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Circadian Changes in Testosterone and Estradiol-17 Glucuronides and Sulfate Steroids during Spawning phase in Fresh Water fish female *Cirrhinus mrigala*

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Abstract : *The aim of this investigation was to know daily changes (circadian) in the blood plasma free (unconjugated) testosterone (TF) and estradiol-17β (E2F) and its conjugated testosterone glucuronide (TG) and testosterone sulfate (TS) as well as estradiol-17β glucuronide (E2G) and estradiol-17β sulfate (E2S) sex steroids in a mature female fish Cirrhinus mrigala (Ham.) during spawning phase using enzyme-linked immunosorbent assay (ELISA) method. The two sex steroids testosterone and estradiol-17β exhibited identical circadian rhythm: a major peak 29.02 ± 3.58 ng/ml occurred at the onset of the dark phase and a minor peak 12.79 ± 1.89 ng/ml was generally observed 4 hour after the onset of light phase . The TG peak (9.23 ± 1.04 ng/ml was in the dark phase whereas TS during 12.00 PM of dark phase. The E2G 7.19 ± .76 ng/ml peak was also indicated high during 04.00 am of the dark phase. Result indicated that during the dark phase elevation of free and conjugated sex steroids have important role for spawning, and maintaining the equilibrium of free and conjugated steroids.*

Keywords: Circadian, Sex steroid, Glucuronide, Sulfate, Hormones, Fish, *Cirrhinus mrigala*.

Introduction

In *Cirrhinus mrigala*, sexual maturation and spawning are endocrinologically regulated by gonadal axis, and sex steroids such as testosterone (T) and estradiol-17β (E2) act on vitellogenesis in females, respectively. Blood sex steroids levels of these hormones are generally high during sexual maturation, but clearly low at the time of final maturation (Divers *et al.,* 2010; Pham *et al.,* 2011; Bahabadi *et al.,* 2011; Friesen *et al.,* 2012 Khodaoust *et al.,* 2013). In contrast to 11-ketotestosterone (11KT) and E2, the blood hormone levels of 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP), which is well known and investigated to induce final maturation including spermiation or ovulation, are remarkably high around the time of final maturation (Endo *et al.,* 2011). Blood gonadotropin levels fluctuations have been studied by few workers (Yamada *et al.,* 2002; Katare *et al.,* 2011).

It is well known that circadian changes in blood hormone concentrations are observed in some fishes (Boujard and Leatherland, 1992; Holloway *et al.,* 1994; Yamada *et al.,* 2000) Thyroid hormones have also shown circadian variations in blood levels of rainbow trout (Boujard and Leatherland, 1992; Boujard *et al.,* 1993; Reddy and Leatherland, 1994; Gomez *et al.,* 1997; However, there are no report on the circadian changes in sex steroids hormone, testosterone and estradiol-17β and their conjugate in matured female major carp *Cirrhinus mrigala* .

The present study was conducted to know the relationship between circadian pattern of plasma levels of testosterone and estradiol-17β and their conjugated forms (glucuronides and sulfates) in *Cirrhinus.*

Materials and Methods

Fish sampling: Adult experimental female fish of *Cirrhinus mrigala* seasonal breeder *was* collected during spawning phase from a pond in Jaunpur Blood sampling was done at every 4 hours for 24 hours morning 4.00 am to midnight 12.00 pm.

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After weighting in single pan balance. Then fish was bled by caudal incision and blood was collected in heparinized glass culture tubes. The blood was centrifuged at 4000 rpm for 15 minutes in a refrigerated testosterone (TF) and estradiol-17β centrifuge at 4^{0}C.The plasma was separated and kept -20^{0}C till further analysis.

Extraction of and ELISA assay of sex steroid hormones: Extraction of unconjugated and conjugated sex steroid hormones was followed as per methods described by Singh and Kime (1995) with some modification. Briefly, The 500µl plasma was extracted twice with 5 ml distilled dichloromethane to give the unconjugated (free) steroid fraction, and the aqueous residue (containing glucuronide and sulfates) was treated with 800 µg β-glucuronidase (Sigma G 0251, from bovine liver, Type B-1, 500 000 units) in 1 ml 0.2M acetate buffer, pH 4.8 for 24 h at 37^{0}C to hydrolyse glucuronide conjugates. After incubation, steroid moieties of the glucuronides were extracted twice with 5 ml dichloromethane. The aqueous phase was extracted twice with 4 ml of water saturated butan-1-ol, and the extract evaporated. Distilled water (20 µl) was added, vortexed, and treated with trifluroacetic acid (TFA) in ethyl acetate (1/100, v/v, 3ml) at 45^0 C for 18 h to hydrolyse sulfate conjugates. Distilled water (1 ml) was added to each tube, shaken, and the organic phase containing the steroid moieties of the sulfates pipetted off. The aqueous residue was re-extracted with a further 3 ml ethyl acetate and the extracts combined and evaporated. Sex steroid hormones- free and conjugated steroid hormone (testosterone and estradiol-17β) assay was done by ELISA Kit (Diametra, Italy).as per standard methods

Statistical analysis : Data was expressed in ng/ml plasma (mean + SEM). For statistical analysis of data analysis of variance and Newman Keul's multiple-range test was employed, at the probability level of 0.05.

Results and Discussion

The analysis of variance indicated that there is significant variation during spawning phases. Time and hormone(testosterone) levels (Time F: 9.16 P < 0.001; Hormone, F: 61.05 P < 0.001; Time × Hormone F: 4.77 < 0.005).

The plasma level of TF was maximum of 8.00 PM and minimum 15.79 ± 2.28 ng/ml at 12.00 midnight. Although its level was also high 14.11 ± 2.10 ng/ml at 4.00 AM of the dark phase. The plasma level of TG was maximum 9.23 ± 1.04 ng/ml at 4.00 AM and minimum 2.00 ± 0.29 ng/ml during 4.00 PM of dark phase. During the dark phase, the level of TG was high 9.23 ± 1.04 ng/ml than the light phase 2.00 ± 0.29 ng/ml). Similarly, TS was also recorded as TG during circadian changes (Fig. 1).

Time and hormone(estradiol-17β) levels (Time F: 14.13 P < 0.001; Hormone, F: 96.06 P < 0.001; Time × Hormone F: 3.63 P < 0.01) Plasma level of E2F was maximum 29.02 ± 3.58 ng/ml during 8.00 PM and minimum 12.79 ± 1.89 ng/ml at 4.00 PM the level of E2G high 16.12 ± 1.49 ng/ml at 4.00 AM but remained low 4.12 ± 0.54 at 12.00 midnight. The E2S level just correspond to the level of E2G. Result indicated that during dark phase the conjugated steroids remain high as compared to light phase (Fig. 2).

In many of reports on circadian changes of blood hormone concentrations (Holloway *et al*., 1994; Gomez *et al*., 1997), have been discussed for one day data. In the present study, serum T and E2 peak was observed in night time in female same as Atlantic salmon on the other hand, a peak of T and E2 in daytime in night and under both phases has also reported in various fishes. This discrepancy of peak time of T and E2 may due to species differences, feeding schedules and developmental stage differences.

In teleost, a catfish *Heteropneustes fossilis* showed increase in blood E2 and T levels at the onset of darkness, and the carp *Cyprinus carpio*

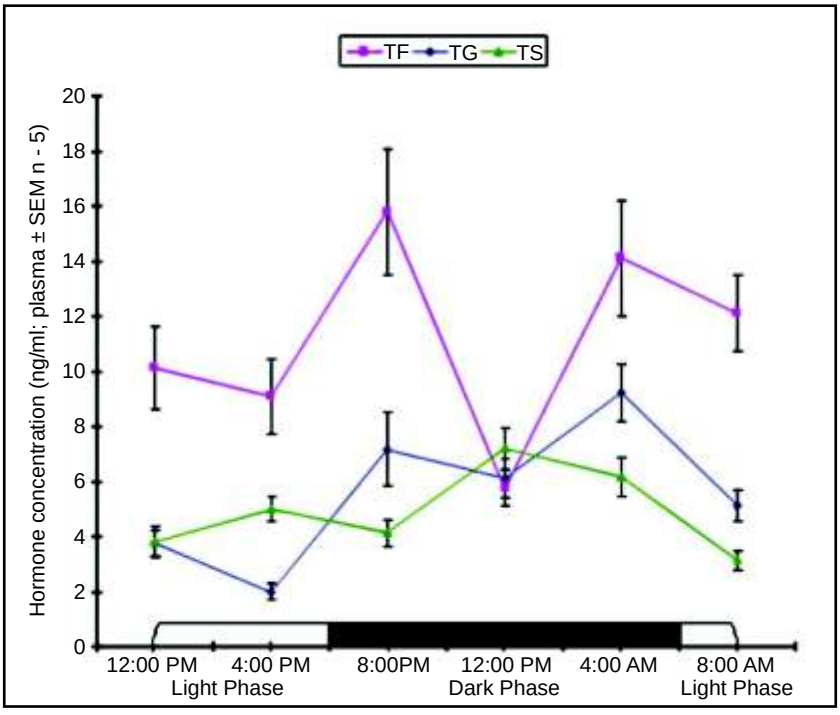

Fig. 1 Circadian changes in plasma levels of testorone free (TF), testosterone glucuronide (TG), and testosterone sulfate (TS), sex steroids in fresh water female major carp, *Cirrhinus mrigala* (Ham.) Analysis of variance two way (ANOVA-TW):

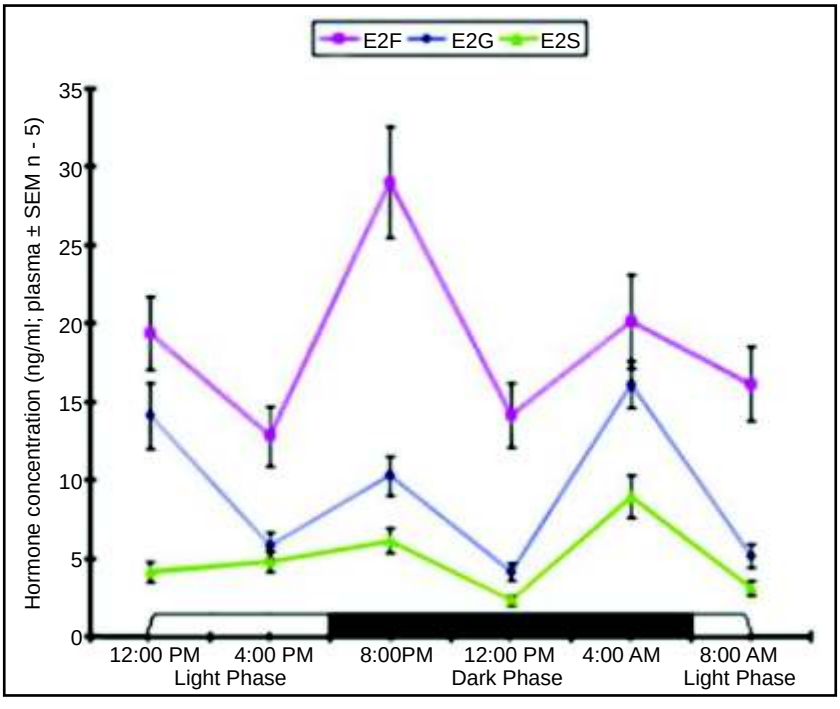

Fig. 2 Circadian changes in plasma levels of estradiol-17β free (E2F), estradiol-17β glucuronide (E2G), and estradiol-17β sulfate (E2S), sex steroids in fresh water female major carp, *Cirrhinus mrigala* (Ham.) Analysis of variance two way (ANOVA-TW):

exhibited diurnal changes in blood T, E2, DHP, plasma T concentrations in both sex were the same, and increased around the onset of darkness. In the present study, production of conjugated sex steroids in darks indicates that glucuronide and sulfate may have important role in pheromonal and sexual behavior as well as spawning in *Cirrhinus mrigala* (Ham.). These reports strongly suggest close relationship between testosterone and feeding or its related behaviors in fishes. This is also supported by the data from T treatment in *Cyprinodon variegatus,* in which aggressive behaviors were enhanced by T administration (Higby *et al.,* 1991). Moreover, environmental light and vision are important for recognizing other individuals in a school, and the feeding activities under relatively low illumination intensity in salmonid fish (Azuma and Iwata, 1996). As it has already been separated that Peak of serum 11KT level in male may be involved in mating behavior including territorial and aggressive behaviors of male char at the time of spawning season. 11-ketotestosterone induces typical male-type spawning behavior in the male goldfish (Kobayashi and Nakanishi, 1999) and in female goldfish (Stacey and Kobayashi, 1996; Kobayashi *et al.,* 1997). Male salmon defend the nesting females from other males, and 11KT levels of male *Oncorhynchus nerka* placed with females are higher than that of male without female (Liley *et al.,* 1993). Moreover, 11KT levels of dominant male rainbow trout are higher than subordinate fish (Cardwell *et al.,* 1996).

In the previous report, catfish shows E2 peak during dark phase of reproductive period. E2 rhythm was observed in female *Cirrhinus mrigala* of the present study. Because of that E2 is synthesized at ovarian follicles during reproductive period, and the serum level was high during vitellogenesis, but low in final maturation of char.

Result have suggested that free and conjugated sex steroids of dark phase (8:00 PM to 4:00 AM) have an important role in spawning.

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