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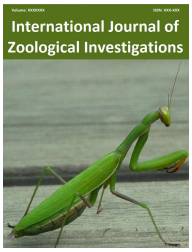

Correlation of Conjugated Sex Steroids with Oocyte Stages During Prespawning Phase of *Heteropneustes fossilis* (Bloch)

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Abstract: Correlation of oocytes with conjugated sex steroid testosterone (glucuronides and sulphates) was assessed during prespawning phase in freshwater female catfish *Heteropneustes fossilis* under laboratory conditions. Injection of testosterone glucuronide (TG) 2 µg/g body weight, testosterone sulphate (TS) 2 µg/g body weight and testosterone glucuronide + sulphate (TG + TS) 4 µg/g body weight was given for 15 days at alternate days. Cumulative doses were 14, 14 and 28mg/kg body weight. Significant increase in oocyte and migrating nucleolus towards periphery was noticed after testosterone glucuronide (TG) treatment. Testosterone sulphate (TS) showed no significant change in oocyte growth. After testosterone glucuronide + sulphate (TG + TS) treatment oocyte became mature, their size increased and nucleolus was migrating towards periphery as after testosterone glucuronide (TG) treatment. Other changes were also noticed like testosterone glucuronide (TG) treatment. GSI showed positive correlation with oocyte sizes after different hormonal treatment except testosterone (sulphate). This experiment revealed that glucuronides played an important role in gonadal development. Simultaneously, it also concluded that TS may function as a potent pheromone in this species.

Key-words: Oocyte, Glucuronide, Sulphate, Testosterone

Introduction

A number of authors have studied correlation between oocyte maturation and different hormones in fish (Cleary et al., 2000; Zohar and Mylonas, 2001; Lee and Yang, 2002; Nagahama and Yamashita, 2008; Yousefian et al., 2009; Heidari et al., 2010; Yelghi et al., 2012). Many authors have studied induced oocyte development in fishes. Impact of different hormones on oocyte development has also been observed in fishes (Senthilkumaran and Joy, 2001; Webb et al. 2002).

Yueh and Chang (2002) had reported that in female black porgy injected with two successive doses of LHRH analog (LHRH-A, 10 and 50 microg/kg of fish) both 17,20β-dihydroxy-4-pregnen-3-one (DHP) and

17,20β,21-trihydorxy-4-pregnen-3-one (20 β-S) were the most effective steroids to induce *in vitro* maturation (e.g. germinal vesicle breakdown, GVBD) in oocytes cultured for either 24 h or 1 min.

In Indian major carp *Labeo rohita*, under stimulation of fish pituitary extract FPE, (as a source of gonadotropin) *in vitro* production of E_2 and T by the vitellogenic follicles were shown to be highest as compared to their production rate in other stages, while the postvitellogenic follicles recorded the highest rate of DHP (17α,20β-dihydroxy-4-pregnen-3-one) synthesis by Sen et al. (2002). Sorensen et al. (1995) had demonstrated that in addition to using the maturational steroid hormone 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) as a potent sex pheromone, the goldfish uses its sulfated metabolite 17,20β-dihydroxy-4-pregnen-3-one 20-sulfate (17,20β-P-20S). Lee et al. (2009) reported that artificial estradiol-17β (E_2) treatment induced sex reversal in some fishes like Japanese pufferfish (*Takifugu rubripes*).

To date, our understanding of the function of testosterone in female reproductive physiology is only marginal although there are indications that testosterone is involved in modulating follicular recruitment, growth, atresia, and ovulation.

A long-term treatment, with T alone or T in combination with GnRHa, increased pituitary gonadotropin II (GtH II) levels 2- and 3-fold, respectively, suggesting that T and GnRHa both stimulate GtH II accumulation (Holland et al., 1998). Chen et al. (2013) have observed that in male zebrafish, 17α,20β-dihydroxy-4-pregnen-3-one stimulates, early stages of spermatogenesis. Testosterone induced maturation of mouse oocytes arrested in meiosis (Gill et al., 2004). Duncan et al., (2012) obtained good quality spawns after GnRHa treatment in wild meagre (*Argyrosomus regius*). In the catfish *H. fossilis*, administration of testosterone (0.25, 0.5, 1 and 2 micrograms/g body weight for 20 days) during mid-preparatory phase (March) increased plasma testosterone, gonado-somatic index, seminal vesicle-somatic index and concentrations of total proteins, fructose and hexosamines in seminal vesicle (SV) and testis in a dose-related manner (Chowdhury and Joy, 2000).

The present study is intended to reveal the correlation of conjugated sex steroid (testosterone glucuronide and testosterone sulphate) with oocyte stages during prespawning phase in female catfish *H. fossilis.*

Materials and methods

Experimental fish

The original research reported herein was conducted under ethical guidelines for the treatment of animals in behavioral research and teaching (Anonymous, 1998). The experimental fish, *H. fossilis* (65-70 g and length 21-22 cm) were collected from a pond of the same brood stock during prespawning phase and supplied with circulating constant flow of dechlorinated tap water. They were fed *ad libitum* with minced goat liver.

Chemicals

Analytical grade chemicals were obtained from E. Merck, Hi Media (India) and EIA-KIT of DIA-METRA, Italy (testosterone and estradiol-17β).

Tissue and plasma collection

After a week of acclimatization during prespawning phase, fishes were bled by caudal puncture and blood was collected in heparinized (1% heparin sodium salt activity 1,00,000 units 140.3 U/mg) glass tubes and centrifuged at 5,000 rpm for 15

minutes at 4^0C for plasma sex steroid (testosterone) analysis by Enzyme-linked immunosorbent assay (ELISA). After decapitation, ovaries were dissected out, washed in saline (0.6% NaCl) blotted and GSI was calculated (gonad weight x 100/ body weight).

Extraction of conjugated testosterone hormone

Conjugated sex steroid (testosterone) hormone was extracted by the method of Singh and Kime (1995).

Treatment with testosterone glucuronide and testosterone sulphate

After 10 days acclimatization following experiments were performed. The freshwater female fish were divided into 5 batches and each comprising of 5 fishes in an earthen pot having 20 L water in each at room temperature (Table 1). All injections (in sesame oil medium) were given intraperitoneally to the fish on alternate day for fifteen day during prespawning phase. Changes in estradiol-17β level were measured against total amount received (14, 14 and 28 mg/kg body weight) of each hormone. At the end of the experiments, fish were bled by caudal incision and blood samples were collected. Plasma was separated and analyzed by enzyme-linked immuno- sorbent assay (ELISA). After decapitation, ovaries were dissected out from fish of each batch, washed in saline (0.6% NaCl) blotted and GSI was calculated (gonad weight x 100/ body weight). A part of ovary was fixed in Bouin's fluid for the histological examination for the purpose of maturation status. The sections were cut at 6 µm and stained with haematoxylin and eosin stain. Oocyte's diameters were measured by trinocular microscope.

Table 1: Treatments of hormones during prespawning phase-

Batches	Hormone treatments
1	Control without any treatment
2	Control fish injected with vehicle
3	Fish injected with Testosterone glucuronide 2 µg/g body weight
4	Fish injected with Testosterone sulphate 2 µg/g body weight
5	Fish injected with Testosterone glucuronide and sulphate simultaneously 4 µg/g body weight

Extraction of sex steroid hormone (E_2)

Estradiol-17β hormone was extracted by method of Singh and Kime (1995).

Data analysis

Data were expressed in ng/ml plasma (mean ± SEM). Results were analyzed by two/one - way analysis of variance (ANOVA TW/OW) by Microsoft Excel tool pack data analysis (ANOVA) two factor with replication (Bruning and Kintz, 1977).

Results

Gonadosomatic index (GSI) and plasma level of estradiol-17β (E_2) level after different hormonal treatment in female H. fossilis in prespawning phase-

Changes in gonadosomatic index (GSI)

Gonadosomatic index increased after testosterone (glucuronide) treatment (2 µg/g body weight) in comparison to control. There was no change in GSI after testosterone (sulphate) treatment (2 µg/g body weight). When testosterone (glucuronide + sulphate) treatment (4 µg/g body weight) was given to fish there was significant increase in GSI (Fig. 1).

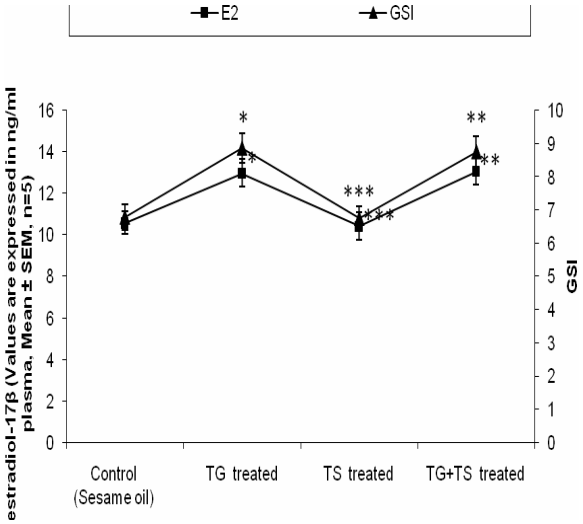

Fig.1: Plasma level of estradiol-17β and GSI in *Heteropneustes fossilis* after different harmonal treatment in prespawning phase. Control versus treated fishes were compared by Student's t-test. The level of significance (P) was *P < 0.01, **P < 0.02, ***P < 0.05. ANOVA (OW): GSI F=8.24 P < 0.001; Estradiol-17β F=5.20 P < 0.001

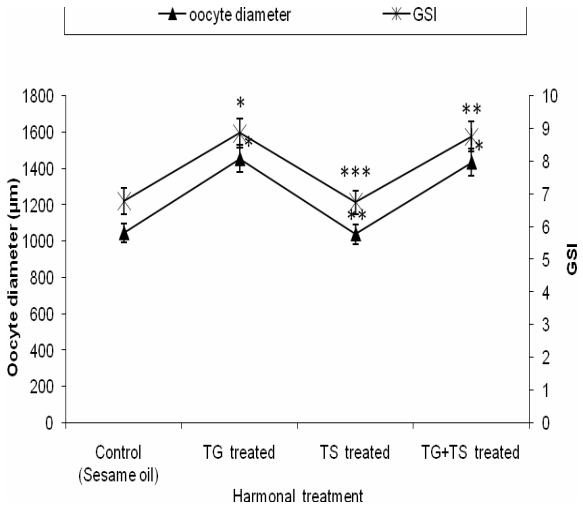

Fig. 2. Oocyte diameter and GSI of *Heteropneustes fossilis* after different harmonal treatment in prespawning phase. Control versus treated fishes were compared by Student's t-test. The level of significance (P) was *P < 0.005, **P < 0.05. ANOVA (OW): GSI F=8.24 P < 0.001; Oocyte diameter F=1.39 P < 0.05.

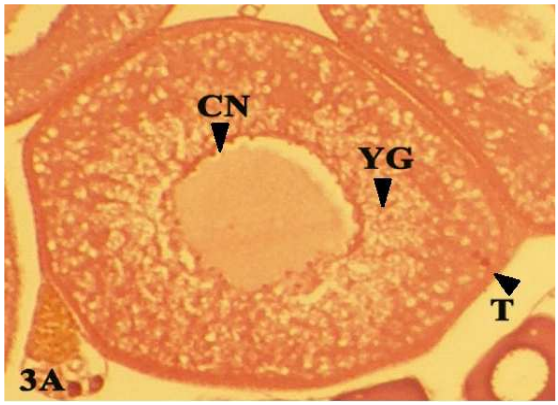

Fig. 3A: T. S. control fish ovary showing mature and vitellogenic oocyte with central nucleolus, yolk globules and theca in prespawning phase. HE x 80.

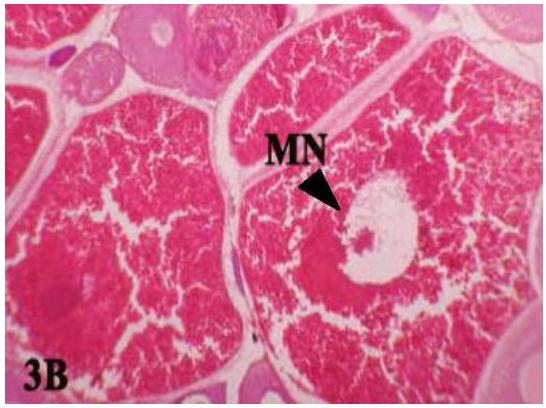

Fig. 3B: T. S. fish ovary after testosterone (glucuronide) injection showing migrating nucleolus towards periphery and bigger size of oocyte. HE x 40.

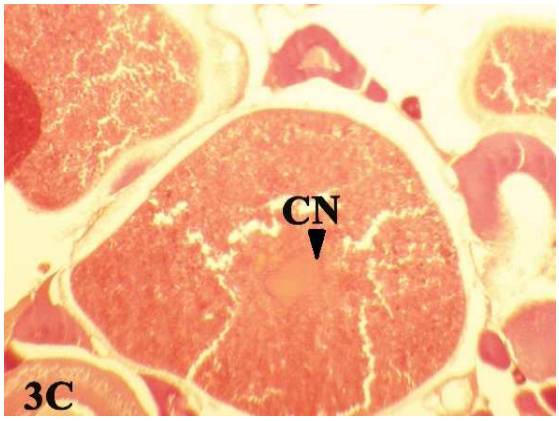

Fig. 3C: T. S. fish ovary after testosterone (sulphate) injection showing mature oocyte and central nucleolus as in control fish. HE x 80.

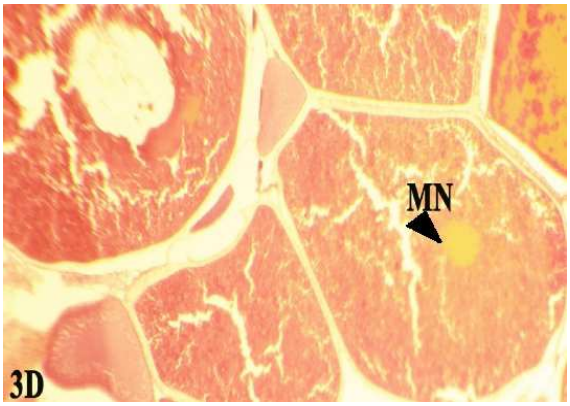

Fig. 3D: T. S. fish ovary after testosterone (glucuronide + sulphate) injection showing migrating nucleolus towards periphery and bigger size of oocyte. HE x 40.

Plasma estradiol-17β (E_2) level assayed by enzyme linked immunosorbent assay (ELISA)

In comparison to control there was significant increase in estradiol-17β level after treatment of testosterone (glucuronide) (2 µg/g body weight) to female *H. fossilis* in prespawning phase. There was no change in estradiol-17β level after treatment of testosterone (sulphate) (2 µg/g body weight) when given to female catfish *H.fossilis* in prespawning phase. When testosterone (glucuronide + sulphate) treatment (4 µg/g body weight) was given to female catfish *H. fossilis*, a significant increase in estradiol-17β level was recorded in prespawning phase (Fig. 2).

Ovary after different hormonal treatment in female H. fossilis in prespawning phase.

During prespawning phase greater number of stage VII vitellogenic oocytes were seen after treatment with testosterone (glucuronide) when compared to control (fig 3a-d). The oocytes were bigger in size. The oocyte diameter reached 1455 ± 74.9 µm, which was the maximum size of the oocytes. Yolk globules and yolk vesicles were in periphery of the cytoplasm. The vesicles gradually joined and became larger. The vitelline envelope was clearly evident. Vitelline membrane which constituted the inner zone of the vitelline envelope was disintegrating by leaving the void spaces from the outer parts. The membrane of the nucleus dissolved and nucleolus was moving towards the periphery. The zona radiata and follicular layer were fully developed and were differentiated as distinct layers. There was no change with the treatment of testosterone (sulphate). After testosterone (glucuronide + sulphate) treatment there were similar changes in oocytes as after the treatment of testosterone (glucuronide). Oocytes became mature and the diameter increased (1435 ±73.65). After the treatment (TG + TS) nucleolus showed migration towards the periphery.

Discussion

The results obtained in present study revealed that changes in plasma levels of gonadal steroid, estradiol-17β (1,3,5(10)-estratriene-3,17β-diol, E_2) are closely correlated with ovarian development and increased gonadosomatic index (GSI) and testosterone glucuronide (TG) is responsible for high level of estradiol-17β (E2) in plasma.

Ovarian development and recrudescence in teleosts are regulated by pituitary gonadotropin (GtH) through the production of ovarian steroid hormones. During oocyte vitellogenesis, the follicular layer of the oocytes synthesizes estradiol- 17 β (E_2), which stimulates the hepatic production of vitellogenin as a yolk precursor (Specker and Sullivan, 1994). The correlation between E_2 and T levels in blood has been found in various teleosts (Pankhurst et al., 1999). These findings implicate T as a substrate precursor of E_2 in most teleosts. In addition, among teleost females, serum E_2 and T exert negative or positive feedback effects on GtH synthesis and secretion

(Schultz et al., 1995). Thus, T seems to play important roles in endocrine control of oogenesis in many teleosts. However, the E_2 synthetic pathway within ovarian follicles has been studied in only a few species.

Specker and Sullivan, (1994) have observed that the primary function of E_2 is the stimulation of hepatic synthesis and ovarian sequestration of vitellogenesis. Conversion of T to E_2 is dependent on the activity of the aromatase cytochrome P450 ($P450_{arom}$) enzyme system. E_2 is also responsible for stimulating hepatic synthesis of egg-shell proteins in teleosts.

In both male and female vertebrates, the gonadal steroid hormone testosterone modulates behavior and physiology related to reproduction and other androgens are essential for normal development and functioning of the reproductive system (Fusani, 2008; Walters et al., 2008).

The ovary contains millions of undifferentiated follicles of which only some will grow to eventually reach the mature state of a preovulatory follicle, whereas others will cease development, become atretic and be resorbed. Although, the mechanisms controlling follicle selection and maturation are still obscure (Johnson and Woods, 2007; McLaughlin and McIver, 2009; Onagbesan et al., 2009), yet there is a potential role for testosterone.

Although, authors have supported the fact that steroid glucuronides play role in oocyte development (Van Weerdt et al., 1991). The role of steroid glucuronides as pheromone are also reported (Lambert and Resink, 1991). A study on pre-ovulatory females of *Barilius bendelisis* (Ham.) revealed that the fish release sex steroids and their conjugates into the water and that a steroid sulphate, of these compounds, functions as a potent sex pheromone which stimulates milt production in conspecific males prior to spawning (Sajwan et al., 1999; Bhatt and Sajwan, 2001). Paitz and Bowden (2011) suggested a different function of estradiol-17β sulphate that Exogenous E_2-S had a significant effect on hatchling sex ratios.

Conclusion

It is evident from the present study that testosterone glucuronide plays an important role in oocyte development. Our study also indicates that testosterone sulphate may function as a sex pheromone in this species.

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The paper is dedicated to late Dr. P.B. Singh.

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