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Blood Metabolic and Hematological Adaptations in Catfish, *Heteropneustes fossilis* in the Conditions of Hypoxia

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Abstract

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; Protein; Haemoglobin; Glucose; Lactate.

Introduction

Hypoxia is a most important environmental phenomenon in the freshwater and sometimes in an area of coastal system in a 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) or may be caused by anthropogenic activities in and around the water bodies.

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.

There is a strong link between hypoxia and fish response that combines behavioural and physiological strategies which can mitigate the effect of exposure to hypoxia. It may limit the energy budget or scope of growth and activity of an organism. In general the responses may be manifested at three levels:

1. Physiological and morphological adjustments that improve the oxygen extraction and delivery to tissues (Jensen *et al.,* 1993; Sollid *et al.,* 2003)
2. Biochemical changes that increase the capacity of tissues to function and survive at low oxygen (Hochachka, 1980; Van den Thillart and Van Waarde, 1985; Hochachka and Somero, 2002).
3. Behavioural to avoid hypoxic areas, utilize well aerated micro environments or reduce activity (Kraemer, 1987; Van den Thillart *et al.,* 1994; Dalla via *et al.* 1998; Wannamaker and Rice; 2000)

Many studies have been conducted by submitting the organisms, especially fishes to hypoxia in order to study intermediary metabolic processes. The first responses of fishes to environmental hypoxia are always related to respiratory and circulatory changes. Respiratory adaptations are well documented for trout and carps (Jones *et al*., 1970).

Changes in enzyme profiles in response to hypoxia have been undertaken in different fishes, air breathing and water breathing both (Shouberidge & Hochachka 1983; Claireaux and Dutil 1992; Sebert *et al*., 1993; Almeida-Val *et al*., 1995). By reducing their metabolic rate during hypoxia fish delay the depletion of glycogen stores as well as the accumulation of toxic levels of lactate in the body.

The blood parameters studied include haemoglobin (Hb), Hematocrit value (Hct) and mean corpuscular hemoglobin concentration (MCHC) from whole blood and lactate and glucose from serum. These parameters have been investigated in many fishes exposed to hypoxia

(Tripathi *et al*., 2013; Muusze *et al*.1998)

Hematological parameters are considered as patho-physiological indicators and are closely related to the responses of fish to environmental and biological factors (Fernandes and Mores, 2003).

Review of Literature

The low oxygen is a major stress in the environment was inferred by the extensive researches of Jones (1952). Kutty (1968) and Bushnell *et al.,* (1984) investigated the effect of chronic hypoxia on fish swimming performance and metabolism. The effect of hypoxia on swimming activity of fishes was supported by Dahlberg *et al.,* (1968), Kutty (1968), Bushnell *et al.,* (1984). Dutil and co-workers (2007) investigated swimming performance of fishes during different periods of hypoxia. Greaney *et al.,* (1980); Taylor and Miller, (2001); Pichavant *et al.,* (2003) studied the effects of chronic (weeks of) hypoxia on oxygen carrying capacity.

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:

1. Either the rate of anaerobic ATP production increases (Pasteur effect) or
2. The ATP rate declines (metabolic depression).

Release of tissue specific enzyme into circulation and changes in haematological parameters have been observed in *Heteropneustes fossilis* when exposed to toxic environment (Bulow *et al.,* 1996).

Gluconeogenesis has been studied extensively in the liver and kidney in various fish species (Saurrez and Mommsen, 1987). The process was studied for the first time in an Indian air breathing fish *Clarias batrachus* by Tripathi and Co-workers (2013). These workers made extensive investigations on metabolic, behavioural and hematalogical responses of this fish to experimentally provoked hypoxia in the laboratory (Tripathi *et al.*, 2013; Kumar A. & Gopesh A. 2015; Kumar A. 2016; Kumar A. 2017).

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:

1. The mechanisms behind the observed effect of hypoxia and improved hypoxia tolerance.
2. If prolonged exposure to sub-lethal conditions can actively import coping mechanisms that are useful for the survival of these fishes.
3. How these stresses (Hypoxia, temperature, hypercapnea, starvation etc.) in the present references affect the fish species of different respiratory habits.

The present piece of work aims to analyze the response of blood and haematological parameters to different degrees of hypoxia in *Heteropneustes fossilis.*

Materials and Methods

Live specimens of *Heteropneustes fossilis (80-90 g 14-16 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 soybean powder. Feeding was stopped 48 h before the start of experiment. All the fishes 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-breath) for collection of different tissues. 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 temperature.

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.

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

Glucose content in different tissues of *Heteropneustes fossilis*

TABLE-1: 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 1). 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≤0.05%) were observed between normoxia and moderate hypoxia in heart, brain, muscle and blood (Fig. 1).

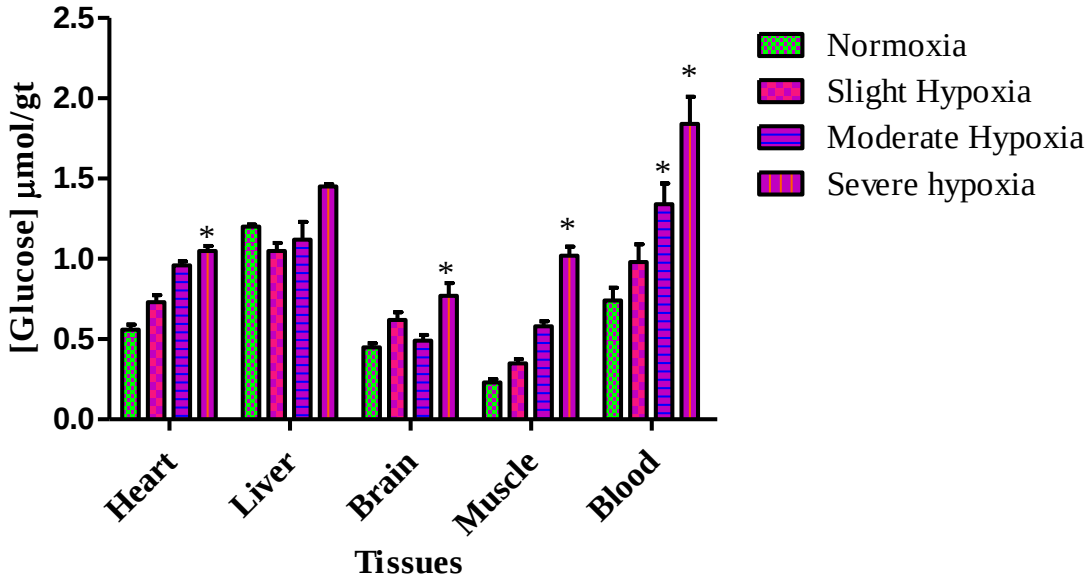

Figure-1: Glucose concentrations in different tissues of *Heteropneustes fossilis* submitted to normoxia and different periods of hypoxia. Error bars are within limits of symbols when not visible. Values are means ± SD, n = 6. * p< 0.05.

Lactate content in different tissues of *Heteropneustes fossilis*

Table-2: **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 2). 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%) andblood (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. 2).

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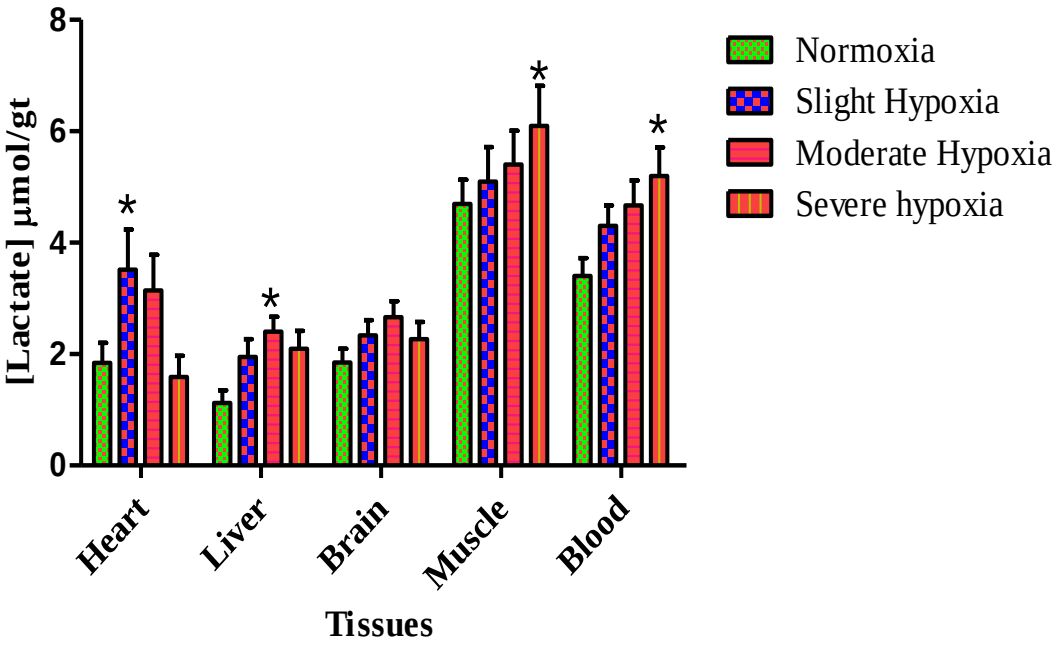


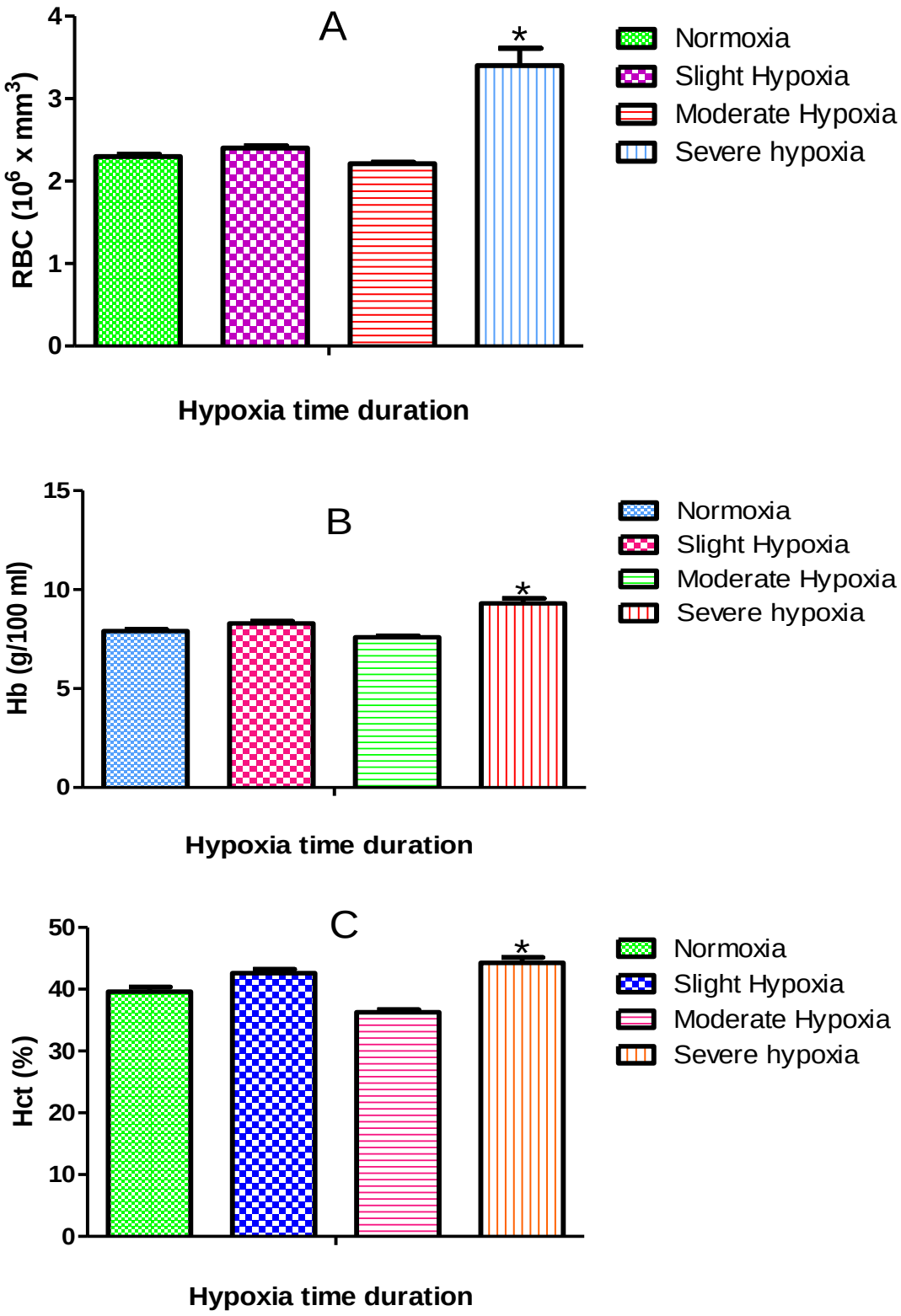
Figure-2: Lactate concentrations in different tissues of *Heteropneustes fossilis* submitted to normoxia and different periods of hypoxia. Error bars are within limits of symbols when not visible. Values are means ± SD, n = 6. * p< 0.05.

Haematological changes

Table-3: **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 3). 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. 3).


A
RBC (10^6 x mm^3)
0
1
2
3
4
*
Normoxia
Slight Hypoxia
Moderate Hypoxia
Severe hypoxia
Hypoxia time duration
B
Hb (g/100 ml)
0
5
10
15
*
Normoxia
Slight Hypoxia
Moderate Hypoxia
Severe hypoxia
Hypoxia time duration
C
Hct (%)
0
10
20
30
40
50
*
Normoxia
Slight Hypoxia
Moderate Hypoxia
Severe hypoxia
Hypoxia time duration

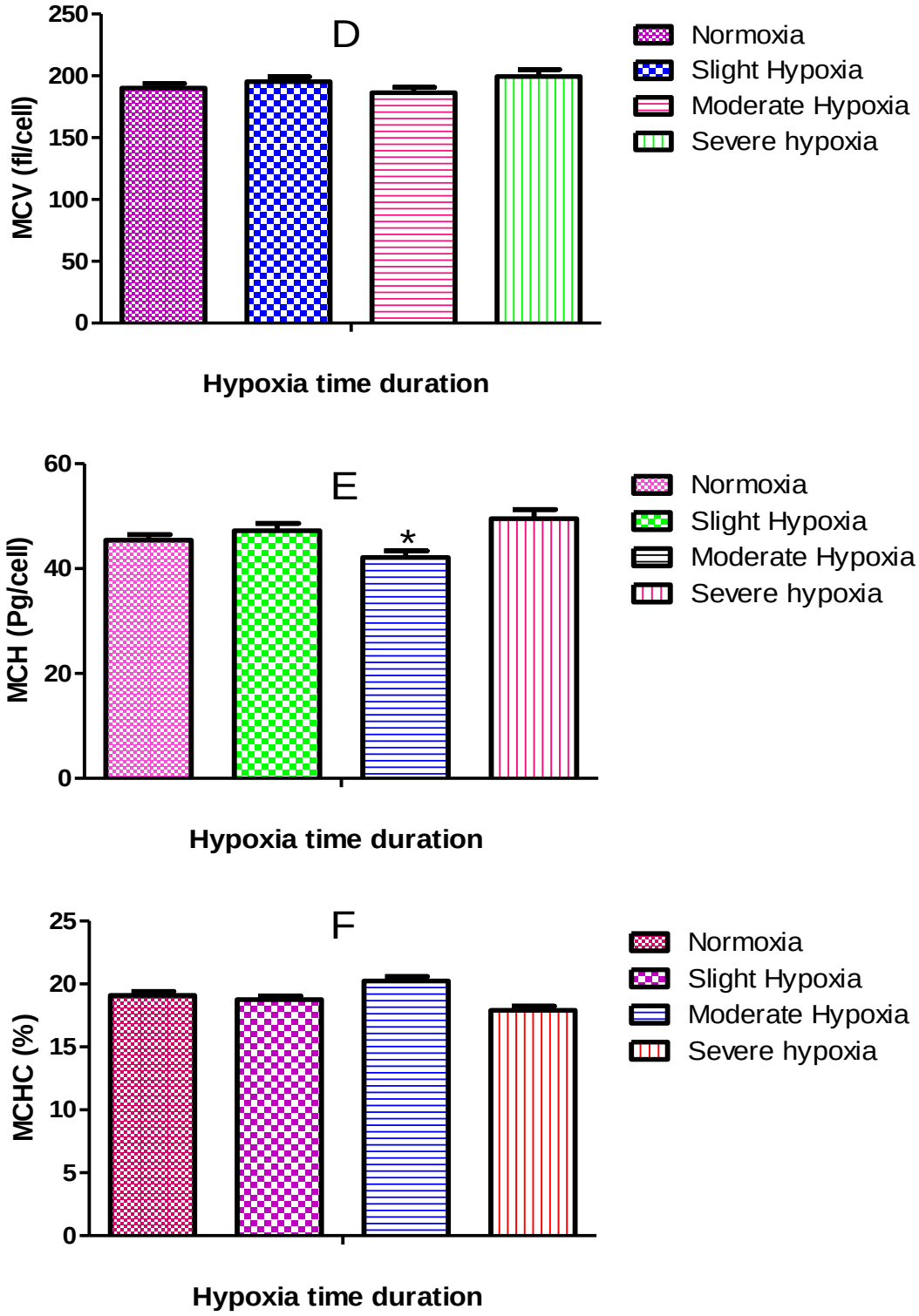


Figure-3: Haematological parameters in blood of *Heteropneustes fossilis* exposed to varying oxygen concentration i.e. different hypoxia stages for 12 hours duration.(A) RBCs (10^6×mm^3), (B) Hb (gm/100 ml), (C) Hct (per deciliter), (D) MCV (fl/cell), (E) MCH (Pg/cell) and (F) MCHC (gm/decilitre).Asterisk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

Discussion

In fish, increase in blood glucose and lactate level and decrease in liver glycogen level, are one of the first signs of stress of carbohydrate metabolism (Wepener, 1990). Stress response in fish is generally caused by an increase in adrenalin which results in mobilization of liver glycogen into blood glucose (Swallow and Flemming, 1970). Cortisol lowers the

liver glycogen and increase in blood glucose during stress conditions. Metabolic consequence of cortisol impairment may be a reduced capacity to mobilize liver glycogen stores (Hontela *et al*. 1995).

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). 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).

There is no significant lactate accumulation in white muscle after slight hypoxia. The other tissues and blood show a significant increase in lactate after moderate hypoxia and then a drop after severe hypoxia. Increase in lactate after hypoxia denotes an increase in anaerobic metabolism as a source of energy. Lactate produced under hypoxia may be transferred to the blood and other tissues such as heart 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, especially 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 during moderate and severe hypoxia.

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.

Glucose and lactate changes during hypoxia are showed in Fig 1-2 in different tissues of *Heteropneustes 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 moderate 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.

In the present study on air-breathing catfishes *Heteropneustes 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. 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).

These changes typically confer an increase in oxygen-binding affinity or increased substrata 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, *C. batrachus* and *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/O2 affinity would be necessary to confirm this (Kumar A. & Gopesh A. 2015; Kumar A. 2016; Kumar A. 2017; Kumar A. 2018; Kumar A. 2019; A. Kumar, A. Gopesh and S. Sundram 2020).

Conclusion

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

Increase in lactate after hypoxia denotes an 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, especially after moderate hypoxia. Muscle and brain do not show variations between hypoxia and normoxia.

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