and estradiol-17β on the concentrations of total phospholipid (TP) in liver, plasma and ovary in control and ovariectomized fish. The level of significance (p): $^{A}p<0.001$. Injections were given 1, 2 and 4 µg g^{-1} body wt. to experimental fish on alternate days for 15 days. Cumulative doses were 7, 14 and 28 mg kg^{-1} body wt. The control versus hormone injected were compared by Students *t*-test: $^{a}p < 0.001$, $^{b}p < 0.005$, $^{c}p < 0.01$ $^{d}p < 0.02$, $^{e}p < 0.05$. NS- not significant at the level of 0.05 ($p > 0.05$)

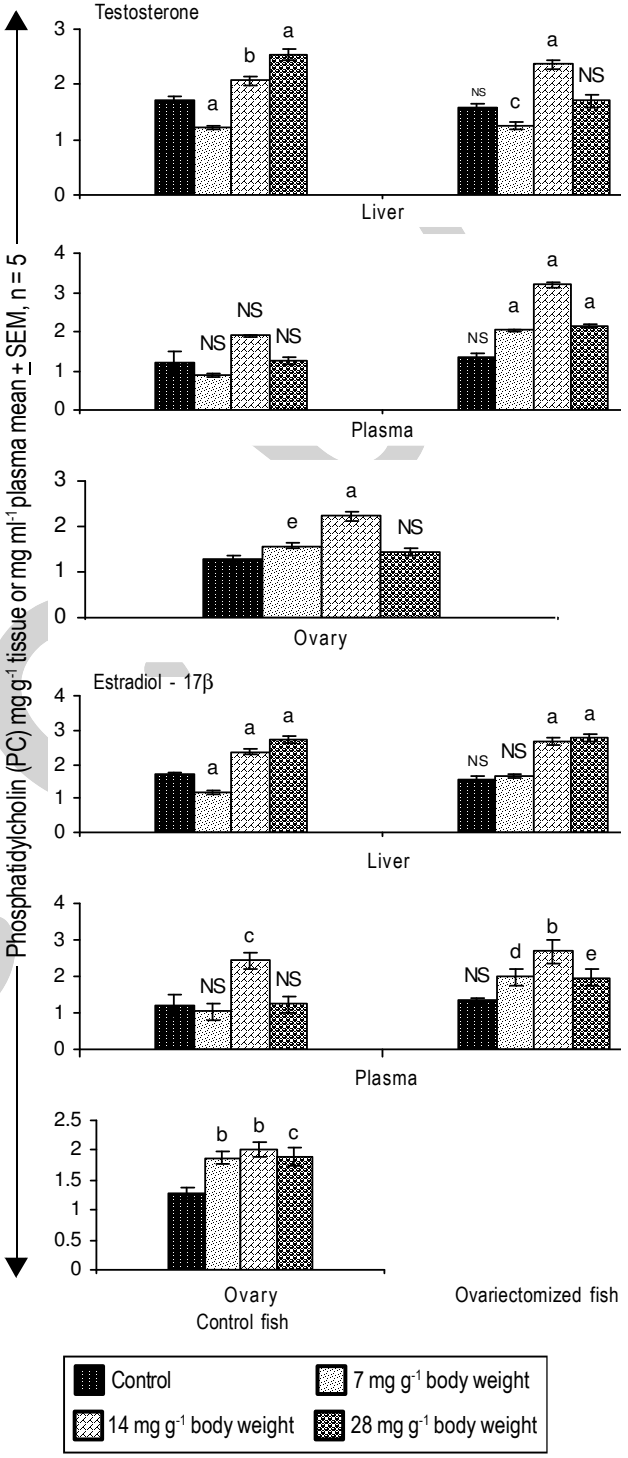

Fig. 2: Effect of testosterone and estradiol-17β on the concentration of phosphatidylcholine (PC) in liver, plasma and ovary in control and ovariectomized fish. Injections were given 1, 2 and 4 µg g^{-1} body wt. to experimental fish on alternate days for 15 days. Cumulative doses were 7, 14 and 28 mg kg^{-1} body wt. The control versus hormone injected was compared by Students *t*-test. The level of significance (p): $^{a}p < 0.001$, $^{b}p < 0.005$, $^{c}p<0.01$ $^{d}p < 0.02$, $^{e}p < 0.05$. NS- not significant at the level of 0.05 ($p > 0.05$)

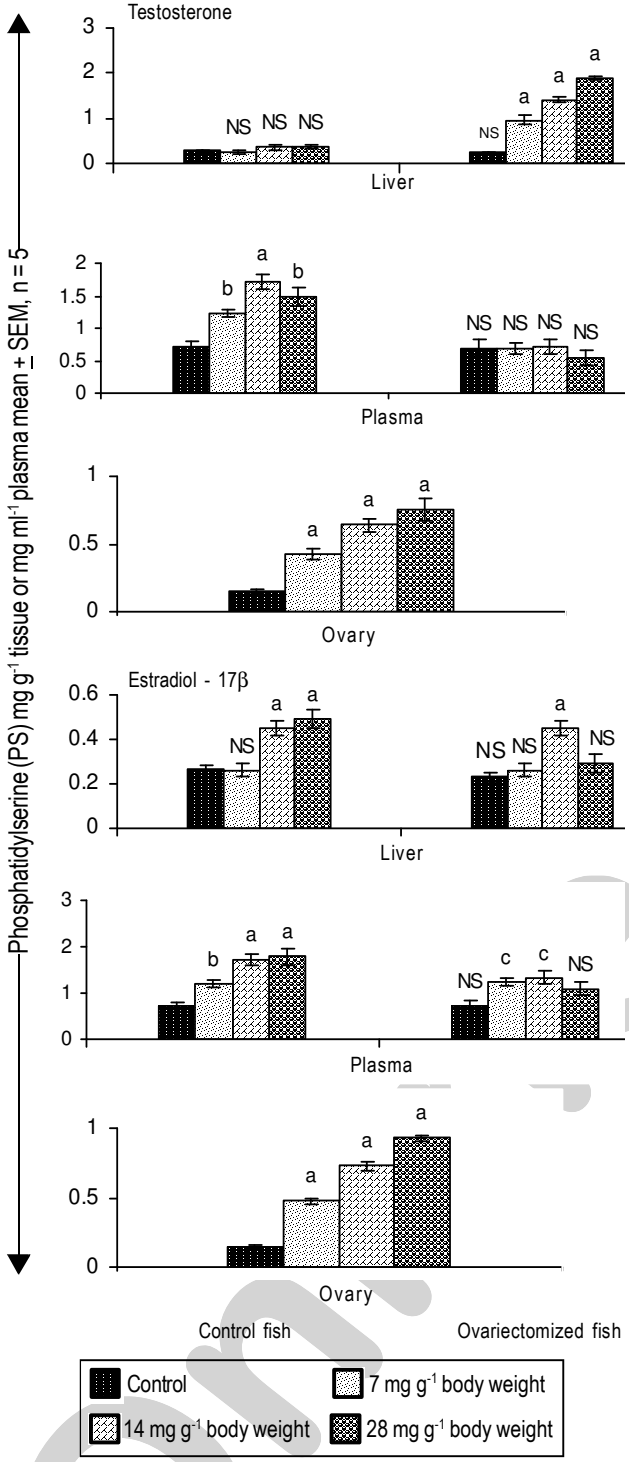

Fig. 3: Effect of testosterone and estradiol-17β on the concentration of phosphatidylserine (PS) in liver, plasma and ovary in control and ovariectomized fish. Injections were given 1, 2 and 4 µg g^{-1} body wt. to experimental fish on alternate days for 15 days. Cumulative doses were 7, 14 and 28 mg kg^{-1} body wt.. The control versus hormone injected was compared by Students *t*-test. The level of significance (p): $^{a}p<0.001$, $^{b}p<0.005$, $^{c}p<0.01$. NS- Not significant at the level of 0.05 (p>0.05)

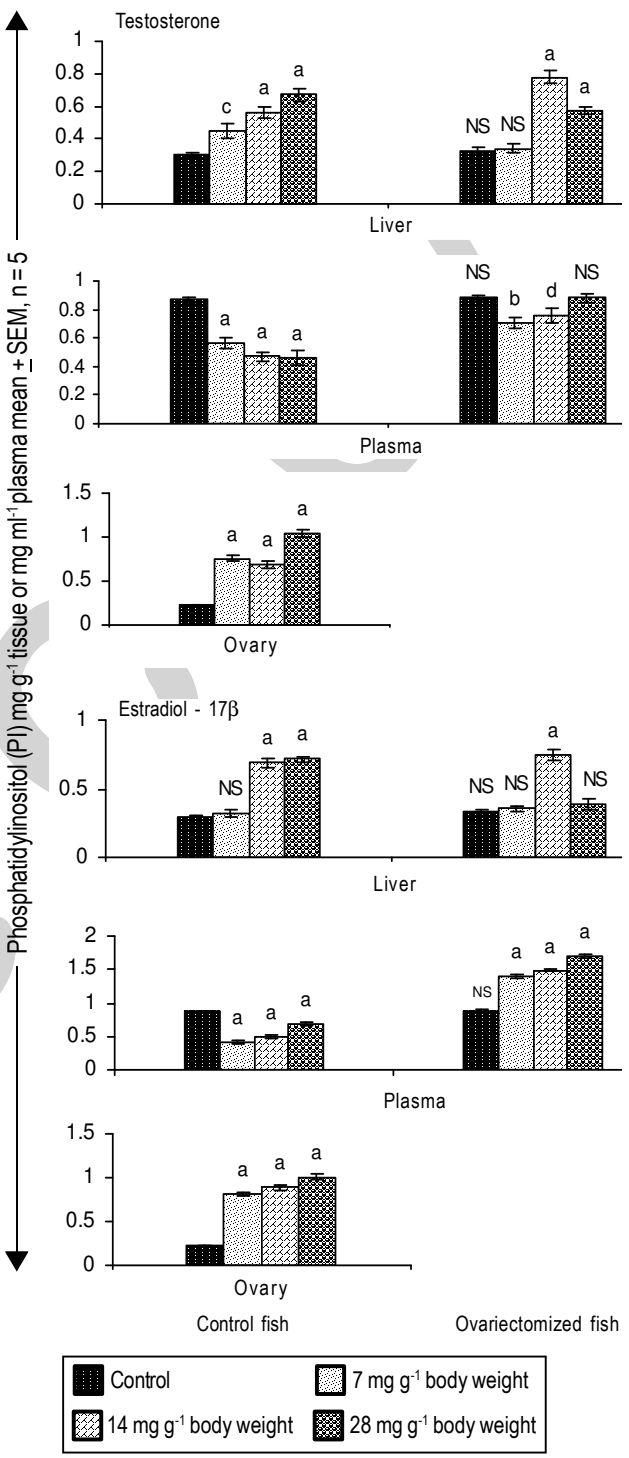

Fig. 4: Effect of testosterone and estradiol-17β on the concentration of phosphatidylinositol (PI) in liver, plasma and ovary in control and ovariectomized fish. Injections were given 1, 2 and 4 µg g^{-1} body wt. to experimental fish on alternate days for 15 days. Cumulative doses were 7, 14 and 28 mg kg^{-1} body wt. The control versus hormone injected was compared by Students *t*-test. The level of significance (p): $^{a}p<0.001$, $^{b}p<0.005$, $^{c}p<0.01$ $^{d}p<0.05$. NS- Not significant at the level of 0.05 (p>0.05)

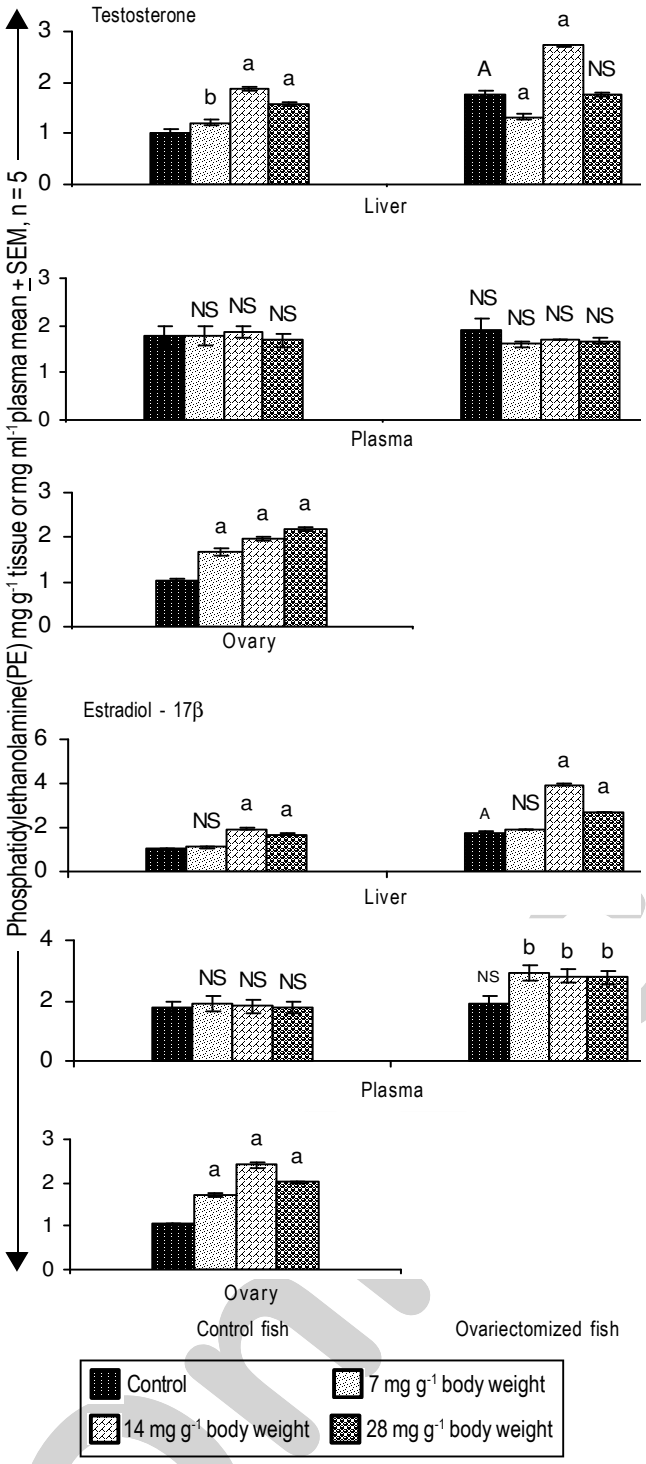

Fig. 5: Effect of testosterone and estradiol-17β on the concentration of phosphatidylethanolamine (PE) in liver, plasma and ovary in control and ovariectomized fish. The level of significance (p): [A]p<0.001. Injections were given 1, 2 and 4 μg g^{-1} body wt. to experimental fish on alternate days for 15 days. Cumulative doses were 7, 14 and 28 mg kg^{-1} body wt. The control versus hormone injected was compared by Students *t*-test: [a]p<0.001, [b]p<0.05. NS- Not significant at the level of 0.05 (p>0.05)

TP and PE were elevated in liver and PC, PS and PI remained unaffected (Fig. 1-5). The response for T and E2 treatment, promoted in liver and its deposition was higher for TP in ovary during prespawning phase. In ovariectomized fish the TP elevation was higher for liver and plasma due to synthesis and release into plasma in response to steroid hormone treatments. Results have demonstrated that hormones play very important role in the lipid biosynthesis and their mobilization under steroid control where T and E2 were the most effective depending upon the dose tested. In the present observation, in ovariectomized catfish, the injections of T and E2 have demonstrated that PC and PE the constituent of phospholipid (a component of vitellogenin) during reproductive growth was sex steroid dependent of its biosynthesis. Lal and Singh (1987) have reported that gonadectomized catfish *C. batrachus* was unable to elevate plasma lipids during prespawning phase but these authors did not study the different phospholipids.

Previous reports have indicated that polar lipids (PC, PS, PI and PE) are a major component of biomembranes. It has been reported that freshwater fish alter the composition of their biomembrane lipids in response to changes in environmental temperature (Henderson and Tocher, 1987; Tocher, 2003). Reports are available that estradiol-17β levels increased coincident with the appearance of yolky oocytes in the ovary and serum estrogen levels were highest during the period of ovulation and spawning in *Leptocottus armatus* (de Vlaming *et al.*, 1984). Lal and Singh (1987) have also reported that estradiol-17β was most potent in inducing, testosterone in suppressing the plasma lipids in gonadectomized catfish, *C. batrachus*. These authors have indicated that progesterone did not affect plasma phospholipids in *C. batrachus*. We have noticed that ovariectomy caused increase in hepatic TP and PE during prespawning phase after T and E2 treatments. The magnitude of elevation in liver and plasma was almost same by T and E2 treatments in ovariectomized catfish. It is suggested that there is a correlation between phospholipids and hormone induction on the synthesis, release of phospholipid and their mobilization from liver to ovary via plasma during gonadal growth.

It has been demonstrated that vitellogenin is considered to be an important carrier of lipids into fish eggs (Mommsen and Walsh, 1988). Phospholipid constitutes approximate 61-70% of total lipid in vitellogenin of *Oncorynchus mykiss* (Norberg and Haux, 1985; Fremont and Riazi, 1988) and 64% in *Hippoglossus hippoglossus* (Norberg, 1995). The phosphatidylcholine (80%) was the principal phospholipid in *Channa punctatus* (Sehgal and Goswami, 2005). In the present study in *H. fossilis*, the PC being the main constituent of phospholipid rather than PS, PI and PE and its biosynthesis was sex steroid dependent. Reports of Leslie and Buckley (1976) have shown that in the goldfish liver, PC was major constituent of total phospholipid, the rest being PE, PI and PS in decreasing order. In the present findings, T and E2 induced stimulation of hepatic biosynthesis of phospholipid for the synthesis of TP, PC, PS, PI and PE during oogenesis required for vitellogenin as well as membrane

lipids supports the previous results. Reports of Sundararaj *et al.* (1982) that purified carp gonadotropin(s) as vitellogenic in hypophysectomized *H. fossilis* during preparatory phase tend to further support our studies in this species. It may be concluded that in *H. fossilis*, T and E2 plays a vital role in regulating lipid dynamics.

In conclusion, retrospect of present finding it is suggested that E2 and T have pronounced effect during prespawning phase in ovariectomized *H. fossilis* in regulation of PC, PS, PI and PE phospholipid biosynthesis and its mobilization to ovaries. In addition to above, among the phospholipids, PC was the main phospholipid in this species whose biosynthesis is under sex steroid dependent. Above findings gives scientific information to understand the sex steroid control of different phospholipid biosynthesis and their mobilization to gonads during gonadal growth.

Acknowledgments

A grant-in-aid from UGC MRP New Delhi-110 016 to PBS former EC-PDF-Fellow (Brussels) is greatly appreciated. The authors are grateful to Dr S.S. Srivastava former DAAD Fellow of this Department and Dr. P.N. Singh, HOD of Department of English for the language editing of this manuscript.

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