Section – 2

Circadian changes in testosterone and estradiol-17β glucuronides and sulfate steroids during spawning phase of *Cirrhinus mrigala* (Ham.)
1. Abstract

The aim of this investigation was to define 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 mature female Cirrhinus mrigala (Ham.) during spawning phase of its annual reproductive cycle. The hormones were measured by enzyme-linked immunosorbent assay (ELISA) method. The two sex steroids (T and E2) exhibited identical circadian rhythm: a major peak occurred at the onset of the dark phase (20.00 hr) and a minor peak was generally observed 4 hour after the onset of light phase (12.00 hr). The TG peak was 04.00 hr in the dark phase whereas TS during 12.00 PM of dark phase. The E2G 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, pheromonal behavior and maintained the equilibrium of free and conjugated steroids in this species during spawning phase.

Key words: Circadian, Sex steroid, Glucuronide, Sulfate, Hormones, Fish, Cirrhinus mrigala (Ham.)
2. 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 (Delahunty et al., 1978; Campbell et al., 1980; Fostier et al., 1983; Bieniarz, et al., 1986; Divers et al., 2010; Pham et al., 2011; Bahabadi et al., 2011). In contrast to 11KT and E2, the blood hormone levels of 17α,20β-dihydroxy-4-pregnene-3-one (17,20BP), which is well known and investigated to induce final maturation including spermiation or ovulation, are remarkably high around the time of final maturation (Goetz, 1983; Young et al., 1983; Endo et al., 2011). Although there are many of endocrinological reports the changes in those hormones in spawning stage have not been well investigated. Blood gonadotropin levels fluctuations have been studied by some workers (Hontela and Peter, 1978; Hontela 1982; Hontela and Peter, 1983a, b; 1984; Yamada et al., 2002 a, b; Zohar and Billard, 1984; Katare et al., 2011).

It is well known that diel changes in blood cortisol concentrations are observed in juvenile Atlantic salmon *Salmo salar* (Thorpe et al., 1987),
immature rainbow trout *Oncorhynchus mykiss* (Bry, 1982; Rance *et al.*, 1982; Laidley and Leatherland, 1988; Holloway *et al.*, 1994), and brown trout *Salmo trutta* (Pickerling and Pottinger, 1983). Thyroid hormones have also shown diel variations in blood levels of rainbow trout (Eales *et al.*, 1981; Laidley and Leatherland, 1988; Boujard and Leatherland, 1992; Boujard *et al.*, 1993; Reddy and Leatherland, 1994; Gomez *et al.*, 1997). Lamba *et al.* (1983) have reported the diel changes in blood sex steroids (testosterone and E2) of catfish *Heteropneustes fossilis*, and Santos *et al.* (1986) showed diurnal changes in blood testosterone (T), E2, 17, 20βP and 17α-hydroxyprogesterone concentrations in carp *Cyprinus carpio*. However, no report on the circadian changes in sex steroids hormone, testosterone and estradiol-17β and their conjugate in matured female major carp *Cirrhinus mrigala* (Ham.).

The present study was conducted to obtain scientific knowledge on the relationship between circadian pattern of plasma levels of testosterone and estradiol-17β and their conjugated forms (glucuronides and sulfates) in *Cirrhinus mrigala* and also for further studies on the mechanisms controlling steroidogenesis in this species. This is the first investigation in any major carp (*Cirrhinus mrigala*) showing the relationship between glucuronide and sulfate sex steroid hormones measured by enzyme linked immunosorbent
assay (ELISA) during annual reproductive cycle. In addition to above, the functional significance of these conjugate steroids during gonadal growth in female fish is unknown.

3. Materials and Methods

3.1 Fish sampling:

Adult experimental female fish of *Cirrhinus mrigala* was collected during spawning phase from a pond cultured by a fish former Banrahia Bagh, Gaurabadshahpur, Jaunpur (UP) fish farm under the conditions of natural photoperiod and temperature. The carp *Cirrhinus mrigala* is a seasonal breeder. Blood sampling was done after every 4 hours for 24 hours to investigate the circadian changes of conjugation of sex steroids during spawning phase of *Cirrhinus mrigala*. The sampling procedure for circadian was followed as described by Lamba *et al.* (1983). Circadian- rhythms with a frequency of one cycle in $24 \pm 4$ Hours have been reported by Halberg *et al.* (1977).

Fish was captured with a hand net and quickly weigh on a top loading 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 centrifuge at 4°C. The plasma was separated and kept -20°C till further analysis of conjugated and unconjugated steroids.

3.2 Extraction of unconjugated and conjugated 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°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 trifluoroacetic acid (TFA) in ethyl acetate (1/100, v/v, 3ml) at 45°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. After extraction of free, glucuronide and sulfate steroids will be assayed for various hormones by ELISA Kit.

### 3.3 ELISA assay:

Methods for sex steroid hormones- free and conjugated steroid hormone (testosterone and estradiol-17β) assay was done by ELISA Kit (Diametra, Italy). Details of methods for ELISA assay for each of hormones were followed as per methods supplied with kit of the each steroid assay. Briefly, the detail has been given in Section 1.

### 3.4 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 (Bruning and Kintz, 1977).

### 4. Result

The analysis of variance indicated that there is significant variation during spawning phases. In the plasma, the level of TF, TG and TS as well
as E2F, E2G and E2S have been reported. The results have been summarized in Table 1, 2 and Fig. 1, 2. The level of TF was maximum of 8.00 PM and minimum at 12.00 midnight. Although its level was also high at 4.00 AM of the dark phase. The plasma level of TG was maximum at 4.00 am and minimum during 4.00 PM of dark phase. During the dark phase, the level of TG was high than the light phase. Similarly, TS was also recorded as TG during circadian changes (Table 1 and Fig. 2).

Plasma level of E2F was maximum during 8.00 PM and minimum at 8.00 AM the level of E2G high at 4.00 AM but remained low at 12.00 midnight. The E2S level just correspondence to the level of E2G. Result indicated that during dark phase the conjugated steroids remain high as compared to light phase (Table 2 and Fig. 2).

5. Discussion

In many of reports on circadian changes of blood hormone concentrations (Leatherland et al., 1974; Eales et al., 1981; Rance et al., 1982; Lamba et al., 1983; Pickering and Pottinger, 1983; Santos et al., 1986; Thorpe et al., 1987; Laidley and Leatherland, 1988; Holloway et al., 1994; Reddy and Leatherland, 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 (Thorpe et al., 1987) and brown trout (Pickering and Pottinger, 1983; Laidley and Leatherland, 1988). On the other hand, a peak of T and E2 in daytime (Garcia and Meier, 1973; Singley and Chavin, 1975), in night (Redgate, 1974), and under both phases (Peter et al., 1978) 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 as suggested by Pickering and Pottinger (1983).

In teleost, a catfish *Heteropneustes fossilis* (Lamba et al., 1983) showed increase in blood E2 and T levels at the onset of darkness, and the carp *Cyprinus carpio* (Santos et al., 1986) 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 as same as Lamba et al. (1983). In addition, changing pattern of serum 11KT concentrations in male was almost the same as that of serum T levels. The high level of serum T and 11KT levels was observed at same clock time for over 3 sampling days, strongly suggesting existence of circadian rhythms of serum T in both sex and 11KT in male in the Japanese char during spawning period. Above authors did not observe the conjugated form sex steroids TG TS as well as E2G and E2S. 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 (Lamba *et al.*, 1983). We have recorded E2 rhythm was observed in female of the present study in *Cirrhinus mrigala*. 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 (Kagawa et al., 1981). The female char have ovulated already in the present study, suggesting less E2 synthesizing activities than vitellogenic stage. This may be the reason for low E2 concentrations and no remarkable changes in the female char after ovulation.

In conclusions, we have investigated circadian changes in plasma T, E2 and its conjugate for the first time and found that there are the circadian variations in plasma sex steroids in Cirrhinus mrigala. Possible involvement of circadian changes of various steroids during spawning phase in spawning behavior such as mating, spawning, territorial and aggressive behaviors of this species has been discussed.
References


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Reddy, PK and JF Leatherland: Does the time of feeding affect the diurnal rhythms of plasma hormone and glucose concentration and hepatic


Circadian changes in plasma level of testosterone free (TF), testosterone glucuronide (TG) and testosterone sulfate (TS) sex steroids in the fresh water female major carp, *Cirrhinus mrigala* (Ham.)

<table>
<thead>
<tr>
<th>Time</th>
<th>Plasma levels of testosterone (ng/ml; mean ± SEM, n = 5)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TF</td>
</tr>
<tr>
<td>12:00 NOON</td>
<td>10.11 ± 1.50</td>
</tr>
<tr>
<td>4:00 PM</td>
<td>9.09 ± 1.35</td>
</tr>
<tr>
<td>8.00 PM</td>
<td>15.79 ± 2.28</td>
</tr>
<tr>
<td>12:00 MID NIGHT</td>
<td>5.76 ± 0.64</td>
</tr>
<tr>
<td>4:00 AM</td>
<td>14.11 ± 2.10</td>
</tr>
<tr>
<td>8:00 AM</td>
<td>12.11 ± 1.40</td>
</tr>
</tbody>
</table>

Analysis of variance two-way (ANOVA-TW): Time, F: 9.16 P < 0.001; Hormone, F: 61.05 P < 0.001; Time × Hormone, F: 4.77 P< 0.005
Circadian changes in plasma level 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.)

<table>
<thead>
<tr>
<th>Time</th>
<th>Plasma levels of estradiol-17β (ng/ml, mean ± SEM, n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E2F</td>
</tr>
<tr>
<td>12:00 NOON</td>
<td>19.37 ± 2.37</td>
</tr>
<tr>
<td>4:00 PM</td>
<td>12.79 ± 1.89</td>
</tr>
<tr>
<td>8:00 PM</td>
<td>29.02 ± 3.58</td>
</tr>
<tr>
<td>12.00 MID NIGHT</td>
<td>14.13 ± 2.10</td>
</tr>
<tr>
<td>4.00 AM</td>
<td>20.11 ± 2.99</td>
</tr>
<tr>
<td>8.00 AM</td>
<td>16.11 ± 2.40</td>
</tr>
</tbody>
</table>

Analysis of variance two-way (ANOVA-TW): Time, F: 14.13 P < 0.001; Hormone, F: 96.06 P < 0.001; Time x Hormone, F: 3.69 P< 0.01
Fig. 1: Circadian changes in plasma levels of testosterone 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): Time F: 9.16 P < 0.001; Hormone, F: 61.05 P < 0.001; Time × Hormone F: 4.77 < 0.005
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): Time F: 14.13 P < 0.001; Hormone, F: 96.06 P < 0.001; Time × Hormone F: 3.63 P < 0.01