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Effects of Hypoxia and Metabolic Adjustments in *Heteropneustes fossilis*, an Indian Air-Breathing Catfish

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Abstract

Dissolved oxygen is one of the most important environmental factors to sustain lives of fish and other aquatic organisms which rely on aquatic respiration alone. In oxygen deficient environment the supply of oxygen is less than required or consumption exceeds supply. Dissolved oxygen in such condition can decline from the levels required by most animal lives generating hypoxic condition.

Low oxygen concentration occurs in a wide range of aquatic systems and range in temporal frequency, seasonality and persistence. These have always been naturally occurring low oxygen habitat but anthropogenic activities related primarily to organic and nutrient enrichment have led to increase in hypoxia and anoxia both in fresh as well as marine system. Freshwater systems are more frequently faced with low oxygen condition and fishes in a tropical country like India are quite frequently exposed to this. The general public is aware of the results of hypoxia as the phenomenon of “Fish Kills” occurring frequently in natural waters.

Keywords: Hypoxia; SDS-PAGE; LDH; Glucose; Lactate

Introduction

Hypoxia is a frequently occurring environmental phenomenon in the freshwater and even coastal system of a tropical country like India. It may be naturally occurring phenomenon due to biological and physical factors (Rosenberg *et al*., 1991; Pihl *et al*., 1992; Hobak and Barnhart, 1996; Wu, 1999) or may be caused due to anthropogenic activities around the water bodies.

In tropical aquatic system such as India, dissolved oxygen in the water of pools, swamps, lakes and rivers may change radically, from almost 100% saturation or sometimes hyper saturation at noon to zero at night of the same day. Sharp seasonal fluctuations also occur frequently occurring.

Under these conditions it is not surprising that air breathing habit seemingly arose as a solution to the main environmental problem specially aggravated by reduced oxygen solubility at different temperatures of tropical waters and in extreme cases, due to drying up of ponds or shallow lakes in which these fishes have to live.

Effect of Hypoxia

Hypoxia can have profound effects on different organisms. In fishes the effect differs in fishes of different respiratory habits. So far the ultimate effect observed at individual and population levels can be enumerated as:

1. Reduced fish growth rates, limiting or productive habitat, increase in mortality of young fish (Burnett and Stickle, 2001).
2. Decrease in feeding habit (Burnett and Stickle, 2001).
3. Decrease in growth rate (Almeida-Val et al., 2000).
4. Increase in ventilation rate (Somero and Childress, 1980; Pelletier et al., 1993).
5. increase in flow of blood on respiratory surfaces
6. Switching from aerobic to anaerobic metabolism
7. Reduction in their overall metabolism
8. Investigation have revealed that hypoxia is a strong and usually positive regulator of gene expressions too (D’Angio and Finkelstein 2000; Prabhakar, 2001; Semenza, 2001).

Review of Literature

Effect of oxygen deficiency on fish had drawn the attention of scientists as early as 1920s and extensive literature is available on fish during that period (Gardner, 1926). A comprehensive study has been made on a number of freshwater, estuarine and marine fishes by Davis (1975) to record the minimum oxygen requirements for survival and growth of fishes. Greaney et al., (1980); Taylor and Miller, (2001); Pichavant et al., (2003) studied the effects of chronic (weeks of) hypoxia on oxygen carrying capacity.

Weber & Kraemer (1983) described that feeding and growth (Cech et al., 1984; Bejda et al., 1992; Hales & Able, 1995; Secor & Gunderson, 1998; Taylor & Miller, 2001) are reduced in fishes when exposed to chronic hypoxia (≤3.0 mg O_2l^{-1}).

Dunn & Hochachka (1986) and Dalla Via et al. (1994) observed in their studies that a metabolic reorganization takes place as a result of hypoxia that tends to follow one of two generalized patterns: (i) either the rate of anaerobic ATP production increases (Pasteur effect) or (ii) the ATP rate declines (metabolic depression). Chabot and Dutil, (1999); Pichavant et al., (2000, 2001) and Zhou et al., (2001) studied the effects of chronic (weeks of) hypoxia on food intake, whereas effect of hypoxia on reproduction has been studied by Wu et al., (2003).

Aim of the Study

Because of the link between urbanization and increased anthropogenic activities and the increase in their adverse effect on aquatic system there is a need to understand the mechanisms behind the observed effect of hypoxia and improved hypoxia tolerance. The present piece of work aims to analyze the response of protein profiling, enzyme assay and blood parameters to different degrees of hypoxia in Cypriniforms, mainly catfishes, which present different respiratory patterns.

Materials and Methods

Live specimens (6 fishes) of *Heteropneustes fossilis* (80-90 g 20-24 cm), were procured from a local market and were acclimatized at normoxia (7.2±0.3 mg/L, DO), at least for a month in tanks of 100 L capacity filled with 25 L of water at 25±3°C. They were fed once a day with processed feed of goat liver or flesh and soyabean powder. Feeding was stopped 48 h before the start of experiment.

All the fishes were held for 12 hrs duration of experimentally provoked hypoxia at three different levels:

1. 65%-40%Oxygen saturation or 5.0±0.3 mg/l to 3.5±0.3 mg/l O_2 (Slight Hypoxia)
2. 40%-20% Oxygen saturation or 3.5±0.3 mg/l to 1.5±0.1 mg/l O_2 (Moderate Hypoxia) and
3. Below 20%Oxygen air saturation or ≤1.5±0.1 mg/l O_2 (Severe Hypoxia)

Three separate experiments were carried out in the closed respirometer (without access to air). Decrease in dissolved oxygen (DO) was accomplished by bubbling nitrogen directly into the water of the experimental tank, or into the reservoir that supplied water to the respirometer. DO probe (WTW, CellOx 325) and pH meter (pH electrode; WTW, SenTix® 41-3) were installed to record dissolved oxygen (DO) and pH.

Lactate dehydrogenase (LDH, EC 1.1.1.27) activity in the cell free extracts of muscle, liver, heart and brain was measured by a NADH linked optical assay following the method of Horecker and Kornberg (1948).

Blood and tissues were treated in 3 volumes of ice-cold 6% perchloric acid (PCA) homogenized in an ice cold bath and centrifuged at 4 ^{0}C. Extracts were used for glucose and lactate determinations. Sigma kits procedures n. 635 and 826-UV were used respectively.

The SDS-PAGE was carried out according to Laemmli (1970) in Mini-PROTEAN Tetra System of BIO-RAD using a 5% (w/v) separating gel. After electrophoresis the gels were stained with coomassie blue R-250 for Visualization of the proteins. Molecular of the protein bands was determined with reference to standards (Genei Marker, PMW).

Fish were anaesthetized prior the collection of blood samples to reduce the handling stress during normoxia. Heparinized blood was used for erythrocyte counts, haemoglobin estimation and haematocrit (Hct) evaluation. Erythrocyte count was made with the help of Neubaur's haemocytometer using standard diluents. Haemoglobin was estimated by the method of Blaxhall and Daisley (1973). [Hct] was determined following centrifugation of microhematocrit capillary tube filled with blood, at 10,000 rpm for 5 min (Assendelft and England 1982). Erythrocytic indices like mean corpuscular volume (MCV) mean corpuscular haemoglobin (MCH) Mean cell haemoglobin concentration (MCHC) was measured by Wells and Weber (1991).

Observation

LDH activity

TABLE-1:

Mean specific activity of lactate dehydrogenase (LDH) enzyme (Units/mg proteins) in different tissues of *Heteropneustes fossilis* subjected to slight, moderate and severe hypoxia for same time duration (12h)

Tissue	Normoxia	Slight Hypoxia	Moderate Hypoxia	Severe Hypoxia
Heart	145.58±13.36	184.29±15.24	212.43±17.39	233.24±18.26
Liver	59.83±6.69	65.60±7.63	68.13±7.97	85.24±8.72
Brain	43.18±6.55	54.52±7.12	65.68±7.27	68.37±8.09
Muscle	178.85±20.62	223.32±22.37	267.23±23.69	294.57±26.34

Highest LDH activity in *H. fossilis* was observed in muscle and lowest in brain during normoxia (Table 1). Maximum increase in LDH activity was found in muscle (64.70%) followed by heart (60.21%) and brain (58.33%) during severe hypoxia. During slight hypoxia maximum increase in LDH activity was observed in heart (26.59%) followed by brain (26.26%) and muscle (24.86%). During moderate hypoxia maximum increase in LDH activity was observed in brain (52.10%) followed by muscle (49.41%), heart (45.91%) and liver (13.87%). Significant change (p≤0.05%) in LDH activities were

observed between normoxia and moderate and severe hypoxia in muscle and in heart it was found between normoxia and severe hypoxia (Fig. 1). No pronounced change was observed in LDH activity in liver and brain during different time duration of hypoxia.

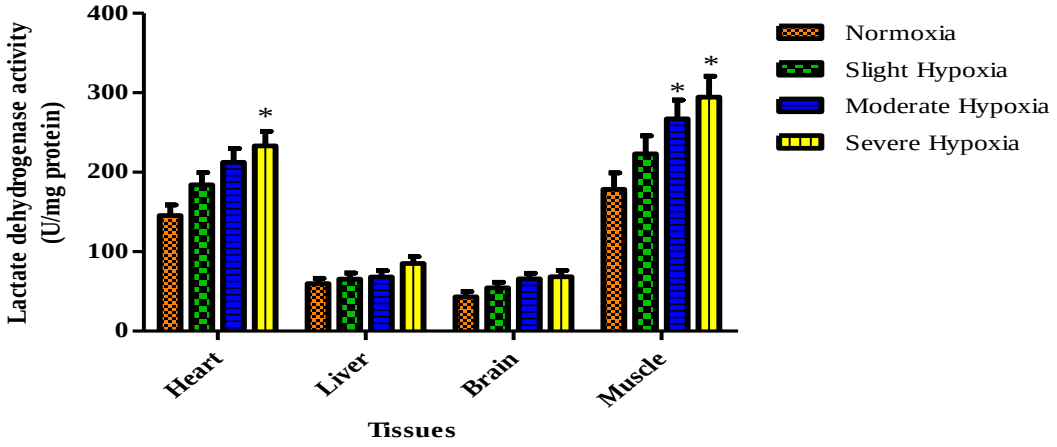


Figure-1: Mean specific activity of lactate dehydrogenase enzyme (U/mg protein) in heart, liver, brain and muscle of *Heteropneustes fossilis* exposed to varying oxygen concentration i.e. different hypoxia period for 12 hours duration. (U, µmole substrate/min; Values are means±s.e.m. n=6). Asterisk (*) represents significant differences ($p<0.05$) between normoxia and 72 hours of hypoxia.

Glucose content in different tissues of *Heteropneustes fossilis*

Table-2: Determination of tissue glucose content in different tissues of *Heteropneustes fossilis* subjected to slight, moderate and severe hypoxia for same time duration (12h)

Tissues	Normoxia	Slight Hypoxia	Moderate Hypoxia	Severe hypoxia
Heart	0.56±0.031	0.73±0.043	0.96±0.025	1.050±0.031
Liver	1.20±0.013	1.05±0.050	1.12±0.11	1.450±0.015
Brain	0.45±0.023	0.62±0.045	0.49±0.037	0.770±0.080
Muscle	0.23±0.020	0.35±0.025	0.58±0.031	1.020±0.056
Blood	0.74±0.080	0.98±0.110	1.34±0.130	1.840±0.170

Glucose concentration was found to be highest in liver followed by blood and heart and lowest glucose content was observed in muscle followed by brain (Table 2). There was increasing trend in glucose concentration in all the tissues except liver during different periods of hypoxia when compared with normoxia. During slight hypoxia maximum increase in glucose content was observed in muscle (52.17%) and brain (37.78%) followed by blood (32.43%). During moderate hypoxia maximum increase in glucose content was observed in muscle (152.17%) followed by blood (81.08%) and heart (71.42%) During severe hypoxia maximum increase in glucose content was observed in muscle and brain followed by heart. The increase in glucose content was found to be 3.5-fold in muscle, 1.5 fold in blood and nearly two fold in heart. Brain and liver showed lowest increase in this stage. Significant changes ($p\leq0.05\%$) were observed between normoxia and moderate hypoxia in heart, brain, muscle and blood (Fig. 2).

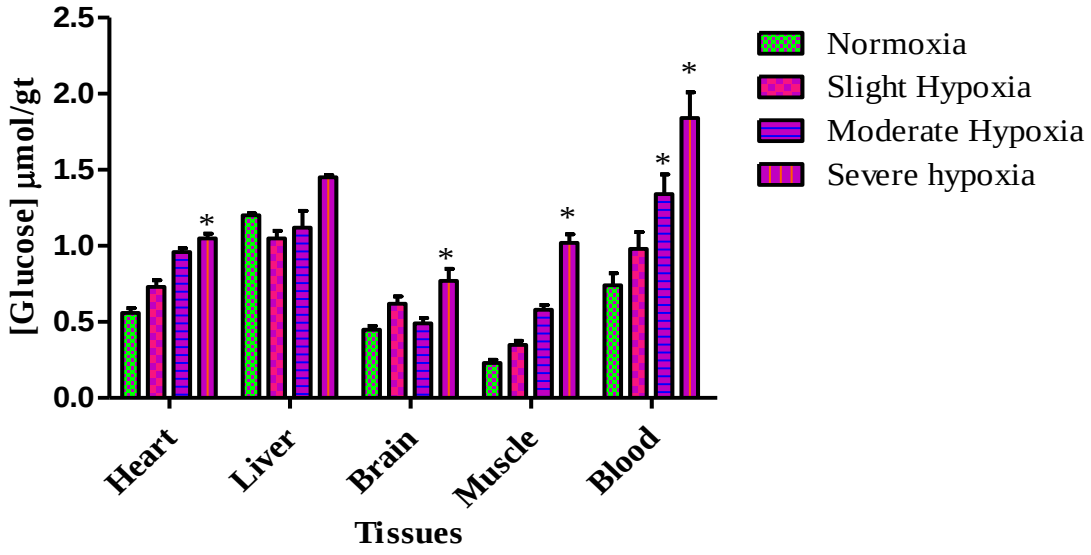


Figure-2: Glucose concentrations in different tissues of *Heteropneustes fossilis* submitted to normoxia and different periods of hypoxia. Error bars are within limits of symbols when not visible. Values are means ± SD, n = 6. * p< 0.05.

Lactate content in different tissues of *Heteropneustes fossilis*

Table 3 Determination of tissue lactate content in different tissues of *Heteropneustes fossilis* subjected to slight, moderate and severe hypoxia for same time duration (12h)

Tissues	Normoxia	Slight Hypoxia	Moderate Hypoxia	Severe hypoxia
Heart	1.84±0.36	3.520±0.72	3.14±0.64	1.59±0.38
Liver	1.12±0.23	1.950±0.32	2.40±0.27	2.10±0.32
Brain	1.85±0.25	2.340±0.27	2.66±0.29	2.27±0.31
Muscle	4.70±0.43	5.100±0.62	5.40±0.61	6.10±0.72
Blood	3.40±0.32	4.300±0.37	4.67±0.45	5.20±0.51

During normoxia highest lactate content was observed in muscle and blood followed by brain and lowest lactate content was observed in liver (Table 3). During slight hypoxia all tissues showed increasing trend in lactate accumulation as the fish rely mostly upon anaerobic respiration for its energy requirements. Maximum increase was observed in heart (90.21%) and liver (59.78%) followed by brain (26.48%). At the moderate hypoxia stage, maximum increase in lactate content was observed in liver (114.28%) followed by heart (70.65%). During severe hypoxia maximum increase in lactate content was observed in liver (87.5%) and blood (52.94%). Significant changes (p≤0.05%) were observed between normoxia and moderate hypoxia in heart and liver, and between normoxia and severe hypoxia in muscle and blood (Fig. 3).

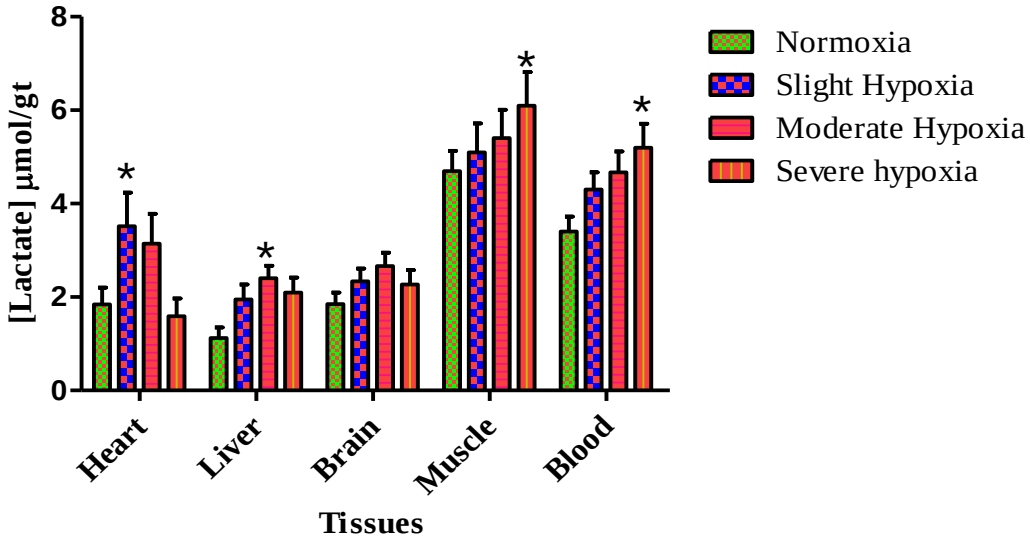


FIGURE-3: Lactate concentrations in different tissues of *Heteropneustes fossilis* submitted to normoxia and different periods of hypoxia. Error bars are within limits of symbols when not visible. Values are means ± SD, n = 6. * p< 0.05.

SDS-PAGE analysis in *Heteropneustes fossilis*

It was observed in SDS PAGE results that during hypoxia 35.1kD and 66.8kD protein bands were absent in heart (Fig. 4). Liver showed two extra protein bands of mol. wt. 45.8kD and 58.4kD while 36.1kD protein band was absent. In brain during experiments of hypoxia extra protein bands having mol. wt. 20.7kD, 32.6kD, 60.2kD and 72.6kD were observed while 14.3kD and 36.0kD proteins were absent. In muscle during hypoxia extra protein bands having mol. wt. 35.4kD and 45.3kD were recorded (Fig. 4).

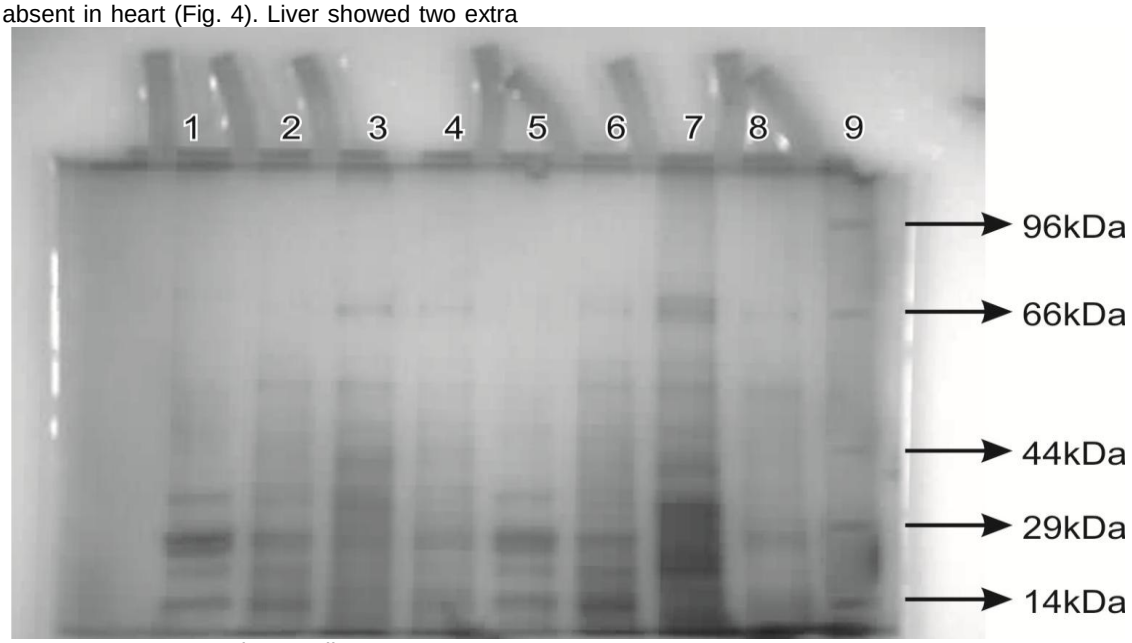


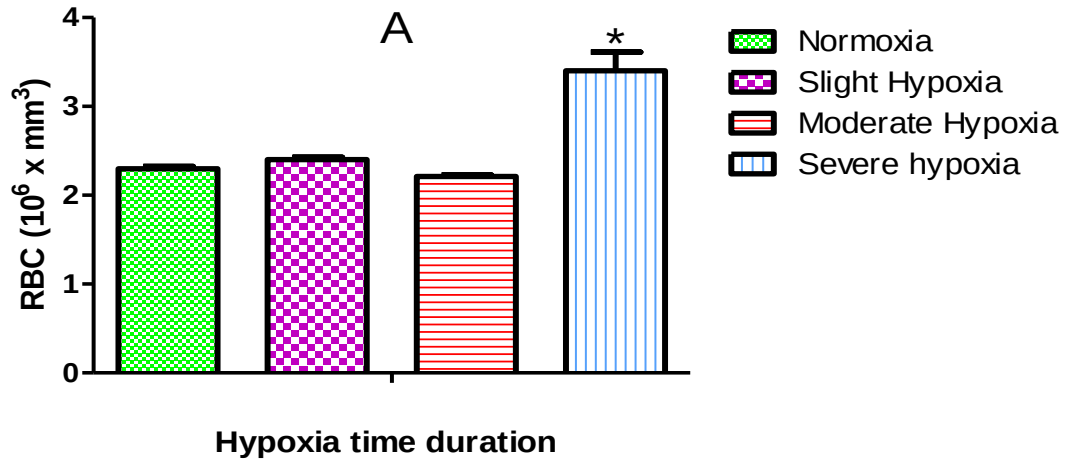
Figure-4: SDS-PAGE (Laemmli, 1970; 12% separating gel) profile of proteins of different tissues of *Heteropneustes fossilis*. Lane 1: normoxia heart, lane 2: normoxia liver, lane 3: normoxia brain, lane 4: normoxia muscle, lane 5: hypoxia heart, lane 6: hypoxia liver, lane 7: hypoxia brain, lane 8: hypoxia muscle and lane 9: mol. wt. Marker (Sigma wide range marker).

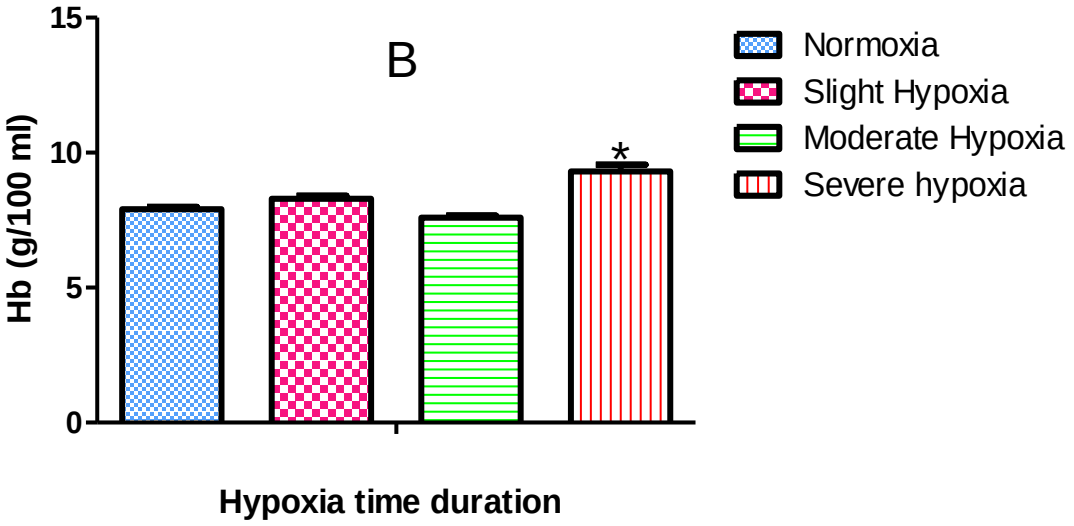
Haematological Changes

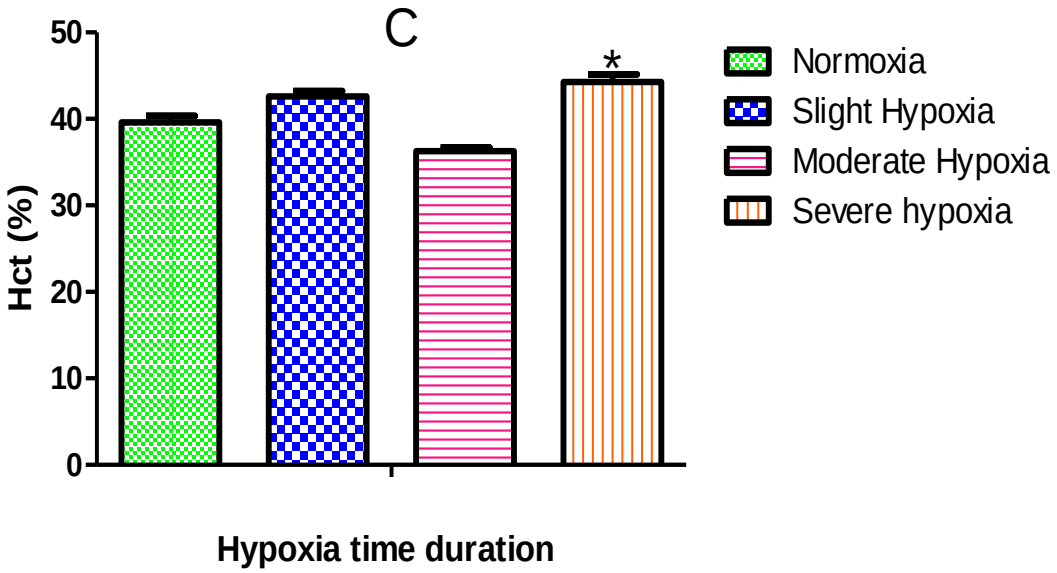
Table-4: Haematological changes in *Heteropneustes fossilis* exposed to different level of hypoxia. Values are mean of three replicates±standard error of mean.

	RBC (10^6×mm^3)	Hb(g/100ml)	Hct (%)	MCV (fl/cell)	MCH Pg/cell	MCHC (%)
Normoxia	2.3±0.026	7.9±0.09	39.6±0.77	190.1±3.8	45.4±1.07	19.09±0.32
Hypoxia						
Slight hypoxia	2.4±0.029	8.29±0.12	42.6±0.62	195.26±4.1	47.2±1.4	18.76±0.29
Moderate hypoxia	2.21±0.018	7.6±0.07	36.29±0.39	186.28±45	42.12±1.27	20.23±0.37
Severe hypoxia	3.4±0.21	9.31±0.25	44.27±0.89	199.45±5.6	49.52±1.74	17.91±0.32

There was an increase (4.34%) in RBC content was observed during slight hypoxia. At moderate hypoxia slight decrease (3.91%) in RBC content was observed. It was further increased (47.82%) significantly (p≤0.05%) at severe hypoxia level (Table 4). Blood haemoglobin (Hb%) level was fluctuate at different stages of hypoxia. It was increased (4.93%) at slight hypoxia and decreased (3.79%) at moderate hypoxia. As water oxygen level was decreased at severely level, Hb content in blood was increased (17.84%). Haematocrit (Hct%) value increased (7.57%) at slight hypoxia and decreased (8.35%) at moderate and increased (11.79%) once again at severe hypoxia. Other haematological parameters like MCH and MCV were increased at slight hypoxia and moderate hypoxia but decreased at moderate hypoxia. MCHC decreased at slight and severe hypoxia level but increased at moderate hypoxia level (Fig. 4)



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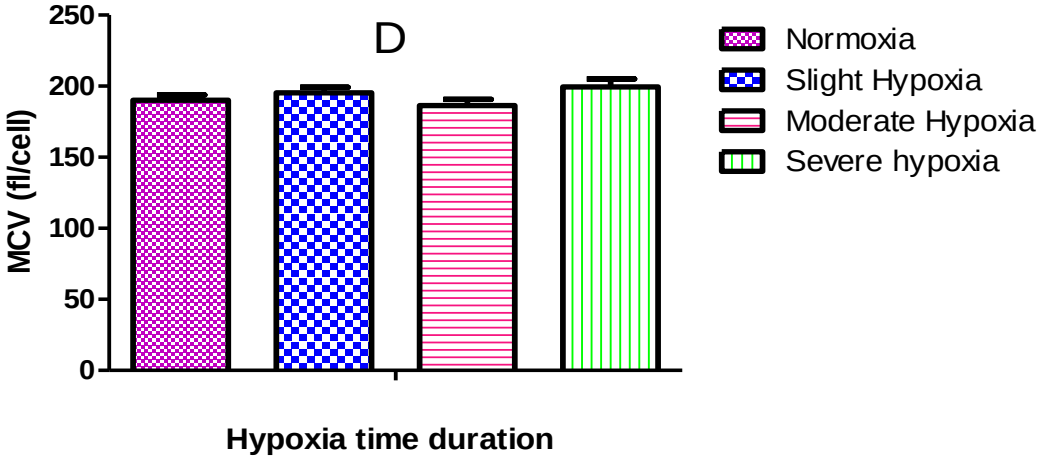
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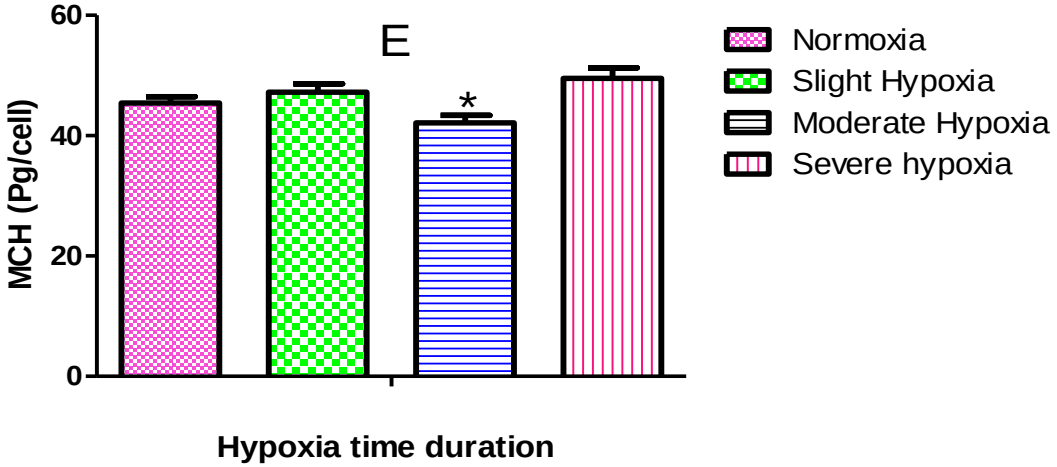
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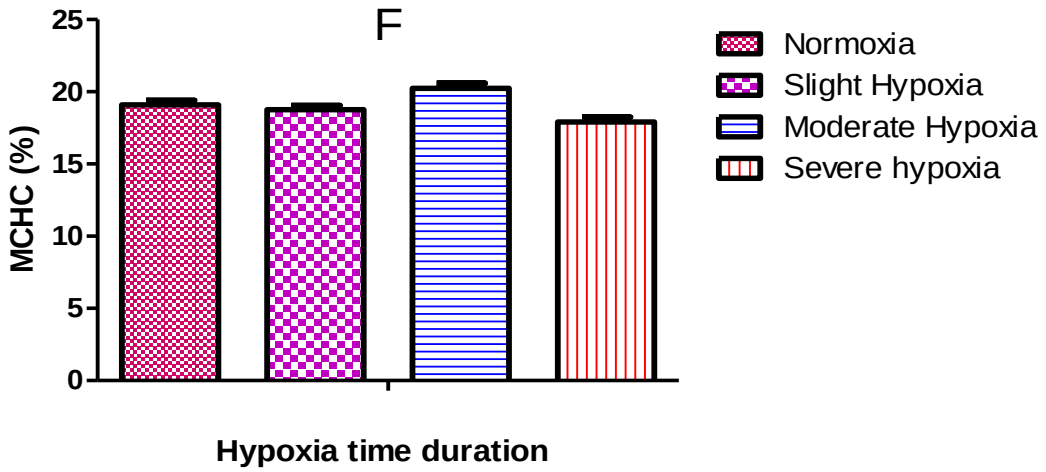

FIGURE-4: Haematological parameters in blood of *Heteropneustes fossilis* exposed to varying oxygen concentration i.e. different hypoxia stages for 12 hours duration.(A) RBCs (10^6×mm^3), (B) Hb (gm/100 ml), (C) Hct (per deciliter), (D) MCV (fl/cell), (E) MCH (Pg/cell) and (F) MCHC (gm/decilitre).Asterisk (*) represents significant differences ($p<0.05$) between normoxia and different hypoxia stages.

Discussion

A lot of stress studies on fish have been made to observe the physiological response. These responses are recorded on two levels, primary and secondary. Primary level included cortisol and catecholamine levels (Bartin and Iwama, 1991), whereas secondary responses included parameters like blood glucose, glycogen, enzyme and proteins.

Brain, liver and heart are known as aerobic tissues which normally tend to avoid anaerobic accumulation of lactate. Therefore the LDH level is adjusted in these tissues according to the degree of exposure to hypoxia (Almeida-Val et al., 2000). The LDH levels observed in different fish species in an investigation has been found to support this observation (A. Kumar and A. Gopesh 2015[1], A. Kumar 2015[2] ; A. Kumar 2019; A. Kumar, A. Gopesh and S. Sundram 2020).

These results, in combination with the absence of lactate accumulation in white muscle, indicate anaerobic metabolism is only beginning to be employed to supplement energy demands at this level of oxygen deprivation, and metabolic depression is an effective way of conserving ATP until fish are faced with almost anoxic conditions (A. Kumar 2016). In other studies with comparable lengths of hypoxia exposure, levels of lactate increased to a greater extent in blood and white muscle (Richards et al., 2007; Wood et al., 2007) than in the current study. This discrepancy in lactate accumulation during hypoxic exposure is most likely a result of the quick entry into hypoxia (~6 h) for these two studies as compared to the gradual transition into hypoxia of our study (~12 h).

Glucose and lactate changes during hypoxia are showed in Fig. 2 and Fig. 3 in *H. fossilis*. Blood did not show significant change in glucose concentrations during hypoxia, which explains the increases and decreases of this metabolite within the tissues only. Liver showed a sharp decrease after four hours of hypoxia and subsequent recuperation, probably due to ASR. The lack of glucose increase in liver supports the conclusion that glycogenolysis was not activated in the slight and severe hypoxia but that glucose was consumed to be re-established to normal values after this period. Muscle, heart and brain showed significant increases in glucose after severe hypoxia probably due to glycogenolysis activation.

Dunn & Hochachka (1986, 1987), both, reported an increase in glucose after hypoxia in trout *Salmo gairedeneri*. According to Walton & Cowery (1982), carbohydrate metabolism is not believed to be a major energy source in fish, but it is reasonable to assume that its importance increases during hypoxia because of its role in activation of anaerobic glycolysis activation.

In fish, increase in blood glucose level and decrease in liver glycogen level, are one of the first signs of stress and carbohydrate metabolism (Wepener, 1990). Stress response in fish is generally characterized by an increase in adrenalin causing mobilization of liver glycogen into blood glucose (Swallow and Flemming, 1970). Cortisol lowers the liver glycogen and increase in blood glucose during stress. Metabolic consequence of cortisol impairment may be a reduced capacity to mobilize liver glycogen stores (Hontela et al. 1995).

Carbohydrate metabolism mainly concerns to fulfill demands of animals by its aerobic and anaerobic segments (Nelson and Cox, 2002). The lactate levels acts as an index of anaerobiosis, which was beneficial for animal in tolerating hypoxic condition.

Under stress condition, with the increase of lactate content there was a decrease in pyruvate content, which suggests a shift towards anaerobiosis as a consequence of hypoxia, leading to respiratory distress (Sambasiva Rao, 1999). Blood lactic acid is

widely used as a biomarker in anoxia and pollutant stress (Srivastava and Singh, 1981).The increase in tissue lactate content is attributed to its involvement in osmoregulation (Sambasiva Rao, 1999).

There is no significant lactate accumulation in white muscle after slight hypoxia. According to Jorgensen & Mustafa (1980) significantly higher values of lactate in muscle are only registered after 21 hours of hypoxia in flounder *Platichtys flesus*. The other tissues and blood show a significant increase in lactate after up to moderate hypoxia and then a drop after severe hypoxia.

Increase in lactate after hypoxia denotes a increase in anaerobic metabolism as a source of energy. Lactate produced under hypoxia may be transferred to the blood and other tissues and even kept to be oxidized after return to normal conditions. The drop in rate of increase in lactate observed in severe hypoxia in all tissues except for muscle, may be due to aquatic surface respiration (ASR) that these fishes perform, specially after moderate hypoxia (Rantin & Kalinin, 1996; Rantin et al., 1998). Muscle and brain do not show variations between hypoxia and normoxia. Farrel & Steffensen (1987) estimated that blood lactate oxidation can fuel approximately 20% of cardiac aerobic metabolism at rest and 100% after exercise, which is consistent with findings of Milligan & Girard (1993), showing that blood lactate is a preferred substrate for cardiac muscle metabolism. This may indicate that, although a very small tissue, cardiac muscle has the potential to play a major role in the clearance of blood lactate.

In the present study on air-breathing catfishes *H. fossilis* an increase in [Hb] and [Hct] and decrease in MCHC in hypoxic conditions with mean values of [Hct] after moderate and severe exposure to hypoxia, suggested the possibility that oxygen carrying capacity of the blood might be enhanced by bringing more red blood cells into circulation (A. Kumar, 2017). These cells are most likely released from the spleen upon adrenergic and/or cholinergic stimulation (Nilsson and Grove, 1974). These hormones serve to increase the transfer of oxygen across the gills and the transport of oxygen, in the blood, to actively metabolizing tissues. During environmental hypoxia, catecholamines are mobilized into the blood when the arterial oxygen content significantly decreases (Perry and Reid, 1974). Evidence from teleost fish suggests that the release of red blood cells via splenic contraction does occur in response to elevated catecholamines (Nilsson et al.1975). Splenic contraction has been well characterized in fishes in response to hypoxia (Lai and Todd, 2006).

Extended holding of large mouthbass, *Micropterus salmoides* at low DO induced an improved ability to transport oxygen in blood relative to fish held at higher oxygen concentrations. Concentrations of both Hct and Hb were significantly higher in Micropterus salmoides held at low oxygen for 50 days relative to fish held at higher oxygen. Hct is the percentage of packed red blood cells relative to the whole volume of blood, but does not account for the size or number of erythrocytes. Hb is a quantification of the O_2 binding protein found in red cells, whereas MCHC is a measure of the Hb in a given volume of packed erythrocytes (Houston, 1990). Increases in Hct and/or Hb are typically caused by an increase in the production of erythrocytes, swelling of the erythrocytes, or a combination of both. These changes are typically a result of catecholamine releases that induce the release of erythrocytes from the spleen (Jensen et al. 1993), or acidosis in the blood, which alters the affinity of Hb to bind oxygen, and can stimulate an increase in erythrocytes (Wells, 2009).

Increases in Hb and Hct concentrations between the air-breathing and non air-breathing groups during an oxygen challenge may have been driven by the release of erythropoietin, the hormone responsible for synthesizing erythrocytes and releasing erythrocyte stores from the spleen. This is evidenced by the increase of erythrocytes numbers (i.e., increase in Hct and Hb) without increasing the amount of Hb per cell volume (i.e., no change in MCHC) in *Clarias batrachus* (Kumar 2018). This is only offered as a potential mechanism as erythropoietin was not quantified. Rainbow trout (*Oncorhynchus mykiss*) subjected to sustained hypoxia (maximum 216 h) had persistent increase in erythropoietin, as well as increased Hb levels (Lai *et. al.*2006), thereby providing an improved ability for oxygen uptake. Additionally, long-term exposure to hypoxia increases both Hb and Hct concentrations for numerous fish species, both air and water breathers (Scott and Rogers, 1981; Tun and Houston, 1986; Petersen and Petersen, 1990 and Timmerman and Chapman, 2004).

Conclusion

These changes typically confer an increase in oxygen-binding affinity or increased substrate for oxygen binding on the erythrocyte, improving performance of fish in low oxygen conditions. Despite Hct and Hb concentrations not differing between control treatments for these two groups, *H. fossilis* acclimated to a low oxygen environment were able to increase those hematological variables relative to the high oxygen group following a low oxygen challenge. It is likely that this increase in Hb and Hct provided an increase in performance during hypoxia, but additional work measuring blood gas concentration and/or Hb/O_2 affinity would be necessary to confirm this.

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