CHANGES IN LACTATE DEHYDROGENASE AND MALATE DEHYDROGENASE ACTIVITIES DURING HYPOXIA AND AFTER TEMPERATURE ACCLIMATION IN THE ARMORED FISH, *Rhinelepis strigosa* (SILURIFORMES, LORICARIIDAE)

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(With 1 figure)

ABSTRACT

Lactate (LDH) and malate dehydrogenase (MDH) of white skeletal muscle of fishes acclimated to 20, 25 and 30°C and thereafter submitted to hypoxia were studied in different substrate concentrations. Significant differences for LDH and MDH of white muscle enzyme activities are described here for the first time in *Rhinelepis strigosa* of fishes acclimated to 20°C and submitted to hypoxia for six hours. LDH presented a significant decrease in enzyme affinity for pyruvate in acute hypoxia, for fishes acclimated to 20°C and an increase for fishes acclimated to 30°C.

Key words: hypoxia, acclimation temperature, lactate dehydrogenase, malate dehydrogenase, armored fish, *Rhinelepis strigosa*.

RESUMO

Mudanças na atividade da lactato desidrogenase e malato desidrogenase durante hipóxia e após aclimatação a diferentes temperaturas no cascudo, *Rhinelepis strigosa* (Siluriformes, Loricariidae)

Foram estudadas a lactato desidrogenase (LDH) e malato desidrogenase (MDH) de músculo branco de peixes aclimatados a 20, 25 e 30°C em diferentes concentrações de substrato e submetidos à hipóxia. Diferenças significativas em atividade enzimática para LDH e MDH são descritas aqui pela primeira vez em *Rhinelepis strigosa* em peixes aclimatados a 20°C e submetidos à hipóxia por seis horas. A LDH apresentou uma diminuição significativa na afinidade enzimática ao piruvato em hipóxia severa de peixes aclimatados a 20°C e um aumento significativo na afinidade enzimática ao piruvato em peixes aclimatados a 30°C.

Palavras-chave: hipóxia, aclimatação à temperatura, lactato desidrogenase, malato desidrogenase, cascudo, *Rhinelepis strigosa*.

INTRODUCTION

The first responses of fishes to environmental hypoxia are related to respiratory and circulatory changes. Many studies have been conducted by submitting organisms to hypoxia in order to study intermediary metabolites and enzymes (Shoubridge & Hochachka, 1983; Claireaux & Dutil, 1992; Sébert *et al*., 1993; Almeida-Val *et al*., 1995) but none of them focused on the effects of acute hypoxia on enzymes of fish acclimated to different temperatures. Hochachka & Somero (1973, 1984) proposed that ectothermic organisms, particularly fish, use adaptive bioche-

mical strategies to obtain metabolic homeostasis during oscillations in dissolved oxygen, in temperature and in some other water physico-chemical parameters. Studies on exposure of fishes acclimated to different dissolved oxygen concentrations did not give a single answer for enzyme responses (Shaklee *et al*., 1977; Almeida-Val & Hochachka, 1993; Almeida-Val *et al*., 1995). There is an extensive background of work in general and specific properties of lactate dehydrogenases (LDH) (Wilson 1977; Graves & Somero, 1982; Panepucci *et al*., 1984, 1987; Coppes & Somero, 1990) and in the soluble form of malate dehydrogenases (cMDH) (Shaklee *et al*., 1977; Schwantes & Schwantes, 1982a, b; Farias & Almeida-Val, 1992; Lin & Somero, 1995a, b). Lactate dehydrogenase (LDH, lactate; NAD-oxidoredutase, EC 1.1.1.27) is among the most extensively studied glycolitic enzyme. In fishes it is usually encoded by three loci, one expressed principally in skeletal muscle (LDH-A), another in heart muscle (LDH-B) and a third one in the eye (LDH-C). Malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) catalyzes the reversible oxidation of malate to oxalacetate requiring NAD^+ as a cofactor. It is involved in gluconeogenesis and lipogenesis, and in the malate-aspartate shuttle during aerobic glycolysis. The mitochondrial form (mMDH) acts in the Krebs cycle (Zink & Shaw, 1968). The present work aimed at understanding how fish enzymes respond to acute hypoxia at different acclimation temperatures.

MATERIAL AND METHODS

The armored fish, *Rhinelepis strigosa*, a facultative air-breather, found in the Mogi-Guaçu River basin, Brazil, is a stenothermal, detritivore-herbivore sedentary fish with moderate economic importance. The habitat temperature in the Mogi-Guaçu River varies from 20 to 30°C during the year. Low temperatures occur only within a short period (June and July) and high temperatures in the middle of summer (January and February). Adult fishes, "Cascudos pretos", *Rhinelepis strigosa*, (wt ≅ 200 g) were net fished in the Mogi-Guaçu River, São Paulo State, Brazil. Fish were kept for at least 30 days at acclimation temperatures of 20, 25 and 30°C ± 1°C in 250 L tanks with water circulation and continuous aeration (P_{WO2} > 130 mm Hg). The tanks were illuminated with natural light and fish fed on lettuce and aquatic plants "ad libitum". Feeding was stopped 24 hours before experiments. After acclimation to the experimental temperatures, six fishes were placed in a special aquarium for 24 hours with proper aeration (P_{WO2} > 130 mm Hg). Oxygen tensions of inlet and outlet water were measured continuously by O_2 electrodes connected to a O_2 analyzer. The water oxygen tensions (Po_2) inside the experimental chamber were gradually decreased until critical oxygen tensions were reached as already determined by Fernandes *et al*. (1995) and Fernandes (personal comunication) and kept at stable levels by bubbling N_2. Fishes were kept in hypoxia during 6 h, then killed with a blow to the head. Tissues were excised and saved frozen at –20°C until needed for use.

Enzyme preparation and assay of LDH and MDH activity

White muscle, heart and brain tissues from fishes acclimated to 20, 25, and 30°C, were weighed and homogenized at ice-temperature with a 9-fold volume of Imidazol 5 mM, KCN 1 mM, pH 7.4 (at 25 °C) buffer. The homogenate was centrifuged at 17,000 g at 5°C for 30 min. The supernatant was used directly as an LDH and MDH source in the kinetic study. LDH and MDH activities were determined by following the oxidation of NADH at 340 nm in a circulating thermobath at 25°C. The reaction mixture was contained in a total volume of 1 ml, 50 mM Imidazol, 1 mM KCN buffer pH 7.4 at 25°C, 0.13 mM of NADH and different concentrations of pyruvate for LDH saturation plots. Substrate saturation plots for oxalacetate were determined for MDH by following the oxidation of NADH at 340 nm. The reaction mixture was contained in a total volume of 1 ml, 50 mM Imidazol, 1 mM KCN, 100 mM KCl buffer pH 7.2 at 25°C, 0.12 mM of NADH and different concentrations of oxalacetate. NADH saturation plots were determined for MDH activity with 0.3 mM oxalacetate and different concentrations of NADH. For obtaining K_M values, mathematical analyses using the Michaelis-Menten model were used with the aid of a computer program, Origin version 4.1. Activity of enzymes were expressed as U/gwt (Unit per gram of wet tissue). One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmole of substrate per minute at 25°C. Non-parametric Mann-Whitney test was used to

estimate differences between experiments with fishes submitted to both hypoxic and normoxic conditions. Rates of MDH/LDH activity were calculated in concentrations of 0.3 mM oxalacetate for MDH and in 1 mM pyruvate for LDH and 0.13 mM NADH for white muscle, heart muscle and brain tissue. Low and high ratios of LDH activity (L/H) were calculated in 1 mM and 10 mM pyruvate respectively for white muscle tissues.

RESULTS AND DISCUSSION

Experiments on fishes submitted to hypoxia showed significant differences in enzyme activity from fishes in normoxia at 20°C. LDH pyruvate saturation plots of white muscle showed significant differences ($P < 0.05$) between hypoxia and normoxia (Fig. 1a). MDH oxalacetate saturation plots of white muscle submitted to hypoxia also showed significant differences $P < 0.01$) in all substrate concentrations from fishes in normoxia (Fig. 1b). MDH saturation plots of white muscle submitted to hypoxia using NADH as a substrate showed significant differences ($P < 0.01$) in all substrate concentrations (Fig. 1c). The fact that MDH using oxalacetate as a substrate and MDH using NADH as a substrate differed in normoxia and hypoxia may reflect its dual role in both aerobic and anaerobic energy metabolism at low temperature in this case, as pointed out by Hochachka & Somero (1984).

Table 1 shows K_M values for hypoxia and normoxia from the above experiments for all temperatures. Except for LDH of fishes acclimated to 20 and 30°C, K_Ms did not reveal significant differences between fishes submitted to hypoxia. Table 2 shows enzyme activities for muscle, heart and brain tissues in normoxia and hypoxia. Significant differences between normoxia and hypoxia were found for white muscle LDH and $MDH_{[OAA]}$, for fishes acclimated to 20°C and, also, for heart muscle $MDH_{[OAA]}$ of fishes acclimated to 25°C. Brain tissue did not show significant differences for enzymes tested. In fishes acclimated to 25°C significantly higher values during hypoxia suggest that MDH has a role in redox regulation during hypoxic stress. Table 3 shows the ratios of MDH/LDH activity which demonstrate the oxidative capacity of the tissues at all temperatures of acclimation (high rates denote high oxidative capacity). These ratios are extremely high (up to 280 times higher than white muscle) for heart muscle of fishes acclimated to all temperatures, showing the importance of this organ for the survival of fish in critical hypoxia situations and at the extreme temperatures found in their habitat. Brain tissue also presented a high ratio (11 times higher than white muscle) at all temperatures of acclimation. Short term hypoxia seems to be more stressful for heart muscle and brain because they need oxygen for their metabolism in order to avoid excessive metabolite accumulation. A high MDH/LDH ratio may cause an attenuated pyruvate to lactate flux and as a consequence carbohydrate metabolism will be largely channeled toward complete oxidation (Almeida-Val & Hochachka, 1995). This will benefit hypoxia situations like in heart muscle acclimated to 20 and 30°C.

The ratio of LDH activity at low to that at high pyruvate concentrations (L/H) is often used as an index of the kinetic poise of LDH (Kaplan & Goodfriend, 1964). L/H LDH ratios for white muscle at different temperatures in normoxia and hypoxia suggest anaerobic organization (Table 4). These values are higher in normoxic than in hypoxic conditions, indicating that an increase exists in the reduction of pyruvate to sustain glycolisis under anaerobic conditions.

The results of K_M values obtained for LDH and MDH of fishes acclimated to 20°C and submitted to hypoxia suggest that naturally intense fluctuations in dissolved environmental oxygen may result in significant changes in enzyme activity, such as the ability of enzymes to respond to acute hypoxia. Lushchak *et al*. (1997) found differences in enzyme activity throughout anaerobiosis and recovery of a sea mussel. Shaklee *et al*. (1977) found significant differences in enzymatic activity for liver LDH and white muscle aldolases in fishes acclimated to different oxygen concentrations.

This ability to respond to hypoxia may have been acquired in times of oxygen deficiency. Experiments at 25 and 30°C did not result in significant changes in enzyme activities and K_M for acute hypoxia except for white muscle of fishes acclimated to 30°C.

It is interesting to notice that these temperatures are those encountered in the environment of the fish almost all year round.

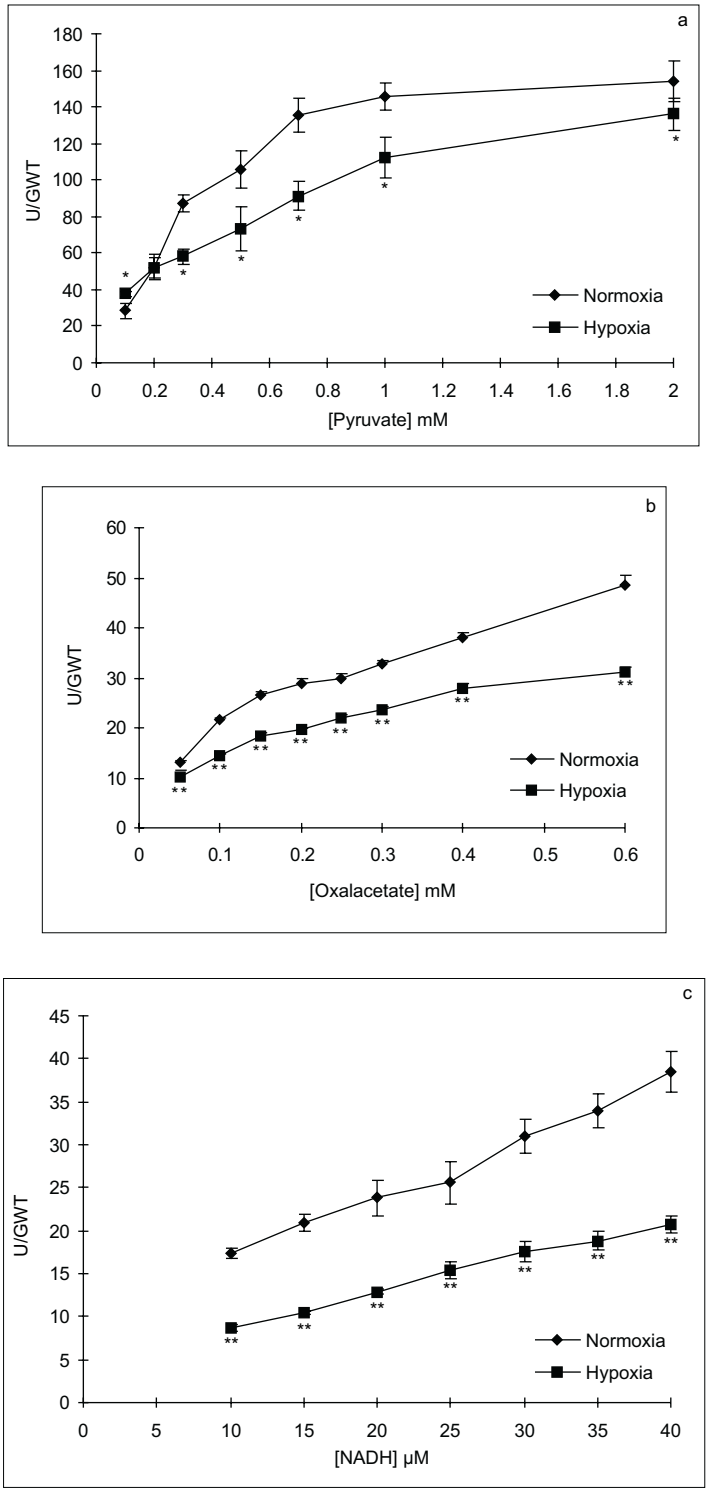

Fig. 1 — Effects of hypoxia on fishes acclimated to 20ºC on: a) LDH activity of white muscle in different pyruvate concentrations, b) MDH activity of white muscle in different oxalacetate concentrations, and c) MDH activity of white muscle in different NADH concentrations. * $P < 0.05$, $n = 5$. Error bars are within limits of symbols when not visible. U, µmole substrate/min. GWT, grams of wet tissue.

TABLE l

K_M values for LDH and MDH for white muscles of fishes acclimated at different temperatures. The values are means ± SD, n = 5 fishes.

20°C		
Enzym	**K_M in Normoxia**	**K_M in Hypoxia**
$LDH_{[PYR]}$ (mM)	0.824 ± 0.14	1.700 ± 0.270*
$MDH_{[OAA]}$ (mM)	0.020 ± 0.005	0.018 ± 0.003 NS
$MDH_{[NADH]}$ (μM)	36.81 ± 11.83	45.360 ± 7.110 NS
25°C		
Enzym	**K_M in Normoxia**	**K_M in Hypoxia**
$LDH_{[PYR]}$ (mM)	1.140 ± 0.22	1.27 ± 0.200 NS
$MDH_{[OAA]}$ (mM)	0.057 ± 0.009	0.046 ± 0.008 NS
$MDH_{[NADH]}$ (μM)	19.040 ± 5.06	22.98 ± 5.130 NS
30°C		
Enzym	**K_M in Normoxia**	**K_M in Hypoxia**
$LDH_{[PYR]}$ (mM)	0.140 ± 0.02	0.830 ± 0.190*
$MDH_{[OAA]}$ (μM)	0.042 ± 0.01	0.059 ± 0.019 NS
$MDH_{[NADH]}$ (μM)	19.070 ± 5.06	23.440 ± 5.490 NS

* P < 0.05, NS, not significant.

TABLE 2

Enzyme activities* in tissues of cascudo preto in normoxia (N) and hypoxia (H) of fishes acclimated at different temperatures. LDH used pyruvate and MDH used oxalacetate as a substrate. The values are mean ± SD, n = 5 fishes.

LDH*						
	20°C		**25°C**		**30°C**	
	N	**H**	**N**	**H**	**N**	**H**
Muscle	187.62*** ±6.59	112.06 ±17.43	104.74 ±35.76	95.66 ±16.64	239.55 ±57.62	330.23 ±105.56
Heart	5.59 ±2.06	4.30 ±2.05	5.47 ±2.20	8.52 ±3.45	13.85 ±9.63	4.22 ±6.24
Brain	41.57 ±4.05	41.92 ±5.96	61.73 ±2.01	54.27 ±8.57	57.81 ±15.55	42.25 ±8.12
MDH*						
	20°C		**25°C**		**30°C**	
	N	**H**	**N**	**H**	**N**	**H**
Muscle	98.87** ±13.37	70.90 ±14.55	69.45 ±16.57	66.44 ±8.89	80.71 ±26.70	74.12 ±22.38
Heart	750.48 ±157.71	842.12 ±148.05	764.95** ±93.12	945.34 107.84	794.86 ±139.86	643.41 ±144.31
Brain	281.67 ±57.33	333.76 ±106.73	345.34 ±67.05	286.5 ±82.74	278.78 ±18.17	269.13 ±19.11

* (U/GWT) U, μmole substrate/min. GWT, g.wet wt. of tissue.
** P < 0.01; *** P < 0.001.

TABLE 3

Comparative MDH/LDH ratios from fish tissues acclimated at different temperatures, n = 5 fishes.

	MDH/LDH*					
	20°C		25°C		30°C	
	N	H	N	H	N	H
Muscle	0.53	0.63 NS	0.66	0.69 NS	0.33	0.22 NS
Heart	134.25	195.84*	139.84	110.95 NS	57.39	152.46*
Brain	6.78	7.96 NS	5.59	5.28 NS	4.82	6.37 NS

(U/GWT) U µmole substrate/min. GWT, g.wet wt. of tissu .
* $P < 0.05$, NS, not significant.

TABLE 4

Rates of white muscle LDH activity in low and high substrate concentrations (L/H) and at different acclimation temperatures. Average of 5 fishes.

Temperature of acclimation	L/H
Normoxia 20°C	0.72
25°C	0.47
30°C	0.73
Hypoxia 20°C	0.48
25°C	0.43
30°C	0.63

A number of studies with many species have shown that fishes frequently respond to change in environmental oxygen levels with changes within hematological parameters and alteration in physiological responses (Randall, 1993; Fernandes *et al*., 1995). Furthermore, fishes are known to avoid low oxygen concentrations (Reynolds & Thomson, 1974). According to M. N. Fernandes & J. R. Sanches (personal communication) no differences were found in *Rhinelepis strigosa* acclimated at different temperatures and submitted to hypoxia for the oxygen carrying capacity of the blood mesured by changes in hematocrit, hemoglobin concentration and red cell count, although changes were found in cardiac frequency, metabolic rate, oxygen uptake, ventilation rate and volume. Probably, hematological changes are subtle while physiological and biochemical adjustments provide the strategy used to deal with changes in oxygen concentrations in natural environments.

Changes in K_M are difficult to explain in short term periods of acute hypoxia. Changes in K_M of pyruvate and NADH for M_4-LDH from shallow and deep sea living species were related with changes in pressure in fishes (Siebenaller & Somero, 1979). According to Greaney & Somero (1980) studies of NADH binding suggest that for M_4-LDHs and other dehydrogenases NADH (NAD) binding sites should remain cofactor-saturated, so that the direction of dehydrogenases function is established by the redox state, i.e. the NADH/NAD ratio of the cell.

Differences in K_M of enzymes have also been attributed to a modulation resulting from changes in the pH milieu (Wilson, 1977; Yancey & Somero, 1978; Walsh & Somero, 1982; Somero, 1983; Coppes *et al*., 1992).

This would explain the higher affinity (lower K_M) of muscle LDH in hypoxia and the lower affinity (higher K_M) of muscle LDH at 20°C in cascudo preto. These hidden strategies such as the ability of enzymes to respond to acute hypoxia may explain differential responses to hypoxia situations which fishes encounter in different environments.

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