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Role of Cortisol and its Effect on Male freshwater fish *Notopterus notopterus* (Pallas) during four Reproductive phases

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ABSTRACT

In the present study serum cortisol level was estimated by radioimmunoassay (RIA) method in the freshwater fish Notopterus notopterus in a single day and also during four reproductive phases. The results indicates that during morning hours 8.30 am (15.75 ± 0.383) cortisol level is low while afternoon 13.30pm (21.08 ± 0.71) and evening 20.30pm (25.50 ± 0.76) hours serum cortisol level is higher. The fish N.notopterus, fed in the morning hours and metabolic activity increases during afternoon and evening hours probably resulting in the increase of serum cortisol level. The reproductive phases were categorized based on the morphology of the gonads observed during different periods. The cortisol estimation during four phases of the reproductive phases indicates that in control fish cortisol level increases during preparatory pre-spawning and spawning. The cortisol administration during preparatory pre-spawning and spawning phases may be because of energy requirements for spermatogenesis and spermiation activities. The cortisol administration during four reproductive phases and estimation of serum cortisol level a day after administration indicate that during preparatory and pre-spawning phases serum cortisol decreases' from that of control. The decrease in serum cortisol indicates its utilization for energy release needed for reproductive activity.

Key words: *Notopterus notopterus, cortisol, hormone, reproductive phases*

INTRODUCTION

Hormones are the dynamically collected by fenestrated capillaries to generate pulses, which are then decoded by target tissues to mount a biological response by Marie *et al*[23]. Glucocorticoids are steroid hormones of the adrenal gland that are an integral component of the stress response and regulate many Physiological processes, including metabolism and immune response. Their release into the blood is highly dynamic and occurs in about hourly pulses, the amplitude of which is modulated in a daytime dependent fashion by Thomas D *et al*[37]. Cortisol is the principle corticosteroid in teleost fishes and its plasma concentration rise dramatically during stress by Mommsen *et al*[26]. Corticosteroids play a major role in the response of the brain to stress by de Kloet *et al*[8].

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One of the most commonly measured indicators of stress in fish is the concentration of the major circulating corticosteroid, cortisol and this hormone can be measured easily and accurately using commercially available radioimmunoassay technique (RIA) by Gamperl *et al*[12]. There is increasing evidence indicating that cortisol directly and/or indirectly plays an important role in intermediary metabolism in addition to its role in other functions by Vijayan *et al*[42.43.25.44] . The above studies tend to correlate the physiological changes associated during stress with cortisol based primarily on the circulating levels of the steroid. The seasonal studies carried out in fish adrenal tissue in relation to reproductive activity indicates that the inter-renal cells are hyperactive during breeding phase of the fish and also it is reported that cortisol is shown to interfere with reproductive function in mature and maturing rainbow trout by Chakraborti *et al*[4]. It is observed that very few studies have directly examined the environmental regulation of cortisol biosynthesis in fish by Mommsen *et al*[26]. Cortisol is present in eggs and larvae of number of species including Japanese flounder *Papatichthys olivaceus* by De Jesus *et al*[9]. Chem salmon *Oncorhhynchus keta*[10.] Hwang *et al*[16], tilapia *Oreochromis massambicus* by (Hwang and Wu, 1993), rainbow trout *Oncorhynchhus mykiss*, Asian sea bass Sampathkumar *et al*[31] and common carp *Cyprinus carpio* by Stouthart *et al*[36]. The cortisol in unfertilized eggs appears to be largely of maternal origin by Feist *et al*[11] and is likely to have been transferred into the growing oocyte adventitiously via vitellogenin similar to other lipophilic hormones such as thyroxin[34]. In addition to this, its role in reproductive activity has been also reported[17], Cook *et al*[6].

Mommsen *et al*[26] have reviewed the dynamics, mechanisms of action and metabolic regulation of cortisol in teleosts describing cortisol is a multifaceted hormone not only chemically, also with physiologically and metabolically. First it is a lipid soluble yet, because of the presence of binding proteins in plasma its physiologically effective concentration may differ substantially from what chemical analysis reveals second, it may exert its action by different modes, one of them is rapid, non-genomic; the other, the genomic route, slower and generally longer lasting and experimentally more accessible. Third, cortisol is always present in vertebrates; even under unstress conditions playing house keeping roles.

The fish *Notopterus notopterus* breeds seasonally and undergoes profound changes in physiological state and may require heavy energy for reproductive activity during the year. These regulated seasonal changes preserve the homeostatic integrity of the fish behaviour and fish physiology over a variety of environmental condition, while also inducing the necessary changes in body condition and reproductive state. Plasma cortisol titers have been shown to vary with both circadian periodicity and seasonally in many vertebrates by Thorpe *et al*[38.29] found such variation in brown trout, *Salmo trutta* by Specker[33] reviewed evidence for the implication of corticosteroid hormones at smolting in salmonids.

In view of cortisol having multifaceted role in both metabolically and physiologically the following studies were undertaken in the fresh water fish *N. notopterus*:- I. Changes in the serum level of cortisol during a day. II. Changes in the level of the hormone during different reproductive phases, estimating serum cortisol in male fish for four phases. III. Cortisol was injected during different phases of the reproductive cycle and the serum hormone level was measured.

MATERIALS AND METHODS

Animals:

Over 200 fish, *N. notopterus* were used for the present study. The day changes in serum cortisol was studied by sacrificing the fish at different times i.e., 8.30am, 13.30 pm and 20.30 pm. The male and female fish were identified only after dissecting the fish. The freshwater fish *N. notopterus* was collected during the four reproductive season of the one year period i.e., preparatory, (Janury-February) pre-spawning, (April, May, June) spawning (August-September) and post spawning (November-December) phases. The fish were collected with the help of local fisherman from Bheema river which is situated around 40 Kms away from Gulbarga University. The live fish were brought to the laboratory and kept in large plastic pool tanks having size 90 cm diameter and 60 cm height. The weight of the mature fish ranged from 80-120 and length 20-25 cm, each tank accommodated 10-15 fish. About 8-10 days were needed for the fish to acclimatize. Fish of both control and experimental groups were fed with live earthworms and small fishes (Gambusia).

Experimental protocols:

Serum hormone (cortisol) level was determined both in control groups during four reproductive phases as well as in the fish injected with cortisol. After ten days of acclimatization. The fish were divided into two groups of 15 during each phase, Group-I served as control and received only olive oil. Group-II received 60 µg/100g body weight (0.6 µg/g body weight). The hormone was injected interperitonally near the caudal region below the lateral line once in a day for 10 days.

After 10 days of treatment, all the experimental and control fish were sacrificed and the serum was processed for cortisol hormone assay by Radioimmunoassay (RIA) technique

Hormone radioimmunoassay (RIA):

Serum cortisol level was measured by Coat-A-Count method using a solid phase radioimmunoassay kit with I^{25} cortisol (TC 02) purchased from DPC® diagnostic products corporation, 5700 West 96th Street Los Angeles.

Procedure:

The animal need not be fasting and no special preparation are necessary to the blood by vein puncture into plain heparinized tubes and separate the serum from the cells. EDTA plasm is not acceptable. Since cortisol exhibits a circadian rhythm, the time of collection should be noted. The procedure calls to 25µL of serum per tube. Before assay allow samples to room temperature (15-28^{0}C).

Serum and heparinized plasma samples are assayed directly, without sample preparation: neither extraction nor predilution is required. There is only one reagent to dispense, and a single 45-minute incubation. No centrifuge is required. Sample and tracer additions can be handled simultaneously, if desired, with the help of an automatic pipettor-diluter. The simplicity of the Coat-A-Count procedure makes it ideal for high-volume testing.

Statistical analysis:

Significance differences among time and each phases were tested by one-way ANOVA, followed by the microcal origin programme (MOP). Significant differences, between the same experimental condition, of the time and difference between the phases were tested by t-test and results were considered significantly different when $P< 0.05$.

OBSERVATION

Single day rhythmic changes in the serum cortisol level was studied by determining serum concentration of cortisol at different periods of time. The serum cortisol was estimated by selecting three timings i.e., at 8.30 am, 13.30 and 20.30 pm in a day and The level of the hormone ranged between 15 µg/dL to 25 µg/dL in fish during a single day. The results are presented in the Table:-2. The results presented in Table:-2 and Fig. 1, indicate that during morning hours cortisol level is low while afternoon and evening hours serum cortisol level seems to be higher. Thus indicating that since fish, *N. notopterus* fed in the morning hours and increases metabolic activity during afternoon hours, which probably results in the increase of serum cortisol level. The fish was found to be active upto evening hours till night, coming to the surface for gulping air and moving continuously.

The gonads (testis) of *N. notopterus* undergoes seasonality of growth and maturation exhibiting spermatogenesis during March, spawning during August and post spawning during November and December Table:-1. The cortisol estimation during four phases of the reproductive cycle is presented in Table:-3 and Fig. 2. The results indicate that cortisol level increases during preparatory pre-spawning and spawning phases. The increase in cortisol during these phases in the fish relates its involvement in the reproductive activity of the fish. In the preparatory phase the fish starts developing gonad. The cortisol level increases during preparatory pre-spawning and spawning phases may be because of energy requirement for spermatogenesis and spermiation activities

The cortisol administration during four phases of the reproductive cycle and estimation of serum cortisol level a day after administration indicate that during preparatory and pre-spawning phase serum cortisol decreases from that of control (Table:-3). This decrease in serum cortisol indicates its utilization for energy release needed for reproductive activity.

Table:-1 Gross characteristics of gonads during different stages of reproductive cycle in the fish *Notopterus notopterus* (Pallas)

Reproductive stage	Testes
I. Preparatory phase (February-March)	Whitish, translucent uneven in size broader at one end.
II. Prespawning phase (April-May)	Increases in size appear turgid, opaque and pink in colour vascularity increases
III. Spawning phase (August-September)	Extensive enlargement in size – bigger with greying pink, under abdominal pressure milt ooses.
IV. Post spawning phase (November-December)	Small in size whitish in colour translucent, a symetrical and uneven.

Table:-2 Concentration of serum cortisol in adult male freshwater fish *Notopterus notopterus* (Pallas) at different times in a day

Time	Male
08.30	15.75 ± 0.383
13.30	21.08 ± 0.71**
20.30	25.50 ± 0.76**

Note: P<0.05 ** P<0.001 *** values are expressed in mean ± S.E., n=6, when compared to morning hours.

Table:-3 Serum cortisol level in the male freshwater fish *Notopterus notopterus* (Pallas) during four reproductive phases

Group	Preparatory	Pre-Spawning	Spawning	Post Spawning
Control	15.50 ± 0.484	25.93 ± 0.625	22.16 ± 0.211	11.25 ± 0.383
Cortisol treated	7.50*** ± 0.713	3.60*** ± 0.036	20.66* ± 0.248	0.85*** ± 0.022

Note: P<0.01 ** P<0.001 *** values are expressed as mean ± S.E., n=6, when compared with control and between the phases.

Fig. 1: Concentration of serum cortisol in adult male freshwater fish *Notopterus notopterus* (Pallas) at different times in a day

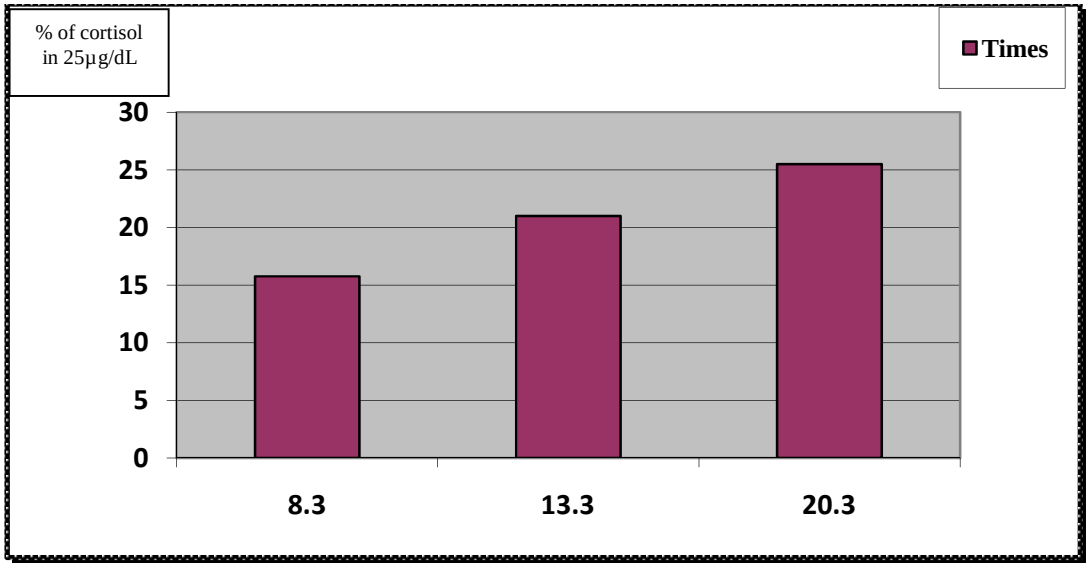
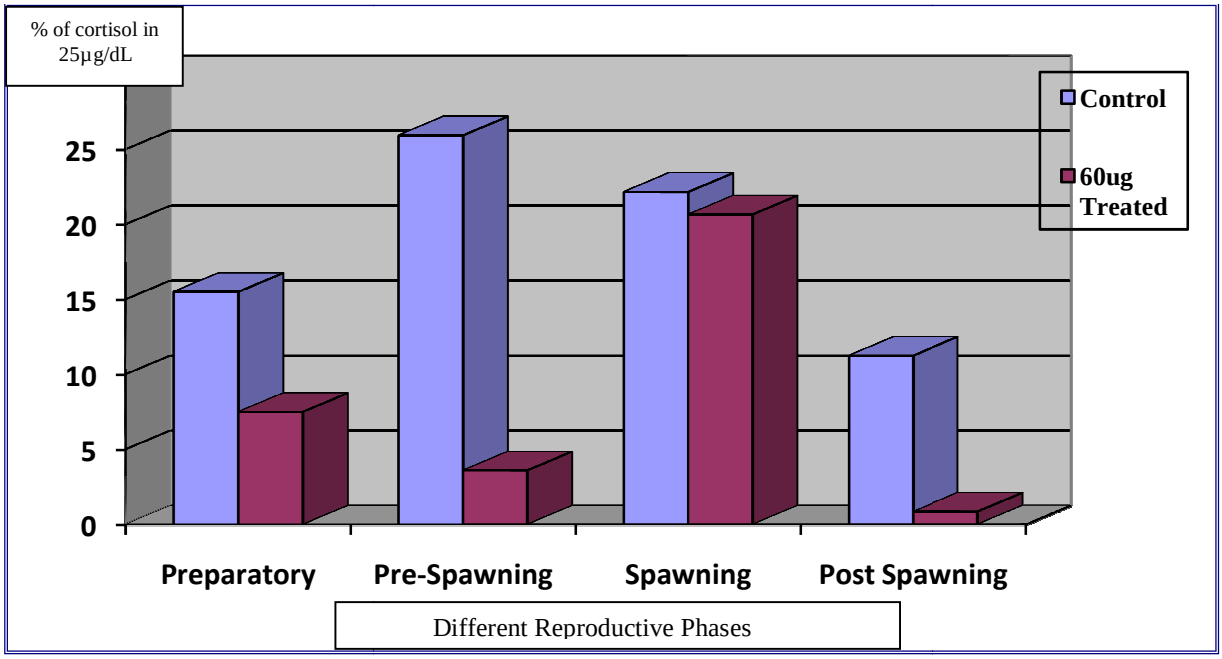


Fig. 2: Serum cortisol level in the male freshwater fish *Notopterus notopterus* (Pallas) during four reproductive phases



DISCUSSION

The plasma cortisol level has been estimated in variety of fishes such as rainbow trout *Oncorhynchus mykiss*, pacific slamon *Oncorhynchus* spp., brown trout *Salmo trutta* and Atlantic salomon, *Salmo solm*[1] Gamperl *et al*[12], Mommsen *et al*[26]. Cortisol level estimated in cyprinids have been compared with the level found in salmonid species and found that it is higher in cyprinids. Cortisol levels in unstressed common carp (*Cyprinus carpio*) has been reported to be ~50-150 mg ml^{-1} rising to ~300-450 mg ml^{-1} during exposure to stressors by Dabrowska *et al*[7] , Van Dijk *et al*[39.30]. In the gold fish (*Carassius auratus*) the cartisol level was found to be between 5 and 300 mg ml^{-1}[-35], between 7 and 110 mg ml^{-1} by Paxtan *et al*[28] and between 25 and 50 mg ml^{-1}[-32]. In *N. notopterus* plasma cortisol was estimated during a day and changes in response to different reproductive stages. The level of cortisol ranged between 15 µg/dL to 25 µg/dL in a single day and the level ranged between 11 µg/dL and 25 µg/dL in different reproductive phases.

Spawning is a critical stage in the life cycle of fishes often involving a substantial energetic investment[18], Clough *et al*[5]. The increase in serum cortisol during pre-spawning and spawning periods of *N. notopterus* reflects cortisol involvement in providing energy requirement for spermatogenesis and spermiation in fish.

Serum cortisol level determined for three period of time in a day in the fish *N. notopterus* indicates that its secretion is low during morning hours and continues to increase during mid day and late evenings, it increases during mid day continues to remain more or less constant during late evening hours. However, the level decreases in the morning hours compared to other day timings. The increase in cortisol level during mid day and after in the fish *N. notopterus* reflects its requirement for metabolic activity after feeding in the morning hours. Similar response of cortisol level increase after eating has been reported[28a].

In the fish rainbow trout (*Oncorhynchhus mykiss*) diurnal plasma profiles of thyroid hormones in relation to cortisol, growth hormone and growth rate was studied by Gomez *et al*[13], the results indicate that no variation in plasma T_3 concentration during one 25 hours period while those of T_4 fluctuated markedly. The diurnal plasma profiles of cortisol are similar marked by one or several increases in concentrations and no relationship was observed between cortisol and TH plasma profiles. Such similar studies on the circadian pattern of hormonal profiles were studied in number of fishes[3.2] Gomez *et al*[13], Holloway *et al*[14].

In *N. notopterus* the serum cortisol exhibits pronounced seasonal cycle. The cortisol level is low at the beginning of the active season i.e., during preparatory phase and rises to a peak by the time it reaches prespawning season (active spermatogenesis) and gradually decreases during spawning periods reaching lower levels during post spawning phases. Cortisol appears to be the principle glucocorticoid in the plasma of the fish *N. notopterus* as reported in other species of teleost fishes by Mommsen *at al*[26].

Seasonal cyclic variation in plasma cortisol level has been studied in teleosts fishes[24]; Venkatesh *et al*[40]. Plasma cortisol levels in juvenile Atlantic salmon, *Salmo solar* L. were higher at night than during the day from September to May, and were higher in the morning than at night from June and August. It is suggested that rising cortisol levels in spring represent a generalised stress response to behavioural and physiological maladaptation at smolting by Thorpe *et al*[38]. As per Mommsen *et al* [26] despite the interest in plasma cortisol measurement as an indicator of stress, few studies have actually measured the kinetics of cortisol in fish. Plasma cortisol concentration reflect the net effect of production and plasma clearance of the hormone. In the fish *N. notopterus* low cortisol level after spawning activity followed by an increase during breading period (Preparatory and Pre-spawning phase) indicates that cortisol is associated with regulation of seasonal changes in energy balance. It is reported that cortisol may play a special role in the regulation of fat metabolism and the prevailing notion assigns a strong peripheral and hepatic lypolytic action to cortisol resulting in increases in plasma un-esterified fatty acids by Mommsen *et al*[26]. The glucocorticoid hormones may also play a special role in the seasonality of fat metabolism by Lamberts *et al*[21]. The adrenal gland (inter-renal) of the fish undergo seasonal changes in size under natural conditions. It is observed in *N. notopterus* that serum cortisol level is having a positive correlation with body weight as body weight during breeding period increases. The correlation of body weight with hormone level did not exist within the samples at specific time of the year. It is suggested that seasonal cycling of *N. notopterus* is correlated with cortisol but that hormonal variations is not likely to play a strong role in individual variation within a season. In addition to energy demands for reproduction and the interesting association of peak levels of cortisol with the energy demanding time of late preparatory and pre-spawning. It is necessary to find out the complex interactions of the adrenocortical system with general seasonal changes of physiological state and energy mobilization.

Plasma levels of steroids reflect the net serum of synthesis, secretion, metabolism and clearance[27]. It is observed that the time of heavy energy demands associated with reproductive activity and seasonal peak of serum cortisol in male during prespawning and spawning phase indicates energy requirement and its utilization for reproductive activity. The peak level of cortisol in male *N. notopterus* was found to be during pre-spawning phase. This difference may be because of hormonal requirement at specific state of the cycle depending on the sex and energy requirement. These corticosteroids are actively involved in fish tissue respiration and are calorigenic in *Clarias batrachus*[22]. Kenagy *et al*[19] have undertaken similar studies in free living degus showing that substantial seasonal changes in plasma levels of cortisol that are associated with changes in body mass and reproduction. Despite the female-male differences in timing of energy demands for reproductive effort, that males have cortisol levels over the course of the year that closely resembles the levels of females. In female yellow-pine chipmunks, *T. amoenus* show pronounced seasonal cycles of plasma cortisol and corticosterone concentration these corticosteroid hormones are low at the beginning of the active season and rise to peak by the time of late lactation and the return to a low level before hibernation[20] and suggested that the seasonal regulation of physiological state, including body mass energy reserves and reproductive function. As it is known that cortisol is involved in the major regulation of intermediary metabolism and normal physiological function in fish, it is likely that increase in the cortisol level during preparatory and pre-spawning phase in male *N. notopterus* indicates that the fish is gearing up for active reproductive activity. Comparative decrease in the level during post spawning may be because of lesser metabolic rate and gonadal regression in vivo conditions.

In the fish *N. notopterus* plasma cortisol level comes down when estimated after ten days of cortisol treatment at the dose of 60 µg/fish. In the untreated male fish the level of hormone is 15.50 ± 0.484 µg/dL during preparatory phase and also in all other phases while after the treatment of cortisol for ten days and subsequent estimation has 7.50 ± 0.713 µg/dL, indicating that plasma cortisol level is reduced even after previous day treatment.

The plasma cortisol concentrations reflect the net effect of production and plasma clearance since the clearance of cortisol is essentially maintained by tissue uptake and catabolism. Because of the lipophilic nature of cortisol, its entry into cells is thought to occur by passive diffusion (Mommsen *et al* 1999). It is likely that the reduction of plasma cortisol after the hormone treatment might result in the utilization of the hormone for other activities through tissue uptake and further metabolic changes. Although liver is the key target for cortisol disposal with the hepatobiliary system as the main route for cortisol clearance[41] Wilson *et al*[45], renal and bronchial routs play subordinate roles is steroid elimination and very little is known about the factors regulating cortisol uptake and metabolism by Mommsen *et al*[26].

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