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INVESTIGATING THE ROLE OF HEAT SHOCK PROTEINS AND MELANOPHORES DURING STRESS CAUSED BY HIGH LIGHT INTENSITY IN INDIAN MAJOR CARP, *CATLA CATLA*

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ABSTRACT: In response to several stressors such as intense light, fish elicit a generalized physiological stress response. *Catla catla* (61.3 ± 2.1 g) were exposed to five different light intensities of 983 ± 162 lx (L1), 1828 ± 324 lx (L2), 2676 ± 409 lx (L3), 3442 ± 648 lx (L4) and 114 ± 4 lx (Lc, control). Higher light intensities resulted in physiological stress, significantly (P<0.05) higher levels of heat shock protein-70 and heat shock protein-90 were found in both hepatopancreas and muscles of fish exposed at L4 treatment compared to the others to cope up with the stressor. A direct relationship was found between the light intensity and the serum glucose level. Significantly (P<0.05) higher glucose level was found in L4 exposed fish compared to the others. Fish at L4 had aggregated its pigments in the melanophores (punctate stage) to reflect intense light. This study could be useful in understanding the aspects of stress induced changes in fish skin melanophores. As a consequence of stress due to intense light, a 12.5% of growth retardation was found in L4 treated fish. Significantly (P<0.05) lower feed conversion ratio was found in Lc than the other treatments. Hence, the present investigation is aimed to evaluate the acute and sublethal effects of light intensities in catla.

INTRODUCTION: One of the hottest areas of current research includes a family of highly conserved stress proteins that are known as Heat Shock Proteins (HSPs). These proteins are ubiquitous occurring in almost all organisms including from bacteria and yeast to humans. Fish, like any other vertebrates, respond to stressful condition by eliciting several physiological stress responses that are characterized by increase in the stress hormones that help the animal to maintain its normal or homeostatic state [1].

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These responses include increase in serum cortisol, glucose and catecholamines levels, branchial blood flow and muscular activity [2]. Fish also respond at cellular level to various stressors. These responses comprise a set of protein changes that actually include increased synthesis of several heat shock proteins [3]. HSPs are of various forms and therefore are categorized into many families on the basis of their molecular weights. There is considerable evidence that HSPs play very important physiological roles in normal conditions and situations that involve both systemic as well as cellular stress [4].

Many researchers have demonstrated that most of HSPs have very strong cytoprotective effects and therefore involved in many of the regulatory pathways and hence act as molecular chaperones for the other cellular proteins [5, 6].

HSPs are generally a set of proteins whose action was induced by heat shock and a variety of other stresses. There are many functional roles of HSPs that have been known today but the exact mechanism for these multiple functions are still not entirely understood. It has been assumed that by determining these mechanisms, it would permit the designing of more precise ways for combating cellular stress such as immunologic diseases, cancer, aging, cardiovascular diseases [7, 8, 9]. HSPs can be used as markers of many cellular injuries and also for the diagnostic and therapeutic purposes.

Light intensity is defined as the amount of illumination at the water surface that influences the aquatic life including both the fish and their prey [10]. Fish larvae are highly selective visual feeders and therefore, are totally dependent on vision in order to capture their prey [10, 11]. The optimum light intensity for feeding is specific to each species and matches their trophic niche and eye structure [12]. In addition to orientate and perform activities such as foraging and breeding, fish also utilize vision in order to avoid predators. Hence, light is often crucial for survival too [13].

There are many similarities between fish and human eye but there is also number of key differences among them [14]. Firstly, fish do not have an eyelid and are unable to change their pupil aperture to protect their retina from high intensity of light. Therefore, alternative protective mechanisms exist in them such as migration of the melanin granules and the photoreceptor mobility [15]. Moreover, unlike mammalian retina, the fish retina can be capable of regeneration [16]. Although, light has been used in the fish industry for a number of years, three important areas of concern have been identified, stress response, impact on immune system and eye damage, all of which compromise fish welfare.

Many animals go through physiological colour changes in order to adapt to their living environment and thus enhance their chances of survival and reproduction. These physiological colour changes are due to the rapid responses of their dermal chromatophores to various environmental stimuli. In most of the cases the response of dermal chromatophores to light occurs either directly or indirectly through the neuronal or endocrinal control [17]. Chromatophore is a light reflecting or pigment containing cell found in the dermis of many vertebrates such as fishes, amphibians and reptiles [18, 19]. In contrast to birds and mammals, fish chromatophores are not the part of epidermis but are situated in dermis and are derived from neural crest cells [20]. When fish exposed to white background will have contracted melanophores while those exposed to black background will have the expanded melanophores [21]. The colour forming pattern in fish skin results from the cooperation of various types of neighbouring chromathophores as well as their positioning in the skin [20].

The goal of present study aims to evaluate the effect of various light intensities on stress response and its relationship with heat shock proteins, melanophores, serum glucose level and growth performance of Indian major carp, *Catla catla*.

MATERIALS AND METHODS:

Experimental Setup and Culture of Test Species: *Catla catla* (61.3 ± 2.1 g) were brought from Chatterrjee Brothers' fish farm, Mogra, West Bengal. Fish were acclimated for 20 days in the laboratory condition and then transferred to glass aquaria. The stocking density was 10 fish aquarium^{-1}. Fish were then exposed to different light intensities of 983 ± 162 (L1), 1828 ± 324 (L2), 2676 ± 409 (L3), 3442 ± 648 lx (L4) and 114 ± 4 (Lc, control) under photoperiod of 12L: 12D which was controlled by automated timer.

The light was provided by Philips tube light (TL 5, 28 W) fixed above each aquarium above 37 cm from the water surface and the interference of light among the treatments were prevented with the help of black carton. Fish were fed twice according to 5 % of the body weight at 9:00 and 17:00 h and the extra feed were collected, dried and weighed to analyze the feed consumption. Fish were harvested after 90 days and euthanized with tricaine methanesulfonate (MS-222, Sigma). The samples (hepatopancreas, muscles and serum) were collected from individual fish and were preserved at -80 °C for various assays.

Water Quality: Various physico-chemical parameters like water temperature, pH, dissolved

oxygen, ammonia, nitrite, nitrate and conductivity were monitored regularly using HQ40d multi parameter (Hach, USA). Nitrite was estimated [22]. The result is shown in **Table 1**.

TABLE 1: WATER QUALITY ESTIMATION IN VARIOUS TREATMENTS DURING 90 DAYS OF EXPERIMENTAL PERIOD. DATA ARE PROVIDED IN MEAN ± SE (n = 3)

Water quality parameters	L1	L2	L3	L4	Lc
	Range (Mean ± SE)	Range (Mean ± SE)	Range (Mean ± SE)	Range (Mean ± SE)	Range (Mean ± SE)
Temperature (°C)	23.7 ± 0.03 - 25.3 ± 0.0	23.3 ± 0.03 - 25.2 ± 0.0	24.6 ± 0.0 - 25.7 ± 0.0	24.9 ± 0.1 - 26.1 ± 0.1	23.3 ± 0.0 - 25.0 ± 0.0
pH	7.74 – 7.83	7.81 – 7.86	7.82 – 7.94	7.54 – 7.85	7.76 – 7.85
Dissolved oxygen (mg L^{-1})	6.1 ± 0.18 - 6.6 ± 0.13	6.2 ± 0.17 - 6.5 ± 0.09	6.5 ± 0.03 - 6.7 ± 0.02	6.0 ± 0.13 - 6.1 ± 0.06	6.7 ± 0.09 - 6.9 ± 0.04
Ammonia (mg L^{-1})	0.23 ± 0.0 - 0.24 ± 0.0	0.43 ± 0.0 - 0.45 ± 0.0	0.24 ± 0.0 - 0.25 ± 0.0	0.46 ± 0.0 - 0.48 ± 0.0	0.22 ± 0.01- 0.24 ± 0.00
Nitrite (mg L^{-1})	0.33 ± 0.0 - 0.34 ± 0.0	0.48 ± 0.0 - 0.5 ± 0.01	0.49 ± 0.0 - 0.5 ± 0.0	0.23 ± 0.02 - 0.29 ± 0.01	0.32 ± 0.0 - 0.34 ± 0.01
Nitrate (mg L^{-1})	5.4 ± 0.1 - 5.7 ± 0.0	5.6 ± 0.03 - 5.8 ± 0.0	5.4 ± 0.05 - 5.6 ± 0.05	5.3 ± 0.01 - 5.4 ± 0.02	5.9 ± 0.1- 6.4 ± 0.01
Conductivity (µS cm^{-1})	1030 ± 0.3 - 1067 ± 0.6	1038 ± 0.6 - 1128 ± 0.6	1071 ± 1.2 - 1087 ± 1.2	1083 ± 0.9 - 1112 ± 0.9	1048 ± 0.3 - 1078 ± 0.6

Survival Rate of Test Species: The fish were carefully monitored and mortality was recorded throughout the rearing period. Survival rate was determined using the formula:

$$\text{Survival rate (\%)} = \frac{(N_0 - N_t)}{N_0} \times 100$$

Where, N_0 and N_t were the initial and final number of fish respectively.

Growth Performance of Test Species: Fish were weighed individually before the exposure of the light intensities and final body weight of each fish was measured at the time of harvest. At the end of the experiment, various growth parameters such as weight gain, Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) were calculated as follows:

Average weight (g) = Total final weight / Total number of fish,

Specific growth rate (%) = [(ln final weight of fish - ln initial weight of fish)/rearing days] × 100

Feed conversion ratio = feed consumed per fish / weight gain

Examination of Melanophores: Scales were obtained by plucking it from the dorsum, located between the pectoral and the pelvic fin of the fish with fine tipped stainless steel forceps [23]. The scales were then immediately placed into the physiological saline solution (128 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5.6 mM D-glucose, 10 mM Tris-HCl, pH 7.2). The scales were then viewed under the microscope (Axio imager, Carl Zeiss, Germany) to observe their characteristics immediately post-denervation.

In each scale, the number of melanophores observed were counted and characterized based on if they were stellate (fully dispersal of the pigment), intermediate (not completely dispersed), or punctate (full aggregation of the pigment).

Preparation of samples: A homogenate (10%, w/v) of hepatopancreas and muscles was prepared in chilled phosphate buffer saline (0.1 M, pH 7.4) using pellet pestles (model Z359971-1EA, SIGMA-ALDRICH, USA) and stored overnight at -20 °C.

After twice freeze-thaw the homogenates were subjected to centrifugation at 5000 × g for 5 min at 4 °C by using cooling centrifuge (3K30, SIGMA, Germany) to get post mitochondrial supernatant (PMS) for various biochemical analyses.

Serum extraction from the blood: Blood was drawn from caudal vein using plastic disposable syringe, fitted with 26 gauge needle. Blood was allowed to clot by leaving it undisturbed for 30 min at room temperature and then centrifuged at 1500 × g for 10 min at 4 °C and serum was separated and transferred to clean tube for serum glucose analysis.

Biochemical Analyses:

Heat Shock Protein-70 (HSP-70): HSP-70 concentration was determined by using fish heat shock protein 70 ELISA kit (catalog no. CSB-E16327Fh, CUSABIO, China). Briefly, 50 µL of PMS (10%, w/v) were added to individual wells of microtiter plate pre-coated with antibody specific to HSP-70 leaving two wells as blank. To this 50 µL of conjugate added except blank, mixed well and then incubated for 60 min at 37 °C. After that each well was aspirated and washed with 200 µL of wash buffer (1 x) and the washing process was carried out thrice. 50 µL of HRP-avidin added to each well except the blank well, mixed and then incubated for 30 min at 37 °C. Again washing process was carried thrice as it was done previously and the plate was inverted on clean tissue paper. 50µL of substrate A and 50 µL of substrate B were added to each well, mixed and incubated for 15 min at 37 °C. Then 50 µL of stop solution was added to each well and optical density was immediately recorded at 450 nm using a microplate reader (model Synergy H1 Hybrid, BioTek, USA). The result was expressed in pg mL^{-1}.

Heat Shock Protein-90 (HSP-90): HSP-90 level was assayed using fish heat shock protein 90 ELISA kit (catalog no. CSB-E16329Fh, CUSABIO, China). 50 µL of PMS (10%, w/v) was added to the respective wells of plate pre-coated with antibody specific to HSP-90 except the two wells that was set as blank. 50 µL of conjugate was added to each well leaving blank well. The solution was mixed well and incubated for 60 min at 37 °C. Each well was then aspirated out, washed with 200µL of wash buffer (1 x). The washing process was done thrice. A 50 µL of HRP-avidin was added to each well except the blank, incubated for 30 min at 37 °C. Again washing process was carried thrice same as earlier. Then 50 µL of each substrate A and substrate B were added to each well, incubated for 15 min at 37 °C. Finally 50 µL of stop solution was added to each well and optical density was recorded at 450 nm. The result was expressed in pg mL^{-1}.

Serum Glucose: Serum glucose was estimated by O-toluidine method [24]. 500 µL of O-toluidine reagent was taken in a tube and 10 µL of plasma was added. The contents were then mixed well and kept in boiling water for 10 min. Then, the tubes were cooled under running tap water for 5 min and the optical density of the test samples were measured at 630 nm within 30 min and the result was expressed as mg mL^{-1}.

Statistical Analysis: The data were expressed as mean ± standard error (SE). A one-way analysis of variance (ANOVA) and Duncan's multiple range test, DMR were used in order to find out the difference among the various treatments [25].

RESULTS:

Survival Rate: In our study the final survival rate of fish was cent percent **Table 2** at all treatments. There was no significant difference between experimental groups. No mortality was recorded at any group during the entire 90 days of light exposure trial.

Growth Performance: Growth performances of *Catla* subjected to different light intensities is shown in **Table 2**. Contrary to feed conversion ratio, the average weight and specific growth rate decreased significantly (P<0.05) as light intensity increased from L1 to L4, and showed no significant difference between L3 and L4. However, the average weight and specific growth rate was maximum at Lc treatment, these were slightly decreased from Lc to L1 but there was no significant difference between these two groups, Lc. Feed conversion ratio of fish was significantly (P<0.05) lower at Lc than any other groups.

TABLE 2: EFFECT OF DIFFERENT LIGHT INTENSITIES ON SURVIVAL RATE AND VARIOUS GROWTH PARAMETERS SUCH AS AVERAGE WEIGHT, SPECIFIC GROWTH RATE (SGR) AND FEED CONVERSION RATIO (FCR) OF CATLA

Parameters	Light intensities treatments				
	L1	L2	L3	L4	Lc
Survival rate (%)	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
Average weight (g)	91.11 ± 0.23^{a}	85.36 ± 0.33^{b}	82.72 ± 0.38^{c}	81.03 ± 0.48^{c}	92.59 ± 1.19^{a}
SGR (%)	0.42 ± 0.003^{ab}	0.35 ± 0.004^{b}	0.32 ± 0.005^{c}	0.3 ± 0.006^{cd}	0.44 ± 0.014^{a}
FCR	0.06 ± 0.0^{b}	0.065 ± 0.001^{b}	0.082 ± 0.001^{a}	0.083 ± 0.003^{a}	0.051 ± 0.002^{c}

Data are provided in mean ± SE (n = 3) with different superscripts are significantly (p < 0.05) different.

Serum Glucose Level: Light intensity had a positive effect on the serum glucose level of catla **Fig. 1**.

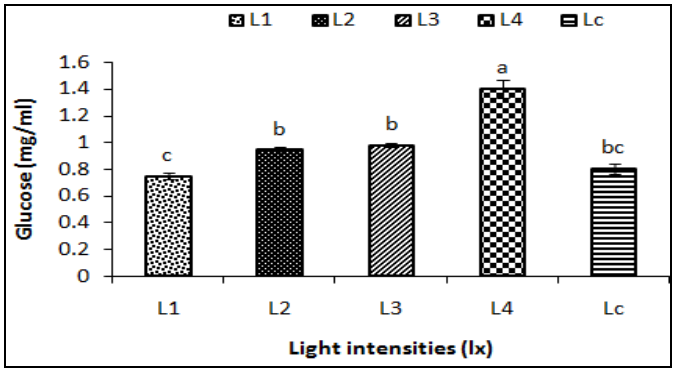

FIG. 1: EFFECT OF DIFFERENT DOSES OF LIGHT INTENSITIES ON SERUM GLUCOSE LEVELS OF CATLA (n = 4). Bars with different superscripts are significantly (P<0.05) different

Fish showed an outstandingly high glucose levels (P<0.05) under L4 treatment, the level gradually decreased with the decreasing level of light intensities. Its concentration was almost the same at L2 and L3 treatments with no significant difference among them. However, its value was markedly (P<0.05) lower at Lc.

Heat Shock Protein-70: HSP-70 level and the light intensity dose had a direct relation among them, shown in **Fig. 2A**. When the level of HSP-70 in hepatopancreas and muscles of catla from various intensities of light regimes was compared, fish collected from L4 treatment showed significant higher values (P<0.05) followed by L3 and L2 groups although, the difference was not statistically significant among these two groups. The least level in both the tissue was observed at Lc treatment (P<0.05). It is interesting to note that the HSP-70 content in muscles was slightly lower than the hepatopancreas in all the common treatments.

Heat Shock Protein-90: HSP-90 showed a positive relationship with the increasing light intensities in both the hepatopancreas and muscles of catla shown in **Fig. 2B**. Although, the level of HSP-90 was highest at L4 treatments (P<0.05) in both the tissues, but its concentration was much more higher in hepatopancreas than in muscles. In addition, catla also exhibited significantly higher (P<0.05) HSP-90 content at L3 group apart from L4 and its value decreased with the decreasing light intensities.

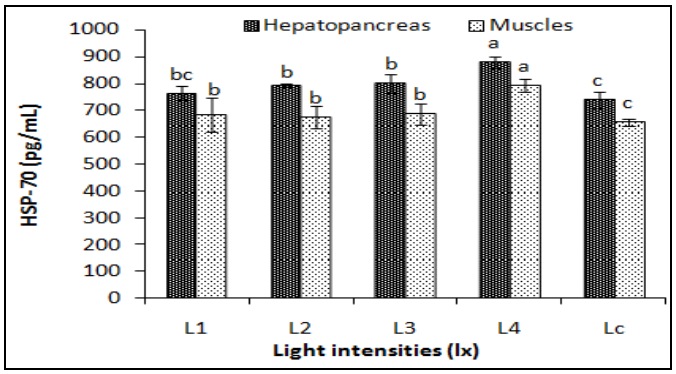

FIG. 2(A): EFFECT OF DIFFERENT DOSES OF LIGHT INTENSITIES ON HSP-70 LEVELS OF CATLA IN HEPATOPANCREAS AND MUSCLES (n = 4). Bars with different superscripts are significantly (P<0.05) different

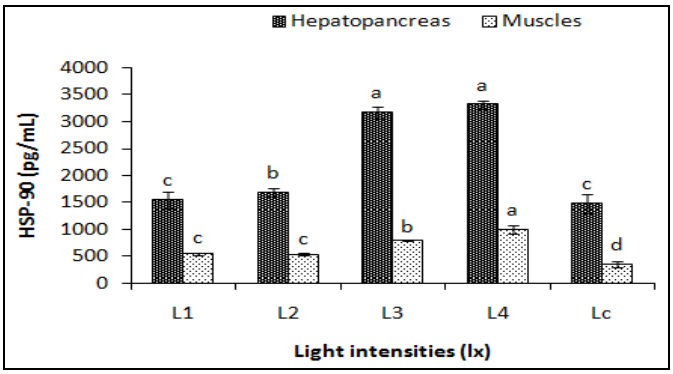

FIG. 2(B): EFFECT OF DIFFERENT DOSES OF LIGHT INTENSITIES ON HSP-90 LEVELS OF CATLA IN HEPATOPANCREAS AND MUSCLES (n = 3). Bars with different superscripts are significantly (P<0.05) different

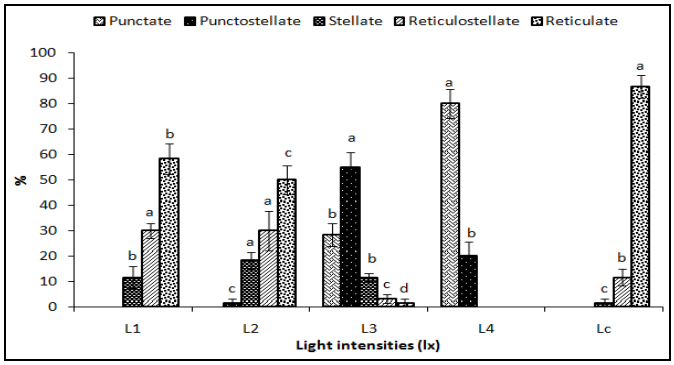

FIG. 3: EFFECT OF DIFFERENT DOSES OF LIGHT INTENSITIES ON DIFFERENT STAGES OF MELANOPHORES IN SCALES OF CATLA (n = 3). Bars with different superscripts are significantly (P<0.05) different

Stages of Melanophores: The stages of melanophores were individually characterized as: punctuate, punctostellate, stellate, reticulostellate and reticulate clearly shown in **Fig. 3**. The majority of reticulate stage (86.7%) was found in Lc treatment followed by L1 and L2, only 1.7% of it was present in L3 and was absent in L4 group. Among the intermediate stages, punctostellate was significantly higher (P<0.05) in L3 group and absent in both Lc and L1 groups, and reticulo-stellate was present in all the treatments except the L4 one. Stellate stage was significantly higher (P<0.05) in L2 treatment and absent in L4 group. Punctate stage was present only in L4 and L3 groups with a majority of 80% in L4 treatment.

DISCUSSION: HSPs are family of highly conserved cellular proteins that are observed in all organisms including fish [3]. In an unstressed cell there is constitutive production of these proteins that are required in protein metabolism so as to maintain the cellular homeostasis [26]. HSP-70 is associated with folding of nascent polypeptide chains and act as a molecular chaperone, repairing and degradating the altered or denatured proteins. HSP-90 helps in supporting of various components of cytoskeletons, enzymes and steroid hormone receptors [27]. In the current study, the level of both HSP-70 and HSP-90 in catla was drastically increased at higher light intensities. The functional explaination for this could be the rise of physiological stress that is caused by intense light and to cope up with this role of HSPs arise for cellular repair. Moreover, HSP 70 also combined with antioxidant enzymes to accelerate the removal of ROS that are generated during stress. Similarly, it was reported that level of HSP 70 in abalones was significantly higher at higher light intensities [28], indicating that HSP 70 may begin to inhibit the activity of key enzymes producing free radicals, thus reducing free radicals by means of feedback.

In aquatic environment serum parameters such as glucose is widely used as stress indicator [29]. Blood glucose can be used as an important parameter of stress response as it is rapid, practicable and quantitative and can be used as an indicator for general state of health. We observed a significant increase in serum glucose level in catla cultured under higher light intensities when compared with the values at lower light intensities.

An increase in the levels of glucose at elevated intensities of light indicates that the normal metabolism got disrupted due to the accumulation of damaged molecules. Similar to our result, level of serum glucose in silver pomfret was elevated in response to stress [30]. These responses thus helped to provide extra energy resources that enable the fish to overcome the stressful condition. Thus, alterations in the glucose metabolism are common response to stress in fish.

In the present study it has been found that fish when cultured under the lower light intensity, its scale showed mostly reticulate (dispersed pigment) melanophores and the fish morphologically became little darker. As the light intensity increased, the pigments in melanophores started aggregating due to this the melanophores started changing from reticulate stage *via* intermediate stages to stellate stage and at L4 treatment mostly punctuate (aggregated pigment) melanophores were found due to this the fish became paler. The major function of fish chromatophores is to protect the body against the intense radiation. The skin colour of fish is generated either by the absorption of light by the pigments present in dendritic melanophores, xanthophores or by the scattering and reflection of light ray by the iridophores [31]. Melanophores that are specific types of chromatophores when aggregate towards the cell center, paling occur and when they disperse through the cytoplasm darkening occur. The translocation of these melanosomes occurs by the activation and deactivation of the motor proteins. Kinesin causes melanosome dispersal while the dynein causes its aggregation along the microtubules [32].

In our study it is perhaps interesting to note that there was 100% survival of catla which shows that light has no significant role in survival of fish. Similar result was found in southern flounder where light intensity did not affect survival rate [33]. Many studies have focused on effect of light intensity on growth performances of fish. In our study we aimed to determine, if growth is affected by light intensity. Culture management should be aim to optimize farming environment to maximize the growth and welfare of fish. Light is one of the major culture management factors that synchronize from embryo development to maturation of fish [34].

Previous studies reveal that inappropriate light intensities lead to poor growth as most fish larvae are visual feeders and need a minimal threshold light intensity to be able to develop and grow normally [10]. In the present study, weight gain was the highest when fish were subjected to Lc, indicating that light intensity had significant effects on the growth of catla. The direct causes for faster growth of rohu larvae at lower light intensity were improved feed intake and feed conversion ratio at this optimum light intensity.

However, the weight gain was minimum at higher light intensity as the active feeding of fish decreased at light intensity that is too low or too much high. FCR was highest at L4. This might also help to explain the reduced weight gain observed in fish under higher light intensity in this study. Similar to our result the better growth of sea bass was at 100 lx [35].

CONCLUSION: The present study indicates that intense light causes sluggish growth due to decreased active feeding and foraging and also due to stress associated problems. The best growth in catla was obtained at 114 ± 4 lx (Lc) than the other treatments. HSPs are involved in various aspects of fish physiology that include stress physiology, endocrinology, acclimation and stress tolerance. Melanophores protect the fish body against the intense radiation either by the absorbing the light or by scattering and reflecting it.

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