SPECIALIZED METABOLISM AND BIOCHEMICAL SUPPRESSION DURING AESTIVATION OF THE EXTANT SOUTH AMERICAN LUNGFISH – *Lepidosiren paradoxa*

MESQUITA-SAAD, L. S. B.,[1,2] LEITÃO, M. A. B.,[1] PAULA-SILVA, M. N.,[1] CHIPPARI-GOMES, A. R.[1] and ALMEIDA-VAL, V. M. F.[1]

[1]Laboratório de Ecologia e Ecofisiologia Molecular, Instituto Nacional de Pesquisas da Amazônia, Alameda Cosme Ferreira, 1756, CEP 69000-000, Aleixo, Manaus, AM, Brazil

[2]Departamento de Medicamentos e Alimentos, Curso de Farmácia, Universidade do Amazonas, Rua Alexandre Amorim, 330, CEP 69010-300, Manaus, AM, Brazil

Correspondence to: Vera Maria Fonseca de Almeida e Val, Instituto Nacional de Pesquisas da Amazônia (INPA), Laboratório de Ecofisiologia e Evolução Molecular (LEEM), Av. André Araújo, 1756, CEP 69083-000, Aleixo, Manaus, AM, Brazil, e-mail: veraval@inpa.gov.br

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ABSTRACT

Lepidosiren paradoxa (pirambóia) is the single representative of Dipnoan (lungfish) in South America. This species is considered a living fossil, in spite of some reports describing this fish as having a very specialized life style. It aestivates during the dry season, and has developed metabolic adaptations to cope with both flooding and drought. The literature describing its tissue ultra-structure shows high glycogen stored in the muscle, suggesting a strong dependence on anaerobic glycolysis. The present paper reports tissue enzyme levels of LDH, MDH, and CS, and isozymic tissue distribution of LDH, MDH, ADH, PGI, SOD, and PGM of 7 aestivating specimens from Lago do Canteiro in the Amazonas River. Animals were caught while burrowed in mud during the aestivation period. Our findings reveal high anaerobic capacity of both skeletal and heart muscles, even during the aestivation period, when enzymes showed suppressed levels compared to those of non-aestivating animals (data from the literature). Isozymic patterns suggest loss of duplicate condition in most analyzed *loci*, a characteristic that occurs mainly in higher vertebrate categories. These data indicate that, compared to the fish group, lungfish may be considered advanced, despite retaining primitive morphological characters.

Key words: lungfish, *Lepidosiren paradoxa*, metabolism, enzymes, isozymes.

RESUMO

Especialização e supressão metabólicas durante períodos de estivação do peixe pulmonado sul-americano – *Lepidosiren paradoxa*

Lepidosiren paradoxa (pirambóia) é o único representante dos Dipnoan (peixes pulmonados) na América do Sul. Essa espécie é considerada um "fóssil vivo", apesar de alguns estudos terem revelado um estilo de vida muito especializado. A espécie pode ser encontrada estivando durante a seca e desenvolvendo adaptações metabólicas para enfrentar as trocas que ocorrem periodicamente em seu ambiente: inundações e secas. As descrições encontradas na literatura sobre as ultra-estruturas dos tecidos revelam alta concentração de glicogênio (substância de estocagem) no músculo, sugerindo forte tendência para a glicólise anaeróbica. O presente estudo descreve os níveis enzimáticos de LDH, MDH e CS nos tecidos do coração e músculo bem como a distribuição enzimática das isozimas LDH, MDH, ADH, PGI, SOD e PGM em 7 espécimes coletadas no Lago do Canteiro, Rio Amazonas, ocasião em que se encontravam estivando. Os animais foram capturados pelos pescadores (armadilha individual)

durante o período em que estavam enterrados na lama do lago durante a "seca". Nossos resultados revelaram alta capacidade anaeróbica dos músculos esquelético e cardíaco no período, quando as enzimas mostram níveis bastante inferiores (indicando supressão metabólica) em relação aos animais ativos (dados obtidos na literatura). Os padrões isozímicos sugerem a perda da condição de *loci* duplicados na maioria dos sistemas isozímicos, característica que ocorre principalmente em vertebrados pertencentes a categorias superiores. Esses dados indicam que, comparado ao grupo dos peixes, o peixe pulmonado pode ser considerado especializado (ou avançado), apesar da manutenção de duas características morfológicas primitivas.

Palavras-chave: peixe pulmonado, *Lepidosiren paradoxa*, metabolismo, enzimas, isozimas.

INTRODUCTION

Lepidosiren paradoxa, known as "pirambóia", is the unique representative of South American Dipnoan group, with true lungs. These three genera are considered to share their ancestray with the Tetrapoda group, all of them belonging to Sarcopterygii (Nelson, 1984; Carroll, 1997).

Past and present patterns of oxygen availability in the Amazon and other continental water bodies may be seen as escalators of selective evolutionary pressure. The currently poorly oxygenated waters in the Amazon Basin are the result of hypoxia and anoxia in the aquatic environment during the Cambrian period, owing to the then low atmospheric O_2-levels. Since then, oxygen depletion has probably been the main limiting factor of aquatic life in general (Randall *et al.*, 1981; Almeida-Val & Farias, 1996). According to Gans (1970), the development of air-breathing systems in vertebrates appeared in fishes when they were skimming the water surface during periods of low oxygen availability. After that, the transition from water- to air-breathing has undoubtedly been, together with the colonization of the terrestrial environments with permanent high O_2-levels, the most important transformation occurring during the evolutionary process of vertebrates. Among teleosts, several optional and obligatory air-breathing patterns have arisen, leading to diversification of morphological, anatomical, physiological, and biochemical adjustments to the new kind of respiration (Kramer *et al.*, 1978; Almeida-Val & Val, 1993; Val, 1993; Val & Almeida-Val, 1995; Almeida-Val & Hochachka, 1995; Almeida-Val & Farias, 1996; Graham, 1997).

Lepidosiren paradoxa, along with other Dipnoi genera, are obligatory air-breathers which probably developed the first true lung among vertebrates. The lungs of lungfish have received considerable attention and a comparison of structures in the different species of *Protopterus* with those of *Lepidosiren* shows that they are paired lungs, although fused at the anterior portion, receiving blood flow via a paired pulmonary circulation (reviewed by Graham, 1997).

The South American lungfish, *Lepidosiren*, is capable of aestivation; it burrows in moist mud and, if conditions are suitable, can survive dry periods through aestivation. The lungfish is normally found in swamps, ponds, and lakes, but usually only near shore areas where swampy conditions prevail (Fink & Fink, 1979; Hochachka & Hulbert, 1978). When fishes are awakened, they use glycogen, which builds up in all tissues during the aestivation period (Dunn *et al.*, 1983). In studying heart and skeletal muscles of *Lepidosiren*, Hochachka & Hulbert (1978) found a very large glycogen store that probably utilized as anaerobic or aerobic energy fuel. When compared with other fishes, enzyme levels in this species proved similar regarding oxidative metabolism, while anaerobic metabolism was abnormally high (Hochachka & Hulbert, 1978). The present paper reports tissue isozymic patterns, heart and skeletal muscle enzyme levels, and some kinetic properties of the main anaerobic enzyme, lactate dehydrogenase.

MATERIALS AND METHODS

Experimental animals

Aestivating *Lepidosiren paradoxa* ($n = 7$) were captured at Lago do Canteiro on Careiro Island in the Amazon River, during the descending water level period (August, 1997), when they were burrowed in the mud. Tissues (skeletal and heart muscle, liver, and brain) were excised in the field, promptly stored

in liquid nitrogen bottles, and transferred to a low temperature (–80ºC) freezer at the Laboratory of Ecophysiology and Molecular Evolution of INPA, in Manaus.

Tissue preparation

Skeletal and heart muscle, liver, kidney, eye, brain, and lung were homogenized in ice-cold phosphate buffer 50 mM, pH 7.0, with a Potter-Elvejhem homogenizer. Dilution rates were established in accordance with tissue size and characteristics. The homogenates were centrifuged at 19,000 g during 30 minutes at 4ºC in a Sorvall RC5B centrifuge. Resulting supernatants were used for enzyme assays and electrophoresis.

Enzyme assays

Maximal activity levels were determined at 25ºC using a Genesys 2 spectrophotometer, following techniques reviewed in Driedzic & Almeida-Val (1996). LDH (E.C.: 1.1.1.27; lactate dehydrogenase) and MDH (E.C.: 1.1.1.37; malate dehydrogenase) enzyme activity was measured following the oxidation of NADH at 340 nm (mM extinction coefficient = 6.22), and CS (E.C.: 4.1.3.7; citrate syntase) enzyme activity through the reduction of free coenzyme-A with DTNB at 412 nm (mM extinction coefficient = 13.6). Assay conditions were based on well-established protocols for fish tissue (Sidell *et al.*, 1987; Moon & Mommsen, 1987; Singer & Ballantyne, 1989). LDH and MDH assays were done using buffer containing 0.15 mM NADH, 1 mM KCN and 50 mM Imidazole, pH 7.4 at 25ºC; reactions were initiated with the addition of different concentrations of pyruvate and oxaloacetate, respectively. K_M and V_{max} were determined for both LDH and MDH using the method of Lineweaver-Burk (plots $1/V_0$ x 1/[S]). CS assays were developed using buffer containing 0.4 mM acetyl CoA, 0.25 mM DTNB and 75 mM TRIS, pH 8.0 at 25ºC; reactions were initiated with the addition of 0.5 mM oxaloacetate.

Electrophoresis

Electrophoreses were carried out in horizontal gels containing 13% (w/v) corn starch prepared according to Val *et al.* (1981). All electrophoreses were performed at 4ºC, applying 5 V/cm for 15-18 h, using a Pharmacia EPS 500-400 (Uppsala, Sweden) power supply. Electrophoreses were used to study the following enzyme systems: LDH (E.C.: 1.1.1.27; lactate dehydrogenase), sMDH (E.C.: 1.1.1.37; malate dehydrogenase – soluble fraction), ADH (E.C.: 1.1.1.1; alchool dehydrogenase), PGM (E.C.: 2.7.5.1; phosphoglucomutase); PGI (E.C.: 5.3.1.9; phosphoglucose isomerase); G6PDH (E.C.: 1.1.1.49; glucose-6-phosphate dehydrogenase); and SOD (E.C.: 1.15.1.1; superoxide dismutase). Staining solutions were prepared according to protocols described in Allendorf *et al*. (1977). Nomenclature adopted for isozymic subunits and structural genes is according to Shaklee *et al*. (1989).

RESULTS

Isozymic electrophoretic patterns of *Lepidosiren paradoxa* are shown in Fig. 1. LDH isozyme patterns are different compared to teleosts, which present two to three LDH *loci*. Lungfish LDH has one well-defined isozyme, predominating in all analyzed tissue, which is probably the product of the LDH-A* *locus*. A second LDH *locus* appeared with very poor resolution and is present in skeletal muscle, heart, and liver. As for most vertebrates, lungfish MDH emerged in two forms: mitochondrial and soluble. ADH, SOD, and PGM are present in this species as a single electrophoretic band, suggesting a non-duplicated state for all these *loci*. Unlike these results, PGI is represented by two *loci*, and is the only isozyme system retaining a certain degree of gene regulation expression (Fig. 1). Tissue restriction appeared for SOD, PGM, and ADH. While SOD could be detected in all tissues except for skeletal muscle, PGM and ADH was restricted to skeletal muscle (Fig. 1). The presence of polymorphism (alleles) was detected only in PGI-A* *locus*.

The skeletal muscle presented a high anaerobic capacity, revealed by the lack of pyruvate inhibition (Table 1), while heart muscle presented a certain degree of inhibition rate, suggesting that this tissue is "protected" against anaerobic metabolism (lactate accumulation), even for short periods of oxygen depletion (during diving). However, LDH Km values obtained for muscle and heart are high compared to those for most teleost fish species ($Km_{(heart)}$ = 2.9 mM and $Km_{(muscle)}$= 3.5 mM), suggesting that both tissues may be anaerobically powered. Other metabolic indexes such as MDH/LDH and LDH/CS also indicate that heart muscle is glycolytic rather than oxidative. These results and the values obtained for enzyme absolute activities are summarized in Table 1.

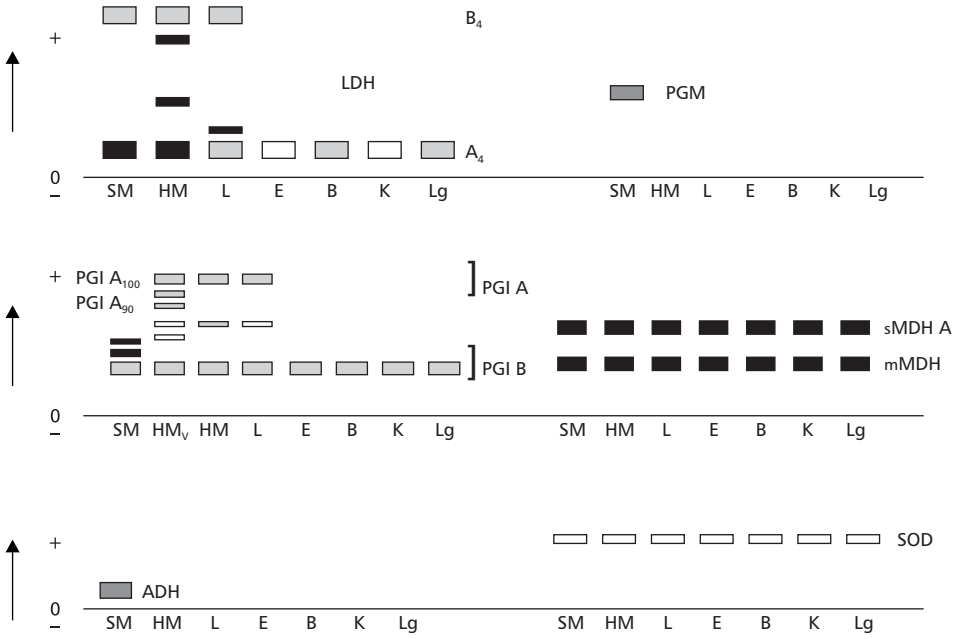

Fig. 1 — Diagramatic electrophoretic patterns for different tissues of *Lepidosiren paradoxa* (South American Lungfish). Skeletal Muscle (SM), Heart Muscle (HM), Liver (L), Eye (E), Brain (B), Kidney (K) and Lung (Lg).

TABLE 1
Enzyme absolute activities (μmoles/min/gram wet tissue) from *Lepidosiren paradoxa*.
LDH Km values are expressed in pyruvate concentration (mM).

Enzymes	Skeletal muscle	Heart muscle
LDH 1 mM	94.65	3.56
LDH 10 mM	151.01	0.53
Pyruvate inhibition rate	0.62	6.73
MDH 1 mM	–	2.71
MDH/LDH	–	0.76
CS	0.83	1.29
LDH/CS	114.04	2.76
LDH Km (mM of pyruvate)	3.5	2.9

DISCUSSION

Several studies have reported tissue expression regulation of LDH and MDH isozymes according to temperature, oxygen availability, and other environmental parameters (Philipp *et al.*, 1983; Val & Almeida-Val, 1995; Almeida-Val *et al.*, 1999). Some environmental characteristics also induce changes in enzyme kinetic properties, e.g., Km and Vmax, and the affinity between enzymes and substrate is inversely related to the apparent Km values (Weiser *et al.*, 1987). In addition, changes in metabolic rates may occur when fish are under stress conditions caused by temperature, oxygen depletion, and pollution, among others. Aestivation causes metabolic depression, termed metabolic arrest by some authors (Hochachka & Guppy, 1987).

In the literature review of the main metabolic biochemistry characteristics of air-breathing fish, the lungfish has been described as retaining heart substrate preferences similar to those of anoxia resistant vertebrates (the aquatic turtle) because they may preferably use carbohydrate instead of lipid (based on enzyme profiles and ultrastructure tissue organization). It has also been suggested that glycogen stores in lungfish heart and skeletal muscles are organized to meet energy needs during periods of aestivation and/or submergence (reviewed by Almeida-Val & Hochachka, 1995). Experiments with submergence and recovery of the African lungfish (*Protopterus*) by Dunn *et al.* (1983), are consistent with a perfectly integrative metabolic response. The main evidence for occurrence of metabolic depression is the abscence of: glycogen depletion, lactate accumulation, change in lactate/pyruvate ratio, change in adenylate concentrations, or significant creatine phosphate depletion in epaxial muscles during submergence (Dunn *et al.*, 1983). Thus, the absolute shutdown in enzyme activity, as observed in the present work, may be expected when fish are in aestivation periods. According to Hochachka (1980), besides the fact that lungfishes may retain relatively high glycolytic power in most tissues, they routinely utilize proteins and amino acids as metabolic fuels. For that reason, the liver and kidneys of these fishes maintain a significant capacity for gluconeogenesis as well as amino acid metabolism. Our findings reveal a high anaerobic capacity of both skeletal and heart muscles, even during the aestivation period. Thus, those glycogen stores may be the main energy fuel reserve during aestivation.

While these findings are consistent with high metabolic organization adapted to a specialized life style and, in particular, to specialized animals, the evolutionary position of lungfish and its isozymic characteristics suggest, a priori, low genetic differentiation when compared to those of other fish groups. LDH and PGI are the only isozyme systems presenting the duplicated gene expression already described for most teleosts (reviewed by Coppes, 1986; Coppes, 1992; Almeida-Val & Val, 1993).

Duplicated structural genes that originated from ancient (500 million years ago) and recent (50 to 100 MY) polyploidization events are considered to be among the main ones that gave rise to isozyme systems in fish (Whitt, 1983). Thus, most enzymes are encoded by two or more gene *loci*. After polyploidization, the diploid state developed further, causing loss of some duplicated genes. The loss of this gene expression has in fact been described for recent polyploid fish species (Buth, 1983; Allendorf *et al.*, 1983). Otherwise, the loss of duplicated gene expression seems to be considered a common evolutionary fate of most vertebrates by many authors (Ohno, 1970; Ferris & Whitt, 1977; Zuckerkandl, 1978; Allendorf *et al.*, 1983; Buth, 1983; Whitt, 1983).

Therefore, the interpretation of our results creates a paradox: although the presence of one *locus* enzyme system may be viewed as a derived state of the whole vertebrate group, it may also be viewed, as in the case of *Lepidosiren*, as a primitive character state due to the non-diverging/non-duplicated *loci*. This last explanation gains further support from the fact that this species is considered to retain most of its ancient.

Several analyzed isozyme systems from *Lepidosiren* (Fig. 1) may be considered as encoded by a single gene *locus*, while most fishes present duplicated gene expression for such isozyme systems, as occurs for sMDH (Schwantes & Schwantes, 1982; reviewed by Coppes, 1990). On the other hand, the presence of some tissue restriction, particularly in PGI, ADH, and PGM, may be viewed as a specialization. According to Whitt (1983), the probability of duplicated gene loss should progressively decrease as duplicate gene products acquire different structural or functional properties.

Crossopterygian fossil records dating from as long as the Devonian period of the Paleozoic era, indicate that this group is nearly as old as the first Actinopterygian groups. Based on the literature, the main genetic characteristics of *Lepidosiren paradoxa* can be summarized as follows: (i) low diploid chromosome number; (ii) absence of chromosomes in the acrocentric state; (iii) enormous size of individual chromosomes; and (iv) high contents DNA (Ohno & Atkin, 1966; Oliveira *et al.*, 1988). These characteristics are all shared with the class Amphibia, although amphibians manifest to a high degree indications of recent polyploidization events. As reviewed by Graham (1997), the Dipnoi belongs to the Sarcopterygian and share a common ancestray with the vertebrate group Tetrapoda. Most reviews report the evolutionary history of lungfishes as parallel to that of the

actinopterygians, which include the whole group of ancient and modern teleosts (Nelson, 1984; Carroll, 1997; Graham, 1997). In fact, the lungfish geological history supports the development of specializations in addition to ancient morphological trait maintenance. According to Hinegardner & Rosen (1972), the more specialized or evolutionary advanced the fishes, the less DNA content in their nucleus. Since it has the highest DNA content described for freshwater fish groups (reviewed by Carvalho *et al*., 1998), *Lepidosiren paradoxa* may be considered as a primitive species.

However, the presence of metabolic and genetic characteristics similar to those of the Tetrapoda (e.g., presence of urea cycle enzymes, true lung development, aestivation capacity, among others) is evidence that lungfish are a specialized group. Meyer & Wilson (1990), studying mitochondrial DNA, suggested that the common ancestor of lungfishes and Tetrapoda already possessed multiple morphological traits preadapting their locomotion, circulation, and respiration for life on land. Regarding metabolic characteristics, found in the present study, which adapt to the fish life style, and the described isozymic tissue patterns, which might be considered differentiated among other fish groups, the data obtained in the present work suggest some degree of specialization in *Lepidosiren paradoxa*.

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