EVOLUTIONARY FEATURES OF HYPOXIA TOLERANCE

IN FISH OF THE AMAZON:

FROM MOLECULAR TO BEHAVIORAL ASPECTS

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Abstract

Fish adaptation to hypoxia does not include, necessarily, the ability to tolerate hypoxia; however, it does include mechanisms they use to avoid hypoxia. Literature reviews on fish of the Amazon shows that they search for oxygen rich environments trying to reach air, water surface layer, or aquatic plant roots when facing hypoxia. While the oxygen lack is considered one of the main causes of air-breathing appearance among fish, the ability to breathe air is not necessarily related to the development of hypoxia tolerance. Additionally, some non air-breathing fish groups do tolerate hypoxia and are able to remain in hypoxic and anoxic environments. This paper will review the main adaptive mechanisms fish developed to survive hypoxic environments and try to correlate changes in innate behavior, morphology and anatomy with physiological and biochemical adaptive mechanisms under an evolutionary perspective. Considering that evolution of hypoxia tolerance is related to genetic mechanisms, we will discuss the studies of genes considered to be involved with hypoxia adaptation: LDH A*, B*, and C*. Data on LDH cDNA seem to be consistent with functional aspects of this isozyme system. Considering that the evolution of hypoxia tolerance in fish may have induced the whole group to adaptive radiation, we suggest it may have contributed to the biological fish diversity in the Amazon basin.

Introduction

The appearance, diversification, and evolution of the fish fauna in the Amazon are associated with the story of hydrographic basin formation (Lundberg, 1998).

Current environmental heterogeneity, caused by flood pulses, different water qualities, with their different physical and chemical parameters, is the main cause of the recent adaptive radiation of the Amazonian fish fauna (Junk et al., 1989; Val, 1993). The 2800 Amazonian fish species already described display a variety of adaptations to their environment that include behavioral, physiological, biochemical, genetic, and evolutionary changes. The appearance of such adaptive traits seam to be related to the intensity and periodicity of the constraint imposed on individuals, populations, species, and groups of species. The number of characters genetically determined is still unknown. However, the description of several adaptive strategies at numerous organic levels have revealed that the selective pressure during evolution may be caused by several chronic constraints such as short- and long-term changes of oxygen, poor ionic waters, acidity, daily and spatial temperature oscillations, among others (Almeida-Val et al., 1999a). Thus, adapting to such ever-changing environment is thought to be the main cause of fish diversity in the Amazon.

Revisiting hypoxia adaptation in fish of the Amazon

While these subjects have already been addressed by several authors, such as the description of regulation on blood physiological parameters, enzyme levels and their tissue expression, ventilation adjustments, adjustments of hematological parameters, ion regulation, and behavior, the relationship between these adaptive strategies and their occurrence among related fish groups is barely understood. The relationship between the Aquatic Surface Respiration (ASR), an innate behavior, and the physiological responses that follow such strategy has been addressed to check its efficiency (Almeida-Val et al., 1993; Val, 1995). However it has not been considered a character worthy of evolutionary studies since it can be a homoplasic character, i.e., its appearance in species was caused either by convergence or parallelism phenomena. There is a lack of information about the genetic bases of such behavior, but there is no reason to consider it a character restricted to some groups of fish in particular, and useful to fish for its adaptation to hypoxic *várzea* lakes. Also, we should presume that its evolution amongst groups might be an important strategy for surviving hypoxia among fish of the Amazon.

The implications of Aquatic Surface Respiration are numerous. So are the implications of air breathing, which may be considered another homoplasic character among fishes. However, in several cases, the diversification of air breathing fishes reflects the successful adaptive radiation of a particular air

breathing morph type, as in the groups Callichthyidae and Clariidae (Graham, 1997). Fossil records indicate that one of the highly specialized living catfish, *Corydoras*, belongs to the family Callichthyidae, indicating a diversity of early Cenozoic callichthyids and loricarioids (Lundberg, 1998), these latter presenting another air-breathing morph, which is not so wide spread. In fact, air breathing occurs in 28 species of the family Callichthyidae, while only seven species from the family Loricariidae are air breathers. The categorization of air breathing organ structure may not be considered useful in phylogenetic studies due to the same reasons we do not use Aquatic Surface Respiration trait, i.e., we cannot distinguish its homology or convergence. However, the presence of an air breathing organ in 49 fish families, all retaining a monophyletic origin from an earlier fish stock, which started with two main vertebrate groups: the lobed fins Sarcopterygian and the ray finned fishes Actinopterygians, may be viewed as the homologous ability for aerial conquest of two diverging groups of vertebrates.

Air breathing has been described in the early literature as a widespread adaptive trait. Rauther (1910) described it as a respiratory adaptation, and subsequent authors have done the same (reviewed by Graham, 1997). According to many authors the development of air breathing among fish is the result of both, habitat and behavioral factors: both hypoxia and emergence have influenced the origin of this character. No other environmental pressure has been so widespread in the aquatic environment or has occurred throughout the vertebrate evolutionary history that could lead to so many separate episodes of air breathing as the low oxygen availability (Johansen, 1970; Graham et al., 1978). However, those who accept hypoxia as the main cause for the development of air breathing in fish may also consider those who believe that air breathing in fish have arisen accidentally (by chance) in fish that were skimming water surfaces (Gans, 1970), or that air breathing has been precipitated by changes in water flows (Hora, 1935).

No matter the reasons or the way air-breathing organs appeared, or aquatic surface respiration developed, fish found a way to avoid low oxygen contents and this adaptation allowed them to explore a wide range of ecological niches. Parallel to these adaptations, most air breathing species had to deal with other type of constraints. Diving into the water bodies and holding their breath for long periods induced metabolic changes, which comprised slowing down total metabolic rates, decreasing oxidative enzyme rates, and increasing their anaerobic ability (reviewed by Almeida-Val and Hochachka, 1995). While these characteristics were first noticed for air-breathing species, further investigations have shown that they are common pictures in fishes of the Amazon, independent

of patterns of respiration or styles of life (Driedzic and Almeida-Val, 1996; West et al., 1999). So, the low oxygen environmental pressure may be also considered the main driving force in the development of long-term metabolic adjustments as well.

Are all air-breathing fish tolerant to hypoxia? Is aquatic surface respiration associated with hypoxia tolerance?

Hypoxia affects air-breathing fish in different ways. Obligate air-breather species are little or non-influenced by water oxygen availability, since they present reduced gill surface areas. Other air-breathing fish species will be affected by hypoxia in different ways; the threshold for water oxygen content in water varies according to the species. For example, among Amazon fishes, the facultative air-breather jeju (*Hoplerythrinus unitaeniatus*) starts breathing air when oxygen drops to 81 mmHg (Stevens and Holeton, 1978a), while the armored catfishes *Hypostomus spp* may search for air when oxygen drops down to 21, 35, or 60, depending upon the experimental temperature (Gee, 1976; Graham and Baird, 1982; Fernandes et al., 1995). The advanced teleost *Symbranchus marmoratus* may tolerate 33 – 69 mmHg before starting to breathe air; but additionally to the fact that this species aestivates during dry seasons, these thresholds may vary according to body size and hypoxia acclimation (Bicudo and Johansen, 1979; Graham and Baird, 1984). When we consider respiratory partitioning of oxygen uptake, the so-called amphibious air-breathing fishes (Graham, 1997) show a variety of patterns, which are affected by age, water oxygen partial pressure, body size, and temperature. Some Amazon fishes like *Arapaima gigas*, which is considered an obligate air breather, may breathe 50 to 100% oxygen via air according to body size and oxygen contents in the water (Stevens and Holleton, 1978b). Following these changes in respiration patterns during growth many physiological and biochemical adjustments have been described to occur in *Arapaima gigas* (personal observations, Souza and Val, 1990).

Aquatic surface respiration is also affected by aquatic oxygen availability. Fishes that possess this innate behavior increase their incursions to the surface when oxygen decreases in the water, and the efficiency of such innate behavior is sufficient to allow blood oxygen loading in *Colossoma macropomum* (Val, 1995). Juveniles of *Astronotus ocellatus*, a cichlid fish that, when adult, tolerates anoxia during 6 hours at 28°C, are able to tolerate hypoxia exposure indefinitely if they are allowed to practice aquatic surface respiration (S.C.Land, personal

communication), but are not able to tolerate long-term hypoxia exposure if denied access to the water surface (Almeida-Val et al., 1999b).

Field studies of fish distribution in the Amazon region have always shown a correlation between oxygen availability and species preferential habitats, migration (particularly lateral migration), and adaptive characteristics, named aquatic surface respiration or air-breathing (Junk et al., 1983; Cox-Fernandes, 1997; Crampton, 1998). The work of Junk and collaborators in 1983 at Camaleão Lake inside Marchantaria Island was pioneer in describing fish movements along the year that could be related to oxygen changes due to water-level oscillations, or flood pulses, as named afterwards by Junk and his co-workers (Junk et al., 1989). During low oxygen-level periods, the diversity of the lake decreased and the remaining species presented some kind of adaptation to cope with episodes of hypoxia or anoxia.

Studying the distribution, migratory behavior, and repertoire of respiratory adaptations of Gymnotiform (electric eels) from Tefé region, Crampton (1998) suggested that all these parameters are directly related to oxygen availability. He also suggested that oxygen availability has an important influence on electric signal design of those species. Whether related or not to oxygen distribution in water, electric eels present a variety of adaptive strategies to avoid hypoxia and some species were described as anoxia tolerant under experimental procedure (Crampton, 1998). Among the studied species, this author described the presence of air-breathing organ in two species (*Electrophorus electricus* is already known as an obligate air-breather – reviewed by Val and Almeida-Val, 1995), the presence of the air gulping behavior in five species, and the presence of aquatic surface respiration in 12 species. Even though hypoxia was chemically induced by sodium sulphide, which may cause drastic changes in ionic homeostasis at the branchial level, methaemoglobin formation, and other physiological effects, the fact that animals could survive anoxia during six hours without breathing air or practicing aquatic surface respiration (impairment caused by experimental design), is evidence that those species are tolerant to hypoxia.

As occurs with air-breathing and aquatic surface respiration, hypoxia tolerance appears in fish as another homoplasic character, which, different from other vertebrate lineages, has multiple independent evolutionary origins and is the consequence of environmental pressure, causing adaptive radiation (Figure 1). It also may be considered a cause for fish diversity, as we have already proposed elsewhere (Almeida-Val and Farias, 1996).

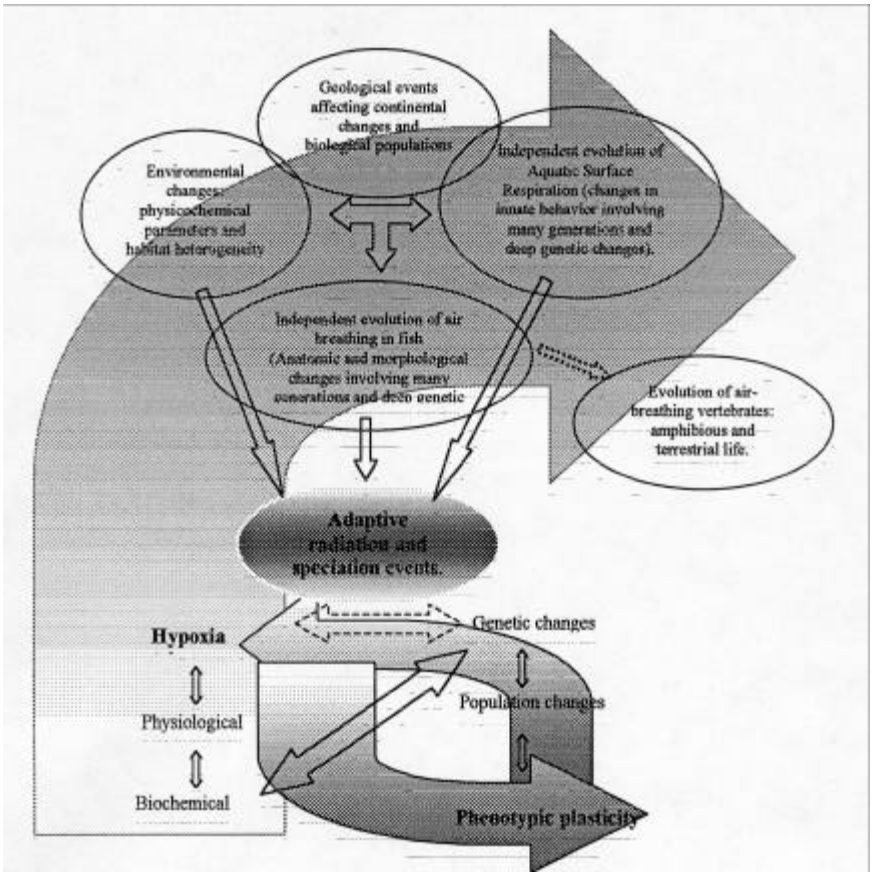

Figure 1. Possible relationships between physico-chemical and biological parameters in the origin of adaptive responses fish developed to survive low oxygen levels and its effects on the diversification and adaptive radiation. The bottom of the figure describes the different ways such adaptive radiation may be observed in biological systems. Big Arrow in the back of the figure shows the direction of ecological and evolutionary relevant events.

How can we follow the evolutionary pathways of hypoxia in fish?

As already stated, the capacity of organisms to deal with environmental changes depends on the magnitude of the change, the time frame in which the change occurs, and the individual genetic constitution, which may be altered over generations by the selection of genetic variants that are suited to cope with the new environmental situation. As a consequence, environmental stress has been considered to be among the most important triggers of change in biological organization and functioning during evolution (Almeida-Val et al., 1999a). As far as morphology and anatomy are concerned, changes in the structure of DNA and proteins may be tolerated without phenotypic effects, that is, *be neutral*. This invariance may be the result of chemical redundancy (degeneracy of genetic code, DNA repair, repeated genes, exchangeable amino acids within protein domains, etc.), or the result of homeostatic reaction (gene regulation via negative feed-back at the level of transcription and translation, physiological homeostasis, pH-buffering, etc.). Changed phenotypic expression, on the other hand, must be accommodated by the chemical reaction chains in which the changed proteins are involved, and which results in changed patterns of growth and differentiation, and hence, of phenotype. Wilson (1976) called our attention to the importance of Gene Regulation events during evolution of plants and animals. At that time the author stated: "*although definitive conclusions are not possible at present, it seems likely that evolution at the organismal level depends predominantly on regulatory gene mutations. Structural gene mutations may have a secondary role in organism evolution*". Thus, changes in form, color, morphology, physiology, and metabolism of many organisms may occur according to regulatory genes and the investigations about the kind of genetic (or metabolic) control over phenotypes under different environmental conditions have revealed that some genes are controlled to be *turned on* or *off* accordingly (Walker, 1979; Smith, 1990; De Jong, 1995; Land and Hochachka, 1995; Hochachka, 1996; Hochachka et al., 1998).

Long term responses to low-oxygen environment involves the suppression of oxidative metabolism in fishes of the Amazon, as first suggested by Hochachka and Randall (1978) and corroborated by Driedzic and Almeida-Val (1995) and West et al. (1999). However, the immediate responses to hypoxia presented by fish of the Amazon have been scarcely studied from the evolutionary point of view (reviewed in Almeida-Val et al., 1999a). Oxygen sensing and its physiological and biochemical consequences in the cell are not fully understood yet, despite some mechanisms having been extensively studied in isolated cell models, which may be cardiac myocytes (Webster et al., 1994), rat liver

hepatocytes (Keitzmann et al., 1992; 1993), or aquatic turtle hepatocytes (Land and Hochachka, 1995). All these studies suggest that some DNA sites are suppressed and some are activated when cells are exposed to hypoxia. Hochachka (1996) summarized all these data and suggested that up or down regulation of certain genes or group of genes are dependent on the intensity of hypoxia constraint and the ability of the model to tolerate this constraint or not. According to his review, a series of messengers (first and second) will be activated by an oxygen sensing mechanism that will affect several 100 nuclear genes and 13 mitochondrial genes when the cells are exposed to moderate hypoxia. However, the exposure to severe hypoxia will down regulate most DNA sites, inducing a decrease in mitochondrial volume densities, a decrease in Krebs cycle enzyme rates, and an increase in the ratios of anaerobic/aerobic pathways. So, up regulation of glycolytic rates are considered to be certain in most hypoxia responsive tissues.

Recent studies on mammalian cells have described that there is a transcriptional factor that coordinates the increased expression of glycolytic enzymes and the decreased expression of aerobic metabolism pathways whose expression is induced by hypoxia: the Hypoxia Inducible Factor 1 (HIF 1) (Firth et al., 1995; Ebert et al., 1996; Jiang et al., 1996; Wang et al., 1995). All these studies were summarized by Hochachka et al. (1998) showing that most glycolytic enzymes are induced, in a second round of gene expression, by HIF 1. The activation of PFK (phosphofructokinase), PGK (phosphoglycerate kinase), and LDH (lactate dehydrogenase) is induced by HIF 1, which in turn, is synthesized after a signal transduction pathway activated by oxygen sensing mechanisms.

Thus, following the evolution of hypoxia tolerance in fish will certainly be possible when we are able to follow the evolution of HIF 1 gene (or genes) and its subsequent distribution among all hypoxia tolerant groups. Hypoxia inducible factors have not been described in fishes. However, as an alternative, we can follow the evolution of the tolerance of hypoxia in fishes by considering the evolutionary trends in the genes that code for the anaerobic enzyme Lactate Dehydrogenase, which have been the subject of many molecular studies. This enzyme is induced in all hypoxia episodes in most vertebrates.

Trends in the evolution of LDH genes reflect trends in the evolution of fish tolerance to hypoxia.

The first evolutionary scheme designed for LDH genes in vertebrates was proposed early in the literature (Holmes, 1972; Holmes and Scope, 1974; Markert et al., 1975). According to this scheme, the ancestral subunit (formed by the ancestral gene) resembles subunit A (coded by LDH-A* gene), and the origin of the current A and B subunits occurred by ancient genome duplication events, as in almost all isozyme systems. Successive and independent duplications lead to the appearance of a third *locus* LDH C*, which is present in fishes, birds and mammals. Subsequent works have described alternative evolutionary schemes for LDH gene system (Li et al., 1983; Rehse and Davidson, 1986; Baldwin and Lake, 1987; Crawford et al., 1989), where LDH C* was considered to have close homology with the ancestral-like protein, rather the LDH A*, as thought before. Based on molecular biology studies, Goldberg (1990) and Stock and Whitt (1992) suggested that LDH C* from teleosts is not homologous to the mammalian LDH C*, suggesting that mammalian LDH C* genes have arisen in mammals as a consequence of gene duplications of LDH A*. Based on a review of structural, functional, and adaptive characteristics of fish LDH, four evolutionary trends may be described:

a) Convergence of LDH-B to LDH-A function, as occurs in turtles and hagfish, both subunits presenting A-type like kinetics. This is typical of good anaerobes (hypoxia tolerant vertebrates);

b) Widespread restriction of LDH B* gene expression, leaving the tissues only with A-type isozymes; a different way to reach the same result as above. It occurs in advanced teleosts, which live in oxygen limiting situations;

c) Non-divergence between A and B subunits. Some fish present this pattern and their metabolism is predominantly aerobic. This trend is supposed to occur in fish that present aquatic surface respiration; and

d) LDH C* widespread distribution in non specialized teleosts as Osteoglossomorpha, Elopomorpha, and Clupeomorpha groups, followed by its possible gene silencing in some fish groups (absence of LDH C isozyme in all Ostariophysan groups), and a strong restriction typically occurring in advanced teleosts (Acanthopterygian groups) (Almeida-Val and Val, 1993).

LDH C* gene silencing (not detected in any tissue) and restriction (detected in only one tissue) in fish; absence in amphibians (except for LDH C in oocytes of *Xenopus laevis*) and reptiles and a restriction in mammals and birds may place this gene as a specialized gene, which may have followed changes during its long evolutionary history, originating from the ancient gene, which was present in the vertebrate ancestor. During evolution this gene was, then, "modulated" inside each vertebrate group. LDH-B* gene restriction to aerobic tissues in most fish, birds and mammals, and its facility to be regulated in some fish (Almeida-Val et al., 1991; 1995) may place this gene in an intermediate position regarding evolutionary LDH system. The LDH A* gene is present in the great majority of fish tissues and it may be considered as the most functional adapted form since it is predominant in anaerobic tissues as skeletal muscles, and is responsible for the sole source of anaerobic ATP/energy in the great majority of fish tissues. So, functional properties of fish LDH support the idea that fish LDH genes do not share the same evolutionary history among other groups of vertebrates.

Studying nucleotide cDNAs sequences encoding LDH subunits A, B, and C from several vertebrate species, Tsuji et al. (1994) suggested that there have been, at least, six LDH gene duplications among vertebrates. Their main conclusions, based on the most parsimonious phylogenetic tree they obtained using cDNA sequences of 32 LDH genes, were described as follows: the tadpole *Xenopus* LDH A*, LDH B* and LDH C* *loci* are most closely related to each other, and originated from 2 more recent gene duplication events; then they are closely related to vertebrate LDH B*. The three cDNA sequences for fish LDH A*, as well as for the single LDH of Lamprey, also seem to be more related to land vertebrate LDH B* than to land vertebrate LDH A*. The mammalian LDH C* (from testis) appears to have diverged very early, prior to the divergence of vertebrate LDH A* and LDH B* *loci*, as already suggested in the literature. The tree corroborates the results obtained by Quattro et al. (1995), which concluded that killifish LDH C* gene is a duplicate of the LDH B* form, duplication that occurred after the divergence of the tetrapods and fish, and that LDH-A isozymes of teleost fish do not support an orthologous relation with LDH-A isozymes from tetrapod vertebrates. While these authors did not consider the results definite, they considered that three, out of these six duplication events, occurred prior to the divergence of the vertebrates. Thus, further cDNA studies of fish LDH gene system are necessary to clarify the real evolutionary pathway in this group, and detect the main duplication events that occurred during fish evolution history.

The Amazon fish fauna is, definitely, a candidate for such analysis, since it is one of the most diversified freshwater fish group on the whole planet, undergoing very similar environmental constraints, including evolutionary history. It includes representatives of non-specialized and specialized groups. The relatedness of LDH evolution with hypoxia tolerance in fishes of the Amazon may be, then, validated by molecular studies.

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EVOLUTION OF METABOLISM:

COMBINING PHYLOGENETIC PHYSIOLOGICAL

AND BIOCHEMICAL INFORMATION

TO STUDY METABOLIC ADAPTATION IN KILLIFISH.

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Introduction

The organizers of this symposium have challenged the participants to combine "morphological, physiological and biochemical traits and fish phylogenies" to understand the evolution of physiological and biochemical traits. My research has focused on identifying physiological and biochemical traits that have evolved in response to selection. The rationale for this has been not only to understand the evolution of these traits themselves, but to use evidence of adaptation to evaluate models of physiological and biochemical function. My interests have been specifically focused on how and which enzymes in metabolic pathways are adjusted in response to environmental challenges, notably temperature.

Temperature has a profound effect on metabolism and enzyme function, with enzyme activities halving for every 10°C drop in temperature. (Hochachka and Somero, 1984; Schmidt-Nielsen 1983). As organisms colonize colder environments, one would expect adaptations that compensate for this reduced activity to evolve. My work has focused on adaptations in cardiac glycolytic enzymes in killifish belonging to the North American genus *Fundulus*. Four species in this genus have independently colonized the steep thermal cline of the North American Atlantic Coast and must cope with over 10°C difference in mean annual temperature over their range (NDOC, 1982).

These fish may compensate for reduced activity at lower temperatures by several different mechanisms. They may increase their cardiac mass or allometric scaling of enzyme activity to maintain power output. At the biochemical level, they may express isozymes with different thermal properties

or they may increase enzyme expression to compensate for the reduced activity of each enzyme molecule (Lin and Somero, 1995; Crawford and Powers, 1992; Pierce and Crawford, 1994; 1997).

My primary question is how many and which enzymes should be adjusted by these various mechanisms during temperature adaptation. Classical biochemical theories predict that there is one "master" regulatory (usually non-equilibrium) enzyme per pathway (Crabtree and Newsholme, 1987) while metabolic control theories argue that all enzymes can modulate flux (Kacser and Burns, 1973; Cornish-Bowden and Cardenas, 1990). Laboratory studies tend to support the classical theories, yet field studies suggest that near-equilibrium enzymes can be important (e.g. Watt *et al*. 1986, Zamer and Hoffman, 1989). I have taken an evolutionary approach, which defines important enzymes as those enzymes whose variation in activity produces physiological consequences that are selectively advantageous or disadvantageous. By identifying enzymes that show evidence of selection and adaptive variation, we can infer that variation in these enzymes has functional consequences. That is, if the variation in enzyme activity is convincingly demonstrated to be due to natural selection, then that variation must affect a biologically important phenotype. However, drift and phylogenetic inertia also affect patterns of physiological and genetic differentiation (Garland and Adolph, 1994). Ideally, studies of physiology and metabolism should attempt to assess the influence of a variety of evolutionary factors on observed patterns of variation (Burggren and Bemis, 1990; Garland and Adolph, 1994). Two of the most common methods for taking into account evolutionary patterns in continuous data are phylogenetic autocorrelation (Cheverud and Dow, 1985; Cheverud *et al*. 1985) and independent contrasts (Felsenstein, 1985; Garland, 1992). These methods incorporate phylogenetic information into analysis of traits of multiple taxa to distinguish between adaptation and other evolutionary forces.

In this paper, I will examine whether the four Atlantic coast species show adaptive changes in heart mass, allometric scaling of enzymes or changes in enzyme levels using both intraspecific and interspecific analyses. Intraspecific analyses will determine population differences in these traits between northern and southern populations within each Atlantic species. These patterns will be compared to the results of interspecific analyses on mean trait values across among fifteen taxa (populations and species) within the genus. Interspecific analyses will be performed with and without phylogenetic information to assess the effect of ignoring phylogeny on the outcome.

Methods

Phylogeny of Fundulus

The genus *Fundulus* is a group of small, North American killifish found in both freshwater and saltwater environments. Most species are distributed along the Gulf of Mexico or throughout the southeastern United States (Wiley, 1986; Cashner *et al*. 1992). Its sister genus, *Plancterus*, ranges from Texas to Kansas. The relationships of the taxa used in this study are based on a combination of morphological and sequence data (Fig. 1; Wiley, 1986; Bernardi and Powers 1995; Pierce and Crawford, 1997).

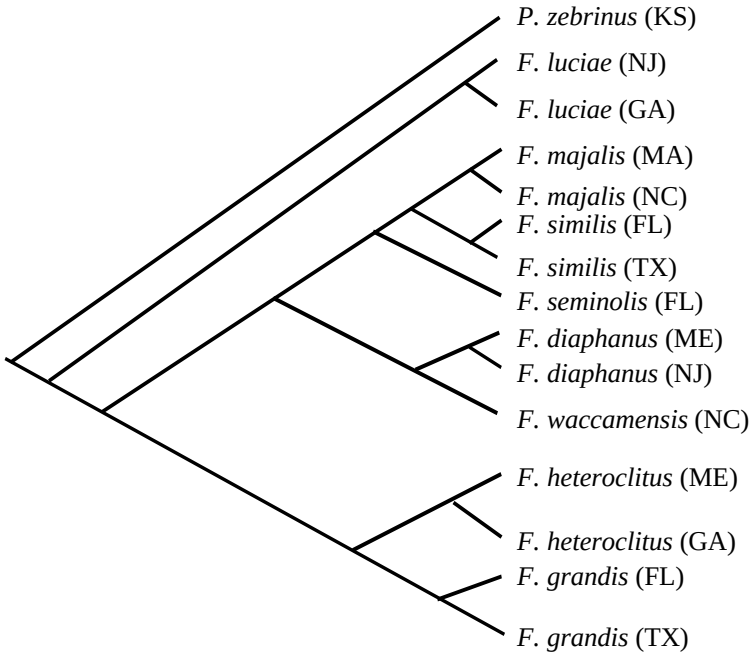

Figure 1. Phylogenetic Relationships of *Fundulus* species. Only species included in this study are shown. Phylogeny modified after Wiley (1986), Cashner *et al*. (1992), and Bernardi and Powers (1995). States of origin for each

Four species are found along the Atlantic Coast: *F. heteroclitus* from the *Fundulus* subgenus, *F. luciae* from the *Zygonectes* subgenus, and *F. majalis* and *F. diaphanus* from the *Fontinus* subgenus. The four Atlantic species each have a sister species in the Gulf of Mexico or in the southeastern United States and thus have independently colonized the Atlantic Coast. Each Atlantic species serve as replicates to identify changes in morphology and enzyme expression that correlate with changes in environmental temperature independently of phylogenetic history (Pierce and Crawford, 1997). Animal collection and handling were described previously (Pierce and Crawford, 1997).

Enzyme Assays

In order to test the competing models of metabolic regulation, all the enzymes in a pathway need to be tested systematically. Choosing only the one or few enzymes that one believes are important introduces an *a priori* bias to the data set generated. Maximal activity assays for all ten glycolytic enzymes plus lactate dehydrogenase were performed as described previously (Pierce and Crawford, 1994; Pierce and Crawford, 1997). Activities are expressed as micromoles of pyridine nucleotide metabolized per minute per milligram total heart protein. These measurements quantify enzyme concentrations, which is one measure of enzyme expression (Pierce and Crawford, 1994). Abbreviations for glycolytic enzymes are given in Pierce and Crawford (1997).

Statistical Analyses

Intraspecific analyses focused on the four Atlantic coast species. Analyses of variance and covariance were used to examine the allometry of body mass, heart mass and enzyme activities (units/mg protein) within and between northern and southern populations in each species.

Interspecific analyses used standard regression analysis to examine the effect of body mass and environmental temperature on mean population enzyme levels. Phylogenetic analyses used two methods, phylogenetic autocorrelation and independent contrasts, as described previously (Pierce and Crawford, 1997). Interspecific analyses included both Atlantic and Gulf coast species, for a total of fifteen taxa (Fig. 1). Phylogenetic autocorrelation is analogous to scaling on body mass and decomposes the measured variance in a trait into a phylogenetic value (the amount of variance explained by genetic distance) and residual, or "phylogeny-independent", values for each taxon (Cheverud *et al*. 1985).

Results and Discussion

Variation in Physical Characteristics

Body mass varied 2-4 fold among individuals within a species and ten-fold among *Fundulus* species. Heart and body mass did not vary consistently between northern and southern populations within species (Table 1). Interspecific analysis of mean body showed no significant correlation with temperature using either standard regression analysis or phylogenetic methods. Body mass did have a significant phylogenetic component, with 47% of the variation in body mass among taxa explained by phylogenetic distance and more closely related taxa tending to have more similar body masses (Pierce and Crawford, 1997). The lack of a consistent north-south mass difference may indicate that size *per se* does not confer an advantage with respect to temperature in *Fundulus* or that other factors may have greater influence on size.

In all but one species, population differences in heart mass could be explained by differences in body mass. Heart mass was proportionately larger in the northern populations of *F. majalis*, which may increase performance at lower temperature. However, because this response evolved in only one species out of four studied, it is not possible to distinguish between adaptiveness and chance differentiation of this trait (Garland and Adolph, 1995).

Table 1. North-South Differences in Body and Heart Mass. ANOVAs identified significant mass differences between northern and southern populations within each species. ANCOVA with body mass as a covariate was used to test whether population differences in heart mass remained after accounting for body size differences.

Species	Body Mass	Heart Mass	Body- Corrected Heart Mass
F. diaphanus	N > S	N > S	none
F. heteroclitus	none	none	N/A
F. majalis	N > S	N > S	N > S
F. luciae	S > N	S > N	none

Scaling of Enzymes with Mass

Mass-specific metabolic traits usually scale negatively with body mass. Recent papers have proposed that this is the result of the physics of branching networks and thus should apply to all biological systems (West *et al*. 1999). However, positive scaling has been reported in fish white muscle for three glycolytic enzymes, PFK, LDH and PYK, even though an aerobic enzyme in the same muscle scaled negatively (Somero and Childress, 1980; Burness et al., 1999). Somero and Childress (1980) noted that fish white muscle contraction is powered primarily by anaerobic glycolysis and thus should function independently of transport during burst activity. They proposed that because the power requirement increases exponentially with size due to the increased drag in water, anaerobic power would also have to increase at a higher exponential rate. It is not clear whether this positive relationship will extend to other muscle types.

In *Fundulus* hearts, we find the scaling relationship between mass and specific activity (per unit total protein) of the different glycolytic enzymes to be complex and not very consistent among species (Table 2). No enzymes scale with body mass or heart mass in *F. luciae* or *F. grandis*. Ten out of eleven glycolytic enzymes show scaling with mass, but never in more than three species. We find the same positive scaling of PFK and LDH with body mass that others have reported, but PYK in two species shows a negative relationship. The Gulf species, *F. similis*, shows negative scaling of seven enzymes. The Atlantic species tend to show positive scaling (with two exceptions, ALD and PYK in *F. diaphanus*), suggesting that perhaps positive scaling of enzyme activity with mass is advantageous along a thermal cline. Alternatively, the lack of consistency among species may indicate that scaling of glycolytic enzymes in this tissue is not very important and may vary due to drift.

Table 2. Intraspecific Scaling of Enzymes with Body Mass†

Enzyme	Scales with body mass in	Relationship
HK	*F. heteroclitus* (A)	+
	F. majalis (A)	+
	F. similis(G)‡	-
PGI	*F. similis*(G)‡	-
PFK	*F. majalis* (A)	+
	F. heteroclitus (p=0.06)(A)	+
ALD	*F. majalis* (A)	+
	F. heteroclitus (p=0.06) (A)	+
	F. diaphanus (p=0.054)(A)	-
TPI	*F. majalis* (A)	+
	F. similis (G)	-
GAPDH	*F. similis*(G)‡	-
PGK	none	
PGM	*F. heteroclitus* (A)	+
	F. similis (G)‡	-
ENO	*F. diaphanus* (A)	+
	F. similis(G)	-
PYK	*F. diaphanus* (A)‡	-
	F. similis (G)	-
LDH	*F. majalis* (A)	+

† no enzymes scaled with mass in *F. luciae* or *F. grandis*

‡ scales with heart mass only

A= Atlantic G= Gulf

The interspecific analyses show substantially different results. Only one enzyme, HK showed a positive relationship with body and heart mass, and three others, PGM, ENO and LDH, showed a negative relationship with body mass (Table 3). In all four cases, the scaling relationship became non-significant after correcting for phylogenetic effects on body mass and enzyme levels (Table 3; $p>0.26$ in all cases). HK, PGM, LDH and body mass have significant positive autocorrelation coefficients, indicating that phylogenetic distance among the taxa explain some of the variation in these traits. Thus apparent mass scaling

across species is confounded with phylogeny and may reflect correlated evolution of variation in body mass and enzyme levels.

Table 3. Interspecific scaling of enzyme activities with body mass before and after phylogenetic correction. Mean population enzyme activities and mean population body sizes were used for all

	Mass scaling relationship	
Trait	Before phylogenetic correction	After phylogenetic correction
HK	+	none
PGM	-	none
ENO	-	none
LDH	-	none

Population Differences in Enzyme Levels

If increasing enzyme levels is a way to compensate for reduced enzyme activity at lower temperatures, then northern populations should have higher levels than their southern counterparts. However, most of the significant intraspecific comparisons show that southern populations have higher enzyme levels. As with the scaling data, the population differences are not always consistent across all four replicate species. PGI is higher in the south in 3 species but higher in the north in the fourth (Table 4). Other enzymes show significant population differences in only one or two species (Table 4).

Again, the interspecific analyses show a very different picture from the intraspecific comparisons. When mean enzyme levels are regressed on mean annual temperature for fifteen taxa, only two enzymes, GAPDH and LDH, show significant or nearly significant relationships with temperature (Table 4; $p=0.033$ and $p=0.074$, respectively). These relationships are both negative, indicating that levels of these enzymes increase as environmental temperature decreases, as predicted.

Table 4. Effect of Mean Annual Temperature on Enzyme Activities. Results of significant ANOVAs and ANCOVAs performed on populations within species are given as intraspecific differences. Interspecific relationships are based on regressions across fifteen

		Interspecific temperature correlations	
Trait	Intraspecific differences	Before phylogenetic correction	After phylogenetic correction
HK**	S>N: *F. diaphanus*	none	none
PGI	S>N: *F. diaphanus* *F. majalis* *F. luciae* N>S: *F. heteroclitus*	none	none
PFK	S>N: *F. diaphanus* *F. majalis*	none	none
ALD	S>N: *F. luciae* N>S: *F. heteroclitus*	none	none
TPI**	none	none	none
GAPDH	none	**negative**	**negative**
PGK**	none	none	none
PGM*	S>N: *F. diaphanus* *F. luciae*	none	none
ENO	S>N: *F. diaphanus*	none	none
PYK**	S>N: *F. majalis*	none	**negative**
LDH**	none	**negative (p=.074)**	**negative**

* significant phylogenetic effect, $p<0.05$

** significant phylogenetic effect, $p<0.01$

Phylogenetic analyses do not substantially change these results, except to increase the significance of the GAPDH and LDH regressions, and phylogenetic autocorrelation indicates that PYK also correlates negatively with temperature (Table 4). Seven enzymes, including LDH and PYK, have a significant phylogenetic component, indicating that 59-79% of the variance in these enzymes can be explained by variation in the genetic distance of the taxa studied. Thus, in this system, natural selection has increased GAPDH, LDH, and possibly PYK, activities in northern taxa, independently of phylogeny.

What are the implications of these results for the regulation of metabolism? Two of the three enzymes, LDH and GAPDH, are considered near-equilibrium enzymes that should not affect flux; yet they appear to have undergone natural selection. If they have undergone selection, than their variation must have functional consequences that affect the survival or reproduction of the fish. The simplest explanation is that they affect metabolic flux, thus supporting metabolic control theories that say any enzyme can be important. This finding highlights the importance of sampling more as many enzymes in a pathway as is feasible - choosing only one or a few enzymes may miss other enzymes that are important.

This argument is based on evolutionary data about enzymes, but recent physiological work by Podrabsky *et al.* (2000) supports these findings. They found that 87% of the variation in oxygen consumption in *F. heteroclitus* hearts could be explained by variation in just three enzymes: GAPDH, PYK and LDH. Thus laboratory physiological measurements agree with the interspecific findings of evolutionary analyses that take into account multiple evolutionary factors.

What has this combination of morphology, physiology, biochemistry and phylogenetics told us about the evolution of physiological and biochemical traits? There are several conclusions that come from this work:

1) A lot of variation in enzyme activity is genetically based and thus subject to the forces that lead to differentiation of species, including neutral drift.

2) Near-equilibrium enzymes can also be important in adaptation and the "marker enzyme" approach may miss important adaptive responses.

3) Where phylogenetic information is available, it allows for a more powerful and informative analysis.

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MECHANISMS OF ADAPTATION TO TEMPERATURE IN FISH LDHS: FROM ANTARCTIC ICE TO THE MEXICAN DESERT

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Introduction

Fish occur in environments representing extremes of temperature, pressure, salinity and desiccation, and to survive these extremes they have evolved a broad array of physiological and biochemical modifications. While the internal milieu can be shielded from some physical factors, such as osmotic stress, others, including pressure and temperature, necessarily affect the organism on the molecular level, and it is here that adaptations to these stresses are most fruitfully studied. In order to understand better how enzymes evolve to cope with varying thermal environments, we have examined the glycolytic enzyme A_4-lactate dehydrogenase (A_4-LDH, EC 1.1.1.27, NAD^+:lactate oxidoreductase) from a diverse array of fishes. In each study, we have compared orthologs from closely related species that occur in differing thermal habitats, in order to ensure that the differences in structure and function we find are adaptive, and not accidents of phylogenetic history. Fish taxa we have studied include notothenioid species from polar Antarctic and temperate South American waters (Fields and Somero, 1998), barracudas (genus *Sphyraena*; Holland et al.,1997) from temperate to tropical waters, and gobies (*Gillichthys* and *Coryphopterus*; Fields and Somero, 1997) from the temperate California subtidal to the intertidal bordering the Mexican Sonoran desert. Our goal has been to determine how much change in environmental temperature is necessary to induce a change in

A_4-LDH function, as measured by apparent Michaelis constants for the substrate pyruvate (K_m^{PYR}), and turnover number (k_{cat}). In addition we have described the changes in structure (i.e., amino acid sequence) responsible for these modifications.

Antarctic notothenioids have adapted to their polar niche in the ~15my since Antarctic waters dropped to below 0°C. In this short period of time their A_4-LDHs have responded by developing lower substrate affinities (higher K_m's) and higher turnover rates (higher k_{cat}'s), as compared to South American notothenioids living in water at ~5-10°C. These functional changes can be ascribed to a limited number of substitutions within the primary structure of A_4-LDH. Similarly, barracuda congeners, some of which live in waters with mean temperatures only ~5°C apart, also show adaptive changes in kinetic parameters. In this case, due to the very limited number of amino acid substitutions among the orthologs, changes responsible for modifying K_m^{PYR} can be identified unambiguously. Among the gobies we studied, again the close relatedness of the species ensured that only a few amino acid substitutions were responsible for the kinetic differences seen between the *Coryphopterus* and *Gillichthys* A_4-LDH orthologs. Surprisingly, two congeners of *Gillichthys*, *G. mirabilis* and *G. seta*, possess orthologs that show distinct substrate affinities and thermal stabilities, and yet have no differences in amino acid sequence. This suggests that enzymes of very closely related species may adapt to changing thermal environments via mechanisms other than alteration of primary structure.

Methods

Sphyraena lucasana and *S. argentea* were collected by hook and line at Guaymas, Mexico (average water temperature 23°C) and La Jolla, CA, USA (18°C), respectively. *Gillichthys mirabilis* and *G. seta* were collected near San Felipe, Baja California Norte, Mexico. *Gillichthys mirabilis* was collected using baited minnow traps from an estuary where temperatures were measured at >30°C, and *G. seta* was collected by dip net from a cobble beach where water temperature as the tide rose exceeded 40°C. Individuals of all species were frozen on dry ice at the site of collection and transported back to the laboratory, where they were stored at –80°C until used. Antarctic notothenioid fishes (habitat temperature ~-1°C) were collected by otter trawl at depths of approximately 100 m from the R/V Polar Duke near Palmer Station, Antarctica. Specimens were frozen immediately at –80°C, shipped on dry ice to the United

States, and stored at –80°C until use. Dr. Ian Johnston of the Gatty Marine Laboratory caught South American notothenioid fishes in Patagonia, and he provided the white muscle samples used in this study as a kind gift.

A_4-LDH was purified from each species using affinity chromatography (Yancey and Somero, 1978). White muscle tissue was homogenized in 50 mM potassium phosphate, pH 6.8, and centrifuged at 10,000 g for 30 min. Potassium chloride and NADH were added to the homogenate supernatant at concentrations of 500 mM and 200 µM, respectively, and the supernatant was passed across an oxamate-sepharose column. After washing with excess NADH, pure A_4-LDH was eluted with 10 mM pyruvate in buffer. Fractions with high A_4-LDH activity were pooled and dialyzed overnight against 4 L 50 mM potassium phosphate, pH 6.8.

The parameters K_m^{PYR} and k_{cat} were determined by measuring the activity of each enzyme spectrophotometrically. Assays were performed in a temperature-controlled cuvette, with 80 mM imidazole (pH 7.0 at 20°C), 150 µM NADH, and eight concentrations of pyruvate chosen to straddle the expected K_m^{PYR} value. Reaction rates were measured at 340 nm, and these rates were input into a computer program (Wilman4; Brooks and Suelter, 1986) along with the corresponding pyruvate concentrations to determine K_m^{PYR} and maximal velocity (V_{max}). To derive k_{cat} from V_{max}, LDH concentrations were measured using a spectrophotometric assay based on Coomassie staining (Pierce Coomassie Plus, Rockford, IL, USA).

In order to obtain derived amino acid sequences of LDH-A from each species, mRNA was purified from white muscle tissue using the Dynabeads poly-thymidine magnetic bead protocol (Dynal, Great Neck, NY, USA). Complementary DNA was prepared from the mRNA using reverse transcriptase, and LDH-A cDNA was amplified using primers based on barracuda (*Sphyraena* spp.) LDH-A (Holland et al., 1997). PCR-amplified LDH-A cDNA was then sequenced using a dye-terminator protocol (Applied Biosystems Prism dye terminator kit) on an Applied Biosystems 373A automated sequencer. Resultant LDH-A sequences were aligned and analyzed using GCG (Oxford Molecular Group, Campbell, CA, USA) and DNAStar (DNAStar, Inc., Madison, WI, USA) software.

Results and Discussion

These studies have had three related goals: i) To describe how A_4-LDH kinetic parameters such as K_m^{PYR} and k_{cat} vary with environmental temperature in marine fishes, ii) To determine the structural differences (i.e., amino acid substitutions) in A_4-LDH orthologs that underlie adaptively modified reaction kinetics, and iii) To explain how these amino acid substitutions among orthologs lead to changes in the kinetic parameters we have measured.

It is clear from measurements of both K_m^{PYR} and k_{cat} that the kinetics of A_4-LDH of marine fishes, like other vertebrates that have been studied (Somero, 1995), are adaptively modified in response to changes in environmental temperature. Figure 1 shows K_m^{PYR} values for representative Antarctic and South American notothenioids (Fields and Somero, 1998), *S. lucasana* and *S. argentea* (Holland et al., 1997) and *G. mirabilis* and *G. seta* (Fields and Somero, 1997) A_4-LDH orthologs in relation to measurement temperature. At any measurement temperature the A_4-LDH orthologs from more cold-adapted species have higher K_m^{PYR} values, indicating a lower affinity to the substrate pyruvate. However,

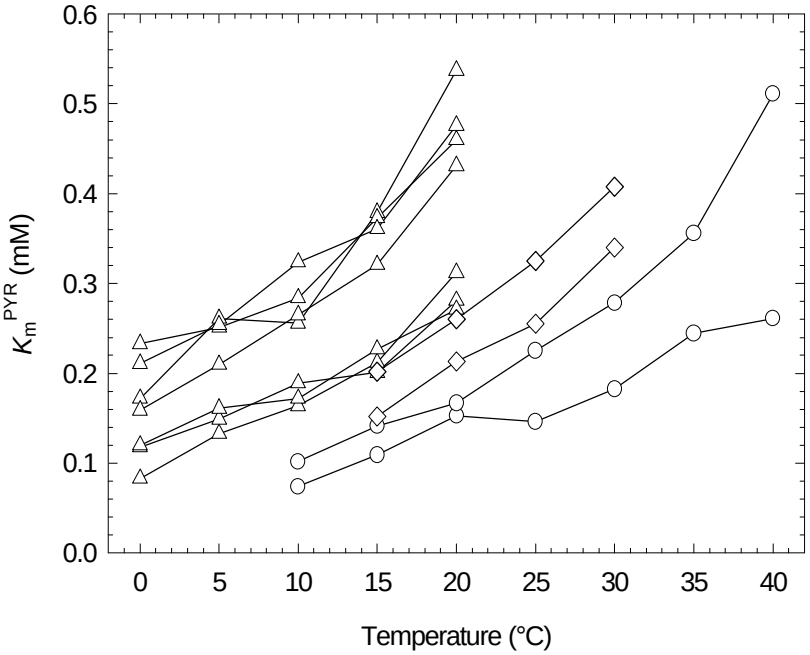

Figure 1. K_m^{PYR} of A_4-LDHs vs. temperature. Antarctic notothenioids -- open triangles; South American notothenioids -- closed triangles; *Sphyraena argentea* -- open diamonds; *S. lucasana* -- closed diamonds; *Gillichthys mirabilis* -- open circles; *G. seta* -- closed circles.

when compared at physiological temperatures (i.e., 0°C for Antarctic notothenioids, 5-10°C for South American notothenioids, 13-23°C for *S. argentea*, 16-28°C for *S. lucasana*, 20-30°C for *G. mirabilis* and 20-40°C for *G. seta*), the K_m^{PYR} values are comparable, indicating compensation to the amount of thermal energy present in the environment.

We also compared k_{cat} values for some of these species, as is shown in Figure 2. In this figure, all k_{cat} values have been corrected to 0°C using a Q_{10} of 2.0. As with K_m^{PYR} values, k_{cat}'s also show compensation for the temperatures each species experiences. In this case, the rate at which any A_4-LDH ortholog

catalyzes its reaction is correlated with environmental temperature; the more cold-adapted enzymes have higher k_{cat} values.

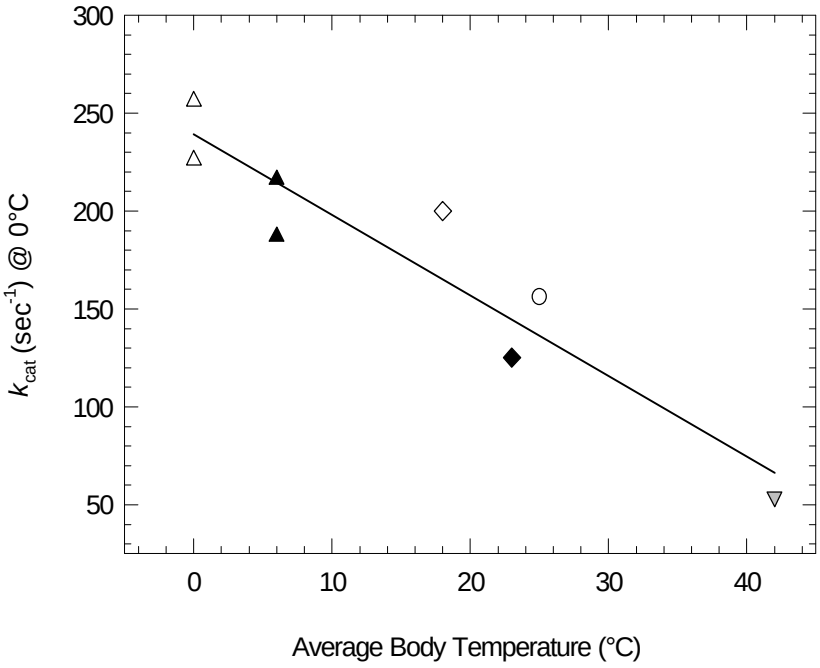

Figure 2. k_{cat}'s of A4-LDHs (at 0°C) vs. habitat temperature. Symbols as in Figure 1; stippled upside-down triangle -- *Dipsosaurus dorsalis* (desert iguana).

In order better to understand these phenomena of temperature compensation in the reaction kinetics of A_4-LDHs, we compared the primary structures (amino acid sequences) of the orthologs of each species. Despite the clear relationship between adaptation temperature and enzyme function, we were not able to identify any general modifications of protein structure that might underlie the changes in kinetics we measured. Table 1 shows a series of structural parameters for A_4-LDH orthologs from fishes, arranged in order of increasing

environmental temperature. Each of the attributes listed has been hypothesized to have some effect on enzyme function at different temperatures, usually by modifying the rigidity of the molecule in question. For example, it is expected that cold-adapted enzymes will have more glycyl residues than orthologs from warmer-adapted orthologs, because glycine, which has no side chain to sterically limit rotation of the polypeptide backbone, allows greater flexibility of protein structure (Matthews et al., 1987). Conversely, it has been hypothesized that cold-adapted enzymes will possess fewer prolyl residues, because the unique structure of the prolyl pyrrolidine ring restricts the rotational freedom of the peptide backbone (Creighton, 1993).

Other structural modifications that are expected to affect enzyme function at different temperatures include the amount of hydrophobic residues, the number of polar residues capable of hydrogen bonding, and the number of charged residues involved in salt bridges (Feller and Gerday, 1997; Jaenicke, 1991). On a secondary structural level, increased proportions of unstructured loops and turns may also lead to greater polypeptide flexibility and increased catalytic efficiency at cold temperatures (Davail et al., 1994).

An examination of each of the columns in Table 1 shows that regardless of the structural parameter chosen, there is no correlation between gross structural features and adaptation to temperature. In other words, at least in the case of A_4-LDH orthologs, there is no particular structural component that has been modified in all species during adaptation to different thermal environments. From these results we can reach two conclusions: i) That in order to alter kinetics to the degree measured in the above studies, the necessary modifications in A_4-LDH structure are relatively small (small enough to be missed by the large-scale comparisons described in Table 1), and ii) that structural adaptation to environmental temperature change can occur a number of different ways. In other words, small substitutions at widely varying sites along the A_4-LDH structure can have similar effects on kinetic parameters.

Table 1. Comparison of polypeptide attributes of LDH-As from some marine and freshwater fishes. All values are given as percent of the entire LDH-A molecule.

Species	Avg. Habitat T°	Helix + sheet	Turn + coil	Glycyl %	Prolyl %	Amino Acid Type				
						Charged	Acidic	Basic	Polar	Hydro-phobic
Chaenocephalus aceratus[1]	-1	85.8	14.2	8.8	3.0	27.8	10.9	10.3	22.4	36.9
Lycodichthys dearborni[2]	-1	85.5	14.5	8.4	3.0	27.4	10.5	10.2	22.6	37.4
Eleginops maclovinus[1]	7	86.5	13.6	8.4	3.0	27.4	10.8	9.9	23.2	37.1
Coryphopterus nicholsi[3]	14	86.8	13.3	8.4	2.7	28.3	10.5	10.8	22.0	37.7

Fundulus heteroclitus[5]	15	87.1	13.0	8.1	3.0	28.3	10.8	10.2	22.3	38.0
Cyprinus carpio[6]	15	83.4	16.6	8.1	3.3	27.9	10.8	10.8	23.1	37.2
Sphyraena argentea[4]	18	82.5	17.5	8.4	3.3	28.9	10.5	11.1	22.9	36.1
Gillichthys mirabilis[3]	20	87.1	13.0	8.1	2.7	28.3	10.5	10.8	22.0	38.0
Sphyraena lucasana[4]	23	86.5	13.6	8.7	3.3	28.6	10.8	10.8	21.1	37.0

1 - Fields and Somero (1998) *C.a.* Accession #: AAC63277, *E.m.*: AAC63283; 2 - AAD48489; 3 - Fields and Somero (1997) *C.n.*: AAC31199, *G.m.*: AAC28855; 4 - Holland et al., (1997) *S.a.*: AAB38886, *S.l.*: AAB38888, 5 - Quattro et al., (1995) Q92055; 6 - AAD40736.

These conclusions can be illustrated more fully by describing the amino acid modifications underlying A_4-LDH temperature adaptation in three groups of marine fishes: Barracudas (genus *Sphyraena*), notothenioids and gobies. In the case of the two *Sphyraena* species shown in Figures 1 and 2 and Table 1 (Holland et al., 1997), the K_m^{PYR} of *S. argentea* A_4-LDH is higher than that of *S. lucasana* at all temperatures tested, but is identical when measured at each species' body temperature (K_m^{PYR} = 0.24 mM pyruvate, 18°C for *S. argentea*, 23°C for *S. lucasana*). These findings indicate adaptation to environmental temperature in each species' A_4-LDH. The deduced amino acid sequences of the LDH-A of these two congeners reveal four amino acid differences in the 331-amino-acid-long molecule. Of these four, site directed mutagenesis studies have shown that only two play a role in modifying the kinetics of the catalytic reaction. These two are, in the direction *S. argentea* → *S. lucasana*, Ala61Val and Ser68Gly. An *a priori* examination of these substitutions would not suggest the presence of temperature adaptation, based on our current limited understanding of protein thermodynamics. Neither of these positions is involved in binding substrate or cofactor, and both substitutions are chemically conservative (that is, each change retains the chemical nature of the original residue, nonpolar to nonpolar and polar to polar, respectively). Thus the function of the enzyme has been adaptively modified by one or two subtle

amino acid residue changes outside the active site of A_4-LDH, although the temperatures the species experience are only ~5°C apart.

A study of notothenioid A_4-LDH orthologs provides conclusions similar to those of the *Sphyraena* study described above. Again, adaptive differences in kinetic parameters, both K_m^{PYR} and k_{cat}, were found in Antarctic notothenioids when compared to South American notothenioids and temperate teleosts in general (Fields and Somero, 1998). In this study, nine Antarctic and three South American notothenioid LDH-A cDNAs were sequenced, and in order to allow an analysis of cold-adaptation in the group as a whole, a consensus sequence of all notothenioid LDH-As was compared to a consensus sequence of orthologs from temperate non-notothenioid teleosts. This comparison revealed 17 amino acid differences, of which nine are non-conservative. We initially hypothesized that some of these non-conservative substitutions would be more important than others in modifying the kinetics of the extremely cold-adapted Antarctic notothenioid A_4-LDHs. These putatively important amino acid changes include additional glycines and fewer prolines in areas of the molecule responsible for controlling the motion of the active site. However, site-directed mutagenesis showed that modification of these amino acids has little effect on K_m^{PYR} of A_4-LDH. Instead, as in the *Sphyraena* study, two conservative substitutions outside the active site seem sufficient to explain the increased K_m^{PYR} found in Antarctic notothenioid A_4-LDHs.

The above two studies illustrate that only very minor changes in structure are necessary to alter kinetics of A_4-LDH in a temperature-adaptive manner. The final study -- of goby confamilials (Fields and Somero, 1997) -- adds support to this conclusion, and further suggests that such adaptive modifications may be possible with no change at all in the primary structure of the molecule. *Gillichthys mirabilis* lives in estuaries where water temperature may exceed 30°C, and *Coryphopterus nicholsi* occurs in the subtidal zone where

temperatures remain between 10 and 18°C. A comparison of the K_m^{PYR}'s of A4-LDHs of these gobies shows the expected temperature compensation (data not shown). As with the studies described above, this difference in kinetics can be traced to a minor substitution, Ala78Gly (from *G. mirabilis* to *C. nicholsi*), which is outside the active site.

A further comparison within the gobies, between *G. mirabilis* and its congener *G. seta*, leads to a more puzzling result. *Gillichthys seta* lives in the rocky intertidal of Northern Baja California, where water temperatures can rise above 40°C. As shown in Figure 1, its A_4-LDH K_m^{PYR} values reflect adaptation to its environment, when compared to those of *G. mirabilis*. Surprisingly, however, the deduced amino acid sequences of the A_4-LDH orthologs of these congeners are identical. The underlying nucleotide sequences show synonymous mutations that have been sequenced repeatedly, indicating that the identity of the amino acid structures is not a spurious finding. Thus, we are left with the conundrum of identical structures providing adaptively different kinetics.

A possible solution to this mystery is suggested by a series of experiments in which both orthologs were exposed to mild denaturing conditions (3M urea) and then allowed to renature (Fields and Somero, 1997). After this treatment K_m^{PYR} values of the A_4-LDH of *G. mirabilis* were unchanged, but the ortholog of *G. seta* now showed K_m^{PYR} values indistinguishable from those of *G. mirabilis.* These experiments suggest that the differences in kinetics between the congeners are based on different conformations of the same molecule, and that these differences can be partially erased by unfolding and refolding.

Conclusions

The three examples of A_4-LDH adaptation to temperature described above illustrate that the "rules" we expect proteins to follow when adapting to different temperatures -- e.g., changes in hydrophobicity, modifications in secondary structure, etc. -- do not necessarily apply when comparisons are made amongst closely-related species. Further, those differences ultimately responsible for large adaptive modifications in kinetics can be quite subtle (i.e., both conservative and far from the active site). These findings agree with other studies (e.g., Giver et al., 1998; Kotik and Huber, 1993), as well as theoretical considerations (Jaenicke, 1991), that suggest there are many ways to modify the kinetics of an enzyme, whether those modifications are performed in a laboratory or are evolutionarily mediated. Thus, if adaptation to novel environments ultimately depends upon random mutations within proteins,

perhaps the multiple pathways apparently available to these molecules makes getting from "here" to "there" more likely than once was expected.

Acknowledgements

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HYPOXIA TOLERANCE IN AMAZON CICHLIDS

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Abstract

Amazon cichlids are amongst the most diverse Neotropical fish groups. They are considered a plastic group from ecological, genetic, and evolutionary point of view. Their radiation may be considered as the result of environmental adaptations they developed during their evolutionary history. Among these adaptive traits, their ability to survive environmental hypoxia and anoxia has been described in several genera and seems to reflect their ecological preferences. The present paper compares behavioral, physiological and biochemical responses of four cichlid species exposed to acute hypoxia.

Introduction

Neotropical cichlids are advanced teleosts and occur in South and Central America and in the south part of North America. Recent reports have described them as a monophyletic clade retaining a fast rate of molecular evolution based upon a significantly higher level of genetic variation compared to their African counterparts (Farias et al., 1999). This group has always been considered a plastic group (Stiassny, 1991). Most authors consider their facility to adapt to heterogeneous habitats, as well as their fast adaptive radiation, as a cause of its numerous speciation events (Fryer and Iles, 1972; Kornfield, 1979, 1984; Stiassny, 1991).

Metabolic adjustments to extremely variable environments have been described as a complement of such phenotypic plasticity, particularly when fishes are exposed to hypoxia, a common event in Amazon water bodies (Almeida-Val et al., 1993; Val and Almeida-Val, 1995; Almeida-Val et al., 1995). The isozymic tissue distribution of Lactate Dehydrogenase (LDH: E.C. 1.1.1.27) represents species' adaptive tolerance to hypoxia and is adjustable to hypoxia exposure (Almeida-Val et al., 1995; 1999).

During our last expedition to Anavilhanas Archipelago in December 1999, our main goal was to establish hypoxia tolerance in cichlids and verify the presence of patterns among species. The present paper compares the preliminary data obtained with four cichlid species found in abundance at that time.

Material and Methods

Animals

Species were captured in Anavilhanas Archipelago at Negro River in the proximities of IBAMA (Braziliam Environmental Agency) floating station (Amazonas, Brazil). INPA's Research Vessel Amanaí II was anchored at 2°43' S and 60°45' W during the whole expedition period (December 1-10, 1999). Dissolved oxygen was monitored at the locality of capture and during different periods of the day. At that time, the amount of dissolved oxygen in the water was not a limiting factor (5.6 ± 0.115 mg/L O_2). The following species were studied: *Heros sp* (body mass 170.0±17.5g, ventricle mass 0.057±0.005g); *Uaru amphiacanthoides* (body mass 218.25±23.3g, ventricle mass 0.067±0.007g); *Satanoperca jurupari* (body mass 181.9±12,4g, ventricle mass 0,091±0,008g) and *Geophagus altifrons* (body mass 202.1±14.3g, ventricle mass 0.058±0.005g). Specimens were kept in outdoor tanks for 24 hours prior to the experimental procedure. After this period, three groups of two specimens (total 6) were exposed to acute hypoxia ($Pw_{O2} \cong 43$ mmHg) (N_2 flushing) in experimental polyethylene aquaria (56 L) at room temperature (26.0±2.0°C). The period of time the animals supported before loosing equilibrium was measured for each animal. Oxygen contents were monitored during the whole experiment using a Digital acid-base analyzer, attached to a PO_2 module Radiometer, PHM72-Mk2. During the experimental period, we measured VO_2 and opercular movements. For control purposes, each experiment was also repeated with animals exposed to normoxia ($Pw_{O2} \cong 160$ mmHg). All experimental procedures took place at INPA's Research Vessel Amanaí II, in December 1999, when river water levels were low.

Blood sample

Immediately after the experiments, blood was collected from the caudal vein into heparinized syringes, transferred to Ependorff tubes and kept on ice. Blood parameters: hematocrit, hemoglobin concentration, and red blood cell counts (RBC) were estimated by classical methods. Plasma glucose was estimated using enzymatic method with commercial kit (Doles®).

Tissue preparation

Immediately after blood collection, animals were killed with a sharp blow to their head followed by severing of the spinal cord, as recommended by animal care associations. Tissues (skeletal and heart muscles) were then excised and promptly frozen in liquid nitrogen. Samples were transferred to laboratory and stored at a - 73^{o}C before analysis. Skeletal and heart muscles were homogenized by hand in test tubes with glass tube rods in 4 vol. (skeletal muscle) or 9 vol. (heart muscle) of medium containing 75 mM TRIS, 1 Mm EDTA, and 1 mM DTT at pH 7.6. The homogenates were centrifuged at 18,000 g for 30 minutes at 4^{o}C in a Sorvall RC5B centrifuge. Supernatants (tissue extracts) were used to estimate enzyme activities and electrophoresis. All assays were performed directly on total extracts or dilutions of total extracts.

LDH activities

Maximum activity of lactate dehydrogenase (LDH; E.C. 1.1.1.27) were determined at 25^{o}C using a Genesys 2 spectrophotometer. Assay conditions followed those described in Almeida-Val et al. (1995). All enzyme assays had a final cuvette volume of 1 mL, and were measured at 340 nm. Composition of the assay media was 0.15 mM NADH, 1 mM KCN and 50 mM imidazole (pH 7.4). The reaction was initiated with the addition of 1 mM pyruvate, or with the inhibitory concentration (10 mM). Enzyme activities were expressed as moles of pyruvate converted per minute per gram of wet tissue.

Data analysis

The results are expressed as means ± SEM. Statistical differences between groups were tested with Student's *t*-test or by one-way ANOVA (Sigma Stat). Significance of difference was accepted when $P<0.05$.

Results and Discussion

VO_2 and Opercular movements

Among all analyzed species, *S. jurupari* had no tolerance to acute hypoxia. The remaining three species tolerate hypoxia for 60 minutes, or more (43 mmHg) (table 1and figure 1)**.**

Table 1. Opercular movements and time to reach disequilibrium of four Amazon cichlids submitted to normoxia and acute hypoxia.

SPECIES (TIME TO REACH DISEQUILIBRIUM)	**Opercular movements (mov/min)**	
	Normoxia	Hypoxia
Heros sp (60 min)	[a] 78.7 ± 1.3	45.8 ± 6.7*
Uaru amphiacanthoides *(85–6.7 min)*	[ab] 70.3 ± 5.3	30.2 ± 6.3*
Satanoperca jurupari *(< 20 min)*	[ab] 72.2 ± 6.5	50.5 ± 4.4*
Geophagus proximus *(67.5–12.2 min)*	[b] 62.4 ± 2.0	37.0 ± 3.7*

Note: Values are given as means ± SEM (mov/min). ***** Represents significant difference between treatments (normoxia and hypoxia). Different letters mean significant differences between means.

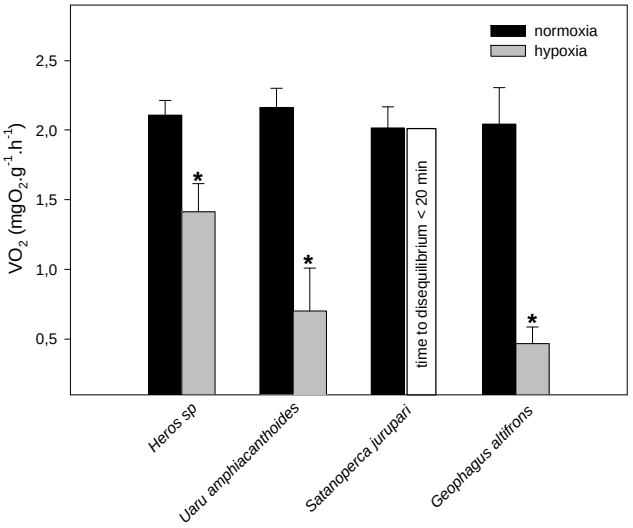

Fig. 1. VO_2 of four Amazon cichlids submitted at normoxia and acute hypoxia. Symbol (*****) represents significant difference for each species in different condition.

Cichlids constitute a group of water-breathing fishes, which are, in the great majority, adapted to hypoxia and anoxia. Most species in this family are considered to inhabit shallow and hypoxic várzea lakes (shallow waters) in the Amazon basin. Such behavior requires a good tolerance to short and long-terms hypoxia episodes (Junk et al., 1983). As already shown for African cichlids (Verheyen et al., 1994), our data are consistent with the hypothesis that, among this family, there are two groups: those that are hypoxia-tolerant and those that are hypoxia-non-tolerant. Regarding the studied species, *Satanoperca jurupari* is the only species considered to be hypoxia-non-tolerant. However, there is no noticeable pattern in the responses among the remaining species.

Hematological parameters

Satanoperca jurupari exposed to hypoxia presented an increase in hematocrit and RBC, suggesting a red blood cell release into this species' circulating blood. The remaining 3 species showed no hematological changes (P>0.05) (table 2).

Table 2. Hematological parameters in four Amazon cichlids exposed to hypoxia (N) and hypoxia (H).

		Ht (%)	Hb (g/dL)	RBC x 10^6.mm^{-3}	Glucose (mg/dL)
Heros sp	**N**	[a] 28.3 ±2.0	4.9 ±0.3	[ab] 1.7 ±0.2	8.8 ±2.4
	H	25.4 ±2.3	[ab] 5.5 ±0.4	1.8 ±0.2	9.0 ±1.0
U. amphiacantoides	**N**	[ab] 25.3 ±1.5	5.1 ±0.4	[ab] 1.7 ±0.1	8.7 ±1.8
	H	28.8 ±2.0	[a] 5.8 ±0.5	2.1 ±0.2	6.8 ±1.9
S. jurupari	**N**	[b] 21.3 ±1.0	4.7 ±0.5	[b] 1.3 ±0.1	6.2 ±1.0
	H	26.9 ±1.6*****	[b] 4.3 ±0.2	1.6 ±0.1*****	8.6 ±1.4
G. proximus	**N**	[a] 27.3 ±1.5	6.0 ±0.4	[a] 2.3 ±0.2	12.8 ±2.41
	H	27.3 ±2.2	[ab] 5.4 ±0.5	2.0 ±0.1	12.3 ±2.3

Note: Symbol (*****) represents significant difference for each species in different conditions, and letters represent significant difference among species in same condition.

LDH activity levels

The exposure to hypoxia induced a decrease in muscle LDH levels from *Heros sp* and an increase in LDH levels of *S. jurupari*. On the other hand, muscle LDH from both *U. amphiacanthoides* and *G. altifrons* did not respond to acute hypoxia exposure. Pyruvate inhibition rates responded accordingly to these changes (table 3). LDH from *Heros sp* was not inhibited by high pyruvate concentrations in both normoxia and hypoxia groups, indicating the reliance of this tissue on anaerobic glycolysis. The same result could be observed in *U. amphiacanthoides* and *Geophagus altifrons*. On the other hand, there was an increase in inhibition rate in muscle LDH from S. jurupari exposed to hypoxia (table 3) showing that the activation of anaerobic metabolism is impaired in this tissue when pyruvate levels increase up to 10 mM.

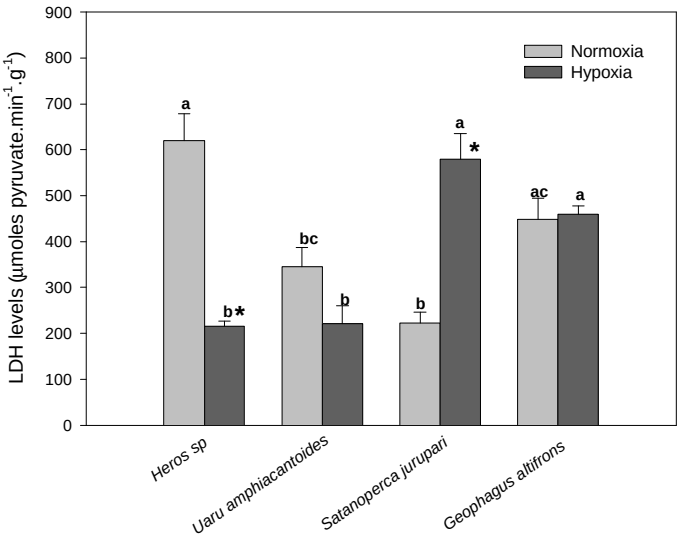

Figure 2. LDH levels in muscle from analyzed species at different experimental conditions. Symbol (*****) represents significant difference for each species in different condition.

Table 3. Enzyme inhibition rates obtained between white muscle (WM) and heart (H) LDH activities at 1 and 10 mM pyruvate concentrations.

SPECIES	**Normoxia**		**Hypoxia**	
	WM	H	WM	H
Heros sp	0.87±0.11	0.74±0.05	0.34±0.03	0.86±0.11
Uaru amphiacanthoides	0.54±0.02	0.59±0.22	0.54±0.04	0.96±0.01
Satanoperca jurupari	0.74±0.08	1.48±0.27	2.84±0.48	1.24±0.22
Geophagus proximus	0.75±0.08	1.18±0.09	0.70±0.01	1.57±0.34

Heart LDH levels of *U. amphiacanthoides* and *S. jurupari* responded to hypoxia exposure in a different way. *Uaru amphiacanthoides* presented an increase in LDH levels indicating that this species' heart activates anaerobic glycolysis, while *S. jurupari* decreases anaerobic metabolism in this organ revealing its metabolic suppression (figure 3). There was no inhibition in LDH levels when 10 mM pyruvate concentration was used in heart of *Heros sp* and *Uaru amphiacantoides*, while heart LDH from *S. jurupari* and *G. altifrons* is inhibited in all analyzed situations (table 3).

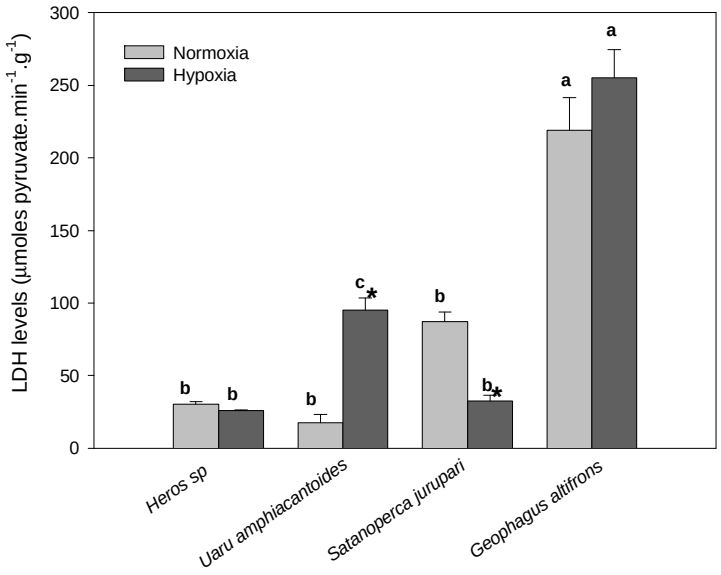

Figure 3. LDH levels in heart from analyzed species at different experimental conditions. Symbol (*****) represents significant difference for each species in different condition. Letters represent differences among species at same experimental procedure.

Cichlid's responses to hypoxia have been related to their preferential habitats and to their ability to regulate tissue expression of the enzyme lactate dehydrogenase (Almeida-Val et al., 1995; 1999). Except by *S. jurupari*, all species regulated their metabolism to tolerate acute hypoxia exposure. Studies with lacustrine and riverine African cichlids also suggested that the species are adapted to tolerate hypoxic environments, and their ability to regulate metabolic rates under gradually increasing hypoxia differs markedly among species (Verheyen et al., 1994). While the experimental design for the present work differs from that used with African cichlids, our data also reveal that acute hypoxia exposure induces metabolic responses that differ markedly among species. In addition, the regulation of LDH levels is also species-specific. Unfortunately, the molecular mechanisms responsible for induction of anaerobic metabolism in the tissues under hypoxia have not been clarified yet.

Considering the myriad of adaptive characteristics already described in fish, particularly tropical species, several options of morphological and anatomical adaptations are linked to metabolic adjustments (reviewed in Val and Almeida-Val, 1995; Almeida-Val and Hochachka, 1995). Amazon fish are supposed to either activate anaerobic metabolism or suppress oxidative metabolism when exposed to hypoxia (Almeida-Val et al., 1993). Among cichlids, these two possibilities are considered based on LDH isozymic distribution in heart of several species according to its preferential habitats (Almeida-Val et al., 1995).

This is the first comparative study about hypoxia tolerance in neotropical cichlids. Thus, more data will be needed before we define patterns of metabolic responses in such group. The present data, plus other enzyme assay measurements (not shown), suggest that there is a species-specific response to hypoxia among cichlids, which may follow species adaptation to preferential habitat.

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TRACKING EVOLUTIONARY TRENDS IN SILURIFORMES

(TELEOSTEI; OSTARIOPHYSI)

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Introduction

Phylogenetic hypotheses have proven to be instrumental in assessing if morphological novelties are advantageously adaptive or exaptations. Darwin (1859) pioneered the use of an evolutionary framework in behavior. The advent of a rigorous method for determining genealogical relationships, cladistics (sensu Hennig, 1966), has triggered more studies on behavior evolution. Phylogenetic works, however, are still in their relative infancy. Large groups are still to be phylogenetically analyzed, and most published studies are preliminary or cover a diffuse array of topics (McLennan, 1994).

In fishes, the vast majority of phylogenetic studies are based on osteological, external morphological or molecular characters. Molecular systematics is attractive for providing a time frame for diversification events: however anatomical/osteological features provide grounds for comparative anatomy, behavior, and analyses of sequence of evolutionary novelties.

The ordinary way to reconcile phylogenetic relationships and behavior is by optimizing the character of interest in a given phylogeny. The main goal is to find the best hypothesis of the evolution of the character that fits in parsimoniously with the given phylogenetic hypothesis. The fact that a derived trait appeared at the same time as the development of a certain function may be the evidence necessary to recognize an adaptation (Greene, 1986; Coddington, 1988). On the other hand, if the function predates the evolutionary novelty, we may almost certainly affirm that the trait is irrelevant for the function - Donoghue (1989, p. 1147) illustrates the issue very clearly and states that

"...besides assessing sequence of character change, cladograms may provide means of testing ideas concerning changes in flexibility during evolution".

The present paper is a preliminary attempt to track putative adaptational traits on Siluriformes based on recent phylogenetic hypotheses. According to available hypotheses of Siluriformes relationships, different tests could be made comparing behavior and morphological or physiological/biochemical traits. Two tentative tests are going to be analyzed: air-breathing capacity and sexual dimorphism.

1.Air-breathing capacity – correlation with swimbladder encapsulation

Swimbladder encapsulation is an overgrowth of bony tissue from skull parts that ended enclosing the bladder in bone. This modification is a synapomorphy for a set of Neotropical catfish families (Baskin, 1973; Schaefer and Lauder, 1986). The physiological importance of such modification has never been properly appreciated. One possible consequence of encapsulation could have been the loss of an organ to perform gas exchange, which could have forced their bearers to develop a new evolutionary pathway such as capturing O^2 from the air, giving rise to the air-breathing behavior in these fishes. In this test, I try to identify the possible correlation between these two traits, air-breathing and gas bladder encapsulation, based on the hypothesis that if these characters co-evolved, one might have predated the other in appearance, the origin of one trait may have been advantageous for the second and it should be present in the same taxa.

2. Sex dimorphism - modifications on skull and mouth parts

In this analysis, I try to evaluate the importance of certain derived features with the presence of sex dimorphism. This analysis is restricted to the armored catfish family Loricariidae because sex dimorphism and morphological novelties in this family have been receiving closer attention lately and some information is currently available in the literature.

Methods

Siluriformes comprise an extremely successful group of freshwater fishes. With around 30 families, only 1 is exclusively marine, 2 have freshwater and marine representatives, and the vast majority occurs exclusively in freshwater. Siluriformes are present in all continents, except Antarctica, being mainly concentrated in tropical areas (low latitudes). Siluriformes originated in the early Cretaceous, before or about the time of the break-up of Gondwana. These fishes have colonized all kinds of water and habitats (except oceanic depths and below-freezing waters) and have certainly a long pathway of historical, phylogenetic and structural constraints still to be uncovered. The high number of Siluriformes species and their morphological and environmental diversity make this group of fishes an excellent source of information on character evolution.

The first step for accomplishing this kind of enterprise is to find robust and reasonably resolved phylogenies. The phylogenetic hypothesis of Neotropical Siluriformes relationships used as the background for this analysis was summarized from de Pinna (1993, 1998). Loricarioidea phylogeny was based on Lundberg & Baskin (1969), Baskin (1973), Schaefer & Lauder (1986), Schaefer (1990), de Pinna (1992, 1998). Loricariinae phylogeny is based on Rapp Py-Daniel (1997). These three sets of cladograms were based on morphological/osteological characters.

Siluriformes are clearly monophyletic (Fink & Fink, 1981, 1996; de Pinna, 1998). Currently, loricarioids include Trichomycteridae, Nematogenyidae, Scoloplacidae, Callichthyidae, Astroblepidae and Loricariidae (Baskin, 1973; Schaefer & Lauder, 1986; de Pinna, 1993, 1998), all exclusively Neotropical. Phylogenetic relationships within loricarioids appear to have been reasonably assessed, as well as the monophyly of its inclusive families.

Based in the Loricariinae phylogeny, some taxonomic names used herein are represented between quotes. These names represent clades strongly supported cladistically, such as, ‘Hemiodontichthyina’ which includes three genera and six species and ‘Planiloricariina’ with six genera and 14 species included.

None of the behavioral characters to be analyzed were used in the reconstruction of the cladograms in order to avoid circularity or excessive weight to the same

features. The characters were mapped in the phylogenies and their evolution optimized using parsimony.

Results

Evolution of swimbladder encapsulation and air breathing behavior

Putative questions:
Is air-breathing related to encapsulation?
Which character originated first?
Are these characters plesiomorphic or apomorphic? For which levels?

Based on the existing available data (Graham, 1997), representatives of Siluriformes considered as air-breathers include Aspredinidae, Trichomycteridae, Callichthyidae, Loricariidae (figure 1), the Asian Pangasiidae and Heteropneustidae and the African/Asian Clariidae. Swimbladder encapsulation is found in Aspredinidae (not complete) and in all loricarioid catfish families Nematogenyidae, Trichomycteridae, Scoloplacidae, Callithyidae, Astroblepidae and Loricariidae (complete) as well as in the African Amphiliidae and the Asian Akysidae, Amblycipitidae, and Sisoridae.

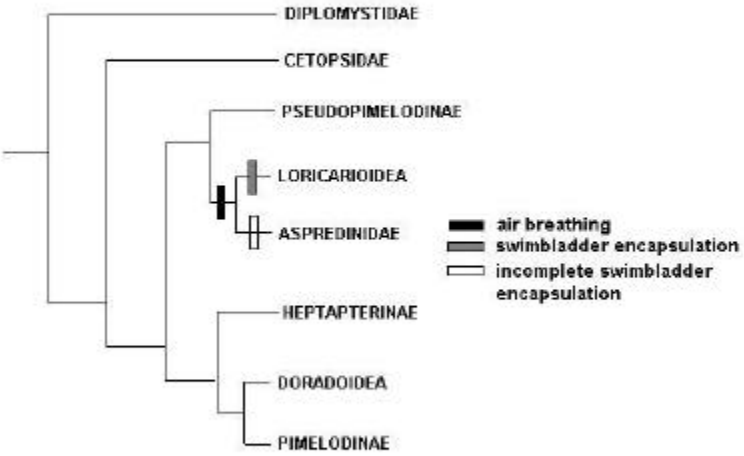

Figure 1. Distribution of air breathing and swimbladder encapsulation among Neotropical Siluriformes (cladogram adapted from de Pinna, 1993, 1998)

In the case of loricarioids, I suggest that air-breathing behavior in these fishes could be related to the encapsulation of the swimbladder. If air breathing had originated as an alternate pathway to compensate for encapsulation of the bladder, we should expect that all families with encapsulated swimbladder should be adapted to breathe air and this is not the case (at least yet). About 50% of loricarioids families are air breathers and have encapsulated bladders. Moreover, aspredinids do not have the complete encapsulation but do perform air breathing (figure 2).

Although we may suspect of some sort of correlation between encapsulation and air breathing among Neotropical catfishes, no correlation can be made for the African/Asian catfishes. Among African/Asian catfish, the families that have air breathers are completely unrelated to the families with swimbladder encapsulation (table 1) since the families that show air-breathing behavior do not have the swimbladder encapsulated and vice-versa.

Table 1. List of Siluriformes families with air-breathing organs (ABO) and encapsulated swimbladders (SB); geographical distribution (SA = South America, AS = Asia, AF = Africa); and number of species (SP)

	AB (ABO)	SB	CONTINENT	# SP
Aspredinidae	+ (mouth)	+	SA	34
Nematogenyidae	?	+	SA	1
Trichomycteridae	+ (stomach)	+	SA	200
Scoloplacidae	?	+	SA	4
Callichthyidae	+ (intestine)	+	SA	172
Loricariidae	+ (stomach)	+	SA	>650
Astroblepidae	?	+	SA	40
Pangasiidae	+ (swimbladder)	_	AS	21
Clariidae	+ (pharyngeal sac)	_	AF + AS	90
Heteropneustidae	+ (pharyngeal sac)	_	AS	2
Amblycipitidae	?	+	AS	8
Akisidae	?	+	AS	15
Amphiliidae	?	+	AF	48
Sisoridae	?	+	AS	91

Based on Burgess (1989), Graham (1997) and de Pinna (1998)

Interestingly, Neotropical Siluriformes that breath air have their guts (stomach or intestine) used as an ABO (air breathing organ), completely different from the African/Asian Siluriformes that have pharyngeal sacs highly specialized (Clariidae and Heteropneustidae) or use the modified swimbladder as an ABO (Pangasiidae) (Graham, 1997). This different level of strategy suggests quite different origins of the air-breathing behavior among silurifomes. African/Asian and Neotropical Siluriformes have been separated for at least 100 million years, probably enough time for development of different air breathing behaviors.

On the other hand, the origin of encapsulation of the swimbladder can be

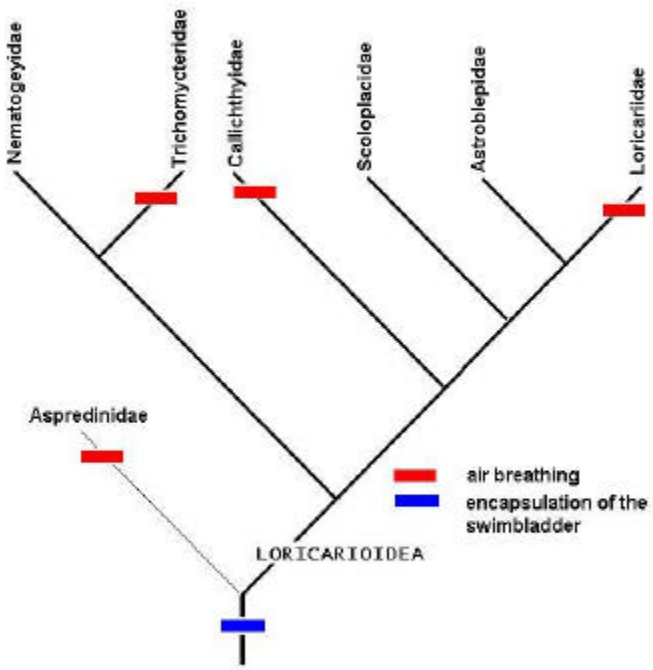

Figure 2. Distribution of air breathing behavior and swimbladder encapsulation among Loricarioidea (cladogram adapted from de Pinna, 1998)

interpreted either as a synapomorphy for a transatlantic group of Siluriformes (South American, African and Asian), as suggested by de Pinna, (1998) or as a homoplasy. The first possibility may be the correct one since the structures involved, lateral expansion of the secondary transverse process of the fifth vertebra and ventrolateral extension of the ventral surface of the Weberian complex, in the bladder encapsulation are the same in these catfishes, being consequently homologous (de Pinna, 1993).

According to the continent, you can draw different conclusions: based on African/Asian catfishes, air breathing and encapsulation are completely unrelated. However, among South American catfishes, encapsulation seems to be related to air breathing.

Encapsulation seems to have predated air breathing in Siluriformes. Air breathing arose at least three times among Siluriformes: derived from modifications of the swimbladder (more primitive), from the guts or from pharyngeal morphological innovations (more specialized). However, we may infer that there is correlation between AB and SB in the Neotropical Siluriformes since both traits seem to have been advantageous for these fishes. The families with air breathing capacity are much more diverse than the families without these traits (table 1). Thus, gut air breathing could be seen as a putative adaptation for catfishes with bladders enclosed in bone.

Sex dimorphism and evolution of the mouth parts in Loricariidae

Loricariidae is a catfish family extremely diverse in the Neotropics, with more than 600 species occurring in all major drainages in South and Central America. These fishes are characterized by an armored body, and very specialized mandibles with suctorial mouth, papillate lips for attaching to the substrate, and great mobility of the mouth parts. This great mouth specialization can be a consequence of the vital functions performed by this structure: the mouth in loricariids functions as an attachment organ, food collector and also sucks water.

Loricariidae are also known by their elaborate sexually dimorphic modifications present in mature males. Some of these sex dimorphisms are pervasive throughout loricariids and are considered generalized traits for the family. Other displays however are very distinctive and present in few selected taxa. Some of these elaborate dimorphic traits also occur on the mouth parts of these fishes, such as lips, teeth, premaxilla, etc. Since the mouth in these fishes has an extraordinarily complex role, I would like to evaluate the possibility of co-occurrence of some of the morphological modifications of the mouth with some behavior characters to assess their adaptive/evolutionary relevance for the group.

Mouth

The generalized condition in loricariids is the presence of large jaws bearing many elongate and asymmetrically cuspidate teeth. Reduction of the number of teeth and size of the jaws occurs in very few unrelated loricariid taxa. Based on the available hypothesis of relationship within the subfamily Loricariinae (Rapp Py-Daniel, 1997), there are two large clades: one with large jaws (Harttiini) and the other with reduced jaws (Loricariini). Jaw reduction is a synapomorphy for the subfamily Loricariinae among loricariids. This reduction involves modifications in many bony structures such as palatine, premaxilla, dentary, maxilla, and branchial apparatus. For all Loricariini, the most remarkable synapomorphy is the reduction of the dentary and the coronoid process.

Within Loricariini, two large clades present different patterns of further jaw reduction (figure 3): the clade 'Hemiodontichthyina' + Limatulichthys + Pseudoloricaria and 'Planiloricariina'+ Loricaria. Hemiodontichthyina + Limatulichthys + Pseudoloricaria comprise loricariines with extremely pointed to slightly prolonged snouts, palatine with large lateral expansion, long maxillae, modified gill-rakers with ossification centers, hypobranchials large and fan-shaped, premaxillae reduced to a thin sheet of bone, and number of teeth reduced (less than 20) until completely lacking (*Hemiodontichthys* and *Reganella*). Planiloricariina + Loricaria include forms with triangular to completely round snouts, palatine extremely elongate and without processes or bone expansions, powerful and completely toothed lower pharyngeal plates, mandibullary teeth round and very reduced in size and number (less than 10) until completely absent (*Planiloricaria*).

In addition to different osteological features, these clades also show different lip morphology. Fishes of the Planiloricariina + Loricaria clade have both upper and lower lip-surface and border covered by strong papilla or long filaments. These fishes have filaments even inside the mouth. Hemiodontichthyina +

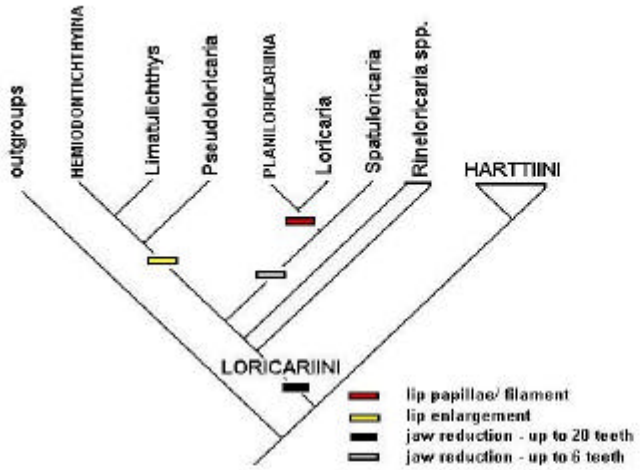

Figure 3. Distribution of mouth-related sexually dimorphic traits and jaw reduction in the subfamily Loricariinae (adapted from Rapp Py-Daniel, 1997)

Limatulichthys + Pseudoloricaria, on the other hand, have the lip-surface almost smooth or covered by delicate papillae, being *Furcodontichthys* the only exception. *Furcodontichthys*' lips are smooth but show few and long filaments on some restricted areas on the lower lip. The border of the lips, however, is straight.

Sex Dimorphism

The generalized sexually dimorphic display in loricariids is the presence of large odontodes (integumentary teeth) on head, fins and body on mature males. Neither Hemiodontichthyina+ Limatulichthys + Pseudoloricaria nor Planiloricariina+ Loricaria males have odontodes enlargement. Rather, these groups have species with other sexually dimorphic traits on the mouth during the breeding season. In the Hemiodontichthyina +L +P clade, males have an outgrowth of lip tissue providing a large soft surface used to carry the mass of fertilized eggs attached to it. This modification is present in almost all representatives, being *Reganella* the only exception. Within the Planiloricariina + Loricaria clade, sexually mature males do not have lip-enlargement but show lip-filaments reduced to papillae, whereas females keep the long lip filaments.

Putative questions:

- Can mouth-related sexually dimorphic traits be evolutionarily related to loricariine jaws reduction?
- Is jaw reduction homologous on both clades?
- What came first? Sex dimorphism or jaw reduction?

It seems that jaw reduction predated the origin of mouth-related sex dimorphism because its origin is early on Loricariini evolutionary history. There are, however, several levels of jaw reduction. We can detect three levels of number of teeth reduction: the first level would be the reduction to up 20 teeth on premaxillae for all loricariinis; second level it would be the reduction to up 6 teeth in the premaxillae (only found in Planiloricariina+Loricaria + Spatuloricaria), and the third level it would be the complete loss of premaxillary teeth, found in representatives of both clades (Hemiodontichthyina + L+ P and Planiloricariina + Loricaria).

Long lip filaments, based on the hypothesis shown in figure 3, did not arise immediately after the first level of jaw reduction, or dentary reduction. Rather, long lip filaments are seen only in a subclade of Planiloricariina+ Loricaria. *Spatuloricaria*, the most basal monophyletic taxon within loricariinis, shows already a strong reduction in the number of premaxillary teeth (up to 6), however, all its representatives have papillate lips (non-filamentous) and the

generalized sex dimorphic ornament of large odontodes on the sides of the head on mature males. Moving up in the cladogram, the next taxon, *Loricaria*, already have sexually mature males with lip filaments reduced to papillae, a mouth-related sex dimorphism. On the other hand, the Hemiodontichthyina + Limatulichthys + Pseudoloricaria clade did not show such a severe reduction in the number of teeth, but rather in the size of teeth. Hemiodontichthyina + L + P have several representatives with up to 20 rather feeble teeth. This clade, however, show lip enlargement on mature males. Homoplastically, both clades have representatives without any teeth on the premaxillae and with different sexually dimorphic traits. In fact, we have three cases: *Spatuloricaria* with few teeth and no mouth-related sex dimorphism, and two clades with jaw reduction involving different anatomical parts carrying different types of mouth-related sex dimorphisms.

Since most species with mouth-related sex dimorphism have reduced jaws, it should be asked if sexually dimorphic mouth-related traits have arisen as a consequence of jaw reduction. If this would be the case, sexually dimorphic mouth-related traits could be seen as adaptative features. Based on the lack of this sort of sex dimorphic trait in *Spatuloricaria*, a jaw-reduced loricariin, one might argue that jaw reduction is decoupled from mouth-related sex dimorphism as a whole. On the other side, it could be suggested that the correlation between jaw reduction and mouth-related sex dimorphism is so strong, that even in non-homologous events of jaw reduction among loricariines, these events were followed by the arousal of different kinds of mouth-related sex dimorphism.

Conclusion

Phylogenetic hypotheses have power in orienting hypotheses of character evolution. The recognition of positive correlation between a morphological novelty and the posterior appearance of a given behavior supports the hypothesis of adaptation. On the other hand, the recognition of independent origins of morphological and behavioral traits, or non-adaptive traits, also provides useful information on character evolution.

The present work demonstrated two different situations of possible evolution of correlated characters among Siluriformes (catfishes). In the first case, swimbladder encapsulation seemed to be correlated with air breathing only within Neotropical Siluriformes. African/Asian catfishes with encapsulated bladders are completely unrelated to air breathers African/Asian catfishes.

Neotropical Siluriformes have been separated from the African/Asian catfishes for a long period (approximately 100 million years), but both groups kept an old morphological novelty, the swimbladder encapsulation. These fishes on separate continents (or in the process of separation) may have certainly be subjected to distinct selective pressures and did probably have enough evolutionary time to develop different adaptations to them. One could also argue that swimbladder encapsulation and air breathing in Neotropical Siluriformes is merely coincidental, without any adaptive value. However, the extreme success of the air breathing-encapsulated Siluriformes might be an evidence to refute this assertion.

In the second case, jaw reduction in loricariids seems to be strongly related to the appearance of mouth-related sex dimorphic traits, since two independent jaw-reduced lineages developed two independent sets of mouth-related sex-dimorphic traits. Due to the temporary or plastic nature of sex dimorphic traits, one might argue that these traits could not be seen as adaptation since they are not "genetically mediated" (Gould, 1984- in West-Eberhard, 1992) and can not be transmitted to the next generation. However, as very well put by Thompson (1991), "plasticity itself is the trait under selection". And, that seems to be the case of sex dimorphism. There is still no evidence to suggest why some groups develop sex dimorphism and others do not. However, the capacity to develop morphological modifications during the reproductive period, other than the gonads, must be a genetically mediated trait and it might confer some sort of advantage.

Certainly, we need more information to consolidate or invalidate the conclusions cited above. Recognition of adaptation has become a very controversial issue and biologists have been treating it with caution. This kind of exercise applied to different groups of organisms and characters helps providing theoretical background for the development of new experiments in order to interpret directions of character evolution.

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INTESTINAL CALCIUM REGULATION

AND THE VITAMIN D_3-SYSTEM

IN TELEOSTS.

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EXTENDED ABSTRACT ONLY – DO NOT CITE

The mechanisms maintaining internal calcium (Ca) homeostasis are fundamentally different between aquatic and terrestrial vertebrates. Thus, the endocrine regulation of Ca balance was probably modified during the water-to-land transition. New hormonal systems and new roles for already present hormones evolved and other hormones disappeared.

One hypothesis, suggesting that the calcium regulatory function of the vitamin D_3 system evolved simultaneously with the transition from water to land, has been put forward (Holick, 1989). However, the current knowledge about the presence and metabolism of vitamin D_3-metabolites in bony fish, together with clear roles for these metabolites in the regulation of Ca-uptake across the fish intestine, suggests an alternative hypothesis (Sundell et al., 1996). The different Ca-regulatory functions of the vitamin D_3-system seem instead to have evolved with the vertebrates, as a result of the Ca-availability in the environment.

Two major patterns of action have been revealed; one predominant in "low" calcium environments like the terrestrial and freshwater (FW) environments and

a second predominant in “high” calcium environments, like the sea. The vitamin D_3-system has well documented physiological roles in intestinal Ca-regulation of both FW and seawater (SW) living teleosts. Both types of fish have the ability to metabolize vitamin D_3 to more polar metabolites, which can be measured in their circulation (Sundell et al., 1996). The classic, genome mediated increase of intestinal Ca-uptake by $1,25(OH)_2D_3$, is present in all FW and SW fish examined (Sundell et al., 1996), except for the icefish *Pagothenia bernacchii* (Fenwick et al., 1994). $1,25(OH)_2D_3$ induces hypercalcemia after 24 h, but not after 12 h, and classical nuclear receptors (nVDR) for $1,25(OH)_2D_3$ are present in the intestine of both FW and SW species (Sundell et al., 1996). The genome mediated response seems to be vital for maintaining functional Ca-transporting mechanisms in the enterocytes, irrespective of the external Ca supply. The rapid, non-genomic actions of vitamin D_3 metabolites, on the other hand, seem to have diverged depending on the availability of Ca in the environment. Transcaltachia, the rapid increase of intestinal Ca-uptake mediated by $1,25(OH)_2D_3$, has been demonstrated in terrestrial animals and FW fish (Larsson, 1999).

These two animal groups are exposed to no or very low concentrations of Ca in the environment. The marine Atlantic cod (*Gadus morhua*), on the other hand, is exposed to Ca-levels exceeding the concentrations in the blood and transcaltachia could not be demonstrated by physiologically relevant doses of $1,25(OH)_2D_3$ in this species (Larsson, 1999). Plasma-membrane-bound receptors (pmVDR) for $1,25(OH)_2D_3$ have also been demonstrated in FW-living fish as well as in chicken and rat, but not in the marine cod (Larsson, 1999). Instead, a second polar metabolite, $24,25(OH)_2D_3$, was discovered to rapidly decrease the intestinal calcium uptake in the cod and pmVDR for 24,25 were demonstrated and biochemically characterized in this species (Larsson, 1999). However, the apparent diversion of the rapid effects of vitamin D_3 metabolites in different environments seems not fully separated, as further studies on the mechanism of action for $24,25(OH)_2D_3$ demonstrate that $24,25(OH)_2D_3$ rapidly decreases the intestinal Ca-uptake by inhibition of Ca-influx through L-type Ca-channels in both the marine cod and the FW-living carp (*Cyrpinus carpio*) (Larsson, 1999).

Furthermore, an increase in intestinal Ca-uptake, through stimulation of Ca-influx simultaneously with a stimulation of Ca-efflux via Na/Ca-exchange, was demonstrated in the marine cod. This effect was not exercised by $1,25(OH)_2D_3$ but instead by the less polar metabolite $25(OH)D_3$ (Larsson, 1999). Thus, bony fish from different environments seem to have the ability to possess both

stimulatory and inhibitory rapid, non-genomic actions of the vitamin D_3-system on intestinal Ca-uptake. However, the potency of the regulatory actions, together with number (B_{max}) and affinity (K_m) of pmVDR's for the different metabolites suggest that the stimulatory effect of $1,25(OH)_2D_3$ is dominant in low Ca environments whereas the inhibitory effect of $24,25(OH)_2D_3$ dominates in a high Ca environments. Data on a euryhaline species, the rainbow trout (*Onchoryhncus mykiss*), transferred between FW and SW supports this hypothesis. In FW, the rainbow trout possessed pmVDR for $1,25(OH)_2D_3$, but not for $24,25(OH)_2D_3$. After transfer from FW to SW, B_{max} and K_m for $24,25(OH)_2D_3$ binding gradually increased to reach a steady high level after 72 h, whereas the specific binding for $1,25(OH)_2D_3$ gradually decreased and was absent after 72 h. Furthermore, enterocytes from rainbow trout in FW were able to increase the Ca-influx in response to treatment with $1,25(OH)_2D_3$, whereas enterocytes from trout in SW were not, and $24,25(OH)_2D_3$ was able to decrease the enterocytic Ca-influx in SW- but not FW-adapted trout (Larsson, 1999).

In conclusion, available data suggest that teleosts possess antagonistic calcium regulatory systems where polar metabolites of vitamin D_3 rapidly control the amount of calcium taken up across the intestine and that the availability of calcium in the environment determines if the regulation is dominated by the stimulatory system governed by $1,25(OH)_2D_3/25(OH)D_3$ or the inhibitory system governed by $24,25(OH)_2D_3$.

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PHYLOGENETIC OCCURRENCE OF ELECTRORECEPTION AND BIOELECTROGENESIS IN TELEOST FISH: EXAMPLES OF CONVERGENT EVOLUTION

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Electric fields in the water can play a major role in the biology of fish through two main biologically relevant circumstances: some fish are able to detect electric gradients in the surrounding medium through a set of specialized sensorial cells, and some fish possess specialized organs that generate electric discharges on a more or less regular basis.

For those fish possessing both Electrosensory and Electrogenic Systems (EES), the electric field generated during an Electric Organ Discharge (EOD) is monitored by an array of electroreceptors distributed over the fish's skin. The two systems working in tandem constitute an effective sensory-motor "unit" that is particularly important for electrolocation and communication. Despite the obvious benefit of having both systems, bioelectrogenesis and electroreception do not always co-occur in the teleosts. There are electroreceptive fish that do not possess electric organs, and there is at least one species of electrogenic teleost (family Uranoscopidae) that has no electroreceptive capability (Bullock et al., 1983).

Electroreceptors can be broadly divided into ampullary and tuberous types according to their morphological and physiological properties. Ampullary electroreceptors detect DC fields and low frequency AC fields. According to the phylogeny of the clades involved and parsimony analysis of character evolution, this type of receptor evolved only within two distantly related fish groups: once

in the common ancestor of Notopteriformes + Mormyriformes (superorder Osteoglossomorpha), and a second time in the common ancestor of Siluriformes + Gymnotiformes (superorder Ostariophysi). The second class, the tuberous electroreceptors, are physiologically most sensitive to the dominant frequencies of the fish's own EOD (Zakon, 1986), and was thought to have evolved twice among teleosts, more specifically only in those clades that also have electric organs: the South American order Gymnotiformes and the African order Mormyriformes. However, a tuberous electroreceptor has been described for *Pseudocetopsis*, a South American catfish of the family Cetopsidae (Andres et al. 1988). Therefore, tuberous organs have evolved independently in three teleost lineages: mormyriforms, gymnotiforms and *Pseudocetopsis*.

Electric organs evolved eight times among teleosts, and five times within the superorder Ostariophysi alone. In the osteoglossomorphs, it is assumed that an electric organ was present in the common ancestor of all mormyriforms. Within the ostariophysans, electric organs evolved in the gymnotiforms' common ancestor and in four siluriform genera: *Clarias*, *Malapterurus*, *Auchenoglanis* and *Synodontis*. The other teleosts possessing an electric organ are *Astroscopus* and *Uranoscopus*, two genera of the perciform family Uranoscopidae (Moller, 1995). Systematic studies (Pietsch, 1989) and morphological examination of the electric organ in the two uranoscopids suggest that they are not homologous. In summary, electric organs appear to have evolved independently in the following teleost lineages: 1) Mormyriformes, 2) Gymnotiformes, 3) *Synodontis*, 4) *Clarias*; 5) *Malapterurus*, 6) *Auchenoglanis*; 7) *Astroscopus*, and 8) *Uranoscopus*.

As one maps the evolution of electric organs and electroreceptors onto teleost phylogeny it is possible to note that, with exception of the uranoscopids, all electric organs evolved in fish that already were electroreceptive. Further, the plesiomorphic electroreceptive teleost lineages had only ampullary organs, as tuberous electroreceptors evolved later in few select electrogenic groups. The development of active electrolocation (an electric organ) on top of an already existing electroreceptive capability likely represents, in terms of costs and benefits, a relatively minor step for a great reward. Because ampullary receptors are physiologically tuned to low-frequency AC and DC signals, in the beginning there must had been a real evolutionary advantage for signals that matched the electroreceptors' tuning curve, suggesting that plesiomorphic waveforms were composed of low-frequency signals such as long lasting monophasic discharges. The development of multiphasic, complex EOD waveform was probably the

best evolutionary option to solve some significant limitations of monophasic pulses. First, it allowed a larger number of possibilities in terms of waveforms, supporting species specificity. Second, by adding fast-frequency components in the EOD, fish were able to broaden the spectral frequency of their discharges, improving the efficiency with which they use their EES to probe the environment. Third, fast frequencies components can shift the spectral peak power frequency of the EODs above ampullary thresholds of electroreceptive predators such as catfishes.

The EOD waveform is the result of complex interaction of several physiological and anatomical features associated with the fish's electric organ. Nonetheless, EODs tend to be a plastic character because they are under constant selective pressure to evolve an ideal compromise between a trait that enhances electrolocation and electrocommunication, ensures species identity through sexual and behavioral segregation, and minimizes the risk of predation.

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NEOTROPICAL CICHLIDS:

ADAPTIVE RADIATION VERSUS GENETIC CONSERVATION

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Cichlids are amongst the most diverse group of fishes. Approximately 1400 species are found distributed across Africa, including Madagascar, South Asia, South and Central America, and the southern part of North America (Kullander & Nijssen, 1989). They are considered a monophyletic group and have been extensively studied because they constitute an outstanding example of adaptive radiation. Chromosome, enzyme (alozyme) and molecular studies have demonstrated a remarkably low level of genetic divergence (or genetic conservancy) in contrast with the high rate of speciation in the whole group of cichlids (Kornfield, 1984; Feldberg and Bertollo, 1985). Comparative studies suggest that neotropical riverine cichlids present faster molecular evolution than tropical lacustrine cichlids (Farias et al., 1999). On the other hand, both neotropical and tropical assemblages are described as plastic groups, adapted to particular niches resulting from fast evolutionary rates, differentiating them from other teleost groups (Kornfield, 1984; Stiassny, 1991).

The family Cichlidae is among the most advanced teleosts that occur in the Amazon basin. Their adaptive radiation includes strategies to perform aquatic

surface respiration, which is an innate behavior that is easily observed in young specimens. Our studies have shown that some species reduce the number of incursions to water surface and increase their anaerobic glycolytic power during growth. This capacity is achieved because there is an increase in the mass specific LDH levels. Changes in the distribution of LDH isozymes in the brain and heart of several Amazon cichlid species help to improve their hypoxia survivorship. Such ability to regulate the enzyme (isozyme) expression has been explained as the result of gene regulation obtained by their phenotypic plasticity (reviewed in Almeida-Val et al., 1999).

Analysis of enzyme/isozyme tissue distribution has been an excellent tool to verify genetic heterogeneity among populations and to determine the amount of species variability. Molecular DNA sequence studies are also used to establish genetic similarities or divergences and have proved to be successful in the analysis of phylogenetic relationship among groups. The neutrality of natural polymorphism and their adaptive character still remain controversial. However, there is no doubt about the fact that evolutionary rates are measured based on nucleotide substitution rates. On the other hand, when studying a group evolution, one may consider that such nucleotide substitution rate may vary depending upon the gene (or group of genes) used to describe genetic divergence between species and so the amount of evolutionary rate suggested to that particular group. From literature, we know that genetic variation, obtained after alozymic studies, varies in number of polymorphic loci. For example, insects may reach 50% of genetic variability, while mammals retain only 20%. Fishes, amphibians and reptiles are all intermediate, and the lowest level is found in avian group with 15 % genetic variability. It is also clear that DNA substitution rates are different among mitochondrial and nuclear genes.
We have analyzed enzyme/alozyme distribution in 14 Amazon cichlid species belonging to different lineages: *Symphysodon discus*, *Heros sp*, *Uaru amphiacanthoides*, *Pterophylum scalare* (Heroines), *Cichlasoma amazonarum*, *Cichlasoma sp*, *Acaronia nassa* (Cichlasomines); *Satanoperca jurupari*, *Geophagus altifrons*, *Geophagus sp*, *Acarichthys heckelli* (Geophagines), and *Cichla monoculus*, *Astronotus ocellatus* and *A. crassipinis*,(these three are all related but do not fit in any lineage) (Farias et al., 1999) .
Our results can be summarized as follows:
lactate dehydrogenase (LDH) presented no polymorphism in all analyzed species and its tissue distribution is according to the distribution of this enzyme in advanced teleosts;
glucose phosphate isomerase (GPI) was polymorphic in 9 species;

alcohol dehydrogenase (ADH) did not present polimorphic loci; and malate dehydrogenase (MDH) presented duplicated MDH-B* locus in all analysed enzymes.

Except by the GPI system, in which polymorphism levels are in accordance with the literature for teleosts, these data show a high level of structural gene conservancy, independent of polymerization number, which are considered to be another determining factor for polymorphism occurrence.
Even though these results are preliminary, such low genetic variability contrasts with the high speciation levels among riverine neotropical group and other molecular and enzyme analysis, which have suggested high evolutionary rates. Considering our previous results about gene regulation under environmental changes and the present data, we can suggest that, at least for Amazon cichlids, genetic variability in structural genes may not be considered a requirement for adaptive radiation, and that their specialization levels will depend upon regulatory mechanisms that allow them a high degree of phenotypic plasticity.

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CIRCADIAN RHYTHMS OF AMYLASE ACTIVITY

IN THREE FISH SPECIES OF THE AMAZON

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Introduction

The Amazon region presents a wide variety of aquatic environments, such as rivers, lakes, *paranás* (channels), *igarapés* (small streams), beaches, *várzeas* (floodplain areas), and *igapós* (flooded forest). These different ecosystems shelter richer ichthyofauna than that of any other river system as well as innumerable sources of food (Val & Almeida-Val, 1995).

Fishes, like other animals, have a lifestyle that includes, in general, species-specific activity, such as feeding and reproductive rhythms (circadian, seasonally and age). The circadian rhythms of the Amazon fish in their habitats are poorly known. Most studies of feeding rhythms in fish have reported strong diel patterns of feeding, suggesting that control of feeding time in some species is not regulated necessarily by natural variations in food availability, but perhaps, for endogenous control (Kadri *et al.*, 1997). This fact pointed out the importance of the circadian feeding rhythms and the need for a better understanding of the internal timing mechanism that governs it (Sanchez-Vásquez & Tabata, 1998). A mean of studying these mechanisms is to determine the rhythmicity of digestive enzymes activities, and other metabolic processes in relation to digestive and feeding frequency. More studies are required before we can draw the relationship of optimal feeding time, endocrine rhythms and other metabolic variables.

Carbohydrates are an inexpensive carbon source and a fundamental macronutrient for fish diet. Amylase promotes carbohydrate hydrolysis down to simple molecules, the monosacharydes, that can be assimilated. The present work reports the Circadian rhythms of amylase activity and their relation with patterns of stomach fullness in three Amazon fish species.

Material and Methods

Collection of samples

The samples were collected during an expedition of our laboratory to Anavilhanas archipelago in December 1999. Adults of jaraqui (*Semaprochilodus taeniurus*), acará *(Geophagus proximus*) and pacu (*Metynnis hypsauchen*) weighing on average 311.19±20.0, 219.51±6.85, 88.72±5.28 cm, respectively, were caught with a small-mesh gill net every other hour during a 24h-cycle. The fishes were killed by a head blow and the spinal cord was punched immediately after capture. Their standard length and weight were measured. Estimates of stomach fullness were based on the subjective "Index of fullness". The gut and pyloric caeca were removed within one hour after capture. The digestive tracts were individually weighed and frozen at -70°C until their use for enzyme analyses.

Preparation of enzyme extracts

The preparations of enzyme extracts were carried out at 4°C. The digestive tracts of each fish were homogenized in 0.02M-phosphate buffer (1:0.5 w/v) at pH 7.0. Homogenates were centrifuged for 15 minutes at 15,000 rpm. The supernatants were taken for enzyme assay. The protein content of the supernatants was estimated using a commercial protein-test Kit (Doles®).

Enzyme assay

Amylase activity was measured according Caraway (1959), using amylase-test kit (Doles®). Amylase activity was followed by the decrease in absorbance (iodine staining power of starch) at 660nm using a Spectronic Genesys^{TM}2 spectrophotometer. Results are shown as means ± SEM. Differences among means were analyzed by one-way analysis of variance, followed by Tuckey's multiple range test.

Results and Discussion

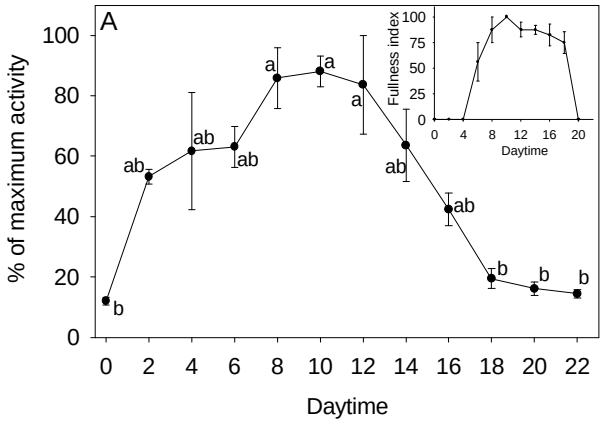
The changes in amylase activity during a 24 hour cycle and their relationships with the index of stomach fullness are presented in Figure 1.

A clear daily rhythm was observed in amylase activity of jaraqui (Fig.1A.). The changes were significant ($P< 0.05$), rising at daybreak and peaking in the morning. Maximum amylase activity was found between 8:00am to noon (0.403±0.023 IU per mg of protein). The relationship between amylase activity and stomach fullness rate show that stomach fullness precedes the amylase activity. High amylase activity in an emptied stomach for 2 to 4 hours may reflect a preparation phase for feeding activity. After the fourth hour of 100% full stomach, the activity gradually decreased until it reached the minimum activity from 6:00pm to midnight, indicating that little or no more enzyme is secreted in the digestive tract and/or that enzymes are partially inactivated.

A different pattern was observed for acará as shown in Fig.1B. Activity levels above 40% of the maximum activity were observed all day long. It seems, therefore, that amylase activity is maintained constant, despite a discrete peak that was observed at 14:00h (0.007±0.001 IU per mg of protein). Amylase activity is not related with stomach fullness rate, in this species.

Similarly, amylase activity found in pacu is maintained constant (mean 30%), as shown in Fig. 1C. The enzyme activity reached a peak at 4:00am (0.007±0.001 IU per mg of protein). As for the preview species, amylase activity is not related with stomach fullness rate in pacu.

Amylase activity in jaraqui is a hundred times higher than observed for pacu and acará. We suppose that high amylase activity in jaraqui is an adaptation to extract high energy levels from detritus, its food source. Acará and pacu are omnivorous fishes; their main sources of food are fruits, seeds and insects. The average of stomach fullness of pacu was maintained above 85% all day long. To digest this food, this species needs to maintain some amylase activity all day long, what contrast with jaraqui.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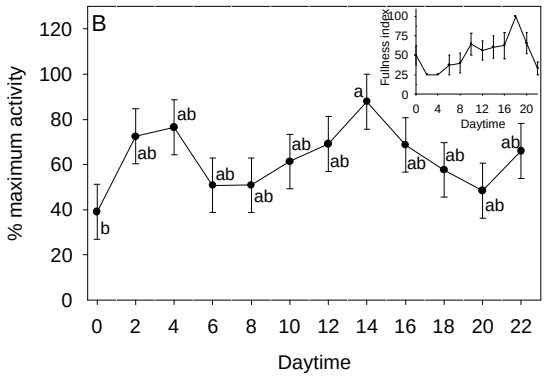
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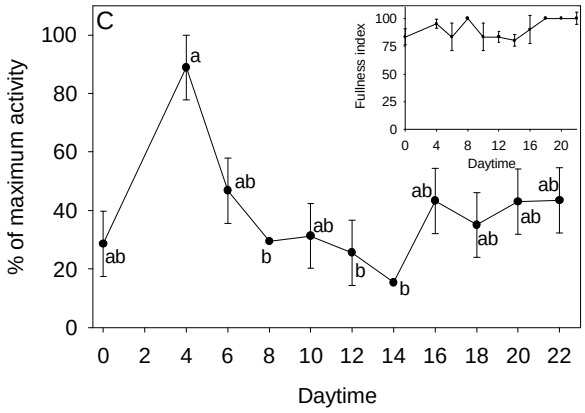

Fig.1. Changes in amylase activity in the digestive tract and fullness index (insert) of *S. taeniurus* (1A), *G proximus* (1B), and *M. hypsauchen* (1C) during 24-hours cycle. Errors bars show standard errors. Values whit different letters are significantly different (ANOVA, Tuckey HSD). The subjective "index of fullness" was expressed in percent of stomach fullness.

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EVOLUTIONARY IMPLICATIONS AND KINETIC PROPERTIES

OF VITAMIN-C SYNTHESIZING ENZYME (GLO) FROM

SOUTH AMERICAN LUNGFISH (*LEPIDOSIREN PARADOXA*).

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Abstract

The ability of vitamin C synthesis in vertebrates has been described in reptiles, birds and some mammalian groups. Such ability is well established in fish belonging to "primitive" groups as the Dipnoans and Crossopterygians. Thus, the ancestors of Sarcopterygians, Actinopterygians, and other groups of modern vertebrates shared the genetic information for synthesis of the enzyme L-gulono-γ-lactone oxidase (GLO), which catalyses vitamin C synthesis. According to literature, the properties of the enzyme GLO from Chrossopterygians correspond to those of the enzyme occurring in amphibians, birds, and mammals, although the occurrence of GLO among modern Actinopterygians (Teleosteans) is still under debate. The South American lungfish resembles modern vertebrates (amphibians and reptiles), regardless of its primitive character among Teleostomi. The present paper describes the properties of the enzyme GLO present in the liver and kidney of the South American lungfish, *Lepidosiren paradoxa*, and compares those properties with the homologous GLO occurring in other vertebrate groups.

Introduction

L-gulono-γ-lactone oxidase (GLO, E.C. 1.1.3.8) catalyzes the final step of the **L**-ascorbic acid synthesis in the kidney of vertebrates such amphibians and reptiles, or in the liver of some mammals and higher order of birds (Chartterjee, 1973). Most fishes, a few avian groups and the primates lost the ability of syntethizing GLO and require a dietary supplement of L-ascorbic acid (Chartterjee, 1973; Birney et al., 1979). Data on the ontogenetic development of GLO in vertebrates are scarce and fragmentary (Jenness et al., 1984). Thus, the ability to synthesize ascorbic acid and tissue distribution of GLO varies phylogenetically among vertebrates. As teleosts are unable to synthesize vitamin C, Chartterjee (1973) suggested that tetrapods developed the pathway for synthesizing ascorbic acid *de novo*. Dykhuizen et al. (1980) reported that the Australian lungfish, *Neoceratodus forsteri*, is able to synthesize ascorbic acid in the kidney and, consequently, the appearance of the information for GLO was prior to the arousal of land vertebrates. As many authors agree, the Chrossopterygian, the amphibians, the reptiles, and the Actinopterygian groups all seem to have arisen from a common ancestor some time before the middle Devonian (375 MYA). Since GLO activity was confirmed in all the above-mentioned groups, except by the Actinopterygian, it is likely that the common ancestor could synthesize vitamin C. The absence of this ability in fishes and anthropoid primates suggests that the information for such enzyme was lost after evolutionary divergence of those groups.

During the last decade, many authors have disagreed about fish ability to synthesize vitamin C (reviewed in Dabrowski, 1994). Such discussions have helped to improve the methodology to assay the enzyme and describe its kinetic properties with more accuracy, which may help establish the homology between the enzymes in several vertebrate groups.

In the present paper we report, for the first time, the activity levels of GLO in South American lungfish (*Lepidosiren paradoxa*) and compare its kinetic properties with other species belonging to other vertebrate groups: a mammalian species – the hamster *Rattus norvegicus*; a reptilian - the Amazon aquatic turtle *Podocnemis expansa*; and an amphibian – the common frog *Bufo marinus.*

Materials and Methods

Tissue homogenate –liver and kidney of 4 lungfishes (*Lepidosiren paradoxa*), liver of 4 rats *(Rattus norvegicus*), kidney of 3 frogs (*Bufo marinus*) and kidney of 3 Amazonian aquatic turtles (*Podocnemis expansa*) were excised from the animals, after they were anesthetized with ether. Tissue was washed twice with 0,85% NaCl solution in the proportion (1:4 - w:v) and homogenized in 50mM potassium phosphate buffer (pH 7.0). The extract was centrifuged at 20.000xg, 4°C at a centrifuge Sorvall RC-5B for 30 min. Supernatant was used for enzyme assays.

Enzyme assays – The increase in the absorbance of DCIP (2,6-Dichlorophenol-indophenol) in the assay mixture was followed at 600 nm at 25°C in a spectrophotometer Genesys 2 Spectronic. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction of 1 µmol of DCIP per min. The extinction coefficient of DCIP at pH 7.0 was taken as 14.5 mM. A cuvette with a 1-cm light path contained 0.16 mM DCIP, 0.16mM PMS (phenazine methosulfate), 200 mM potassium phosphate buffer, 400 mM L-gulono-γ-lactone, 20 µl of enzyme solution (tissue extract), and 50mM phosphate buffer (pH 7.0) in a final volume of 1.0 ml and assayed for enzyme activity as described previously by Sugisawa et al. (1995). The blank contained all solutions, except L-gulono-γ-lactone. The reaction started with the addition of substrate and the enzyme activity was measured as the initial reduction rate of DCIP. Three enzyme reactions were done for each individual and situation (substrate, temperature, and pH) and the values are expressed as means ± SEM. Enzyme activity values were converted to µmoles of substrate (**L**-gulono-**γ**-lactone) oxidized and transformed in ascorbic acid per hour and per gram of fresh tissue. Lineweaver-Burk equation was applied after obtaining saturation plots and Km and Vmax were obtained by the equations of the linear regressions (95% confidence intervals).

Results and Discussion

Gulono Lactone Oxidase (GLO) maximum activity values (µmoles **L**-Gulono-**γ**-lactone.$g^{-1}.h^{-1}$), Km (mM of **L**-gulono-**γ**-lactone) and saturation curves from *Lepidosiren paradoxa*'s liver and kidney homogenates, *Rattus norvegicus*' liver, *Bufo marinus*'s kidney, and *Podocnemis expansa*'s kidney are presented in table 1 and figure 1.

Table I – Maximum activities (µmoles **L**-Gulono-**γ**-lactone.g^{-1}.h^{-1}) and Km (mM) values obtained for liver and kidney homogenates of the four studied species.

Lepidosiren paradoxa (liver) n=4	*Lepidosiren paradoxa* (kidney) n=4	*Rattus norvegicus* (liver) n=4	*Bufo marinus* (kidney) n=3	*Podocnemis expansa* (kidney) n=3
235,5 ± 21,00	274,5 ± 29,19	229,9 ± 10,20	213,00 ± 45,79	192,6 ± 16,21
35,72	35,60	13,18	68,54	63,02

*Data are expressed in Means±SEM
** Km values obtained by Lineweaver-Burk equation.

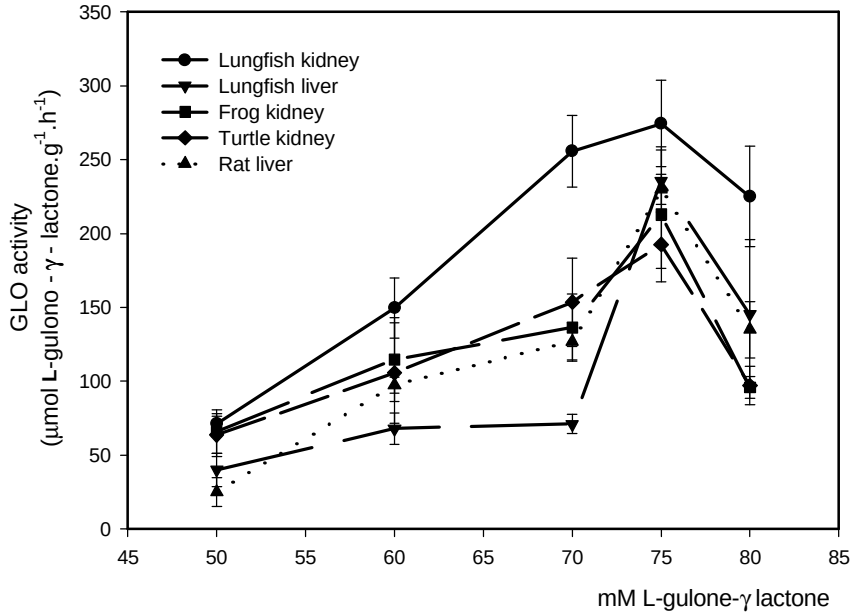

Figure 1. Substrate saturation plots for GLO levels in liver and kidney of *Lepidosiren paradoxa* compared with the other analyzed species.

The enzyme saturation curves and kinetic properties of all analyzed species are similar. GLO activities were similar in the kidney and liver in lungfish, suggesting that ascorbic acid synthesis occurs in both tissues. Km values were lower in lungfish tissues, compared with turtle and frog kidney and higher than the Km values of mammal liver. Enzyme Km values represent the inverse of enzyme-substrate affinity and, therefore, it may be considered as the enzyme functional ability for product synthesis. Based on higher activities at all substrate levels (Figure 1) and low Km values (Table 1) we can suggest that (excepting rat liver) lungfish kidney GLO is a very efficient enzyme compared to the other vertebrates analyzed.

The importance of retaining an efficient site to produce vitamin C in lungfish is probably related to its life style and respiration mode. During the dry season this animal remains burrowed into the mud and its oxygen demand may decrease. Low metabolic rate may remain in vital organs, such as kidney, heart and brain. Animal arousal involves an increase in metabolic levels, oxidative metabolism and, consequently, an increase in the amount of oxygen reactive species due to the return of high levels of air intake. Synthesizing antioxidants such as vitamin C should help to avoid possible tissue or cellular damage in both kidney and liver. Whether one gene or duplicate genes encode for GLO, still remains under investigation.

Enzyme thermostability was tested after tissue extract exposure to different temperatures during 5 min, using 75 mM potassium phosphate buffer. Activities were measured as above described and results are presented as enzyme activity levels obtained at the different tested temperatures (Figure 2). While expecting a higher sensitivity in rat liver enzyme, due to the fact that this species is endothermic, the GLO from lungfish liver and turtle kidney were affected by temperature more rapidly than the other groups.

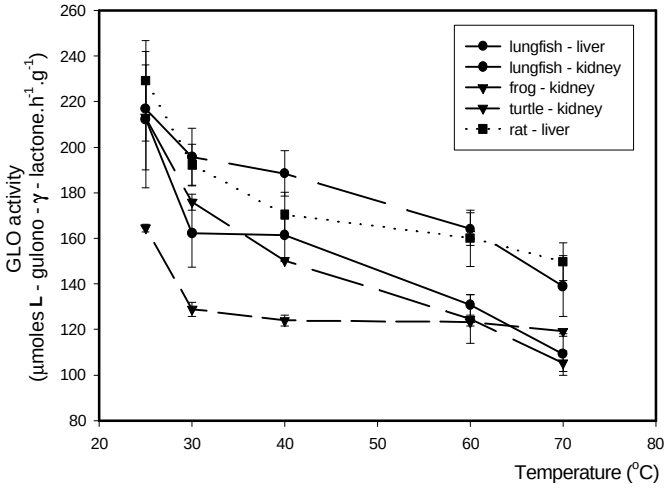

Figure 2. Temperature effects on maximum activity levels of the enzyme GLO from kidney and liver of *Lepidosiren paradoxa* compared with the other analyzed species.

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Results are presented in figure 3. Higher activities were obtained at pH 7.0 for all analyzed species and tissues. All enzymes presented the same reaction to pH variation indicating a good degree of homology between them.

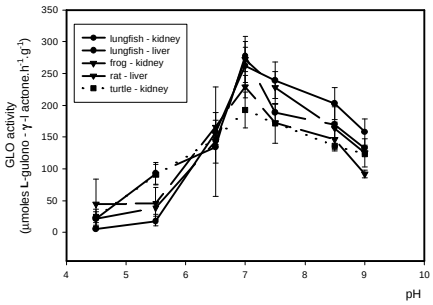

Figure 3. pH effects on maximum activity levels of the enzyme GLO from kidney and liver of *Lepidosiren paradoxa* compared with the other analyzed species.

The homology between GLO enzymes from lungfish and other vertebrates can be suggested from the above-described data. To date, there was no evidence that Dipnoi possessed the genetic information for GLO enzyme in the liver. Liver GLO have appeared only in eutherian mammals (Dykhuizen et al., 1980). These authors were unable to detect ascorbic acid formation in the liver of the Australian lungfish *Neoceratodus* and in the liver of Amphibian. Thus, this work is the first to describe the presence of GLO in two different organs (kidney and liver) of a primitive vertebrate, indicating that this information is prior to terrestrial vertebrate differentiation. The existence of more than one ancient loci encoding for the enzyme GLO deserves now new investigations.

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BIOCHEMICAL CHARACTERISTICS

OF LIVER AND BRAIN MONOAMINE OXIDASE

FROM PACU (*PIARACTUS MESOPOTAMICUS)*

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EXTENDED ABSTRACT ONLY-- DO NOT CITE

Monoamine oxidase (MAO) catabolizes biogenic amines in animal tissues. It has been involved in the inactivation of neurotransmitters like serotonin and dopamine in brain. Exogenous amines like tiramine are also catabolized by MAO in liver, kidney, and intestines. MAO is an integral protein of the outer mitochondrial membrane. MAO type A is inhibited by clorgyline and preferentially deaminates serotonin and noradrenalin. MAO type B is inhibited by deprenyl and preferentially acts on phenylethylamine and benzylamine. Both forms can act upon dopamine and kynuramine. Studies with teleost such as rainbow trout (Salmo gaidneri), carp (*Cyprinus carpio*) and *goldfish (Carassius auratus*) suggested that these species have only one isoform type A, whereas birds, amphibians and mammalians have both isoforms. In our study, we have

determined the biochemical characteristics (incubation time, temperature, protein concentration and pH variation) and kinetics parameters, KM and Vmax values for hepatic and cerebral MAO from a Brazilian fish, pacu (*Piaractus mesopotamicus*). MAO was determined fluorimetrically using kynuramine as substrate. Apparent Michaelis constant (KM) was similar in these tissues but MAO activity in liver was 6.5 times higher than in brain. Moreover, we have observed the inhibitory effects of clorgyline and deprenyl on MAO activity from liver and brain of this species. Clorgyline was much more effective than deprenyl in reducing MAO activity.