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Physiological and Molecular Responses of a Bottom Dwelling Carp, *Cirrhinus mrigala* to Short-Term Environmental Hypoxia

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Abstract

An experiment was conducted to monitor temporal changes in the gene expression of a specific molecular marker, HIF-1α in response to a 15-day exposure to chronic hypoxia in a bottom dwelling carp, *Cirrhinus mrigala*. The expression level of HIF-1α mRNA was increased in gills of *C. mrigala* in response to 0.5 mg/L, dissolved oxygen (DO) for 15 days with the time of exposure, whereas it remained unchanged in fishes exposed to normoxia (DO, 6.5 mg/L). Based on the first experiment it is found that HIF is expressed at moderate level 72 hours, and it peaks at 7 and 15 days, indicating that 72 h is a tolerable time limit for survival under hypoxia for the particular species. Thus, a second experiment was conducted to evaluate the effect of hypoxia (0.5 mg/L) for 72 h and assessed different parameters such as metabolic enzymes, antioxidant enzymes and haematological parameters. The haematological parameters, such as RBC count, Hb and Hct values increased significantly ($P<0.05$) during exposure to hypoxia. Metabolic enzyme activities, especially the enzymes of the anaerobic glucose metabolism, such as HK, LDH and G6pase activities and the antioxidant enzymes, such as SOD, CAT and GPX activities have also revealed increasing trend in hypoxic conditions. We conclude that 72 h under acute hypoxia (0.5 mg/L) is sufficient to potentiate haematological, metabolic and antioxidant responses and the tolerance to hypoxia in mrigal is mediated by the activation of the transcription factor, HIF-1α.

Keywords: HIF-1α, Haematological parameters, Antioxidant enzymes, Metabolic enzymes

Introduction

The aquatic environment is always vulnerable to environmental stressors caused by changes in the physical and chemical parameters of water. Both subtle and apparent changes in physicochemical parameters of water such as dissolved oxygen, temperature, pH, hardness, salinity, ammonia and other dissolved gases may cause stress to the fish (Harper & Wolf, 2009). Other potential environmental stressors include heavy metals, insecticides, herbicides, fungicides, and defoliants. Natural changes in water quality, as occurs during low tide in tide pools, may stress the organisms that live in such environments (Galhardo & Oliveira, 2009). Of these various stressors, limited availability of dissolved oxygen, more appropriately described as hypoxia is found to be the one that can alter the aquatic environment within a short period. Environmental hypoxia is one of the recent challenges to be faced by the aquatic environment, which eventually affects the economically important fish fauna. Many of the coping mechanisms to hypoxia are subjected to extreme intraspecific and interspecific variations. The temperate species such as salmon and trout fall under the category of hypoxia-sensitive species and the carps such

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as common carp belong to hypoxia-tolerant species (Farrell, Gamperl, & Birtwell, 1998). In this context, it will be meaningful to study stress tolerance of fish to environmental changes, particularly those which can reduce the availability of oxygen i.e. about the hypoxic stress conditions which has an ill impact on aquaculture production. Mrigal (*Cirrhinus mrigala*) is one among the indian major carp, which is a bottom dwelling species unlike the other Indian major carp species, rohu and catla. India and Bangladesh are the major producers of mrigal and it production constitutes 8.7% of the total carp production across the globe (FAO, 2014). However, there are very few studies on the threshold of hypoxia tolerance and the biochemical adjustments of during stress exposure in this species.

There is a growing concern over hypoxia in the present climatic conditions as the global warming will further exacerbate the problem by reducing the oxygen solubility in water. Thus, it has become increasingly important to understand mechanisms by which the fishes survive under hypoxia. At the molecular level, the most common transcriptional response to hypoxia is the expression of hypoxia-inducible factor (HIF) as a central effecter mechanism coordinating cellular responses to oxygen tension level (Wang & Semenza, 1995). HIF-1 is a heterodimeric transcription factor formed by the association of a constitutively expressed beta subunit and an inducible alpha subunit, which is rapidly degraded in the presence of oxygen level (Wang & Semenza, 1995). Furthermore, the metabolic and haematological responses during hypoxia are mediated by HIF, which is well studied in several fish species (Kajimura, Aida, & Duan, 2006; Rahman & Thomas, 2007; Rytkonen, Vuori, Primmer, & Nikinmaa, 2007; Terova et al., 2008). However, the time of exposure to hypoxia and its intensity determine the threshold and magnitude of these responses, which is critical to enable the survival of the fish in the hypoxic water. With this background, two experiments were designed; the first one to determine the time for eliciting moderate surge in the HIF expression, and the second one to evaluate their physiological response during the exposure to hypoxia for the pre-evaluated time period in the first experiment.

Material and Methods

Experimental Animal

Mrigal fingerlings, with an average weight of 5.56 ± 0.43 g, were transported from the local fish farm, Maharashtra, India with sufficient aeration. They were acclimatized under aerated conditions for 15 days and during acclimation, fish were fed to satiation with a diet having 30 % crude protein (CP). Feeding was stopped 24 h before the exposure to hypoxia.

Hypoxia Exposure Trial and Sample Collection

Twenty-four aquarium tanks containing eight fishes (6 ±0.81 g) each of 50L capacity and after seven days of acclimation were exposed to severe hypoxia (0.5±0.2 mg/L), with the allocation of 3 tanks (replicates) for each sampling interval. The sampling intervals were 1h, 3h, 6h, 12h, 24h, 72h, 168h and 360h. Another set of 3 tanks were maintained at normoxia (DO, 6.5±0.2 mg/L). The hypoxia was created in aquarium tanks by bubbling nitrogen gas into airtight aquarium chambers (0.5±0.2 mg/L). The survival during the 15-day exposure period was above 90% and it did not vary among different time intervals. Three fishes from each tank were sampled

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when the sampling time was attained. For RNA isolation, gills were isolated and snap frozen in liquid nitrogen and stored for RNA isolation.

Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from the gill tissues using an RNAeasy Mini Kit (Qiagen, Cat #: 74104) following the protocols recommended by the supplier and reverse transcribed into cDNA by using the kit (Thermo scientific, Cat #: K1621) following the manufacturers' instructions. Briefly, cDNA synthesis was performed with 5 mg of total RNA using avian myeloblastosis virus reverse transcriptase (Promega) and oligo(dT) primers (Invitrogen) as per the manufacturer's recommendations. This cDNA was used as a template for quantitative realtime PCR.

Quantitative Real-Time PCR (qRT-PCR)

An aliquot of the cDNA was amplified with each of the primer set (Table1) designed basd on the available HIF sequence (Accession no: JF951958.1). qRT-PCR was performed with Roche, Light Cycler LC480 real-time PCR system using DNA binding dye maxima R-SYBR green. The reaction mix was prepared under dim light. Each reaction was prepared to 10µl per reaction volume contains, 5µl Maxima R SYBR green PCR master mix (2x), 1µl Forward and Reverse qRT-PCR Primer (2.5 pmol), 1µl template cDNA (1µg/µl) and 2µl of nuclease free water. Species-specific primers (Table 1) were used for qRT-PCR. qRT-PCR conditions included a 10-min activation and denaturation step at 95°C followed by 40 cycles of 15s at 95°C, 30s at 56°C and 30s at 72°C. The RT-PCR analysis of HIF1α of *Cirrhinus mrigala* was done for all the time intervals. The gene β-actin was used as internal control and zero-day reading was used as the external control. Analysis was done to three individuals from each group in triplicates (hence, n=9 for each treatment group). Relative mRNA level was calculated by $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{internal control}})n^{th}\text{ day} - (Ct_{\text{target gene}} - Ct_{\text{internal control}})0^{th}$ day (n=time interval).

Second Hypoxia Exposure and Sampling

The second trial was conducted in 8 aquarium tanks (50L) and a total of 64 fishes were distributed to 8 aquarium tanks. A hypoxia exposure (0.5±0.16 mg/L) for 72 hours was conducted in 4 aquarium tanks (quadruplicates) of 50L capacity with each containing 8 fishes (7 ±0.21 g). Another set of 4 tanks (quadruplicates) were kept under normoxia (DO, 6.5±0.2 mg/L). The preliminary trials have shown that the experimental animals go off feed below 1 mg/L dissolved oxygen level, which made it impossible for feeding during hypoxia exposure. Water quality parameters were maintained in the optimum range during the period (water temperature 27.8-30.5°C; pH:7.2-7.8; ammonia nitrogen; 0.11-0.15 mg l−1; nitrite nitrogen; 0.002-0.005 mg l−1; nitrate nitrogen; 0.02-0.06 mg l−1; Photoperiod; 12 h light: 12 h dark). The water was exchanged 30% daily with the hypoxic water maintained in separate storage tanks. The survival of the fishes did not vary significantly from the control group. Three fishes from each tank were sampled from each tank and gills, liver and muscle samples were collected.

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Estimation of Haematological Parameters

Blood (20 µl) was added to 3980 µl of RBC diluting fluid and mixed in a glass test tube to get a uniform suspension. Ten microliters of this solution were loaded into a Neubauer's counting chamber without trapping any air bubbles. The red blood cells were counted in five groups of squares. The following formula is used to calculate the number of RBC per mm^3 of the blood sample;

$$\text{Number of RBC}/\mu\text{l} = \frac{\text{N}\times\text{Dilution}}{\text{Area counted}\times\text{Depth of fluid}}$$, Where N is the total number of red blood cells counted in 5 squares of the haemocytometer.

The haemoglobin content of blood was analyzed following the cyanmethaemoglobin method using Drabkins Fluid (Qualigens). Blood (20 µl) was mixed with 5 ml of Drabkin's working solution. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The following formula was used to calculate the final haemoglobin concentration.

haemoglobin content (g %) = [OD (T)/ OD (S)]*[251/1000]*60, Where, OD (T) = Absorbance of test and OD (S) = Absorbance of standard.

The haematocrit values were estimated with the haematocrit reader. Briefly, the blood was drawn and the capillary tubes were two-third filled with the blood. The tubes were then centrifuged for 5 min and the percentage of the packed cell volume was determined by the hematocrit tube reader. The erythrocyte fragility was assayed using the method of Draper and Csallany (1969). Three drops of blood were added to 3 mL saline phosphate buffer, pH 7.4. After centrifuging for 10 minutes at 5000 X g the supernatant was removed and the cells were resuspended in 3 mL of buffer. The tubes were incubated in a water bath at 37°C for 4 hours and again were centrifuged. Aliquots of each supernatant were transferred to a 3-mL spectrophotometer tube and the optical density (OD) was read at 415 nm against a buffer blank. The haemolysis of the sample was calculated as the percentage of completely haemolysed blood in the buffer.

Metabolic Enzymes

The hexokinase (HK) activity was measured by the method of Crane and Sols (1955). The reaction mixture consisted of 0.2 mL ATP-$MgCl_2$ mixture, 0.2 mL histidine buffer mixture, 0.2mL glucose, and 0.3mL distilled water and 0.1mL tissue homogenate. One unit of enzyme activity is that amount which can catalyze the phosphorylation of 1µmole of glucose in 15 minutes at 30°C. The pyruvate kinase (PK) activity was assayed by the method of Bergmeyer, Gawehn, and Grassl (1974). The total 2.952 mL of reaction mixture comprised of 0.80 ml of imidazole buffer (pH 7.6), 0.10 mL of PEP, 0.25 ml of NADH, 0.20 ml of magnesium sulphate, 0.10 mL of ADP, 0.002 mL of LDH and 0.1 mL of tissue homogenate. The enzymatic activity was expressed as unit mg protein/ min at 37^0C where one unit will convert 1.0 µmol of phospho (enol) pyruvate to pyruvate per minute at pH 7.6 at 37°C. The lactate dehydrogenase (LDH) activity was assayed by the method of Wroblewski (1955). The

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total 3 ml of the reaction mixture was prepared by mixing 0.1 mL of NADH solution, 0.1 ml of sample and 0.1 mL of sodium pyruvate with 2.7 mL of 0.1 M phosphate buffer (pH 7.5). The enzymatic activity was expressed as unit's mg protein/ min at 25^0 C where 1 unit was equal to Δ0.01OD/ min. The glucose-6-phosphatase (G6Pase) activity in the liver tissue was estimated by the method of Swanson (1955). The assay mixture consisted of 0.3 mL of malate buffer (pH 6.5), 0.1 ml of 0.1M glucose-6-phosphate solutions and 0.1 mL of tissue homogenate. One unit of enzyme is defined as the amount that releases 1 µg of phosphorus per min.

Antioxidant Enzymes

The superoxide dismutase (SOD) activity was estimated by the method of Sun and Zigman (1978). The reaction mixture consisted of 50 µl of sample, 1.5 ml phosphate buffer and 0.5 ml epinephrine. One unit is defined as the amount of SOD required to achieve 50% inhibition of the auto-oxidation of epinephrine. Catalase (CAT) was assayed by the method of Takahara et al. (1960). To a reaction mixture of 2.45 ml phosphate buffer (50 mM, pH 7.0), enzyme sample was added and the reaction was started by the addition of 1.0 ml of H_2O_2 solution. Enzyme activity was expressed as nanomoles H_2O_2 decomposed / min / mg protein. Glutathione peroxidase (GPX) activity was assayed by the method of Lawrence and Burk (1967) using hydrogen peroxide as the substrate. The oxidation of GSH by H_2O_2 was coupled to NADPH oxidation in the presence of exogenous glutathione reductase (GR) to maintain substrate concentration and expressed as nmol NADPH oxidised/min per mg protein. Enzyme activity was expressed as mmoles of GSH converted /min/mg protein.

Statistical Analysis

Statistical significance of different parameters was analyzed using independent sample t test and the significance among different time intervals of first experiment was analysed by one way ANOVA via SPSS 22.0 for Windows. Levene's Test was performed to assess the homogeneity of variances and equal variances were assumed for whole data. All data presented in the text, figures and tables are means ± standard error and statistical significance for all statistical tests was set at $P<0.05$.

Results

HIF Expression

The real-time expression analysis of HIF 1α in gill tissues of *Cirrhinus mrigala* has clearly revealed that the gene is expressed in the gills even during normoxic conditions (Fig.1). However, the expression levels were quite low in the gill tissues under normoxia (0 h) compared to those at hypoxia, and thus expression at 0h was taken for baseline correction to quantify the relative change in the expression at the time intervals. It was evident that HIF 1 α expression increased with respect to time; however the moderate mRNA levels were maintained upto 72 hours and afterwards showed a sudden peak in the mRNA levels was observed at 7^{th} and 15^{th} day (Fig. 1).

Haematological Parameters

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The RBC count (RBC), Haemoglobin (Hb) Haematocrit (Hct) and erythrocyte fragility (EF) were represented in Table 2. Hypoxia had significant ($P<0.05$) effect on all the haematological parameters studied in this experiment. The significantly highest ($P<0.05$) RBC count, Hb content and Hct values were observed in hypoxia exposed treatment group as compared to the normoxia treatment group. There was significant ($P<0.05$) changes in EF values (Table 2) and they were significantly higher ($P<0.05$) in the hypoxia exposed group compared to normoxia treatment group.

Metabolic Enzymes

Activities of selected metabolic enzymes such as HK, PK, LDH and G6Pase activities in liver and muscle tissues are shown in Table 3. There was a significant increase ($P<0.05$) in HK and LDH activities in the hypoxic treatment group compared to normoxia treatment group in both liver and muscle tissues. However, there was no significant difference ($P>0.05$) in PK activity in muscle and liver tissues between the two treatment groups. Hypoxia significantly increased G6Pase activity in liver when compared to normoxia treatment group.

Antioxidant Enzymes

Significant increases ($P<0.05$) in CAT, SOD and GPX activities in gills were observed in hypoxia treatment group compared to normoxia treatment group (Table 4). Similarly, there was significantly higher ($P<0.05$) antioxidant enzyme activities in the liver tissue of the hypoxia treatment group compared to that of normoxia treatment group.

Discussion

HIF (Hypoxia-inducible factor)-1 is a heterodimeric helix-loop-helix transcription factor which acts as the master regulator of hypoxic responses, and it consists of two subunits, HIF-1 α and HIF-1β (Wang & Semenza, 1995). Protein stability of the α subunit (HIF-1α) is regulated by a family of oxygen dependent prolyl hydroxylases and its expression is regulated by oxygen at the protein level (Maxwell et al., 1999). From the present study, it was found that the HIF expression in gills was increased within one hour itself and remained at moderate levels for 72 h during exposure to hypoxia. The expression level has shown a peak response at 168 h; then the same level of expression was continued up to 360 h. A gradually increasing HIF expression with respect to time has been reported by Terova et al. (2008) in European sea bass (*Dicentrarchus labrax*) when exposed to a dissolved oxygen (DO) level of 4.3 mg/L for 15 days. However, from our study, it is evident that *C. mrigala* shows only a moderate response up to 72 h, which reveals its endurable time limit of exposure to acute hypoxia. This difference in temporal expression pattern signifies that the species of the present study can better tolerate short-term hypoxia, up to 72 h without the aid of a high HIF expression. Also, HIF expression pattern suggests that HIF-1 α is involved in the adaptive response to hypoxia in *C. mrigala* and helps the fish to survive under short-term hypoxia which is in agreement with several earlier studies (Kajimura et al., 2006; Rahman & Thomas, 2007; Rytkonen et al., 2007). It was also observed that the response of HIF-1 α to low environmental oxygen

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tensions was quite rapid as the expression level increased even at one-hour exposure to 0.5±0.16 mg/L oxygen level. It was also evident that the HIF-1 α was expressed under both normoxia and hypoxia, however lower level of expression was observed in normoxic conditions, as observed with other species (Terova et al., 2008)

The erythrocytes play an important role in adaptive mechanisms against hypoxia, which are primarily meant for improving oxygen delivery to compensate for the reduced supply of oxygen. Increasing red blood cell mass is an obvious way to improve the delivery efficiency of oxygen to the tissues. There are reports that hypoxia induces erythropoiesis in fish via the release of erythropoietin hormone (Gracey, Troll, & Somero, 2001). External hypoxia induces amitotic division of mature erythrocytes in fish (Murad, Everill, & Houston, 1993; Soldatov, 1996). In this study, it was found that there was an increase in RBC count in the blood implying the prevalence of hypoxia-induced erythropoiesis in *C. mrigala*. However, it is reported that there is no hypoxia responsive elements in the erythropoietin (*Epo)* gene of Fugu in the 5' or 3' flanking regions, although there was some indication that hypoxia increases appropriate splicing of *Epo* transcripts (Chou, Tohari, Brenner, & Ven, 2004). In this context, there is an explicit need for further studies to elucidate the mechanisms behind hypoxia-induced erythropoiesis in fishes. The fish species of the present study, *C. mrigala* responded quickly to hypoxia by exhibiting erythropoiesis within 72h of exposure itself. In contrast, haematocrit, haemoglobin and erythrocyte concentrations increase in some species after several weeks of exposure to hypoxic conditions (Frey, Weber, van Aardt, & Fago, 1998; Timmerman & Chapman, 2004). In another species, *Pagothenia borchgrevinki*, exposure to low oxygen levels resulted in a significant (66%) rise in haemoglobin concentration after 11 days (Wells, Grigg, Beard, & Summers, 1989). However, red blood cell count of rainbow trout is increased immediately during hypoxia due to the release from the spleen and then later due to erythropoiesis in response to the hormone, erythropoietin, produced by the kidney (Lai, Kakuta, Mok, Rummer, & Randall, 2006).

The increased fragility of erythrocytes of *C. mrigala* during hypoxia signifies the excessive oxidative stress on the erythrocyte membranes. Since hypoxia strengthen itself by involving oxidative stress (Behn, Araneda, Llanos, Celedon, & Gonzalez, 2007), it results in excessive membrane damage of erythrocytes. Hypoxia-induced oxidative stress has been shown to affect membrane lipid diffusion and anion exchange in erythrocyte membranes (Celedon, Gonzalez, Sotomayor, & Behn, 1998). Clanton (2007) further explained that generation of reactive oxygen species (ROS) during hypoxia might cause injury to the tissues.

The activity of the first regulatory enzyme of glycolysis, HK was up-regulated in different tissues like liver and gills during hypoxic stress which is consistent with findings of Zhou, Randall, Lam, Ip, & Chew (2000) in common carp. However, there was no evidence of regulation of PK activity by hypoxia, which may be due to the low sensitivity of this enzyme to allosteric control and may keep constant activity levels during hypoxia and normoxia. There are no reports available on the direct regulation of PK by the transcription factor, HIF-1α. LDH is one of the enzymes of glycolytic pathway responsible for the anaerobic conversion of pyruvate to lactate, and it is ubiquitously present in most tissues. During the anaerobic metabolic shift, caused by hypoxia, there may be an up-regulation of LDH activities in different tissues (Borowiec, Darcy, Gillette, & Scott, 2015). The present study revealed an increase in LDH activity in both liver and muscle tissues after 72h of hypoxia exposure. However, muscle LDH remained unchanged after exposure to hypoxia in killifish *Fundulus heteroclitus* (Greaney,

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Place, Cashon, Smith, & Powers, 1980), However, there was an increase in activities of liver LDH in the above fish, *Fundulus heteroclitus* during hypoxic conditions (Borowiec et al., 2015). This may be due to an increase in lactate level during hypoxia, as lactate is the substrate for gluconeogenesis in liver. Under long-term hypoxia, as glycogen reserves decline, liver gluconeogenesis must be activated to supply glucose required for anaerobic metabolism. This enhanced gluconeogenesis is mediated through the action of cortisol and catecholamines (Vijayan, Pereira, Grace, & Iwama, 1997). This confirms that there is an up-regulation of anaerobic metabolic pathways in *C. mrigala*, species selected for the present study. Several studies have demonstrated changes in glycolytic enzyme activities and increased LDH activities during hypoxia (Rees, Bowman, & Schulte, 2001). G6Pase is an enzyme which catalyses the conversion of glucose-6-phosphate to glucose, exclusively present in the liver. The higher G6Pase activities during hypoxia in our study may be a result of increased glycogenolysis or increased gluconeogenesis. This was a shred of evidence for the increase in glycogenolysis in the liver during hypoxia, potentiated by increased glycogen mobilisation and production of blood glucose to meet the energy requirement (Vijayan et al., 1997). The survival under hypoxia is greatly contributed by upregulation of the pathways such as anaerobic glycolysis and gluconeogenesis and further, hypoxia-induced upregulation in the expression of glycolytic enzymes is mediated by HIF-1α (Semenza, 2006) which was shown an increased expression in the first trial of the present study.

Our study indicated that antioxidant enzymes such as CAT, SOD and GPX activities increased in both gill and liver tissues when exposed to hypoxia. Although aerobic respiration is reduced at hypoxic conditions, there would be an increased rate of ROS production at the tissue level (Clanton, 2007). The lipids are more prone to damage by ROS, due to the presence of unsaturated fatty acids. Lipid peroxidation products do not only mark the oxidative damage to lipids; they are also possibly involved in triggering the up-regulation of antioxidant enzymes (Lushchak & Bagnyukova, 2007). The main components of the cellular defence system against ROS production are antioxidant enzymes and hypoxia appears to trigger SOD, CAT and GPX activities (Cooper, 2002). As the antioxidant enzyme activities are increased during hypoxia in the present study, it is rational to infer that the oxidative stress due to the formation of ROS might have been increased in hypoxic conditions. It was found that hypoxia increased the activities of SOD and CAT activities in liver of common carp, *Cyprinus carpio* (Lushchak, 2011). In another species, rotan *Percottus glenii*, upregulation of antioxidant activities was evident during hypoxia (Lushchak & Bagnyukova, 2007) while exposure to hypoxia caused an up-regulation in glutathione-S-transferase activity in medaka, *Oryzias latipes* (Oehlers, Perez, & Walter, 2007). The activities of catalase and glutathione peroxidase were also responded to reducing levels of oxygen in the clam, *Corbicula fluminea* (Vidal, Basseres, & Narbonne, 2002). The mRNA levels of antioxidant genes were increased during hypoxic conditions (De Zoysa et al., 2009) in disk abalone, *Haliotis discus.*

From the above study, it is logical to conclude that this bottom-dwelling species, *C. mrigala* can tolerate acute hypoxia for about 72 h by potentiating metabolic, haematological and antioxidant mechanisms which are mediated by a substantially enhanced HIF α expression. These findings disclose that the carp, mrigal can be a potential candidate species for aquaculture in the climate change era.

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Table 1. Primers used for relative quantification of HIF 1 alpha expression by realtime PCR

Primers	Sequence
Forward primer (HIF)	CGAGAGATGCTGGTCCACAG
Reverse primer (HIF)	AGAACCTTCCACGTAGCAGG
Forward primer (β-actin)	GGCTGTGCTGTCCCTGTA

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Reverse primer (β-actin	GGCTGTGCTGTCCCTGTA

Table 2. Responses of haematological parameters to environmental hypoxia in *Cirrhinus mrigala*.

Parameter	Normoxia	Hypoxia	P value
RBC (*10^6)	1.39^a±0.12	1.91^b±0.18	0.002
Hb (g/dl)	4.83^a±0.15	8.45^b±2.25	0.001
Hct (%)	20.30^a±1.48	27.20^b±1.74	0.001
EF (%)	7.29^a±0.76	34.27^b±0.16	0.001

RBC-Red blood cell count Hb-Haemoglobin , Hct-Haematocrit, EF-Erythrocyte fragility
Values represent means± SE, n=12, Different superscript in same row signify statistical differences

Table 3. Responses of metabolic enzymes to environmental hypoxia in *Cirrhinus mrigala*.

Enzymes	Tissue	Normoxia	Hypoxia	P value
HK	Liver	0.91^a±0.04	1.96^b±0.41	0.003
	Muscle	0.75^a±0.09	1.83^b±0.03	0.002
PK	Liver	17.26±0.88	18.67±1.26	0.151
	Muscle	37.42±2.10	39.84±1.21	0.148
LDH	Liver	2.59^a±0.29	5.64^b±0.17	0.001
	Muscle	5.45^a±0.51	9.02^b±0.27	0.001
G6Pase	Liver	8.67^a±0.78	18.25^b±1.64	0.001

HK (Hexokinase): Units/min/ mg protein at 37 ^{0}C, PK (Pyruvate Kinase): Units/min/mg protein at 37 ^{0}C, LDH (Lactate dehydrogenase): Units/min/ mg protein at 37 ^{0}C , G6Pase (Glucose 6 phosphatase): µg phosphrous released/min/mg protein at 37 ^{0}C
Values represent means± SE, n=12. Different superscripts in same row signify statistical differences (*P<0.05*)

Table 4. Responses of antioxidant enzymes to environmental hypoxia in *Cirrhinus mrigala*.

Treatments	Catalase[1]		Superoxide Dismutase[2]		Glutathione peroxidase[3]	
	Liver	Gill	Liver	Gill	Liver	Gill
Normoxia	5.21^a±0.12	8.4^a±0.21	19.81^a±0.31	28.04^a±3.86	1.16^a±0.21	3.78^a±0.30
Hypoxia	11.31^b±0.11	22.69^b±2.51	25.19^b±1.23	42.51^b±6.05	2.54^b±0.31	7.04^b±0.17
P value	0.002	0.001	0.004	0.001	0.021	0.001

[1]Catalase: mmol H2O2 decomposed /min/ mg protein at 37 ^{0}C. [2]Superoxide dismutase : µ mol/min/ mg protein at 37 ^{0}C; [3]Glutathione peroxidase: mM substrate converted/min/mg protein at 37 ^{0}C.
Values represent means± SE, n=12. Different superscript in same column signify statistical differences

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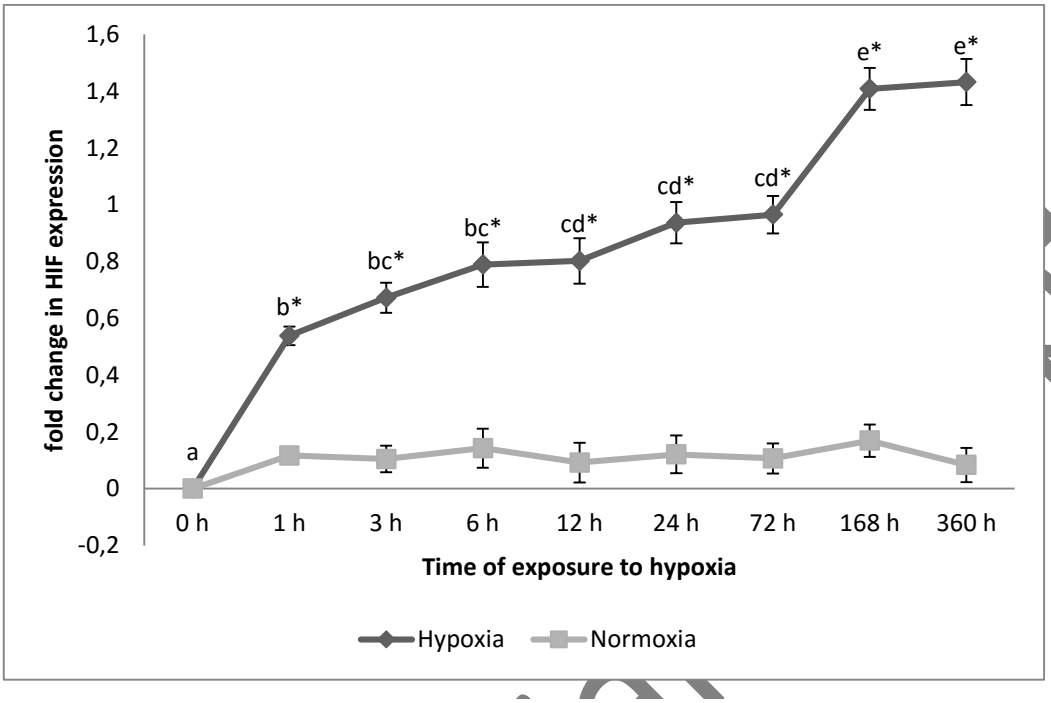

Figure 1. Analysis of expression HIF 1alpha in *Cirrhinus mrigala* by realtime PCR. Values represent means± SE of fold change in HIF 1 alpha mRNA levels (fold change of expression) (n=9), *P<0.05*, Timepoints without a common superscript in the line diagram (hypoxia) signify statistical differences. Asterisks indicate significant difference between normoxia and hypoxia groups where **P < 0.05*