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ORIGINAL ARTICLE

Chromaffin cell activity in *Heteropneustes fossilis* exposed to artificial photoperiod

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The study deals with the effect of artificial photoperiod on the interrenal and chromaffin tissues and physiological stress parameters in the teleost, *Heteropneustes fossilis*. Fishes were exposed to photoperiods of continuous illumination 24L:0D and continuous darkness 0L:24D for a short period (24hrs) and a long period (10 days) following which the histology of the tissues was carried out for morphometric measurements of the interrenal and chromaffin cells and blood was analyzed for the physiological stress parameters (plasma glucose, plasma chloride, plasma protein and N:L ratio). No changes in the physiological variables were observed following any of the treatments for short periods. Plasma glucose, plasma chloride and plasma protein levels increased significantly ($p<0.05$) following the 24L:0D exposures for ten days. Significantly the interrenal cells showed no noticeable change in size following any of the long term exposures whereas the chromaffin cells were found to be significantly ($p<0.05$) increased in size after the 0L:24D treatment for long period of ten days. The findings strongly indicate that long exposures to artificial photoperiod activates the chromaffin cells which possibly direct the stress responses. A serotonin mediated activation of the chromaffin tissue is suggested in this catfish known for exhibiting photoperiod dependence in several of its activities.

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Like in other vertebrates, in fish too, photoperiod plays an important part in governing various behavioural and physiological activities. It acts as the zeitgeber regulating circadian rhythms in activities like feeding, locomotion, onset of reproduction cycles, etc. and maintaining endogenous and/or exogenous rhythms. Many fishes exhibit a daily light/dark diel pattern tuned to the natural photoperiod with melatonin/serotonin levels playing a key role in maintaining these rhythms (Meissl *et al.*, 1978; Pevet, 1979; Iigo *et al.*, 1991). Melatonin with its high titre during scotophase (night) and low titre during the photophase (day) serves as an internal temporal bench mark in general use among vertebrates (Menaker, 1982) and as a biological clock oscillator as demonstrated in some fishes like goldfish and pike (Iigo *et al*., 1991).

Recently some studies have reported stress induced responses in fish following exposure to artificial/altered photoperiod. Physiological changes have been observed in plasma cortisol, blood cell indices, plasma lactate, glucose, plasma protein, in several fish species exposed to artificial photoperiod condition for long duration (Biswas *et al.,* 2004, Almazan Rueda *et al.*, 2005; Valenzuela *et al*., 2008). Srivastava & Choudhary (2010), observed stress related changes in the leucocyte profile- an important stress biomarker, of a nocturnal catfish, *Clarias batrachus* exposed to artificial photoperiod (24L:0D) for a short duration of 24 hrs.

It is widely known that under stressful conditions, fish show certain responses which vary from stressor to stressor. These responses are mediated by the HPI axis (the hypothalamic-pituitary-interrenal axis) resulting in the release of stress hormones via a cascade of events. The stress hormones are secreted by the interrenal cells and the chromaffin cells which comprise the vertebrate adrenal homolog in teleosts (Chester-Jones & Mosley, 1980). The interrenal cells produce the corticosteroids. The chromaffin cells which are under sympathetic stimulation from the hypothalamus release the catecholamines for associated adaptive and regulatory activities involving regulation of metabolic disturbances and osmoregulation, ion-control, sugar balance and other physiological adjustments.

A relationship between the hypothalamic-pituitary-interrenal axis (HPI)–the teleost homolog of the mammalian (HPA axis) and the brain serotonergic system (5-HT) has also been suggested in fish (Winberg and Nilsson,1993; Winberg *et al.,* 1997). Stressful conditions induce hyperactivity in the metabolism of brain serotonin both in mammals (Khan, 1986, Khan and Kalra,1988) and in fish (McIntyre *et al.*, 1979; Winberg *et al.*, 1997). Also, the brain serotonergic system plays a key role in integrating autonomic, behavioural and neuroendocrine stress responses in fishes as well as in mammals (Winberg *et al*., 1997).

While the influence of artificial/altered photoperiod upon the stress variables has been reported in some fishes, its impact upon the tissues involved in the generation of stress responses remains uninvestigated. The present study investigates the stressor influence of artificial photoperiod of continous illumination LL (24L:0D) and continous darkness DD (24D:0L) upon an Indian catfish, *Heteropneustes fossilis* for a period of 10 days with particular reference to the activity of the interrenal and chromaffin tissues.

MATERIALS AND METHODS

Experimental animals

Live adult specimens of *Heteropneustes fossilis* of average body weight 30-50 g and average length 15-25 cm were purchased from the local market and maintained in the laboratory under natural photoperiod 14L:10D. The fishes were maintained in aerated aquaria (dissolved oxygen levels 8-10mg/l, pH 7.5-8.5 and water temperature 26-30°C) in disease free condition and water was changed daily. The fishes were fed with chopped goat liver on alternate days and were acclimatized for a month before they were put on experiment.

Experimental protocol

Five batches of healthy fishes (for each n =5; b wt- 35±2.5 g and length- 25±5 cm) were selected for experimental procedures. While one was used for control, two batches were selected for 24hrs exposure to photoperiods 24L:0D (continous illumination) and 0L:24D (continous darkness), and the other two batches for exposure to the two

photoperiods, each for a period of 10 days. Three replicates were performed for each experimental exposure.

For Continuous illumination and Darkness experiments.

Aquaria were fitted with hood (cover) containing CFL light. (25w:2900 lux intensity) and for continuous darkness the aquaria were kept in dark chambers and covered with black paper.

Fixation and Histology of tissues

After 10 days of exposure, the fishes were gently netted out of the experimental aquaria and anaesthetized with ethyl p aminobenzoate (Across, Germany) at a concentration of 0.35g/l. While anaesthetizing the exposed fishes, care was taken that anaesthesia was given under the same photic conditions as the experimental condition. They were dissected out and the head kidney was removed from the fish and fixed as quickly as possible in Orth's fluid for 24 hrs. This fixative enables the chromaffin cells to be more easily differentiated (Nandi, 1962). Pemanent slides were pepared by dehydration through a graded series of ethanol and staining with Azocarmine- G Solution & Aniline blue-Quinoline yellow solution.

Morphometry

Morphometric measurements for about 10 cells each of interrenal and chromaffin cells per fish, were done using ocular micrometer under high magnification and then averaged for agreement within a 15% difference. Cell size measurements of the interrenal and chromaffin cells were done only in fishes which were exposed to the artificial photoperiod for ten days.

Blood sampling

Blood was drawn from the caudal vein of the anaesthetized fish, collected in heparinized tubes and aliquots were used for plasma analysis and smear preparations. About 2 ml of the blood was centrifuged at 6000g for 5 min to obtain the plasma. After the blood samples were taken depuration of the fishes was carried out in fresh water for 3-4 hrs after which the fish were returned to the aquaria.

Determination of N:L ratio

Blood smears were prepared immediately from whole blood which were then air-dried. They were stained with Wright's and Leishman's stains and observed for differential leucocyte counts (DLC) by counting 200 cells and expressing the leucocytes as a percentage. Identification of the lymphocytes, thrombocytes and neutrophils was done according to Ellis (1977) and subsequently neutrophil:lymphocyte (N:L) ratio was obtained.

Plasma analysis

Plasma obtained by centrifugation of the blood was analysed for glucose (glucose-oxsidase method) and chloride (thiocyanate method) using diagonostic kits (Siemens Medical Solutions Diagnostic Ltd., India). Plasma protein was determined by the Lowry method (Lowry *et al*.,1951).

Statistical Analysis

The data are presented as mean ± s.d.values. Student t-test was used to calculate significant values. A difference of $p<0.05$ was accepted as significant.

RESULTS

Exposure to artificial photoperiod for short duration (24 hr period)

24L:0D

Exposure to artificial photoperiod of continuous illumination 24L:0D did not elicit any significant

changes in plasma glucose or plasma chloride. A lower value of plasma protein was obtained. A higher neutrophil: lymphocyte (N:L) ratio of 0.22 as compared to normal value of 0.095 was obtained but the value is not significantly different. (Table 1).

24D:0L

No significant changes were observed in plasma glucose, plasma protein and chloride levels in fishes exposed to artificial photoperiod of continuous darkness 24D:0L. N:L ratio 0.186 was slightly higher than the control but not significantly different. (Table 1).

Exposure to artificial photoperiod for longer duration (10 days)

24L:0D

Fish exposed to artificial photoperiod of continuous illumination for a period of ten days showed significantly ($p<0.05$) higher levels of plasma glucose, chloride and protein as compared to control. The N:L ratio was comparable to control. (Table 2).

The chromaffin cells appeared larger in size. However there was no change in the size of the interrenal cells. (Table 3). The nature of the cytoplasmic material and the staining characteristics of the interrenal cells or chromaffin cells did not appear to be different from that of the control cells.

24D:0L

Exposure to continuous darkness for a period of 10 days produced nominal changes in the blood variables. Plasma glucose, and protein values showed slight increases, but the changes were not significantly different. Plasma chloride levels remained almost the same as compared to that of the control. N:L also were slightly higher but not significant as compared to control. (Table 2).

The chromaffin cells were predominantly seen and the cell size was significantly larger ($p<0.05$) as compared to that in the control. The cytoplasm of the cells appeared granular. There was no change in the size of the interrenal. (Table 3). The cells also did not show any changes in the cytoplasmic material or its staining characteristics.

Table 1: Haematological Parameters of *Heteropneustes fossilis* exposed to normal photoperiod (14L :10D) and artificial photoperiods (24L:0D and 0D:24L) for 24 hrs.

Parameters	Control 14L : 10D	Continuous illumination 24L : 0D	Continuous Darkness 0L : 24D
N : L ratio	0.095 ± 0.121^{a}	0.22 ± 0.112^{a}	$0.186 \pm .089^{a}$
Plasma Glucose (mg/dl)	70.45 ± 10.052^{a}	55.55 ± 6.21^{a}	66.33 ± 12.14^{a}
Plasma Chloride (mEq/l)	82.42 ± 15.094^{a}	102.23 ± 11.23^{a}	96.63 ± 7.25^{a}
Plasma Protein (g/dl)	4.066 ± 2.36^{a}	3.20 ± 1.46^{a}	2.84 ± 2.34^{a}

Values are expressed as mean $\pm$ s.d. Different superscript lower case letters in lines indicate significant difference ($p < 0.05$). All values are mean values of data for three replicates in each parameter.

Table 2: Haematological Parameters of *Heteropneustes fossilis* exposed to normal photoperiod (14L :10D) and artificial photoperiods (24L:0D and 0D:24L) for a period of 10 days.

Parameters	Control 14L : 10D	Continuous illumination 24L : 0D	Continuous Darkness 0L : 24D
N : L ratio	0.095 ± 0.121^{a}	0.31 ± 0.11^{a}	0.141 ± 0.018^{a}
Plasma Glucose (mg/dl)	62.0 ± 8.37^{ac}	106.39 ± 10.98^{b}	52.005 ± 8.59^{ac}
Plasma Chloride (mEq/l)	85.40 ± 10.35^{ac}	110.68 ± 7.54^{b}	80.125 ± 17.52^{ac}
Plasma Protein (g/dl)	4.066 ± 2.36^{ac}	9.83 ± 2.25^{b}	6.65 ± 1.89^{ac}

Values are expressed as mean $\pm$ s.d. Different superscript lower case letters in rows indicate significant difference ($p< 0.05$). All values are mean values of data for three replicates in each parameter.

Table 3: Cell diameters of Interrenal and chromaffin cells of head kidney of *Heteroneustes fossilis* exposed to normal (14L:10D) and artificial photoperiod (24L:0D and 0L:24D) for 10 days.

Photoperiod	Interrenal Cell	Chromaffin cell
Control (14 L : 10D)	0.0194 ± 0.314^{a}	0.0207 ± 0.016^{b}
24 L : 0D	0.0184 ± 0.011^{a}	0.03 ± 0.006^{ab}
0L : 24D	0.0184 ± 0.025^{a}	0.036 ± 0.001^{ac}

Values are expressed means $\pm$ s.d. Different superscript lower case letters in columns indicate significant differences ($p<0.05$). All values are mean values of data for three replicates in each parameter.

DISCUSSION

A characteristic observation of the present study is that there was consistently no change in the size of the interrenal cells of *H. fossilis* following exposure to artificial photoperiods of 24L :0D or of 24D:0L for ten days. The results do not conform to other observations on interrenal cell activity in fishes exposed to stress. Studies on the histological changes in the interrenal tissues in response to stress are still few, yet in most of the investigations, increased interrenal activity was found to be always associated with stress in fish. Bromage and Fuchs (1976) indicated histological changes in the interrenal (steriodogenic) cells of the head kidney in response to detergents while little or no changes were observed in chromaffin cell activity. Similar results in interrenal activity were found in gold fish exposed to detergent treatments (Oguri, 1960, Mahon *et al*., 1962) and exposure to cold stress (Mahon *et al.,* 1962). Variations in number and structure of the interrenal cells have been found to occur in response to physiological changes in the body (Sufi *et al*., 1978). The present findings indicate that prolonged exposure to artificial photoperiods - 24L:0D and 0L:24D in *H. fossilis* does not evoke any change in the interrenal cells. Increased interrenal cell sizes are suggestive of chronic activation of the HPI (hypothalamic-pituitary-interrenal) axis (Noakes and Leatherland, 1977). In majority of teleost fish species, cortisol the primary stress hormone is produced by the interrenal tissues following exposure to an environmental stressor. Definitely exposure to prolonged artificial photoperiods in *H. fossilis* fails to activate the HPI axis as revealed in the present study.

However, an interesting observation in this study not earlier reported in fish, is the increased size of the chromaffin cells following exposure to both the 24L:0D and 0L:24D photoperiods and their predominance in the fishes exposed to 0L:24D photoperiods. Changes in the chromaffin cell size has not been observed in any of the stress related studies in fish (Oguri, 1960; Bromage and Fuchs, 1976). Artificial photoperiods are manipulations into the natural light/dark 14L:10D cycles which cause imbalances in the naturally occurring melatonin/serotonin metabolic cycles. resulting in fluctuating levels of serotonin during 24L:0D and 0L:24D. In teleosts, chromaffin tissues are associated with the synthesis, storage and secretion of the catecholamines (adrenaline and adrenaline) (Reid *et al*, 1998) and the control of the chromaffin cell activity and catecholamine secretion involves cholinergic and non-cholinergic neural stimulation as well as endocrine humoral control (Reid *et al.*, 1998). In fish, serotonin is also known to have stimulatory effect on catecholamine release (Fritsche *et al*., 1992) by interacting with the HT receptors on chromaffin cells to initiate the release

of chromaffin adrenaline (Fritsche *et al*., 1993). The increased size of the chromaffin cells following exposure to prolonged artificial photoperiods points invariably to their activation by the resulting changing levels of serotonin.

Marked changes in blood biochemistry was observed in the fishes exposed to artificial photoperiod of continuous illumination for long duration of ten days. Plasma chloride levels were significantly higher than the control ($p<0.05$). Plasma glucose and plasma protein levels were also found to be significantly higher ($p<0.05$) than control after exposure to 24L:0D for ten days. However, exposure to 0L:24D photoperiod for the longer period of ten days did not elicit any noticeable change in the stress parameters as was also observed following exposures to the short period of 24 hrs. The findings of the present study are in agreement with other studies which have also reported changes in haematological parameters following exposure to artificial photoperiod in fish for long duration (Biswas *et al.*, 2004, Valenzuela *et al*., 2008). Srivastava and Choudhary, (2010) reported increased plasma chloride levels and altered N:L ratio- an important stress biomarker in the catfish, *Clarias batrachus* following exposure to 24L:0D for 24 hrs. The observations of the present study compliment those seen in *C. batrachus* as in both the cases changes were seen following the 24L:0D exposure, although for different durations. Interestingly both the catfishes are nocturnal. These physiological changes can be attributed to the fluctuating levels of serotonin under different photoperiods and their subsequent effects on the chromaffin cells triggering adrenal humoral stress responses.

In fish different kinds of stresses e.g. exposure to predator (Winberg and Nilsson, 1993a), hierarchy dominance and agonistic behavior (Winberg and Nilsson, 1993b) are reported to elevate brain serotonergic activity which is known to integrate autonomic, behavioural and neuroendocrine stress responses in fish as well as in mammals (Winberg *et al.*, 1997). Hypothalamic serotonergic system was found to mediate photoperiod dependant gonadal activity in female *Heteropneustes fossilis* (Senthikumaran and Joy, 1994). In the same study, day night variations in 5HT (serotonin) was observed and it was seen that 40 days of exposure of the catfish to total darkness inhibited 5HT whereas exposure to continuous light for the same length of time elevated serotonin levels. Although serotonin levels have not been estimated in the present study, variations in the stress variables and changes in the size of chromaffin cells under different photoperiods can be discussed in this light of fluctuating serotonin levels. Ten days of 24L:0D elevates serotonin levels causing greater stimulation of chromaffin cells to secrete catecholamines that are responsible for the observed physiological changes in the blood. Elevated catecholamines levels under stressful conditions are known to modulate various physiological responses that serve to enhance cardio-respiratory and metabolic functions (Wendelaar Bonga, 1997; Reid *et al*., 1998; Perry and Gilmour 1999). Ten days of exposure to continuous darkness inhibits serotonin levels causing no stimulation of the chromaffin cells. There was no release of catecholamines and therefore no physiological changes were observed. The increased size and granular content of the chromaffin cells observed under 0L:24D possibly represents the storage phases of these cells due to the non-release of catecholamines.

Heteropneustes fossilis is a crepuscular,

nocturnal catfish which shows photoperiod dependence in several of its activities viz. locomotor activity (Srivastava, 2003a), secretory patterns in the pineal gland (Srivastava, 2003b) and gonadal activity (Senthikumaran and Joy, 1994). The present investigation highlights the effect of photoperiod on the chromaffin activity and suggests a possible serotonin mediated activation of these tissues as the underlying mechanism. However, further studies on quantitative estimation of serotonin and catecholamine levels will be useful in corroborating the present findings.

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