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Comparative Account of Effects of Hypoxia and Metabolic Adjustments in Two Catfishes *Clarias batrachus* and *Heteropneustes fossilis*

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Abstract

In spite of several studies, not much attention has been directed towards the role of their enzymes in the metabolic adjustments to hypoxia and compare the same between fishes of two different respiratory habits. The present work aims to analyze the metabolic adjustment to different degrees of hypoxia in two catfishes which present different respiratory patterns and make a comparative study between the two.

Because of their rich evolutionary history and current ecological diversity of these specific groups of teleosts the study of the fishes of this group has been particularly informative in elucidating the responses of animals to hypoxia.

Keywords: Hypoxia; SDS-PAGE; LDH; MDH; Protein Bands.

Introduction

Throughout the world large areas of fresh and coastal waters are becoming polluted that lack sufficient oxygen, one of the basic building blocks of life. This condition is called “Hypoxia”. Hypoxia means “low oxygen” in aquatic ecosystems, low oxygen usually means a concentration of less than 2-3 mg of O_2/litre of water (Mg/l) in marine and 4-5 mg O_2/l in fresh water systems. These waters are usually acidic, rich in CO_2 and H_2S (Almeida-Val and Hochachka, 1995) but are observed to be varied in oxygen concentrations. Hypoxia is frequently accompanied by hypercapnia (elevation of CO_2 in water) acidification of the body tissue, including blood (Burnett and Stickle, 2001). It may be a 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 due to anthropogenic activities around the water bodies.

Review of Literature

Effect of oxygen deficiency on fish had drawn the attention of scientists as early as the 1920s and extensive literature is available on fish during that period (Gardner, 1926). Story of studies of adaptations of fish to low oxygen was extended by investigation undertaken in swamps (Carter and Beadle, 1931). 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. 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), Bushnell et al., (1984). 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 O2l-1).

Dunn & Hochachka (1986) and Dalla Via et al. (1998) 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) and

Pichavant et al., (2000) studied the effects of chronic (weeks of) hypoxia on food intake.

Aim of the Study

This study aims to analyze the comparative responses of aerobic and anaerobic enzyme activity and protein profiling to different degrees of hypoxia in two different catfishes, *Clarias batrachus* and *Heteropneustes fossilis*.

Materials and Methods

Live specimens (6 fishes) of *Clarias batrachus* and *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 soybean powder. Feeding was stopped 48 h before the start of the 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). Malate dehydrogenase (MDH; E.C. 1.1.1.37) activity was determined by conversion of oxaloacetate to malate (Somero and Childress 1980).

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 weight of the protein bands were determined with reference to standards (Genei Marker, PMW).

Observation

Lactate dehydrogenase (LDH) activity in *Clarias batrachus:*

Table-1: **Mean specific activity of Lactate dehydrogenase (LDH) enzyme (Units/mg proteins) in different tissues of *Clarias batrachus* subjected to slight, moderate and severe hypoxia for same time duration (12h)**

Tissue	Normoxia	Slight Hypoxia	Moderate Hypoxia	Severe Hypoxia
Heart	112.58±8.36	124.29±9.24	130.43±12.69	138.24±13.26
Liver	19.83±3.69	22.60±3.63	23.13±3.97	25.4±4.72
Brain	53.18±13.55	64.52±7.12	65.68±9.27	67.37±8.26
Muscle	245.55±27.62	263.32±25.37	307.23±23.68	318.57±31.34

Highest LDH activity was observed in muscle and lowest in the liver during normoxia (Table 1). During slight hypoxia the maximum increase in LDH activity was observed in the brain (21.32%) followed by the liver (13.96%) and heart (10.4%). During moderate hypoxia the maximum increase (21.32%) in LDH activity was observed in muscle (25.11%) followed by the brain (23.50%), liver (16.64%) and heart (15.85%). Maximum percentage increase in LDH activity was found in muscle (28.56%) followed by liver (28.08%) and brain (26.68%) during severe hypoxia. Significant changes (p≤0.05%) in LDH activities were observed between normoxia and severe hypoxia in muscle and heart (Fig 1).

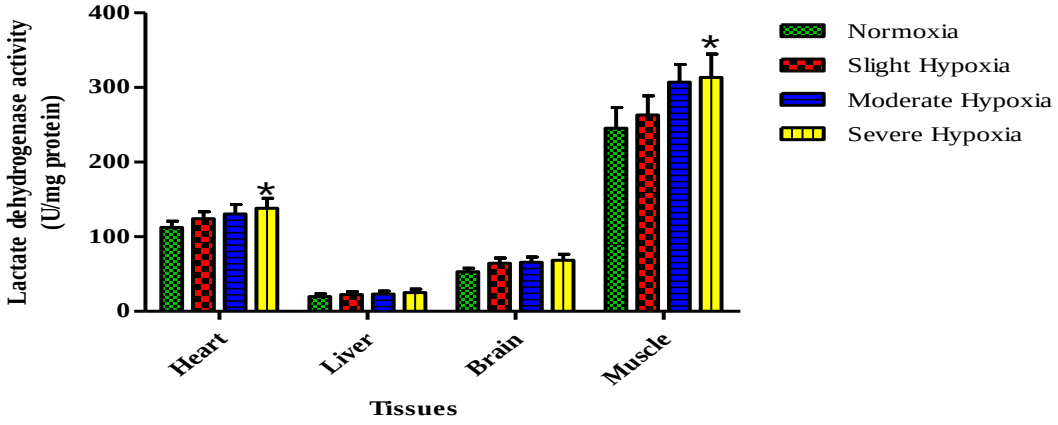

Figure-1: Mean specific activity of lactate dehydrogenase (LDH) enzyme (U/mg protein) in heart, liver, brain and muscle of *Clarias batrachus* exposed to varying oxygen concentration i.e. different hypoxia period for 12 hours duration. (U, µmole substrate/min; Values are means±s.e.m., n=6). Asterisk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

Lactate dehydrogenase (LDH) activity in *Heteropneustes fossilis*

Table-2: 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 the brain during normoxia (Table 2). Maximum increase in LDH activity was found in observed in the heart (26.59%) followed by the brain (26.26%) and muscle (24.86%). During moderate hypoxia the maximum increase in LDH activity was observed in the brain (52.10%) followed by muscle (49.41%), heart (45.91%) and liver (13.87%). Significant change (p≤0.05%) in LDH activities were muscle (64.70%) followed by heart (60.21%) and brain (58.33%) during severe hypoxia. During slight hypoxia the maximum increase in LDH activity was observed between normoxia and moderate and severe hypoxia in muscle and in heart it was found between normoxia and severe hypoxia (Fig. 2). No pronounced change was observed in LDH activity in the liver and brain during different time duration of hypoxia.

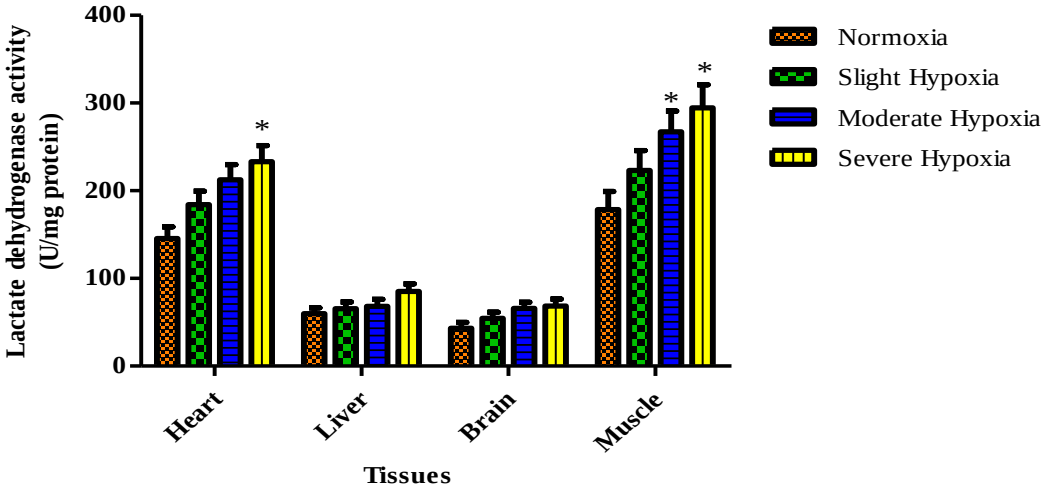

Figure-2: Mean specific activity of lactate dehydrogenase enzyme (U/mg protein) in heart, liver, brain and muscle of *Heteropneustes fossilis* exposed to varying oxygen concentration i.e. different hypoxia period for 12 hours duration. (U, µmole substrate/min; Values are means±s.e.m., n=6). Asterisk (*) represents significant differences (*p*<0.05) between normoxia and 72 hours of hypoxia.

Malate dehydrogenase (MDH) activity in *Clarias batrachus*

Table-3: Mean specific activity of Malate dehydrogenase (MDH) enzyme (Units/mg proteins) in different tissues of *Clarias batrachus* subjected to slight, moderate and severe hypoxia for same time duration (12h)

Tissue	Normoxia	Slight Hypoxia	Moderate Hypoxia	Severe Hypoxia
Heart	165.71±12.62	147.32±14.37	142.23±10.69	123.57±9.73
Liver	128.67±8.55	117.18±11.21	114.38±9.27	108.96±10.43
Brain	45.58±6.36	44.29±7.24	42.43±10.39	40.24±8.26
Muscle	40.83±9.69	38.60±10.63	37.13±9.97	41.26±6.21

Highest MDH activity was observed in the heart followed by liver and lowest in muscle during normoxia (Table 3). Maximum decrease in MDH activity was found in the heart (25.42%) during severe hypoxia. MDH activity was observed to be decreased in all the tissues suggesting that the aerobic metabolism during hypoxia is depressed. During slight hypoxia the maximum decrease in MDH activity was

observed in the heart (12.24%) followed by the liver (9.1%). No pronounced changes were observed in the brain muscle during this period. During moderate hypoxia a decrease in MDH activity was observed in the heart (14.16%) and liver (11.10%) followed by muscle (9.06%). MDH activity in different tissues did not show significant differences between normoxia and slight and moderate hypoxia. Significant changes (p≤0.05%) observed between normoxia and severe hypoxia in heart and liver (Fig. 3).

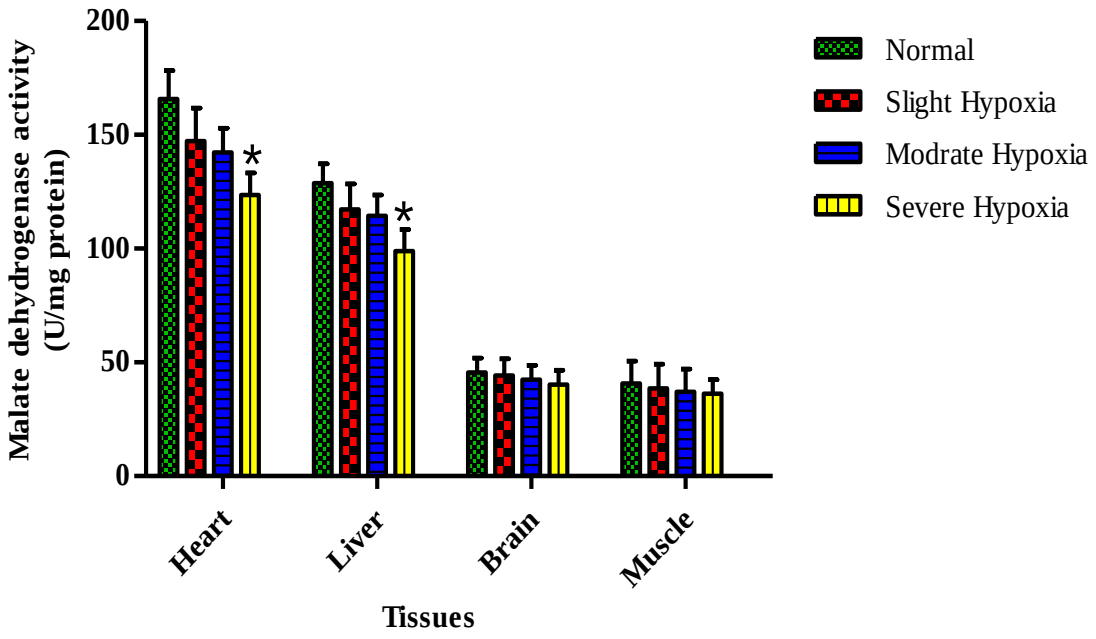

Figure-3: Mean specific activity of Malate dehydrogenase (MDH) enzyme (U/mg protein) in heart, liver, brain and muscle of *Clarias batrachus* exposed to varying oxygen concentration i.e. different hypoxia period for 12 hours duration. (U, µmole substrate/min; Values are means±s.e.m., n=6.) Asterisk (*) represents significant differences (p<0.05) between normoxia and severe hypoxia.

Malate dehydrogenase (MDH) activity in *Heteropneustes fossilis*

Table-4: Mean specific activity of malate dehydrogenase (MDH) 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	145.71±12.62	127.32±11.39	122.28±12.69	93.57±10.34
Liver	80.67±9.55	67.18±8.21	59.38±9.27	45.96±6.43
Brain	95.58±6.45	82.29±7.24	80.43±10.39	70.24±8.26
Muscle	50.83±9.69	43.60±10.63	41.13±9.97	35.26±6.27

Highest MDH activity was observed in the heart followed by the brain and lowest in muscle during normoxia (Table 4). During severe hypoxia the decrease in MDH activity was found to be highest in the liver (42.99%) followed by heart (35.78%) and muscle (28.92%). During slight hypoxia the maximum decrease in MDH activity was observed in the liver (16.72%) followed by muscle (14.17%). During moderate hypoxia the maximum decrease in MDH activity was also observed in the liver (26.39%) followed by muscle (19.09%). MDH activity in different tissues did not show significant differences between normoxia and slight and moderate hypoxia. Significant changes (p≤0.05%) observed between normoxia and severe hypoxia in heart, liver and muscle (Fig.4).

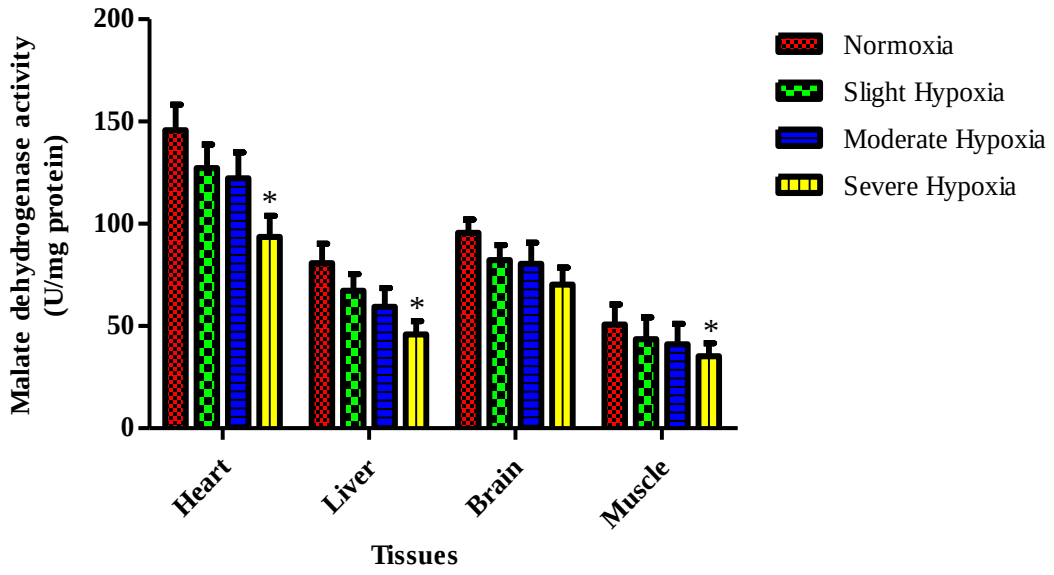

Figure-4: Mean specific activity of malate dehydrogenase enzyme (U/mg protein) in heart, liver, brain and muscle of *Heteropneustes fossilis* exposed to varying oxygen concentration i.e. different hypoxia period for 12 hours duration. (U, µmole substrate/min; Values are means±s.e.m., n=6). Asterisk (*) represents significant differences (p<0.05) between normoxia and different periods of hypoxia.

SDS-PAGE analysis in Clarias batrachus

Table-5: Molecular weight (kDa) of protein/peptide bands obtained from different tissues of *Clarias batrachus* subjected to hypoxia for same time duration (12h)

Lane 1 NH	Lane 2 NL	Lane 3 NB	Lane 4 NM	Lane 5 HH	Lane 6 HL	Lane 7 HB	Lane 8 HM
14.7	29.1	14.3	14.8	14.7	-	14.3	14.8
17.3	35.5	20.6	25.6	-	35.5	-	25..4
22.5	38.4	29.0	29.7	22.5	-	29.0	29.7
29.0	44.6	34.1	35.4	29.0	-	34.1	31.4
55.1	55.7	54.2	44.3	44.0	55.7	-	-
70.8	70.0	60.5	68.1	55.1	70.0	-	68.1
	72.4	70.4	72.2	70.8	72.4	-	72.2

Marker protein in lane-9 as shown in figure-5.NH-Normoxia Heart; NL- Normoxia Liver; NB- Normoxia Brain; NM-Normoxia Muscle.

HH-Hypoxia Heart; HL- Hypoxia Liver; HB- Hypoxia Brain; HM- Hypoxia Muscle.

In hypoxia heart 17.3kDprotein bands were absent and 44.0kD extra protein bands were found (Table 5). In hypoxia liver extra protein band of 72.4kD mol. wt. was present while 29.1kD, 38.4kD and 44.6kD mol. wt. proteins were absent. In hypoxia brain 20.6kD, 34.1kD, 54.2kD, 60.5kD and 70.4kD mol. wt. protein bands were absent while the extra protein band having mol. wt. 44kD was observed. In hypoxic muscle protein band of 44.3kD mol. wt. was absent (Fig.5).

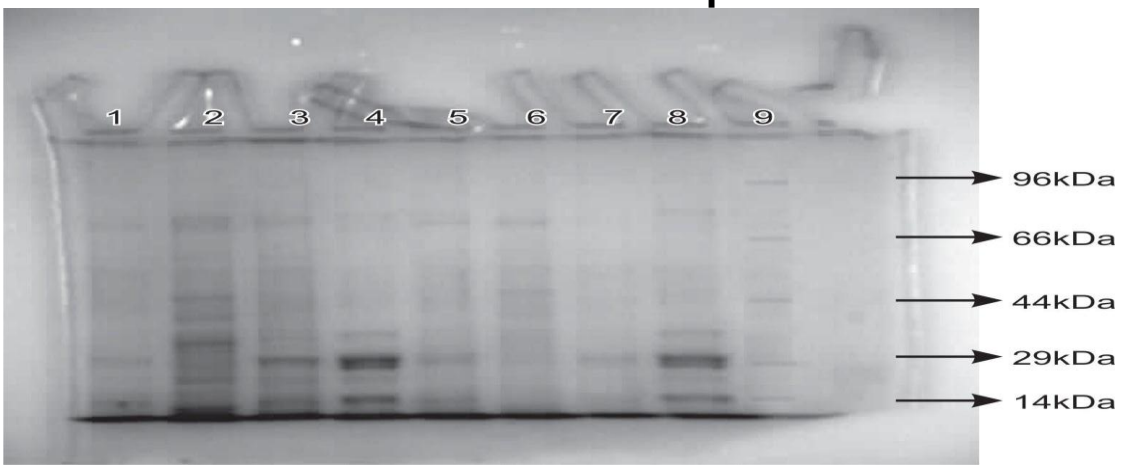

Figure-5: SDS-PAGE (Laemmli, 1970; 12% separating gel) profile of proteins of different tissues of *Clarias batrachus*. Lane 1: normoxia heart, lane 2: normoxia liver, lane 3: normoxia brain, lane 4: normoxia muscle, lane 5: hypoxia heart, lane 6: hypoxia liver, lane 7: hypoxia brain, lane 8: hypoxia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

SDS-PAGE analysis in *Heteropneustes fossilis*

Table-6: Molecular weight (kDa) of protein/peptide bands obtained from different tissues of *Heteropneustes fossilis* subjected to hypoxia for same time duration (12h)

Lane 1 NH	Lane 2 NL	Lane 3 NB	Lane 4 NM	Lane 5 HH	Lane 6 HL	Lane 7 HB	Lane 8 HM
14.7	14.1	14.3	14.8	14.7	14.1	20.7	14.8
20.3	20.8	29.6	29.7	20.3	20.8	29.5	29.4
25.5	29.4	36.0	35.4	25.5	29.4	32.6	-
29.0	36.1	40.1	44.3	29.0	45.8	40.1	-
35.1	55.7	45.2	55.1	-	55.7	45.2	55.1
66.8	-	55.5	66.9	-	58.4	55.5	66.9
		66.4			-	60.2	
		-				66.5	
		-				72.6	

Marker protein in lane-9 as shown in figure-5. NH- Normoxia Heart; NL- Normoxia Liver; NB- Normoxia Brain; NM- Normoxia Muscle; HH-Hypoxia Heart; HL- Hypoxia Liver; HB- Hypoxia Brain; HM- Hypoxia Muscle.

In hypoxia heart 35.1kD and 66.8kD protein bands were absent (Table 6). In hypoxia liver two extra protein bands of mol. wt. 45.8kD and 58.4kD were present while 36.1kDprotein band was absent. In hypoxia brain 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 hypoxia muscle extra protein bands having mol. wt. 35.4kD and 45.3kD were present (Fig. 6).

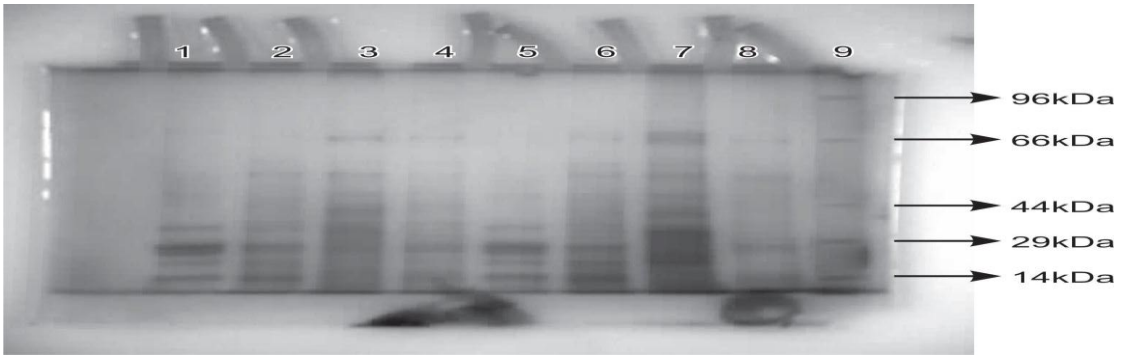

Figure-6: SDS-PAGE (Laemmli, 1970; 12% separating gel) profile of proteins of different tissues of *Heteropneustes fossilis*. Lane 1: normoxia heart, lane 2: normoxia liver, lane 3: normoxia brain, lane 4: normoxia muscle, lane 5: hypoxia heart, lane 6: hypoxia liver, lane 7: hypoxia brain, lane 8: hypoxia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

Discussion

Brain, liver and heart are known as aerobic tissues which normally tend to avoid anaerobic accumulation of lactate while muscle is known as anaerobic tissue. Therefore the LDH level is adjusted in these aerobic 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, A. Gopesh and S. Sundram 2020 and A. Kumar 2021).

The LDH level in *H. fossilis* shows more significant changes than the *C. barachus*. 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 2015; A. Kumar 2016; A. Kumar, A. Gopesh and S. Sundram 2020; A. Kumar 2021). In other studies with different degrees 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.

Increase in LDH activity 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, specially after moderate hypoxia (Rantin & Kalinin, 1996).

The specific activities of enzymes of glycolysis (LDH) and gluconeogenic (MDH) were found to be tissue specific and species specific too. Strongly suppressed by hypoxia, the white muscles reflected decreased energy demand of the tissue during sustained hypoxia. In contrast, several enzymes specific activities were higher in liver tissue after exposure to hypoxia, suggesting increased capacity for carbohydrate metabolism.

The activity of gluconeogenic enzymes (MDH) was observed to be lower in liver tissue in decreasing order in both the fishes. The decreased activity of this enzyme in the liver is known to be coupled with increased protein catabolism in skeletal muscle (Martinez *et al*., 2006).

Increased levels of glycolytic (LDH) enzymes in the muscles have been correlated with burst swimming capacity of fish (Somero and Childress, 1980; Pelletier *et al*., 1993). In white muscle anaerobic pathways support burst swimming activity (Almeida-Val *et al*., 2000). In case of air-breathing fish *Clarias batrachus* and *Heteropneustes fossilis,* it can be correlated with frequent movement of fish to the surface at the onset of hypoxia. The reduced level of LDH under the condition of sustained hypoxia can be attributed to the constant “surfacing behaviour” of the fish when negligible movement is observed.

Enzymes MDH is known to catalyse the reversible oxidation of malate to oxaloacetate requiring NAD^+ as a cofactor. Found both in cytoplasm and mitochondria, the two forms are recorded to play roles in the gluconeogenesis, lipogenesis, in malate-aspartate shuttle during aerobic glycolysis and in the Kreb’s cycle (Almeida-Val *et al*., 2000). Increase in MDH levels in the liver observed in the present investigation is suggestive of a role in increased glycogen synthesis as the liver is the known organ of gluconeogenesis. Its increased level in heart is also significant as the heart is an organ which depends on glucose as an important metabolic fuel. Its increased levels in the brain are probably due to an increase in oxidative powered capacity of this organ during the condition of long lasting stress.

In *C. batrachus*, a facultative air breather, the hypoxia was found to be associated with activation of anaerobic respiration in response to oxidative stress caused by hypoxia which was reflected by increased levels of LDH in muscle and liver and decrease in MDH levels in heart and liver after exposure to different durations of experimentally provoked hypoxia (A. Kumar 2015; A. Kumar 2016; A. Kumar, A. Gopesh and S. Sundram 2020; A. Kumar 2021). These physiological alterations are accepted to be correlated with its capacity to tolerate hypoxic conditions as observed earlier in *C. batrachus* (Tripathi *et al*, 2013).

In *Clarias batrachus* there are more protein bands found in the heart and liver than the brain and muscle during hypoxia which shows more metabolically active tissues. While in *Heteropneustes fossilis* there are less protein bands found in hypoxia heart and muscle tissue than the liver and brain during hypoxia. These results of protein metabolism of *Heteropneustes fossilis* in comparison to *Clarias batrachus* shows more metabolically activeness of the fish.

Conclusion

Because the different tissues of *Heteropneustes fossilis* has more active aerobic enzymes (MDH) and anaerobic enzymes (LDH) and also more metabolically active protein bands than the *Clarias batrachus* we can say that the *Heteropneustes fossilis* is more tolerant to graded hypoxia than the *Clarias batrachus.*

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