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Dr. Kaushlendra Kumar
Former Research, Department of Zoology, LNMU, Darbhanga, Bihar, India

Correspondence
Dr. Kaushlendra Kumar
Former Research, Department of Zoology, LNMU, Darbhanga, Bihar, India

Physicological studies to acute hypoxia in the air-breathing cat fish

Dr. Kaushlendra Kumar

Abstract
Hypoxia is an environmental stressor caused by normally large temporal and spatial variations in oxygen content of water and influences fish behaviour, survival, growth and reproduction. The main goal in this paper is to determine the mass specific metabolic rate (VO_2) of *C. batrachus* and to study the mechanism of adaptation to acute hypoxic periods.

Keywords: Physicological and air-breathing cat fish

Introduction
The antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) act to eliminate these ROS produced within the cell, while the processes of gene expression, apoptosis and signaling are affected by glutathione level within cell and tissue. The Species from the family of Clariidae are known for their air-breathing capabilities and their ability to survive the adverse conditions of frequent oscillations in oxygen content in their habitat, as they use air-breathing mechanisms to avoid hypoxia.
The Indian catfish, *Clarias batrachus*, commonly known as 'Mangur' is a freshwater air-breathing teleost species, endemic to the Indian subcontinent and has a fairly common distribution in freshwaters of the plains throughout India. It inhabits wetlands, swamps, rivers ponds and tanks, and is well adapted to adverse ecological conditions, such as dissolved oxygen changes in the same habitat during different seasons of the year [1-5]. *C. batrachus*, known to be hypoxiatolerant, is a facultative air breather at normoxia [6-9]. And it was hypothesized for the present study that the hypoxic conditions will be associated with activation of anaerobic respiration and oxidative stress in *C. batrachus*.

Materials and Methods
Superoxide dismutase (SOD) activity was determined in serum, liver, gill and muscle tissues. All tissue lysates were centrifuged at 1500g for 5 min at 4 °C, and the supernatant was incubated on ice until analyses. Only serum samples were diluted (1:5 times) for SOD activity assay. The activity of SOD was measured using colorimetric measurement Assay kit (Cat no. 706002, Cayman Chemicals Ltd, USA), following the manufacturers protocol. The assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine absorbing maximum at 460 nm, which was determined with Sun Rise A 5082 ELISA plate reader (TECAN, Salzburg, Austria). SOD activity was reported as units per milligram protein.

Statistical Analysis
There was a steady decline in mass-specific metabolic rate (VO_2) of *C. batrachus* as shown in figure 1 with decreasing DO concentration, where no point of sharp decrease in VO_2 with varying DO was observed.

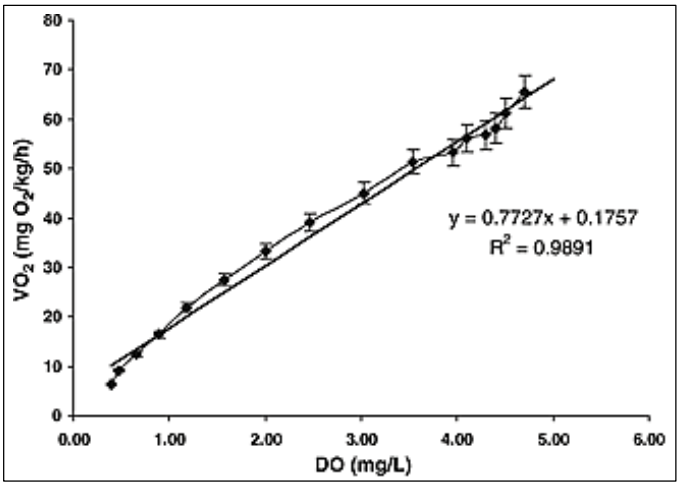

Fig 1: Representative graph showing VO_2 versus [O_2] curve for *Clarias batrachus* respiration with declining dissolved oxygen concentration. Values are expressed as mean ± standard deviation

When calculated from start of the experiment to 16 h or till suffocation of fish, VO_2 reduced from 65.6±3.52 mgO_2/kg/h at 4.8±0.1 mg/L (11.99±0.24 kPa) to 6.45±1.35 mgO_2/kg/h at 0.37±0.1 mg/L (0.87±0.24 kPa) at 25°C. The best fit curve to describe the relationship between VO_2 and DO was a straight line for pooled values with simple linear regression modeling (y00.7727x+0.175; R200.989).

Furthermore, there was no statistical support for justifying two lines in the plot (i.e. no breakpoint where a regulation pattern was superseded by a conforming pattern) that can permit us to calculate pCrit.

Hematological and blood metabolite levels

[Hct] and [Hb] were found to be significantly increased ($p<0.01$), with respect to normoxic control groups by 1.64- and 1.37-fold, respectively, following 12 h exposure at experimental hypoxic level, while MCHC significantly decreased by 1.20-fold ($p<0.05$) following 6 and 12 h of hypoxia exposure as shown in table 1.

Table 1: Quantitative estimation of hematocrit (Hct), hemoglobin (Hb) and mean corpuscular hemoglobin concentration (MCHC) in blood of *Clarias batrachus* subjected to progressive and acute hypoxia without access to air (at 0.98 mg/L≈18 mmHg) for different time intervals

Treatments	Time period at pCrit (h)	Hct (%)	Hb (gm%)	MCHC (gm%)
Normox		25.0±4.00[a]	9.83±0.55[a]	39.8±4.24[a]
PH	0	18.3±4.73[a]	8.43±1.10[a]	47.2±7.12[a]
H1	1	18.0±2.00[b] (–28.0%)	9.50±1.81[a]	53.5±9.81[c] (+34.4%)
H2	2	20.3±3.21[a]	7.93±1.31[b] (–17.3%)	39.0±1.92[a]
H3	3	22.0±3.61[a]	8.83±1.38[a]	40.2±0.78[a]
H6	6	32.2±0.76[b,c] (+28.8%)	10.6±0.35[c] (+8.0%)	32.9±0.42[b] (–17.2%)
H12	12	41.0±4.00[c] (+64.0%)	13.5±1.05[c] (+36.9%)	32.9±0.65[b] (–17.3%)

The blood metabolite levels suggested a particular trend under experimental hypoxia as shown in figure 2.

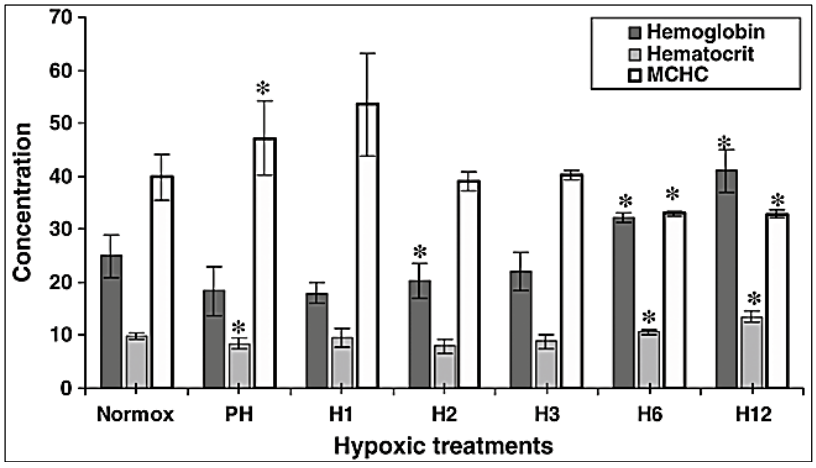

Fig 2: Hematological parameters of *Clarias batrachus*, mean hemoglobin concentration (gram per deciliter), mean hematocrit concentration (per deciliter) and mean cell hemoglobin concentration (gram per deciliter) in normoxia (Normox), progressive hypoxia (PH) and after 1 h (H1), 2 h (H2), 3 h (H3), 6 h (H6) and 12 h (H12) at 0.98±0.1 mg/L, dissolved oxygen. Mean±standard deviation
Asterisk (*) represents significant differences ($p<0.05$) between normoxia and acute hypoxia

Table 2: Quantitative estimation of glucose and lactate concentration in serum of *Clarias batrachus* subjected to progressive and acute hypoxia without access to air (at 0.98 mg/L≈18 mmHg) for different time intervals

Treatments	Serum glucose (mg%)	Serum lactate (mg%
Normox	54.4±7.63[a]	46.0±0.27[b]
PH	143±23.4[b,c] (+)	129±0.25[c] (+)
H1	123±33.3[b,c] (+)	89.0±0.29[b]
H2	187±73.6[c,d] (+)	72.0±0.35[b]
H3	58.3±10.7[a]	56.0±0.10[b]
H6	97.3±25.7[b] (+)	18.0±0.33[a] (–)
H12	55.3±9.29[a]	46.3±0.35[b]

Significant increase in serum glucose levels were observed for group PH (2.63-fold) and after 1 h (2.26-fold; $p<0.01$), 2 h (3.44-fold; $p<0.001$) and 6 h (1.79-fold; $p<0.05$) as shown in table 2 at experimental hypoxic level as shown in time figure 3.

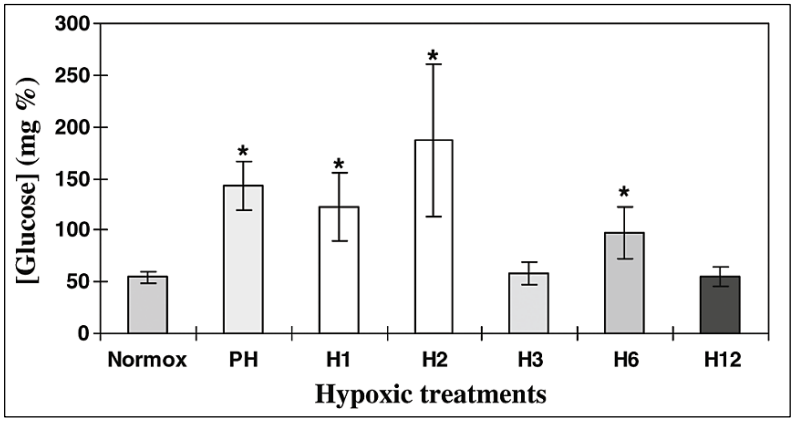

Fig 3: Mean serum glucose concentration (milligram per deciliter) of *Clarias batrachus*, under normoxia (Normox), progressive hypoxia (PH) and 1 h (H1), 2 h (H2), 3 h (H3), 6 h (H6) and 12 h (H12) at 0.98±0.1 mg/L, dissolved oxygen. Mean±standard deviation. Asterisk (*) represents significant differences ($p<0.05$) between normoxia and acute hypoxia.

Serum lactate concentration initially increased significantly (2.80-fold; $p<0.05$) following PH and 1 h of hypoxia, while it decrease at 6 h (2.56-fold; $p<0.05$) and stabilized at its initial level at 12 h at experimental hypoxic level as shown in figure 4.

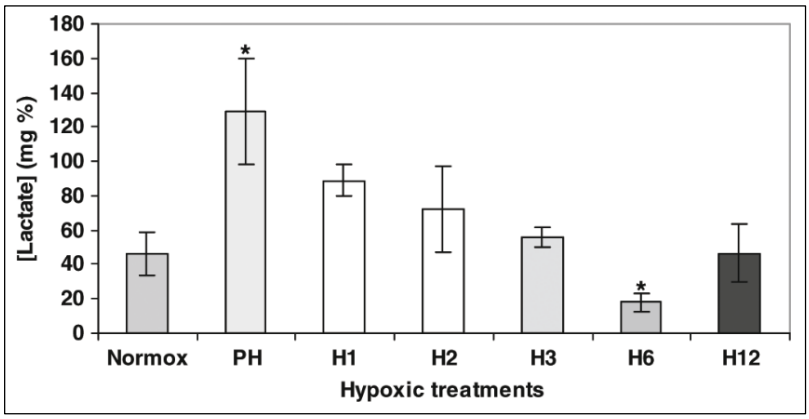

Fig 4: Mean serum lactate concentration (milligram per deciliter) of *Clarias batrachus*, under normoxia (Normox), progressive hypoxia (PH) and 1 h (H1), 2 h (H2), 3 h (H3), 6 h (H6) and 12 h (H12) at 0.98±0.1 mg/, dissolved oxygen. Mean±standard deviation. Asterisk (*) represents significant differences ($p<0.05$) between normoxia and acute hypoxia.

Lactate dehydrogenase activity

LDH activity ranged from 9.63 to 104 U/mg protein in the gill and from 12.9 to 27.8 U/mg protein in the liver as shown in table 3.

Table 3: Mean specific activity of lactate dehydrogenase (LDH) enzyme in different tissues of *Clarias batrachus* subjected to progressive and acute hypoxia without access to air (at 0.98 mg/L≈18 mmHg) for different time intervals

	Lactate dehydrogenase enzyme (Units/mg protein)			
Treatments	Liver	Muscle	Gill	Serum
Normox	12.9±5.30[a]	11.8±1.00[a]	22.3±14.0[a,b]	31.2±1.39[a,b]
PH	18.5±5.06[a,b]	13.8±0.43[a]	104±6.28[d] (+)	37.9±7.67[b]
H1	16.1±7.68[a,b]	13.7±1.37[a]	11.4±3.42[a]	36.3±1.98[a,b]
H2	24.6±1.59[a,b]	16.8±3.34[a]	45.8±18.8[b,c]	28.8±2.39[a,b]
H3	21.2±3.35[a,b]	20.6±5.23[a]	18.9±4.53[a,b]	37.3±1.99[a,b]
H6	14.8±1.40[a]	16.7±1.46[a]	9.63±5.45[a]	27.7±3.61[a]
H12	27.8±0.57[b] (+)	19.8±7.23[a]	75.0±18.8[c,d] (+)	30.7±2.32[a,b]

It was significantly higher in gill tissue at PH (4.66-fold) and 12 h (3.36-fold) and in liver at 12 h (2.15-fold) of hypoxia as shown in figure 5.

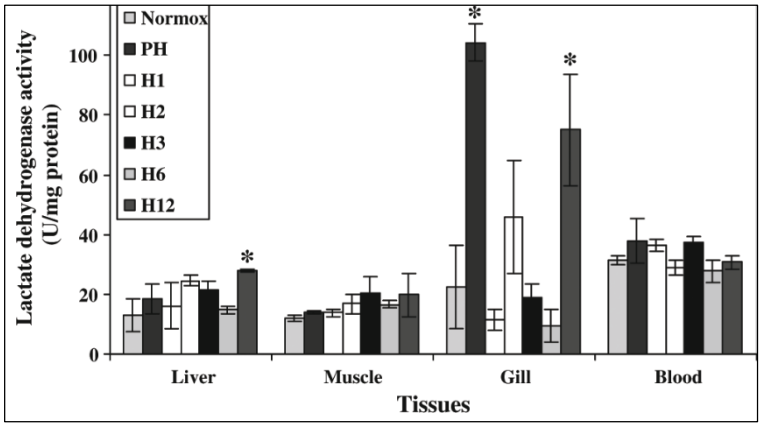

Fig 5: Mean specific activity of lactate dehydrogenase enzyme (U/mg protein) in liver, muscle, gill, and blood of *Clarias batrachus* in normoxia (Normox), progressive hypoxia (PH) and after 1 h (H1), 2 h (H2), 3 h (H3), 6 h (H6) and 12 h (H12) at 0.98±0.1 mg/L, dissolved oxygen. Mean ± standard deviation. Asterisk (*) represents significant differences ($p<0.05$) between normoxia and acute hypoxia

There was no significant change observed in other tissues.

Total glutathione

Total glutathione concentration ranged from 138 to 204 µM/mg protein in liver tissue, 4.66 to 69.6 µM/mg protein in muscle and 34.7 to 103 µMNmg protein in gill tissue as shown in table 4.

Table 4: Total glutathione (GSH) concentration in different tissues of Clarias batrachus subjected to progressive and acute hypoxia without access to air (at 0.98 mg/L≈18 mmHg) for different time intervals

	Total GSH (µM/mg protein)			
Treatments	Liver	Muscle	Gill	Blood
Normox	196±43.4[a]	11.7±4.98[a]	72.9±34.4[a]	bd
PH	274±72.3[a]	6.58±4.18[a,b]	204.4±121.5[a]	bd
H1	138±38.2[a]	4.98±1.36[a]	34.7±6.54[a]	bd
H2	205±36.7[a]	7.78±5.28[a,b]	79.3±22.9[a]	bd
H3	159±28.5[a]	4.66±0.32[a]	87.5±22.8[a]	bd
H6	162±45.7[a]	46.9±9.59[a,b]	42.3±6.80[a]	bd
H12	252±66.1[a]	69.6±11.63[b] (+)	103±15.1[a]	bd

Total glutathione level did not change significantly in any tissue, except for muscle, where a significant increase (5.95-fold; $p<0.05$) was observed at 12 h of hypoxia as shown in figure 6, in comparison to normoxia. In serum, glutathione activity was below detection limit.

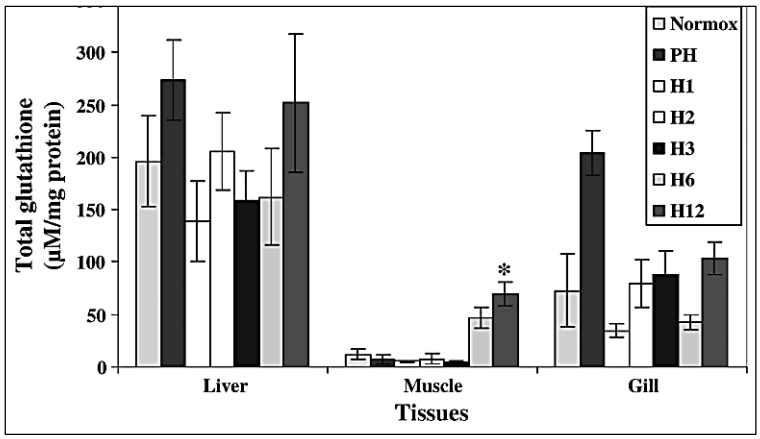

Fig 6: Total glutathione concentration (µM/mg of protein) in liver, muscle and gill of *Clarias batrachus* in normoxia (Normox), progressive hypoxia (PH) and after 1 h (H1), 2 h (H2), 3 h (H3), 6 h (H6) and 12 h (H12) at 0.98±0.1 mg/L, dissolved oxygen. Values are expressed as mean±standard deviation. Asterisk (*) represents significant differences ($p<0.05$) between normoxia and acute hypoxia.

Superoxide dismutase activity

SOD activity ranged from 0.18 to 0.92 U/mg protein in liver, 0.99 to 2.97 U/mg protein in muscle, 0.14 to 3.26 U/mg Mean specific activity values of LDH, are mean±SD, n=3; statistical differences among treatments are indicated by dissimilar letters. Whereas (+) represents significant increase in experimental hypoxia as compared to normoxia. Superscripts a, b, c, d indicate homogenous subsets (Tukey's post hoc test). protein in gill and 0.14 to 0.65 U/mL in serum as shown in table 5.

Table 5: Mean specific activity of superoxide dismutase (SOD) enzyme in different tissues of *Clarias batrachus* subjected to progressive and acute hypoxia without access to air (at 0.98 mg/L≈18 mmHg) for different time intervals

	SOD activity (Units/mg protein)			
Treatments	Liver	Muscle	Gill	Blood
Normox	0.92 ± 0.32^{b}	0.99 ± 0.39^{a}	$0.53\pm0.19^{a,b}$	$0.43\pm0.14^{b,c}$
PH	0.24 ± 0.15^{a} (–)	1.16 ± 0.84^{a}	3.26 ± 1.15^{c} (+)	0.65 ± 0.14^{c}
H1	0.18 ± 0.07^{a} (–)	2.13 ± 0.32^{a}	0.14 ± 0.07^{a}	0.14 ± 0.03^{a} (–)
H2	0.37 ± 0.17^{a} (–)	2.00 ± 0.19^{a}	$0.89\pm0.12^{a,b,c}$	0.16 ± 0.01^{a} (–)
H3	$0.54\pm0.15^{a,b}$	2.97 ± 1.37^{a}	$0.97\pm0.19^{a,b,c}$	0.18 ± 0.03^{a} (–)
H6	0.19 ± 0.05^{a} (–)	2.15 ± 0.89^{a}	$1.02\pm0.02^{b,c}$	0.20 ± 0.04^{a} (–)
H12	0.57 ± 0.21^{a} (–)	2.32 ± 0.88^{a}	$1.43\pm0.30^{b,c}$	$0.32\pm0.14^{a,b}$

After PH, the highest mean SOD activity was observed in gill tissue, which was significantly increased (6.15-fold; $p<0.05$), while it was significantly decreased ($p<0.05$) in liver (PH; 3.84-fold, 1 h; 5.13-fold, 2 h; 2.49-fold, 6 h; 4.85-fold and 12 h; 1.61-fold) and serum (1 h; 3.07-fold, 2 h; 2.69-fold, 3 h; 2.39-fold and 6 h; 2.15-fold), as compared to normoxic control groups (figure 7). There was no difference in muscle SOD activity at any of the time interval.

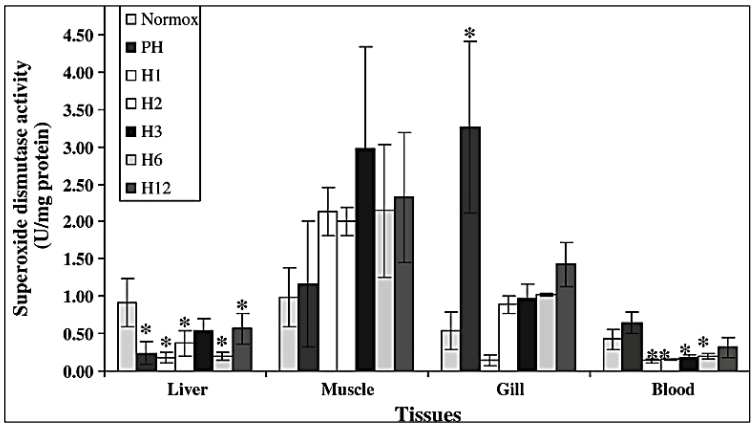

Fig 7: Mean specific activity of superoxide dismutase enzyme (Units/mg protein) in liver, muscle, gill, and blood of *Clarias batrachus* in normoxia (Normox), progressive hypoxia (PH) and after 1 h (H1), 2 h (H2), 3 h (H3), 6 h (H6) and 12 h (H12) at 0.98±0.1 mg/L, dissolved oxygen. Mean ± standard deviation. Asterisk (*) represents significant differences ($p<0.05$) between normoxia and acute hypoxia

Catalase activity

CAT activity ranged from 10.9 to 55.9 U/mg protein in serum, 111 to 303 U/mg protein in gill, 25.4 to 69.2 U/mg protein in muscle and 419 to 559 U/mg protein liver as shown in table 6.

Table 6: Mean specific activity of catalase (CAT) enzyme in different tissues of *Clarias batrachus* subjected to progressive and acute hypoxia without access to air (at 0.98 mg/L≈18 mmHg) for different time intervals

Treatments	Catalase activity (Units/mg protein)			
	Liver	Muscle	Gill	Blood
Normox	390±26.6^{a,b}	26.2±4.40^{a}	157±79.4^{a,b}	20.7±2.41^{a,b,c}
PH	443±32.8^{b}	25.4±4.12^{a}	111±48.6^{a}	10.9±2.98^{a}
H1	421±44.7^{a,b}	28.6±7.08^{a}	194±34.0^{b,c}	23.0±4.48^{a,b}
H2	419±12.4^{a}	36.4±5.34^{a}	142±51.1^{a}	30.3±17.3^{c,d}
H3	452±59.2^{a,b}	49.9±9.17^{a}	303±48.1^{d} (+)	33.2±4.48^{b,c,d}
H6	559±15.7^{b}	69.2±10.0^{b} (+)	257±32.0^{c,d} (+)	55.9±28.7^{e} (+)
H12	423±86.1^{b}	53.9±25.3^{a}	217±28.1^{c,d} (+)	33.2±15.7^{c,d}

Significant increase ($p<0.05$) in catalase activity was detected in gill (3 h; 1.93-fold, 6 h; 1.64-fold and 12 h; 1.38-fold), muscle (6 h; 2.64-fold) and serum (6 h; 2.70-fold) as shown in figure 8.

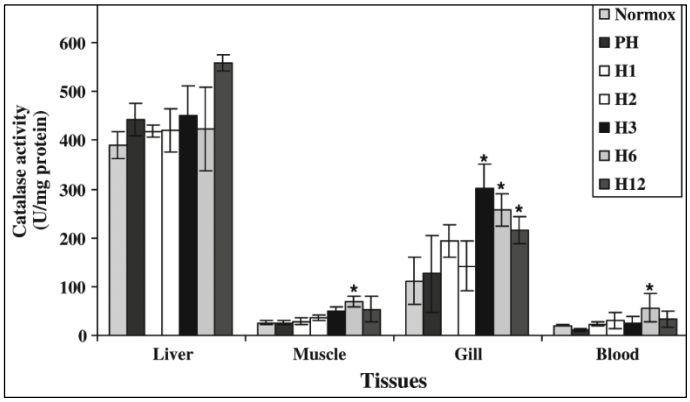

Fig 8: Mean specific activity of catalase enzyme (U/mg protein) in liver, muscle, gill, and blood of *Clarias batrachus* in normoxia (Normox), progressive hypoxia (PH) and after 1 h (H1), 2 h (H2), 3 h (H3), 6 h (H6) and 12 h (H12) at 0.98±0.1 mg/L, dissolved oxygen. Mean±standard deviation. Asterisk (*) represents significant differences ($p<0.05$) between normoxia and acute hypoxia.

Results and Discussion

In the present study, it was observed that in the facultative air-breathing catfish, *C. batrachus*, oxygen consumption rate decreased linearly with decrease in environmental oxygen, and it was not able to regulate oxygen consumption in hypoxic conditions, thus indicating that *C. batrachus* can be classified as an oxyconformer. In previous reports, oxyconforming response to lowering oxygen levels have been reported in fishes like Galaxias maculatus and *Acipenser naccarii* as well as in a sea worm, *Sipunculus nudus*.

Conclusion

On the basis of the results presented here, it was found that the *Clarias batrachus*, an air-breathing fish, is an oxyconformer, and the exposure of fish to different periods of experimental hypoxia resulted in adjustment of oxygen carrying capacity, metabolic depression and antioxidant defense system. These physiological alterations might be correlated with its capacity to tolerate hypoxic conditions. Although these broad outlines of adaptation for hypoxic survival are recognized through this study, understanding of signals involved in these interrelated processes needs to be further explored.

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