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METABOLIC AND PHYSIOLOGICAL ADJUSTMENTS TO LOW OXYGEN AND HIGH TEMPERATURE IN FISHES OF THE AMAZON

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NÍVIA PIRES LOPES

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I. INTRODUCTION

Adaptations of organisms to long- and short-term environmental changes are one of the basic concepts of evolution ([Futuyma, 1986](#); [Pigliucci, 1996](#); [Rose and Lauder, 1996](#); [Hochachka and Somero, 2002](#)). These adaptations involve genetic changes that will result in either metabolic/physiological

adjustment to short-term changes (e.g. gene regulation of LDH isoforms), or in changes at population and species levels (e.g. overall down-regulation of energy metabolism). During the evolution, individuals must cope with short- and long-term variations of the same physical parameters, i.e., temperature, pressure, and oxygen. In most cases, functional responses involve adjustments in metabolic processes which depend on the genetic make-up and may, in addition, result in anatomical and morphological variation (Almeida-Val, Val *et al.*, 1999). Evolutionary changes rely on genetic mutation and selection (in the broad sense), but a quantitative assessment of genetic variation alone fails to consider the phenotype range of variation of any given genotype (Schichting and Pigliucci, 1993, 1995). Thus, these two adaptation processes are interdependent: metabolic adaptation and (long-term) genetic changes will alter different spectra – the spectrum of selection is altered by physiological changes, and the spectrum of physiological and metabolic patterns will be altered by genetic mutation over evolutionary time (Walker, 1983; Walker, 1997; Almeida-Val, Val *et al.*, 1999). Currently, the interplay between metabolic and genetic adaptation may be the reflection of gene regulation processes: regulatory loci directly respond to specific environmental stimuli by triggering a specific series of “changes” (Pigliucci, 1996) and, in consequence, induce metabolic adjustments during the transcriptional phase. Subsequently, other changes in metabolism, which may be post-transcriptional, may take place to allow the fine adjustments that allow for a perfect interaction between organisms and environment. In fact, the discovery of regulation of many genes reconciles the apparent paradox between “unity *versus* diversity” suggested by Hochachka (1988), in which the relative constancy of chemical structure opposes with genotypic and phenotypic diversity within and between species.

The adaptation of Amazonian fishes to warm and hypoxic waters is of special interest, primarily because of the number of commercially valuable fish that can die in the course of a day as a consequence of occasional cold fronts that break into the Amazon during the southern winter. These cold fronts cause a turnover between the more oxygen-rich, cooled down surface water and warmer, oxygen-depleted deep waters in *várzea* (floodplain) lakes and *igapós* (flooded forests) during the periods of high water levels (Junk *et al.*, 1983; Val and Almeida-Val, 1995; Almeida-Val, Val *et al.*, 1999). Historically, floodplains of the Amazon were deforested for agricultural production by Pre-Colombian natives and later by European immigrants. Thus, this phenomenon seems to be the result of habitat destruction by humans since under intact forest canopies, no (or only insignificant) cold-front fish kills occur, because temperature differences between surface and deep water are less pronounced. However, this phenomenon is not

exclusive from Amazonian *várzea* lakes and may also occur in other kinds of water bodies, in which thermoclines occur.

Amazonian ecosystems are specially suited for studies of individual metabolic adaptations in relation to genetic speciation, because along with tremendous species richness (estimated *ca.* 3000 species of fish, see Chapter 1, this volume – after Roberts, 1972; Böhlke *et al.*, 1978; Rapp-Py-Daniel and Leão, 1981), there are vast areas of pristine habitats in an “equatorial hot climate”, with open lakes partly covered by aquatic macrophytes, and inundation of high-canopy forests that are subject to drastic diurnal and seasonal changes of oxygen levels.

Earlier studies showed that long- and short-term changes in oxygen are both determinants of fish distribution in Amazonian water bodies and that hypoxia tolerance is particularly common in fishes of the Amazon. This fact, along with associated tropical temperature regimes, has driven fish species through a series of adjustments at different levels of biological organization, e.g., ethological, morphological, anatomical, physiological, metabolic, and molecular, which are combined to produce phenotypic plasticity that allows them to adapt to the pulsating nature of the basin (Junk *et al.*, 1983; Val and Almeida-Val, 1995; Almeida-Val and Farias, 1996).

II. ENVIRONMENTAL CHALLENGES

Past and present patterns of oxygen availability in Amazonian water bodies may be seen as putative escalators of evolutionary selective pressure. Hypoxic and anoxic conditions were prevalent in the aquatic environment during the Cambrian period, owing to the low atmospheric oxygen levels at that time. At present, the poorly oxygenated waters of the Amazon basin result from different phenomena. Thus, since the Cambrian geological period, oxygen depletion has been a limiting factor for aquatic life in general (Randall *et al.*, 1981; Almeida-Val and Farias, 1996; Almeida-Val, Val *et al.*, 1999). South America and Africa appeared after Gondwanaland broke up in the southern hemisphere during the Cretaceous. During the Tertiary period the Andean Mountains were folded up on the western part of South America, inducing remarkable changes in the Amazon basin. The Pacific drainage of the upper tributaries of the Amazon River was cut, and the whole Amazon basin became oriented towards the Atlantic. Consequently, a completely new set of habitats became available, and seasonal oscillations in river water levels became the main driving force of the Amazon basin (reviewed by Val and Almeida-Val, 1995). Flood pulses (Junk *et al.*, 1989), i.e., annual oscillations of water levels, produce an average crest of 10 m between November and June in central Amazonian (see Chapter 1, this volume).

These flood pulses inundate a large area and make several new habitats available to fish in the flooded forest (*igapó*) and in the floodplain areas (*várzea*). Such flood pulses also cause the appearance and disappearance of many other aquatic formations such as *paranás* (channels), *igarapés* (small streams), and beaches. During low water levels, the receding water leaves behind small discrete water bodies, i.e., temporary lakes, while during high water levels, these water bodies are all interconnected. Changes in chemical, physical, and biological parameters occur, and such predictable inundations affect virtually all living organisms of the Amazon.

Although attention must be drawn to the oscillations in dissolved oxygen in water, investigations of temperature effects on metabolism in Amazonian fish are unavoidable. The main reason for this is that these fish spend their life cycles at high temperatures and have reorganized their metabolism in different organs to acclimate to these conditions, as well as undergone adjustments at the level of the whole organism, e.g., differential metabolic rates and differential scaling patterns. Water temperatures in the Amazon basin typically range from ~ 25 to 32°C , although under some conditions temperatures may be higher (Kramer *et al.*, 1978). This is in marked contrast to water in the north-temperate zone, which approaches 0°C in winter and does not exceed 25°C in summer. A life cycle spent at low temperatures is often associated with elevated activities of key enzymes of energy metabolism. In contrast, acclimation of fish to high temperatures always results in lower activity levels of enzymes associated with aerobic energy (Guderley and Gawlicka, 1992; Johnston *et al.*, 1985; Jones and Sidell, 1982; Way-Kleckner and Sidell, 1985). Furthermore, there is also a propensity for aerobic oxidation to be enhanced, especially the use of fatty acids, to meet energy demands at low temperatures (West *et al.*, 1999). In addition to temperature differences, fishes living in the Amazon drainage basin may cope with lower levels of oxygen. The bottom of the water column in the Amazon *várzea* lakes and *igapós* may be hypoxic or even anoxic, and dissolved oxygen can reach 0 ppm (mg/l) at night and oversaturated values during the following day in floodplain areas (Almeida-Val, Val *et al.*, 1999; Val, 1996). In general, Amazonian fishes deal with these conditions through metabolic depression, anaerobic metabolism, surface-skimming, air-breathing, or some combination of these adaptations (Almeida-Val *et al.*, 1993; Almeida-Val and Hochachka, 1995). Aerial respiration and a high anaerobic potential are not mutually exclusive; there are occasions when air breathing is inappropriate and the solution may be anaerobic energy production. Furthermore, the combination of anaerobic power and metabolic suppression, as evidenced by other good anaerobes (Hochachka, 1980) can also be found in Amazonian fishes (Almeida-Val *et al.*, 2000).

This chapter will focus on the main effects of environmental changes on energy metabolism of fishes of the Amazon. To accomplish this goal, we will review both the effects of natural environmental changes and discuss some of the metabolic responses of fish to different thermal regimes (temperature changes) and oxygen depletion (naturally occurring hypoxia and anoxia). This chapter will also consider the differential scaling properties of metabolism, and transcriptional and post-transcriptional changes in enzymes with key metabolic functions, such as LDH, in response to the main aquatic parameters, which are considered of great importance in regard to fish metabolism and aquatic ecosystems of the Amazon: temperature and oxygen. As will become evident throughout this chapter, it is not always possible to deal with each parameter separately, since aquatic ecosystems of the Amazon comprise a mosaic of situations and environmental conditions. This impairs considerations of just one phenomenon by itself and necessitates a more integrative treatment of many of the issues that will be addressed in the following sections. Chapter 9 deals with adaptations to acidic and ion-poor water conditions, these being other important environmental challenges in the Amazon basin.

III. EFFECTS OF TEMPERATURE ON FISH METABOLISM

[Hochachka and Somero \(2002\)](#) stated that “biogeographic patterning indicates that temperature is a major determinant of habitat suitability.” In fact, distribution patterns of organisms reflect temperature gradients or discontinuities and can be observed in both aquatic and terrestrial habitats, spatially and temporally, influencing all types of organisms. Species replacement can be seen with changes in latitude as well along vertical gradients in temperature at given latitude; good examples are temperature changes in the transitions from subtidal to intertidal marine habitats and from low to high elevations in mountainous regions. Some aquatic animals typically show diurnal vertical migrations and select the appropriate time of the day for foraging. The effects of temperature on organisms are universal among different species, but vary according to the thermal regime to which the species is acclimated. Basically, temperature affects every aspect of the organism’s physiology, and consequently imposes strict limits on where life can occur ([Hochachka and Somero, 1984, 2002](#); [Prosser, 1991](#)). The dynamics of temperature changes and their consequences differ between temperate climates and tropical regions, particularly in aquatic ecosystems. As water temperature from temperate regions rises towards the upper tolerance limits, the animals must cope with decreased dissolved oxygen and increased oxygen demand due to elevated metabolic rates and, consequently, elevated

maintenance costs. Thus, regulated physiological and metabolic parameters such as oxygen consumption rates, blood and tissue oxygenation, acid-base status and cellular energy levels may show substantial changes before harmful effects occur (Pörtner, 1993; Pörtner and Grieshaber, 1993; Sartoris *et al.*, 2003). In tropical regions, the dynamics of temperature oscillations at different times of the day and throughout the year are different. As mentioned above, temperatures may drop overnight and increase during the day in *várzea* lakes and *igapós*. However, oxygen levels do not change inversely, as occurs in temperate lakes. At night, when temperature drops, other phenomena take place, and the water column may become completely anoxic (Val and Almeida-Val, 1995; Almeida-Val, Val *et al.*, 1999; Chippari Gomes, 2002). Therefore, the imbalance that occurs between energy consumption and oxygen-dependent energy production in temperate fishes at extreme temperatures may be aggravated in tropical species due to the complexity of changes in physical-chemical water parameters.

Most fishes are organisms whose body temperatures conform to the temperature of the aquatic environment and are thus considered ectotherms (Hochachka and Somero, 1973, 1984, 2002). Ectotherms lack anatomical and physiological means for maintaining a thermal gradient between the external medium and the body. As stated by Hochachka and Somero (2002), a primary source of the difficulty faced by aquatic ectotherms in avoiding thermal equilibration with their medium is the requirement for gas exchange at respiratory surfaces. Metabolically produced heat is lost rapidly at respiratory surfaces during uptake of O₂ and elimination of CO₂ and other waste products, such as ammonia. This phenomenon may be less pronounced in tropical fishes due to higher medium temperatures. However, other environmental challenges may impose energy expenditures at the respiratory surfaces, e.g. low pH, ion-poor waters, and diurnal oxygen depletion (see Chapters 6, 7 and 8 for details on this issue). Thus, Amazonian fishes face other problems regarding temperature, since many water- and air-breathing fishes depend on the water surface layer to breathe, which imposes increased exposure to higher temperatures as well as higher radiation levels. For ectotherms, rates of respiration, feeding, growth, and locomotion are strongly influenced by changes in environmental temperature on both daily and on a seasonal basis. Such effects of temperature on rates of biological activity can be quantified by determining the specific temperature coefficient (Q_{10}) of a process, i.e., the effect that a change in 10 °C will have on the rate being measured. As repeatedly described in the literature for many processes such as rates of respiration and enzymatic activity, Q_{10} values near 2.0 or slightly higher are observed when thermal effects are studied within the normal ranges of body temperatures. Outside this range, Q_{10} values may deviate sharply from 2.0 (reviewed by Hochachka and Somero, 2002). For fishes, the

temperature coefficients of metabolic rates lie mostly within a range of 0.05 to 0.10, which corresponds to Q_{10} values of 1.65 to 2.70 (Jobling, 1994).

The effects of temperature on fish metabolism have been studied extensively, and a vast body of literature exists regarding metabolic thermal compensation, which quantifies the effects of temperature on rates of oxygen consumption by differently adapted and differently acclimated ectotherms (see Pauly, 1998; Hölker, 2003 for different reviews on this subject). According to Hölker (2003), the patterns generated by growth-related processes, such as mortality, reproduction and rate of food consumption, can be explained by acclimation temperatures, i.e., latitudinal fish distribution. Reviewing the FishBase 98 for several parameters, Hölker (2003) stated that tropical fishes, which, on average, live in a range of *ca.* 20–30 °C, occupy higher trophic levels and consume more food than their colder-water counterparts. This author suggested that, on average, tropical fishes tend to be smaller than temperate fishes, and that metabolic rates will be higher in warm-water fishes. However, studies have revealed a different picture for Amazonian fish species.

Among fishes of the Amazon, metabolic rates of exclusively water-breathing species vary along a spectrum from “sluggish”- to “athletic”-type behavior patterns. As expected, comparisons between Amazon and temperate fish species suggest that the more sluggish a fish is, the less oxygen is consumed per unit weight (reviewed in Val and Almeida-Val, 1995). Regardless of the high acclimation temperatures, Amazon fishes will experience a suppressed aerobic capacity (Almeida-Val and Hochachka, 1995; Driedzic and Almeida-Val, 1996; West *et al.*, 1999), and this is directly reflected in their metabolic rates (Figure 10.1). A simple comparison among three sets of data obtained with respiration rates (whole organism oxygen consumption) as a function of increased body mass was plotted according to the allometric relationship $VO_2 = aM^b$, where a = log mass coefficient, M = log-body mass, and b = mass exponent. Variations of b were adopted as described by Wootton (1990) for fishes in general, including Antarctic, temperate, and tropical fishes; by Hammer and Purps (1996) for tropical fishes, particularly facultative air-breathing species; and for the anoxia-tolerant Oscar (*Astronotus ocellatus*), as described in Almeida-Val *et al.* (2000). Figure 10.1 shows a clear trend towards metabolic suppression rates in fishes as they become naturally acclimated nearer the Equator. These data can be interpreted as being temperature-related or as being an adaptive characteristic to typically hypoxic waters of the tropics. In either case, there is a good reason to believe that a metabolic gradient of adaptation occurs and may be biogeographically linked to latitude.

According to Hochachka and Somero (2002), the temperature-linked biogeographic patterning found in nature is a clear manifestation not only

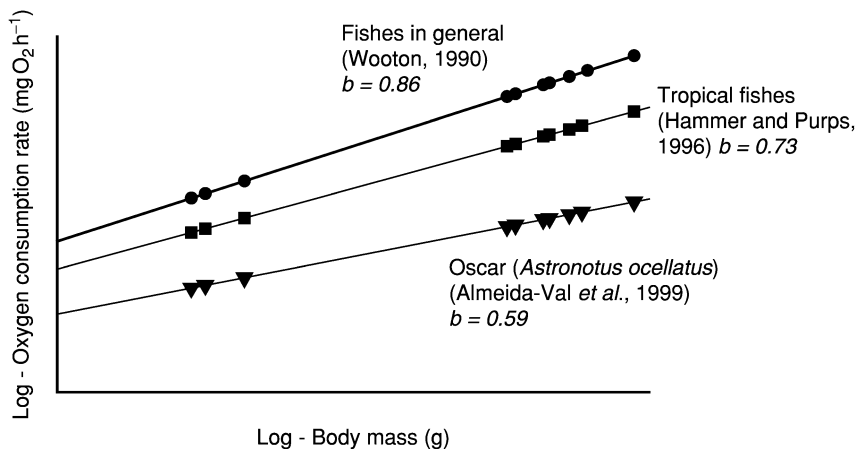


Fig. 10.1 Relationship between body mass (g) and mass-specific oxygen uptake (specific routine metabolic rate – $\dot{V}O_2 = \text{mg O}_2 \text{ g}^{-1} \text{ h}^{-1}$) from *Astronotus ocellatus* (Almeida-Val, Paula-Silva et al., 1999), fishes in general (Wootton, 1990), and tropical fishes (Hammer and Purps, 1996). Values were determined by measuring oxygen consumption in a completely sealed chamber and recording the change in oxygen concentration within the chamber over a period of time (approximately 2 hours) as described by Almeida-Val et al. (1999). Values refer to each individual and are plotted against its respective body mass, according to the allometric relationship described as follows: $\dot{V}O_2 = aM^b$, where: a = log mass coefficient, M = log-body mass, and b = mass exponent. Comparison of b exponents from *Astronotus* with other groups of fishes reveals a decrease in the reliance on aerobic metabolism as the animal grows when compared with b exponents obtained for fishes in general and tropical fishes. There are two possible (although not exclusive) explanations for this finding: (i) *A. ocellatus* retains a higher ability in suppressing its metabolic rate as an evolutionary adaptive response to the chronically hypoxic environment; and (ii) this is an adaptation to different thermal regimes over evolutionary time. See text for more details.

of the pervasive effects of temperature on all organisms, but also of the success of organisms in adapting to thermal variation. In fact, investigations of activity levels of enzymes of energy metabolism in heart and muscle of Amazonian teleosts in comparison with north-temperate teleosts revealed that the general differences between these two groups of fishes are in fact due to their thermal history (West et al., 1999).

IV. ENZYME LEVELS REFLECT THE NATURAL HISTORY OF FISH

Genes for glycolytic enzymes are thought to be regulated by being linked to common inducing or repressing signals, during long-term evolutionary processes (Hochachka et al., 1996). Thus, the capacity of glycolytic regulation of organisms will be indicated by enzyme levels of organs and tissues,

and will reflect life style and respiration patterns of the organism. In an earlier review, we compared the metabolic profiles of Amazon fishes to those of temperate species and suggested that fish of the Amazon generally show down-regulated enzymes, whereas an up-regulation of relative glycolytic capacity can be observed in both anaerobic and aerobic tissues, regardless of respiration type (Almeida-Val and Hochachka, 1995). Comparing rates of fish heart enzyme activities of anaerobic and oxidative metabolism, i.e., the ratio of lactate dehydrogenase to citrate synthase (LDH/CS), north-temperate teleosts show lower anaerobic power than tropical fishes, regardless of their respiratory type (reviewed by Almeida-Val, Val *et al.*, 1999). Besides the fact that such different patterns occur in different species, one might hypothesize that phenotypic and gene regulatory plasticity may have preceded speciation during the evolution of these species, thus allowing for down-regulation of enzyme levels in tropical fish. To better assess this hypothesis, it is necessary to review some comparative studies performed on Amazonian and north-temperate teleosts using enzyme activity levels of energy metabolism of different tissues, in particular, the heart (Driedzic and Almeida-Val, 1996; West *et al.*, 1999).

Among the Amazonian fishes, the activity levels of enzymes required for anaerobic glycolytic metabolism do not correlate with the ability of ventricular strips to maintain force development in the face of an anoxic challenge (Bailey *et al.*, 1999; West *et al.*, 1999). Furthermore, the activity levels of these enzymes (i.e. phosphofructokinase – PFK, pyruvate kinase – PK, and LDH) are not higher in the water-breathing, anoxia-tolerant oscar (*Astronotus ocellatus*) and tambaqui (*Colossoma macropomum*) relative to the facultative air-breathers acari-bodó (*Liposarcus pardalis*) and tamoatá (*Hoplosternum littorale*) and the obligate air-breather pirarucu (*Arapaima gigas*). The activity levels of hexokinase (HK), which catalyzes the first step in glucose breakdown, was higher in the two above-mentioned armored catfish species, which showed a greater capacity to maintain ventricular-strip contraction under cyanide poisoning than did the oscar and tambaqui, which failed (Bailey *et al.*, 1999). Thus, this data set revealed that enzyme activity neither presented a consistent pattern that could be associated with anaerobic glycolysis and whole-animal hypoxia tolerance, nor with the ability of isolated hearts to maintain performance under conditions of impaired oxidative phosphorylation. Similar results were achieved in red muscle, which will deserve a special section further. Because levels of enzymes associated with anaerobic glycolysis showed no elevated values in hearts with high resistance to anoxia in those studies, the final conclusion was that the general differences in enzyme activities between Amazonian and north-temperate teleosts, i.e., higher levels in the latter group, are most likely due to their thermal histories. Here, long-term changes in environmental

temperature have driven the evolutionary changes among groups of fishes. Enzyme activity levels are often higher in Antarctic species than in north-temperate teleosts (Crockett and Sidell, 1990; Driedzic, 1992) and within north-temperate teleosts when they are acclimated to low as opposed to high temperature (Jones and Sidell, 1982; Johnston *et al.*, 1985; Way-Kleckner and Sidell, 1985; Guderley and Gawlicka, 1992). The enzyme activity levels reported by West *et al.* (1999) were determined at a common assay temperature of about 25 °C, which is 10 °C higher than the acclimation temperature of north-temperate teleosts. Their enzyme activity levels are typically 1.5- to 2.2-fold higher in hearts and 3- to 4-fold higher in red muscle than those from Amazonian species. Q_{10} values for the activity of the enzymes are highly variable with respect to enzyme and species (Crockett and Sidell, 1990; Bailey *et al.*, 1991; Sephton and Driedzic, 1991). According to West *et al.* (1999), if the *in vitro* enzyme activity levels of north-temperate teleosts were decreased by *ca.* 2-fold to bring them into the physiological range they were acclimated, the resultant activity levels would be similar in the two groups of animals and they should therefore have the same metabolic capacity in their tissues at their respective acclimation temperatures. Thus, thermal regimes and fish life history are both determinants of enzyme activity levels: the lower the thermal regime of a species, the higher the expression of many of the enzymes associated with energy metabolism.

The above discussion refers to mean activity levels of enzymes involved in energy metabolism in the heart of fishes from different thermal zones, regardless of phylogeny and respiration types among species (Driedzic and Almeida-Val, 1996; West *et al.*, 1999). As mentioned above, a short literature review on metabolic characteristics of air- versus water-breathers revealed similar results that can be summarized as follows: (i) enzyme-level adjustments in air-breathing fishes are lower in terms of their absolute enzyme activity levels in pathways of both aerobic and anaerobic metabolism, and (ii) glycolytic rates are up-regulated in relation to oxidative tissue capacities of air-breathers, which varies on a tissue basis (Almeida-Val and Hochachka, 1995).

However, after reviewing those absolute enzyme values we suggested, based on a tissue-by-tissue comparison, the heart is a metabolic “hot spot” in many Amazonian air-breathers (Almeida-Val and Hochachka, 1995). Hearts of the species *Lepidosiren paradoxa* (Amazonian lungfish), which are very oxidative and have a high mitochondrial content, contrast with those of *Arapaima gigas* (pirarucu), whose hearts show lower oxidative power. However, while *A. gigas* hearts are less oxidative than lungfish hearts, they maintain an impressively high oxidative potential in their muscles, compared to muscles of most water-breathers (reviewed by Almeida-Val and Hochachka, 1995). Although lungfishes are phylogenetically specialized

compared to teleosts, they show similar metabolic patterns when compared to the advanced teleost *Symbranchus marmoratus* (the South American swamp eel, or muçum), which has a similar life style and burrows into the mud during the dry season. Both species sustain high anaerobic potential in their hearts, with LDH activity levels up to 5-fold higher than those of most mammalian hearts and higher than most fish hearts. Heart LDH activities in *Arapaima gigas* and *Osteoglossum bicirrhosum* (aruanã) are comparable to tuna heart LDH levels, which are half of lungfish levels (reviewed by Almeida-Val and Hochachka, 1995). The reason for such high LDH levels in hearts of lungfish and muçum, different from other interpretations, where LDH is viewed just as an anaerobic power enzyme, is thought to be related to their recovery from estivation, since their heart type kinetics (LDH-B₄ orthologs – isoform predominant in vertebrate heart muscles), or high pyruvate inhibition rates, and higher trends towards lactate–pyruvate reversible conversion, will guarantee an efficient lactate back-conversion when oxygen becomes available upon arousal from estivation. In fact, during estivation, *Lepidosiren paradoxa* shows a suppression of metabolic characteristics, slowing down LDH levels in the heart from >1000 units per gram of wet tissue when awake (Hochachka and Hulbert, 1978) to <100 units per gram of wet tissue when estivating (Mesquita-Saad *et al.*, 2002). Thus, one may conclude that the substrate preference of the heart and the muscle is carbohydrate, not lipid-based (Hochachka, 1979; Hochachka and Hulbert, 1978).

Although air-breathing fishes have certain characteristics that resemble the biochemical strategies of diving aquatic mammals, mainly with regard to relative hypometabolism during diving (Burggren *et al.*, 1985; Dunn *et al.*, 1983) or estivation (Mesquita-Saad *et al.*, 2002), the preferential use of oxygen-efficient carbohydrate metabolism, which appears to be fundamental for some air-breathing fishes, may become less significant when fat fuels become more important under particular conditions. At this point, it is obvious that substrate (fuel) preferences deserve some attention.

V. FUEL PREFERENCES IN TROPICAL VERSUS TEMPERATE FISHES

In most fishes, cardiac ATP production under aerobic conditions is usually supported by a mixed catabolism of exogenous glucose and fatty acids. Studies on fuel availability, performance of isolated hearts, rates of oxygen consumption and ¹⁴CO₂ production by intact hearts and isolated mitochondria, along with *in vitro* enzyme activity levels support this idea (Driedzic, 1992; Driedzic and Gesser, 1994). In many species of north-temperate teleosts, seasonal decreases in temperature from about ~15–25 °C

in summer to $\sim 0\text{--}5^\circ\text{C}$ in winter result in an enhancement of aerobic-based fatty acid metabolism in heart (Way-Kleckner and Sidell, 1985; Sephton and Driedzic, 1991; Bailey and Driedzic, 1993). An extension of this characteristic can be seen in Antarctic fishes, which spend their life cycle at 0°C . Crockett and Sidell (1990) and Sidell and Crockett (1995) described a strong reliance of cardiac energy metabolism on fatty acids associated with substantially higher *in vitro* activities of mitochondrial enzymes. A comparative analysis of activity levels of enzymes required for the use of glucose and glycogen in anaerobic metabolism (Table 10.1) suggests that within the Amazonian fishes, the enzymes PK and LDH do not differ between facultative air- and water-breathing species. However, the activity level of HK is higher, which may be correlated with the use of glucose as fuel for aerobic-based metabolism in the heart. According to Bailey *et al.* (1991), high activity levels of HK are consistent with the indirect arguments that extracellular glucose is used by fish heart as a metabolic fuel under oxygen limitation. Driedzic and Bailey (1999) suggested that hearts of fish have the ability to maintain high levels of performance in the absence of oxidative metabolism. According to these authors, under oxygen-limiting conditions, lactate is produced and, similar to other tissues, intracellular glycogen is mobilized (Dunn *et al.*, 1983; van Waarde *et al.*, 1983; Driedzic, 1988). However, extracellular glucose is also critical in extending heart viability. Although intracellular glucose levels are generally maintained at very low levels in fish hearts, during periods of oxygen limitation, an increase in glucose in the heart was described in lungfish (Dunn *et al.*, 1983), in goldfish (Shoubridge and Hochachka, 1983) and in the small Amazon cichlid (Almeida-Val and Farias, 1996), achieving levels similar to blood. HK may be an important rate-controlling site to achieve the right energy production under anaerobiosis. The results obtained by (West *et al.*, 1999) with regards to enzyme activity levels in the hearts of Amazonian fish species, combined with the results obtained by Bailey *et al.* (1999) on the cardiac performance of isolated hearts submitted to anoxia, using the same species as study models, have revealed that the ability of heart strips of the armored catfish *Hoplosternum littorale* (tamoatá) to recover from cyanide poisoning may be related to translocation of glucose transporters from intracellular sites to the plasma membrane. This in turn allows a greater uptake of glucose and support of the energy demands under anoxic conditions, e.g., cyanide poisoning (Driedzic and Bailey, 1999). In subsequent studies, no other armored catfish showed similar levels for HK (Lopes, 2003), suggesting that tamoaá deserves further attention regarding fuel preference studies and anoxia tolerance in their tissues and organs.

Another point that emerges from this discussion is that, regardless of temperature acclimation and the differential means of enzyme activity levels,

Table 10.1
Enzyme Activity Levels in Heart Muscle of Temperate and Tropical Fish Species

Species	HK	PK	LDH	HOAD	CS	References
Temperate fishes						
<i>Perca flavescens</i>	23.5 ± 2.4	158.7 ± 9.9	255 ± 11	7.6 ± 0.7	26.2 ± 3	West <i>et al.</i> , 1999
<i>Oncorhynchus mykiss</i>	25.6 ± 1.5	71.2 ± 2.6	307 ± 18	17.8 ± 2.3	47.8 ± 3.1	West <i>et al.</i> , 1999
<i>Anguilla rostrata</i>	14.7 ± 0.5	90.0 ± 0.7	726 ± 55	10.3 ± 0.5	38.9 ± 3.0	West <i>et al.</i> , 1999
<i>Myxine glutinosa</i>	1.70	35.9	114.4	1.78	6.92	Driedzic, 1988
<i>Squalus acanthias</i>	3.81 ± 0.18	8.85 ± 0.2	–	–	21.12 ± 0.8	Sidell and Driedzic, 1985
<i>Gadus morhua</i>	4.92 ± 0.15	46.5 ± 0.9	–	–	9.6 ± 0.4	Sidell and Driedzic, 1985
<i>Morone saxatilis</i>	14.8 ± 1.2	37 ± 2.6	–	–	5.94 ± 0.4	Sidell and Driedzic, 1985
<i>Macrozoarces americanus</i>	2.45	36.34	127.8	1.79	12.78	Driedzic, 1988
<i>Ictalurus punctatus</i>	13.2 ± 14	58.8 ± 3.6	423 ± 11	5.6 ± 0.1	12.5 ± 0.3	West <i>et al.</i> , 1999
Tropical fishes						
<i>Colossoma macropomum</i>	3.0 ± 0.3	76.5 ± 6.9	573 ± 11	8.7 ± 1.0	13.9 ± 3.0	West <i>et al.</i> , 1999
<i>Hoplosternum littorale</i>	20.2 ± 2.1	60.0 ± 2.0	235 ± 14	10.7 ± 0.4	20.3 ± 1.5	West <i>et al.</i> , 1999
<i>Arapaima gigas</i>	10.1	27.3 ± 2.8	256 ± 30	2.4 ± 0.4	23.0 ± 2.0	West <i>et al.</i> , 1999
<i>Satanoperca aff. jurupari</i>	–	96.6 ± 5	52 ± 6	–	–	Chippari Gomes, 2002
<i>Cicla monoculus</i>	–	12.1 ± 1	13 ± 1	–	–	Chippari Gomes, 2002
<i>Geophagus aff. altifrons</i>	–	113.5 ± 6	89.2 ± 14	7.6 ± 0.5	–	Chippari Gomes <i>et al.</i> , 2000
<i>Astronotus ocellatus</i>	2.4 ± 0.3	46.2 ± 8.1	134 ± 3	5.8 ± 0.4	10.4 ± 0.7	West <i>et al.</i> , 1999
<i>Astronotus crassipinnis</i>	–	55 ± 0.5	8.6 ± 0.5	–	19.8 ± 5	Chippari Gomes <i>et al.</i> , 2000
<i>Symphysodon aequifasciatus</i>	–	41.9 ± 5	33.4 ± 2	–	20.3 ± 2	Chippari Gomes <i>et al.</i> , 2000
<i>Glyptoperichthys gibbiceps</i>	1.4 ± 0.1	27.9 ± 0.87	21.6 ± 0.5	6.8 ± 0.5	79.6 ± 3.7	Lopes, 2003
<i>Platydoras costatus</i>	2.2 ± 0.7	66.7 ± 9.6	492 ± 114	1.36 ± 0.2	–	Lopes, 2003
<i>Calophrys macropterus</i>	1.3 ± 0.17	57.2 ± 1.4	635 ± 15	18.8 ± 0.7	32.7 ± 1.9	Lopes, 2003
<i>Prochilodus nigricans</i>	1.46	29.1	135.3	6.36	19.98	Lopes, 1999
Comparison between the two groups						
Range (temperate)	2.45–25.6	8.4–158.7	114–726	1.78–17.8	6.92–47.8	
Range (tropical)	1.3–20.2	12.1–113.5	8.6–635	1.36–18.8	10.4–79.6	

Enzyme activity is expressed as $\mu\text{mol min}^{-1} \text{g}^{-1}$ wet weight (mean ± SEM).

as described by West *et al.* (1999), the magnitude of ranges of enzyme activity levels is very similar between tropical and temperate teleosts (Table 10.1). This reveals once again that inspecting these data very closely may reveal more important characteristics than an attempt to establish general patterns will, particularly when dealing with the mosaic of changes in aquatic ecosystems that the animals face in tropical areas. In fact, most studies on whole animals that have been done in our laboratory with Amazonian fish species subjected to some level of oxygen depletion (acute hypoxia, graded hypoxia or anoxia) revealed that the animals showed alterations in plasma glucose and lactate levels resulting from the usage of glucose reserve mobilization and anaerobic-based lactate production (Table 10.2). Furthermore, after these experimental procedures with different Amazonian fish species, most of which were considered hypoxia-tolerant, it became clear that anaerobic glycolysis took place in most species and that this response is combined with metabolic depression in some species, mainly in those that were already known to be hypoxia-tolerant (Table 10.2).

Glucose mobilization may occur even in facultative air-breathers such as the armored catfish *Glyptoperychthys gibbiceps*, which is not necessarily related to anaerobic metabolism activation, since lactate levels are significantly decreased (Lopes, 2003). Following these studies, a detailed study using *G. gibbiceps* as a model was performed in our laboratory. This species was compared to a closely related armored catfish, *Lipossarcus pardalis*, by MacCormack *et al.* (2003). We observed that no changes in heart rate occurred under conditions of controlled hypoxia in aquaria, under natural hypoxia in a simulated pond, and in field cage sites. When denied aerial respiration under hypoxia in laboratory aquaria, *G. gibbiceps* increased gill ventilation rates, but neither *G. gibbiceps* nor *L. pardalis* exhibited alterations in heart rate, suggesting that bradycardia is not one of their strategies against hypoxia. On the other hand, *G. gibbiceps* was hyperglycemic under normoxia (mean plasma glucose ranging from 16.88 to 31.24 $\mu\text{mol ml}^{-1}$) and extremely large increases were observed under hypoxia (29.84 to 51.11 $\mu\text{mol ml}^{-1}$). Unlike graded hypoxia responses, where lactate decreases (Lopes, 2003; Table 10.2) or natural hypoxia in a simulated pond, where lactate does not change (MacCormack *et al.*, 2003), the plasma lactate levels of this species increased from 1.55 ± 0.81 to $65.91 \pm 7.48 \mu\text{mol ml}^{-1}$ when submitted to acute hypoxia in indoor aquaria and denied access to air (MacCormack *et al.*, 2003). Therefore, the reliance on extracellular glucose as a metabolic fuel under oxygen limitations may constitute one of the strategies to better deal with oxygen limitation and may occur in addition to other responses. It may be dependent upon respiratory pattern and species phylogeny.

After investigations of metabolic responses of the common carp to prolonged hypoxia, Zhou *et al.* (2000) concluded that metabolic depression

Table 10.2
Changes in the Plasma Amounts of Glucose and Lactate After
Different Levels of Oxygen Deprivation

Species	Glucose	Lactate	References
<i>Colossoma macropomum</i>	(N) 75.68 (GH) 135.13*	1.60 4.55*	Chagas, 2001
<i>Platydoras costatus</i>	(N) 36.83 ± 6.48 (AH) 43.86 ± 1.44	0.47 ± 0.01 0.89 ± 0.09*	Lopes, 2003
<i>Calophysus macropterus</i>	(N) 50.87 ± 12.04 (AH) 196.64 ± 26.42*	0.37 ± 0.06 0.35 ± 0.05	Lopes, 2003
<i>Hoplosternum littorale</i>	(N) 57.73 ± 4.94 (GH) 70.61 ± 8.40*	3.60 ± 0.47 4.43 ± 1.02*	Lopes, 2003
<i>Glyptoperichthys gibbiceps</i>	(N) 37.26 ± 2.61 (AH) 174.31 ± 30.53*	0.91 ± 0.03 0.20 ± 0.11*	Lopes, 2003
<i>Liposarcus pardalis</i>	(N) 59.46 ± 3.74 (GH) 95.54 ± 11.70*	1.62 ± 0.11 14.11 ± 1.47*	Lopes, 2003
<i>Astronotus ocellatus</i>	(N) 41.08 (GH) 29.01	1.50 16.5	Muuszé <i>et al.</i> , 1998
<i>Astronotus crassipinnis</i>	(N) 67.73 ± 4.70 (GH) 232.26 ± 16.80*	1.49 ± 0.33 17.11 ± 0.73*	Chippari Gomes, 2002
<i>Symphysodon aequifasciatus</i>	(N) 23.64 ± 4.81 (GH) 93.64 ± 23.91*	1.52 ± 0.44 7.61 ± 0.69*	Chippari Gomes, 2002
<i>Heros sp.</i>	(N) 89.00 ± 24.73 (AH) 85.87 ± 9.88	1.93 ± 0.36 11.14 ± 1.08*	Chippari Gomes <i>et al.</i> , 2000
<i>Uaru amphiacanthoides</i>	(N) 88.02 ± 17.99 (AH) 70.56 ± 18.88	2.63 ± 0.93 14.52 ± 0.64*	Chippari Gomes <i>et al.</i> , 2000
<i>Satanoperca jurupari</i>	(N) 111.58 ± 37.29 (AH) 156.04 ± 37.37	0.91 ± 0.05 9.69 ± 0.09*	Chippari Gomes <i>et al.</i> , 2000
<i>Geophagus altifrons</i>	(N) 147.05 ± 16.29 (AH) 139.20 ± 21.93	1.44 ± 0.57 10.17 ± 1.26*	Chippari Gomes <i>et al.</i> , 2000

Glucose content is expressed as mg/plasma dl, lactate levels is expressed as $\mu\text{mol/l}$.

* $P < 0.05$; (N) normoxia; (AH) acute hypoxia; (GH) graded hypoxia.

allows this species to reduce accumulation of lactate and save on the use of energy reserves in the face of hypoxic stress. It is important to consider that for fish inhabiting water bodies that are frequently hypoxic, as in the Amazon, natural selection will favor the evolution of adaptive strategies such as varying enzyme activity levels, modifications in enzyme kinetics, and metabolic depression. These biochemical adaptations, together with a decrease in locomotor activity, occur along with a reduction of oxygen

consumption rates, as observed in several experiments realized in our laboratory (Muusze *et al.*, 1998; Chippari Gomes, 2002; Lopes, 2003) as well as in others (Dunn and Hochacka, 1986; Zhou *et al.*, 2000). Chronic exposure to hypoxia could, therefore, induce a suppressed metabolic rate in tropical animals, as already mentioned above (Figure 10.1).

Fuel utilization under metabolic suppression and under oxygen deprivation in systems that use metabolic arrest involves the mobilization of anaerobic pathways. The use of carbohydrate reserves such as glycogen or amino acids, which are the main fuels available for fermentation in animals, is almost always necessary (Hochachka and Somero, 2002). Lipid reserves are used when oxygen is not necessarily limiting, such as in the burrowing lungfish (Dunn *et al.*, 1983), which uses lipid as the major fuel during the early phases of estivation. When this reserve becomes depleted, proteins are mobilized and amino acids serve as precursors for gluconeogenesis and for catabolic substrates. Dunn *et al.* (1983) suggested that glycogen reserves are conserved to save muscle energy during arousal and escape.

According to Hochachka and Somero (2002), most biologists realized early that fishes differ greatly in their patterns and capacities of locomotion, ranging from fast-start, burst-swim specialists, to species that can swim steadily but slowly for intermediate or long periods, and finally to species that can swim for long periods of time and long distances. Extensive studies have shown that the biochemical machinery of red and white muscles is adapted and coordinated in its physiological adjustments, affecting fuel and oxygen supply capacities, and can be so extensive that it can mix-up the distinction between white and red muscle. For some groups, such as scombroid fishes, mitochondrial enzyme concentrations per gram of white muscle can be higher than its homologous (orthologous) enzyme levels in red muscle of some sluggish Amazon fishes. In red muscle of some Amazon fishes, the concentrations of enzymes in anaerobic metabolism may be higher than in the white muscle of more hypoxia-sensitive fishes from more oxygen-rich, usually colder, waters. To better address this issue, we will review some data on red and white muscle biochemical machinery in the following section, presenting new data about neotropical fishes, particularly Amazon fishes.

VI. RELATIVE AMOUNT OF RED MUSCLE IN FISH AND ITS ADAPTIVE ROLE

Red and white muscle fibers are often spatially differentiated in fish. The slow oxidative fibers in red muscle contrast with white muscle, which is anaerobic and displays exceptional compositional homogeneity. The relative amount of these fibers changes according to several characteristics

of the species, such that in some species, like the fast pike, the entire swimming musculature is a uniformly white (fast-twitch muscle) glycolytic system, with red muscle fibers (slow-twitch muscle) extremely reduced (Moyes *et al.*, 1992). Among fishes of the Amazon, some of the highest relative anaerobic rates in metabolism occur in *Osteoglossum bicirrhosum* (aruanã, or water monkey), i.e., LDH/CS (Citrate Synthase) ratios can reach 800. The aruanã has one of the lowest ratios of red to white muscle among studied Amazon species. A cursory inspection of red:white muscle ratios among fishes of the Amazon reveals an interesting picture regarding environmental adaptations (Table 10.3). It is clear that fishes with long time/long distance swimming habits have more slow-twitch red muscle and, therefore lower ratios of white to red muscle (WM/RM). On the other hand, fish species with fast-twitch, burst-swimming activity show such a low amount of red muscle that ratios between fast and slow muscle fibers can reach the extreme of nearly absent red muscle fibers in the total skeletal muscle. This is the case in an ornamental fish, the cichlid discus *Symphysodon aequifasciatus* (Table 10.3). In fact, high WM/RM ratios occur in the whole cichlid family, with the exception of *Satanoperca acuticeps*. Cichlids constitute a family that sustains highly specialized reproductive habits. Most species retain territorial and show aggressive parental care, and consequently require a high capacity for fast-twitch muscle fibers to allow for bouts of burst swimming. The cichlids are also considered a hypoxia-tolerant family as a whole; this characteristic may occur in different degrees in each group of species (Chippari Gomes, 2002). Fishes with a higher proportion of red muscles fit into the category of either active species, such as the Serrasalminidae (piranhas and pacus), or species with long and continuous swimming habits, such as fish occurring in large schools in the Amazon region, e.g., characids and prochilodontids. In an intermediary position, other fish families with moderately active lives or facultative air-breathing habits will have intermediate WM/RM ratios (Table 10.3). Red muscle fibers have a well-developed blood supply, high myoglobin and mitochondria contents, high concentrations of lipids and cytochromes, and high activities of respiratory and citric acid cycle enzymes. Therefore, red muscle fibers show active aerobic metabolism, using both carbohydrates and lipids as substrates (reviewed by Van Ginneken *et al.*, 1999). However, the bulk of muscle tissue in most fish species consists of white muscle, which depends mainly on anaerobic glycolysis for its energy supply.

Biochemical comparisons between both kinds of muscle fibers are well described with regard to amino acids, phosphorylated compounds, and enzyme activity levels and most authors refer to them as clearly different when responding to a stressor, particularly hypoxia (Van Ginneken *et al.*, 1999; Hochachka and Somero, 2002). Comparative analyses of pairs of species,

Table 10.3
Relative Amounts of Red Muscle and White Muscle,
Represented as the % of Total Weight, and the Ratio Between the Two

Species	Red muscle somatic index	White muscle somatic index	WM/RM ratio
Osteoglossidae			
<i>Osteoglossum bicirrhosum</i> (n = 1)	0.82	30.94	37.7
Characidae			
<i>Triportheus flavus</i> (n = 1)	2.20	26.8	12.2
<i>Triportheus albus</i> (n = 1)	2.92	28.99	9.9
Curimatidae			
<i>Curimata inornata</i> (n = 4)	3.24	33.89	10.5
<i>Psectrogaster amazonica</i> (n = 3)	1.77	31.14	17.6
<i>Psectrogaster rutiloides</i> (n = 2)	2.14	23.57	11.0
Serrasalminidae			
<i>Pigocentrus nattereri</i> (n = 3)	1.95	18.74	9.6
<i>Mylossoma duriventre</i> (n = 4)	4.04	22.41	5.6
<i>Metynnis hypsauchen</i> (n = 4)	3.91	19.17	4.9
Anostomidae			
<i>Leporinus friderici</i> (n = 2)	2.58	33.94	13.2
<i>Rhytiodus microlepis</i> (n = 1)	2.11	25.80	12.2
Callichthyidae			
<i>Hoplosternum litoralle</i> (n = 12)	0.71	10.49	14.77
Loricariidae			
<i>Liposarcus pardalis</i> (n = 8)	1.57	11.36	7.24
Doradidae			
<i>Corydoras</i> sp (n = 7)	2.19	9.15	4.18
Cichlidae			
<i>Satanoperca acuticeps</i> (n = 2)	1.26	14.36	11.4
<i>Cichlassoma severum</i> (n = 1)	0.84	22.71	27.0
<i>Cichla monoculus</i> (n = 4)	0.69	33.87	40.1
<i>Geophagus altifrons</i> (n = 15)	0.50	25.28	50.6
<i>Uaru amphiacanthoides</i> (n = 3)	0.64	27.67	43.1
<i>Astronotus crassipinnis</i> (n = 23)	0.39	22.73	58.3
<i>Satanoperca jurupari</i> (n = 15)	0.77	40.46	52.5
<i>Symphysodon aequifasciatus</i> (n = 19)	0.06	28.18	469.1

even those showing similar metabolic trends, suggest they vary on a species-specific basis, reflecting many characteristics, including the type of swimming performance and particular mode of life (reviewed by [Johnston, 1977](#)). As shown in [Table 10.3](#), it is clear that a correlation exists between fish mode of life and the degree of red muscle development in the myotome, and such correlation may occur in nature due to the fact that the basic metabolic

differentiation of red and white muscles in fish may be under environmental evolutionary pressure.

To better address this issue, we have compared two closely related fish species that were acclimated differently: *Prochilodus scrofa* (curimbatá) and *Prochilodus nigricans* (curimatã). These two congeneric species have different life styles, live under different thermal regimes (i.e., different latitudes; the former species lives close to the Tropic of Capricorn and the latter is closer to the Equator), and have different migratory habits. *Prochilodus scrofa* inhabits Paraná-Pardo basin located at the southeast region of Brazil and has short-distance, fast-swimming habits, “running” upstream large rivers during the spawning season, which requires a high anaerobic potential. On the other hand, *Prochilodus nigricans* inhabits the Amazonian basin and has long-distance, low-speed no-stopping swimming habits (year-round migration habits), requiring more of an endurance-type, low-twitch, oxidative muscle fiber, similar to red muscle. Evolution has driven these two species to develop different amounts of muscle fibers, which is illustrated in [Figure 10.2](#). Once again, a long-term environmental pressure, combined with the development of different habits and differential adaptation to different kinds of habitats can result in long-term metabolic and morphological adjustments, both depending on the evolutionary genetic processes of adaptation, as discussed at the beginning of this chapter. Evolutionary history has thus played an important role in establishing biochemical characteristics in the muscle fibers of these species.

The analysis of muscle enzyme activity levels fits perfectly within their modes of life as well. Absolute activity levels of enzymes of glycolytic pathway (HK, pyruvate kinase – PK, and LDH), citric acid cycle (CS, citrate synthase), mixed functions (malate dehydrogenase – MDH), and lipid metabolism (beta-hydroxyacyl-CoA dehydrogenase – HOAD) in heart, red muscle, and white muscle of these two closely related species are summarized in [Table 10.4](#). The activity levels of these enzymes were measured under saturated conditions of substrates as described elsewhere ([Moyes et al., 1989, 1992; Driedzic and Almeida-Val, 1996](#)). Considerable species differences can be observed in the absolute activity levels of the enzymes, reflecting their different metabolic profiles, which can be associated with the life history of each species. HK activities are higher in heart compared to white muscle levels in both species. Except for the heart, the long-swimming species *P. nigricans* shows lower HK values than the higher-speed, burst-swimming species *P. scrofa*. HK, as already mentioned, is unique among glycolytic enzymes because its activity in vertebrate muscles is directly related to the preferential utilization of glucose from liver rather than muscle glycogen for energy production, i.e., a preferential usage of free glucose. For fish species swimming upstream, such as *P. scrofa*, anaerobic power is very important, since the animal must maintain high energy production in a low oxygen-loading environment, the white muscle fibers. Indeed, white muscle

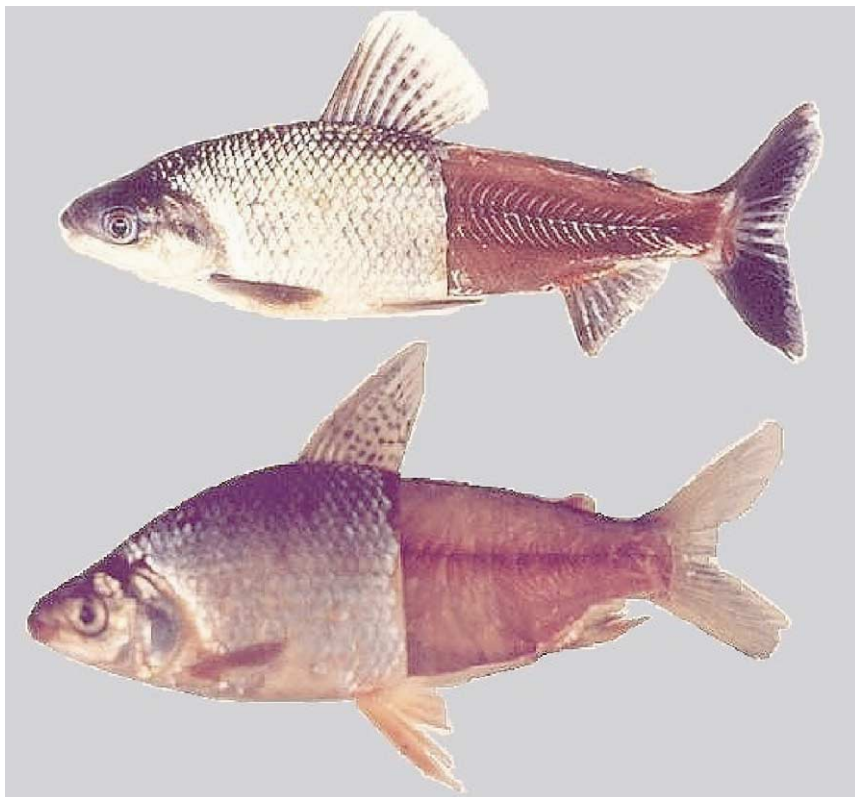


Fig. 10.2 Comparison of two congeneric species acclimated to different thermal regimes (i.e., different latitudes), showing differences in relative amount of red muscle: *Prochilodus scrofa* (curimbatá) bottom and *Prochilodus nigricans* (curimatã) top. The former has short-distance, fast-swimming habits, “running” upstream in large rivers during the spawning season, which requires a high anaerobic potential (higher amount of white muscle); the latter has long-distance, low-speed no-stopping swimming habits (year-round migration habits), requiring more of an endurance-type, low-twitch, oxidative muscle fiber, similar to red muscle. See [Table 10.4](#) and text for differences in metabolic profiles of red muscles of the two species.

fibers from this species retain higher anaerobic capacities ([Table 10.4](#)), which can be seen through the differences in glycolytic enzymes, mainly PK and LDH activity levels.

The picture that emerges from these data is that *P. nigricans* has the ability to endure continuous swimming with a higher amount of mitochondria-rich red muscle, metabolically adapted to preferential usage of carbohydrate in an oxygen-rich environment, since red muscle fibers are up-loaded with blood, as mentioned earlier ([Figure 10.2](#) and [Table 10.4](#)).

Table 10.4
Absolute Enzyme Activities (V_{\max}) of Heart, Red Muscle, and
White Muscle of the Congeneric Species of *Prochilodus*

Enzyme levels	<i>Prochilodus nigricans</i>	<i>Prochilodus scrofa</i>	<i>P</i> values (<i>t</i> student)
Heart			
HK	1.47 ± 0.12	1.001 ± 0.04	0.0017
PK	29.68 ± 1.09	25.78 ± 0.5	0.0043
LDH (1 mM)	137.52 ± 5.27	123.98 ± 2.93	0.038
LDH (10 mM)	246.64 ± 10.03	248.18 ± 7.92	0.905
CS	18.85 ± 0.44	13.66 ± 0.22	<0.001
MDH	190.14 ± 2.87	185.99 ± 4.07	0.415
HOAD	6.58 ± 0.24	7.98 ± 0.18	0.0002
Red muscle			
HK	1.53 ± 0.09	5.05 ± 0.31	<0.001
PK	11.85 ± 0.54	25.63 ± 0.6	<0.001
LDH (1 mM)	143.96 ± 21.89	174.77 ± 6.31	0.003
LDH (10 mM)	127.91 ± 5.65	236.07 ± 11.76	<0.001
CS	23.87 ± 0.41	13.03 ± 0.36	<0.001
MDH	105.33 ± 1.19	67.76 ± 1.7	<0.001
HOAD	10.03 ± 0.34	5.8 ± 0.19	<0.001
White muscle			
HK	0.016 ± 0.002	0.936 ± 0.034	<0.001
PK	406.24 ± 3.93	475.85 ± 5.73	<0.001
LDH (1 mM)	561.96 ± 21.89	951.82 ± 28.97	<0.001
LDH (10 mM)	573.12 ± 21.32	519.43 ± 13.32	0.046
CS	3.51 ± 0.12	3.09 ± 0.12	0.023
MDH	47.71 ± 1.79	82.74 ± 2.45	<0.001
HOAD	9.71 ± 0.45	1.49 ± 0.14	<0.001

Enzyme activity is expressed as $\mu\text{mol min}^{-1} \text{g}^{-1}$ wet weight (mean \pm SEM).

Source: After [Lopes, 1999](#).

Also, a higher level of lipid metabolic enzyme HOAD is present in the oxidative red fibers, as is a higher amount of CS, which is also higher in white muscle of *P. nigricans*. The result of a lower blood perfusion and consequently lower oxygen loading is evident in *P. scrofa*, which retains a higher anaerobic capacity in both red and white muscles ([Table 10.4](#)). In some way, these results change the notion that a life cycle spent at low temperatures is often associated with elevated activities of key enzymes in energy metabolism, i.e., the higher the latitude at which a fish occurs, the higher its enzyme activity levels, as discussed in previous sections. When the comparison is made between two closely related species living at different latitudes, the species adapted to higher latitudes and lower temperature

regimes generally shows lower absolute enzyme levels than the one living closer to the Equator at higher temperature regimes. Furthermore, the absolute enzyme levels are tissue-specific and are adapted to the metabolic profile that fits in their evolutionary life histories, which suggests that in terms of general patterns, congeneric or confamilial species should be compared to generate a better picture of the role of evolution in the establishment of metabolic profiles and morphological adjustments, which depend upon long-term, structural genetic changes during evolution.

VII. OXYGEN DEPRIVATION AND ITS CONSEQUENCES IN AMAZON FISHES

Oxygen depletion in water (hypoxia) is a common phenomenon in nature, which may be caused by human activities or may have natural origins. Acute pollution episodes may cause mortality and/or permanent damage to aquatic organisms. On the other hand, constant pollution activities may induce a decrease in oxygen availability in water bodies over several years and can result in changes in species distribution and cause severe decreases in population sizes. Paradoxically, this may result in animals adapted to the new hypoxic conditions. This may be the case because some fishes have adapted to hypoxic conditions due to their evolutionary history, which will enable them to better cope with the new situation and have better chances of survival in a polluted site. Furthermore, natural episodes of hypoxia occur globally in different environments and may have different causes and effects in aquatic organisms that inhabit different ecosystems. Hypoxia occurs naturally below the frozen surface of lakes, especially when photosynthesis decreases due to snowfall and oxygen consumption decreases under winter ice due to nitrification (Van Ginneken, 1996); hypoxia also occurs in some large lakes such as Lake Tanganyika due to strict stratification (Coulter, 1991); and hypoxic conditions may also occur in aquaculture ponds due to overstocking (Boyd and Schmitton, 1999). In the waters of the Amazon, flood pulses occur annually (Junk *et al.*, 1989; Val *et al.*, 1998) and cause oscillations of several physical-chemical factors resulting in the seasonal variance in oxygen availability (see Chapter 1). In aquatic ecosystems of the Amazon, episodes of severe hypoxia can occur, and oxygen levels may drop down to values below $2.0 \text{ mg O}_2 \text{ l}^{-1}$, lasting up to several months at a time (Val *et al.*, 1986; Val *et al.*, 1998). To survive such conditions, fish of the Amazon have developed a series of coordinated metabolic adjustments, which, combined with morphological and anatomical changes, have resulted in a number of solutions to avoid stress caused by hypoxia (Val and Almeida-Val, 1995). In the case of the Amazon, the amount of oxygen is altered due to the interactions of many characteristics and processes, including photosynthesis, respiration

of aquatic macrophytes and phytoplankton, light penetration, organic decomposition, molecular oxygen diffusion, wind, water body depth and shape, and temperature. Both long- and short-term changes in oxygen are determinants of fish distribution in Amazonian water bodies (Almeida-Val, Paula-Silva *et al.*, 1999; Almeida-Val, Val *et al.*, 1999).

The critical oxygen tension (P_c – critical pressure) is the tension of oxygen in water below which oxygen consumption by the fish begins to drop. The critical oxygen tension, or threshold, varies among fish species. In the past, two definitions were given for critical oxygen tension: “incipient limiting level” and “no-effect oxygen threshold,” which reflected the level at which fish populations were not impaired in their growth – development – activities (Davis, 1975). Minimal values were described for populations of trout (55% air saturation, AS), carp (50% AS), and eel (35% AS). Some authors have proposed the concept of “incipient lethal level”, or the level that the animals can resist for some time but eventually die, probably because of the activation of anaerobic metabolism, which would result in metabolic imbalance (Fry *et al.*, 1947; Davis, 1975). Other studies have described P_c as a parameter that is dependent on metabolic demands and the ability of the animal to supply oxygen to its tissues. Thus, all of these indices (P_c , incipient limiting level, and incipient lethal level) should be described under defined experimental conditions. Today, many more accurate approaches are used in studies of hypoxia tolerance, particularly in fish, and it is well known that conformity and regulation are two mutually exclusive metabolic conditions in fish when exposed to graded hypoxic conditions. Studies on the effects of hypoxia on fish have increased markedly in the last decades and many reviews are available (Nikinmaa, 2002; Wu, 2002). Most processes are now elucidated and may be summarized as follows: adaptive responses to hypoxia shown by fish are (i) escape reaction; (ii) adaptations of circulatory and ventilation systems; (iii) reduction of activity to standard metabolic rates (SMR); (iv) activation of anaerobic glycolysis; (v) phosphocreatine depletion; (vi) metabolic depression (reduction of activity bellow standard metabolic rates); and (vii) release of inhibitory neurotransmitters in brain (reviewed in Van Ginneken, 1996). Recent molecular studies have revealed that gene regulation and signal transduction are common in vertebrates exposed to hypoxia and also occurs in fish (Soitamo *et al.*, 2001; Powell and Hahn, 2002). Considering the current literature, we can affirm that evolution played a crucial role shaping many mechanisms to help fishes to better cope with low oxygen environments.

A. How Fish Became Tolerant to Hypoxia

The evolution of fishes occurred independently from other vertebrates for several hundred millions of years, and the periods of low oxygen pressure are coincident with the appearance of important groups of fishes. Among

these are groups with adaptations to breathe air, such as the lungfishes (Dipnoan). Moreover, modern groups like the Osteoglossomorpha, Ostariophysi and Plecomorpha, which have representatives living in natural hypoxic waters, also developed some adaptations to breathe air and tolerate hypoxia. Among modern Ostariophysi, the current hypoxic environments of tropical waters have resulted, in recent history, in the development of facultative air breathing. Three families of neotropical armored catfishes have developed adaptations in their digestive system to breathe air – these adaptations are coincident with the encapsulation of the swim bladder (Rapp Py-Daniel, 2000). The two main groups of fishes have appeared in periods of limiting oxygen availability. Both Chrossopterygian (lobed-fin fishes) and Actinopterygian (ray-fined fishes) are thought to have developed air-breathing habits and hypoxia tolerance since early in their evolutionary history. Fish arose more than 500 MYA. As reviewed recently, the evolution of fish occurred independently from other vertebrates for several hundred millions of years (Nikinmaa, 2002) and hypoxic environments dominated the first geological eras after fish arose (Berner and Canfield, 1989; Graham, 1997). The current values of atmospheric oxygen were reached around 200 MYA. Thus, the origin of fishes and half of their evolutionary history occurred under conditions of oxygen depletion.

From that period through to the present, some environments on earth have remained hypoxic, if not constantly, at least periodically. Aquatic ecosystems such as shallow and warm waters of the tropics are commonly found to be hypoxic and even anoxic during some periods of the day (Junk, 1984; Val, 1995; Almeida-Val, Val *et al.*, 1999). Not surprisingly, these environments are among the most diverse in the world (see Chapters 1 and 2).

The amount of dissolved oxygen varies in response to the interactions of many characteristics and processes in the waters of the Amazon. Seasonal variations and daily and spatial oscillations may occur and induce complex patterns of oxygen distribution. During most of the year, anoxic conditions are observed at night (Figure 10.3). However, plant cover, depth and sunlight play important roles in oxygen availability during the day. Hypoxia tolerance therefore became particularly common among fishes of the Amazon, and we believe that such chronic hypoxic conditions drove fish species through a series of adjustments at different levels of biological organization (ethological, morphological, anatomical, physiological, metabolic, and molecular) that were combined to produce phenotypic plasticity, allowing them to survive the pulsating nature of the basin (reviewed in Almeida-Val, Val *et al.*, 1999).

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

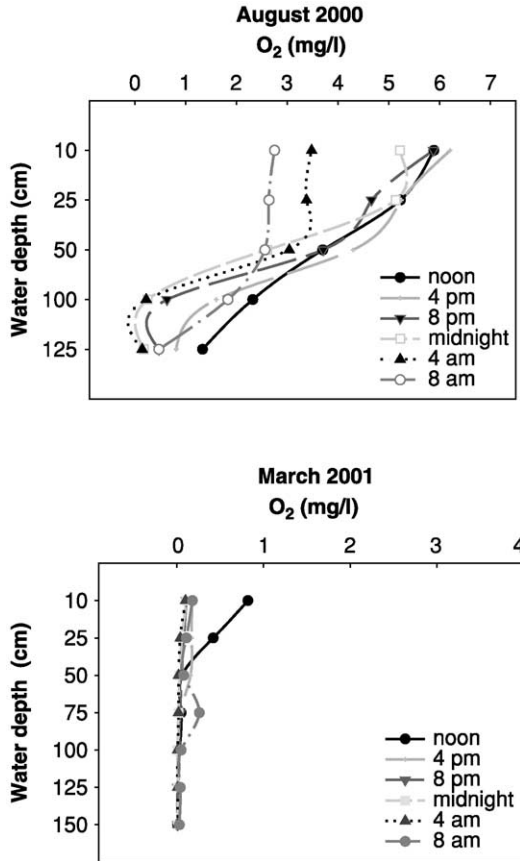


Fig. 10.3 Oxygen distribution in the water column of *várzea* lakes near Catalão Lake at two different times of the year. Data were collected in the right side of the Rio Negro, inside the lake just before where the Rio Negro meets the Rio Solimões. The two boxes show that the amount of dissolved oxygen varies with depth, time of the day, and time of the year. These seasonal variations and daily and spatial oscillations may induce complex patterns of oxygen distribution. Also, during different times of the year, anoxic conditions can always be observed at night. (Original data obtained by [Chippari Gomes, 2002.](#))

1998). Current environmental heterogeneity, caused by flood pulses, different water types, and physico-chemical parameters, are the main causes of recent adaptive radiation in fish of the Amazon ([Junk et al., 1989](#); [Val, 1993](#)). The 3000 fish species described to date in the Amazon display a variety of adaptations to their environments that include behavioral, physiological, biochemical, and genetic changes. The time course of the appearance of such adaptive traits seems to be related to the intensity and periodicity of the

constraints imposed upon each individual, population, species, and group of species. However, the description of several adaptive strategies at numerous taxonomic levels has revealed that the selective pressure during evolution may be caused by several chronic constraints such as short- and long-term changes in oxygen, water pH, ion-poor waters, acidity, and daily and spatial temperature oscillations, among others (Almeida-Val, Val *et al.*, 1999). Thus, adapting to such ever-changing environments is probably the main cause of fish diversity in the Amazon.

B. Levels of Responses to Hypoxia

Five levels of response to hypoxia are commonly described in aquatic ecosystems: ecological, behavioral, physiological, biochemical, and molecular. At the ecological level, results may be different according to environment characteristics. However, for most environments, an increase in hypoxia episodes may be devastating because it may cause mass mortality, defaunation of benthic populations, declines in fisheries production, permanent damage to part of the aquatic environment, changes in community composition, and, as an ultimate consequence, a decrease in animal diversity. On the other hand, chronic hypoxic situations, such as are common in the Amazon basin, have caused several adaptations at different levels of biological organization, thereby inducing increased species diversity. Also, seasonal changes in species composition may occur as a result of different oxygen availabilities. The classic study of Junk *et al.* (1983) showed that during low oxygen conditions, the species remaining in the lake were mostly those that could breathe air or those with some kind of hypoxia tolerance. Among water-breathers, cichlids always remain inside hypoxic lakes, and are a group considered to be tolerant of hypoxia and anoxia (Junk *et al.*, 1983). The occurrence of cichlid species in Catalão Lake was investigated during one year and was correlated with oxygen availability (Chippari Gomes, 2002). Variance in oxygen availability occurs throughout the year. During periods of low water, oxygen levels drop substantially, reaching concentrations below 1 ppm. Although the number of captured species varied during the year, we cannot imply that cichlids species richness was related to oxygen availability (Chippari Gomes, 2002). However, the abundance of cichlids was higher when oxygen reached the lowest level of the year (Figure 10.4). As hypoxia-tolerant fishes, cichlids remain in hypoxic and anoxic waters, while more sensitive species escape. Other species that may remain in hypoxic lakes have the option to breathe air or skim the water surface. The tambaqui has the ability to expand its inferior lips to help direct more oxygenated water through its gills. In the wild, the occurrence of lips is inversely related to oxygen availability (Figure 10.5).

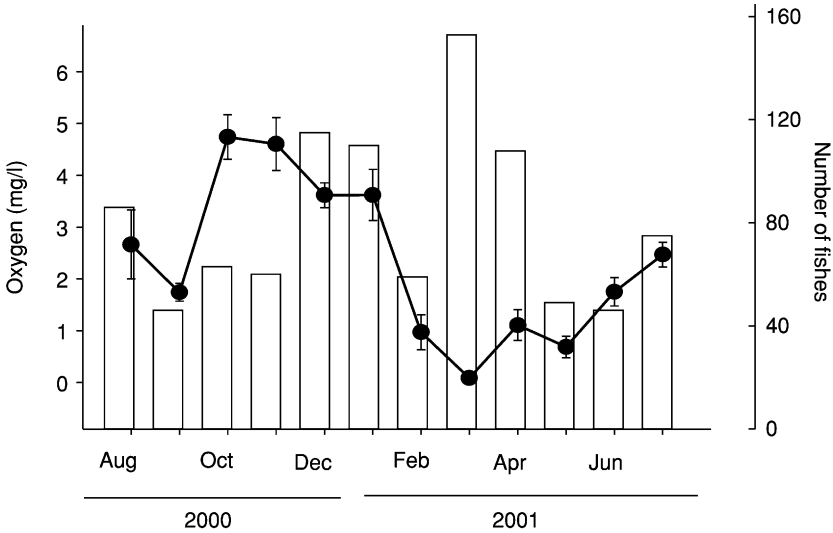


Fig. 10.4 Relationship between oxygen distribution (closed round symbols) during the year (values obtained at noon) and the abundance (number of fishes – open bars) of cichlids captured near Catalão Lake. The highest abundance of fishes is coincident with the lowest oxygen availability, which can be explained as their ability to occupy and stay in hypoxic environments, which is not observed in any other group of fish. (Original data obtained by [Chippari Gomes, 2002.](#))

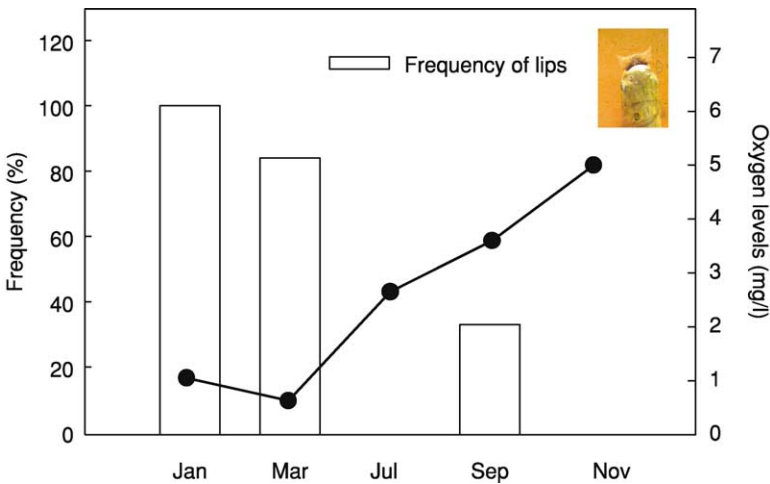


Fig. 10.5 Relationship between frequencies of animals captured with swollen lips and oxygen availability where they were captured. Tambaqui swells its inferior lips (inlet) to better capture the surface water layer, which is richest in oxygen. (Redrawn from [Val and Almeida-Val, 1995.](#))

Field studies of fish distribution in aquatic ecosystems of the Amazon basin have always correlated oxygen availability with preferred species habitats, migration (particularly lateral migration), and adaptive characteristics, namely air breathing or aquatic surface respiration (ASR) (Junk *et al.*, 1983; Cox-Fernandes, 1989; Crampton, 1998). The pioneering work of Junk and collaborators (1983) at Camaleão Lake on Marchantaria Island described fish movements throughout the year that were related to changes in environmental oxygen levels. Another interesting study by Crampton (1998) investigated the distribution, migratory behavior, and respiratory adaptations of electric eels (*Gymnotiformes*) in Rio Tefé, located in the upper Amazon basin. This author suggested that all these parameters were directly related to oxygen availability, and that oxygen availability may have an important influence on the design of electric signals in the eels. Regardless of whether this is related to oxygen distribution in the water, electric eels of the Amazon have a variety of adaptive strategies to survive episodes of hypoxia. Some species were even described as anoxia-tolerant under field and experimental conditions. Among the studied species, Crampton (1998) described air-breathing organs in two species, air-gulping in five species, and ASR in 12 species. However, these data were based on chemically induced hypoxia (forced oxygen reduction by using sodium sulfite in the metabolic chamber water). Thus, these findings must be confirmed under different conditions, since sodium sulfite may cause drastic changes in ionic homeostasis, and can induce sulfohemoglobins formation, which results in other physiological effects such as physiological impairment of oxygen transfer to tissues. In spite of this, the fact that some water-breathing species could survive anoxia for 6 hours without ASR, which was inhibited by the experimental design, is good evidence that those species are tolerant of hypoxia. As with air breathing and ASR, hypoxia tolerance appears in fish as a homoplastic characteristic, which, different from other vertebrate lineages, has multiple independent evolutionary origins and is the consequence of multiple environmental pressures, causing adaptive radiation.

Reviewing the subject of hypoxia tolerance in fishes, Hochachka and Somero (2002) suggested that except for the Ostariophysi, within which are several groups known to be notably hypoxia-tolerant, the distribution of hypoxia tolerance within the phylogeny appears sporadic, which seems to be consistent with multiple independent origins of hypoxia-tolerance mechanisms in teleost fishes, different from other hypoxia-tolerant vertebrate groups such as turtles and pinnipeds (reviewed by Hochachka and Somero, 2002). When considering air breathing as a defense adaptation against hypoxia, many (and possibly all) air-breathing fishes – described as “the first diving vertebrates” – display impressive hypoxia defense mechanisms similar to those described for aquatic turtles (Almeida-Val and Hochachka, 1995).

The Ostariophysi comprise four fish Orders that are predominantly tropical. Three out of four Orders occur in tropical and subtropical hypoxic waters. The Gymnotiformes (electric eels), Siluriformes (catfishes), and Characiformes (suckers, tambaqui, pacu) retain hypoxic-tolerant species with many representatives in the Amazon basin. Such hypoxia tolerance results from different kinds of adaptational changes. The fourth group, Cypriniformes (goldfish, carp), does not occur in South American basins but is strongly hypoxia-tolerant due to metabolic adaptations (Van Waarde *et al.*, 1993). Many authors have suggested that air breathing is an indicator of hypoxia defense adaptations and of hypoxia tolerance. As such, the examination of the phylogeny of the group also suggests that this trait has evolved numerous times within the fishes (Val and Almeida-Val, 1995). According to Hochachka and Somero (2002) and Val and Almeida-Val (1995), while in some lineages this may have involved common descendants, in many others it is clear that hypoxia tolerance has evolved independently. Because of the large number of fish species that are known to be hypoxia-tolerant, these authors suggested that it is clear and compelling that hypoxia tolerance has arisen independently many times within this group of organisms. What we now need is a detailed evolutionary analysis of hypoxia adaptations in fishes analogous to that presented for diving in pinnipeds (Mottishaw *et al.*, 1999). However, the possibility of many traits originating by parallel convergent evolution in response to similar environmental challenges appears to be the key for beginning to understand how comparable complex physiologies independently evolved within a group as diverse as fishes. One of the best examples is the development of the ASR in response to the chronic hypoxic waters of Amazonian *várzea* lakes (Almeida-Val, Val *et al.*, 1999).

C. Appearance of Aquatic Surface Respiration and the Development of Air-Breathing Organs

While these subjects have already been fully investigated by several authors through a description of the regulation of 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 poorly understood. Studies on the relationship between aquatic surface respiration (ASR), an innate behavior, and the physiological responses that follow such a strategy have been addressed to evaluate its efficiency (Almeida-Val *et al.*, 1993; Val, 1995). This adaptation may be considered a homoplastic characteristic among fish since it has appeared in different phylogenetic groups in response to the same environmental pressures. Such behavior is useful for fish inhabiting hypoxic *várzea*

lakes, and its evolution among several groups may be considered one of the main surviving hypoxia strategies among fishes of the Amazon. The cichlids (Perciformes) are among the most advanced teleosts that occur in the Amazon basin; they are considered a highly specialized group with a high degree of adaptive radiation and are considered to have a faster evolutionary rate compared to their African counterparts (Farias *et al.*, 1998). Several cichlid species show ASR, a behavior that is more noticeable in young of the species. During growth, some species, e.g., *Astronotus ocellatus*, reduce the number of incursions to the water surface and increase their anaerobic glycolytic power as a result of increased mass-specific LDH levels, improving their survivorship under hypoxic conditions (Almeida-Val, Paula-Silva *et al.*, 1999; Almeida-Val *et al.*, 2000). LDH isoforms also change accordingly in the brain of this species. It is important to state, however, that LDH scaling properties have also been described in non-hypoxia-tolerant fish groups, apparently due to an increase in burst swimming capacity (Burness and Leary, 1999).

The implications of ASR are numerous, as are the implications of air breathing, which may be considered another homoplastic characteristic among fishes. However, in several cases, the diversification of air-breathing fishes reflects the successful adaptive radiation of a particular air-breathing type, as in the groups Callichthyidae and Clariidae (Graham, 1997). Fossil records indicate that one of the highly derived living catfishes, *Corydoras*, belongs to the family Callichthyidae, indicating an early Cenozoic differentiation between callichthyids and loricarioids (Lundberg, 1998). The latter is another air-breathing type, which has not been considered to have spread as successfully as the callichthyids. In fact, air breathing occurs 28 times in the Callichthyidae, while only seven species in the Loricariidae have been described as air-breathers.

Similar to ASR, ABO structure cannot be categorized, because no one can distinguish its homology or convergence. The presence of ABO has been described in 49 fish families, all of them presenting different solutions for breathing air. It is most probable that these solutions appeared among ray-finned fishes in response to the same environmental pressure: hypoxia. Air-breathing habits were described early in the literature as a widespread adaptive trait. In 1910, Rauther described them as respiratory adaptations, 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: hypoxia and emergence. Both traits have influenced the origin of this characteristic. It is postulated that 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 episodes of air breathing as low oxygen availability (Johansen, 1970; Graham *et al.*, 1978). Some researchers have

suggested that air breathing in fish arose accidentally (by chance) in fish that were skimming water surfaces (Gans, 1970), or that air breathing was precipitated by changes in water flow (Hora, 1935). However, these interpretations are rather rare.

Regardless of why or how ABO appeared and how ASR developed, fish have found a way to live in environments with low oxygen content. These adaptations allowed them to explore a wide range of ecological niches, and yet most air-breathing species must deal with other types of constraint. Almeida-Val and Hochachka (1995) pointed out that diving into the water bodies and holding their breath for long periods induces metabolic changes in fish, i.e., slowing down total metabolic rates, decreasing oxidative enzyme rates, and increasing anaerobic ability. While these characteristics were first noticed in air-breathing species, further investigations have shown that low metabolic profiles are common in fishes of the Amazon, independent of respiration patterns or life style (Driedzic and Almeida-Val, 1996; West *et al.*, 1999; Table 10.1). Thus, the environmental pressure imposed by low oxygen availability may be considered the main driving force in the development of long-term metabolic adjustments.

Hypoxia affects air-breathing fishes in different ways. Obligate air-breathing species are not strongly influenced by water oxygen availability, since they have reduced gill surface areas. Other air-breathing fishes are affected by hypoxia in different ways; the threshold for oxygen content in the water varies by species. For example, among Amazonian fishes, the jeju (*Hoplerythrinus unitaeniatus*), a facultative air-breather, starts breathing air when oxygen drops to 81 mmHg (Stevens and Holeton, 1978a), while the armored catfishes (*Hypostomus spp.*) may seek air when oxygen drops down to 21, 35, or 60, dependent upon the experimental temperature (Gee, 1976; Graham and Baird, 1982; Fernandes and Perna, 1995). The swamp eel (*Symbranchus marmoratus*), an advanced teleost, may tolerate 33–69 mmHg before starting to breathe air; but in addition to the fact that this species estivates during dry seasons, these thresholds may vary according to body size and hypoxia acclimation (Bicudo and Johansen, 1979; Graham *et al.*, 1987). When we consider respiratory partitioning of oxygen uptake, the so-called facultative 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, such as *Arapaima gigas*, which is considered an obligate air-breather, may breathe 50 to 100% oxygen via air depending on body size and oxygen content in the water (Stevens and Holeton, 1978b). Following these changes in respiration patterns during growth, changes in physiological and biochemical parameters have been observed in *Arapaima gigas* (Salvo-Souza and Val, 1990). Recent investigations in our laboratory revealed that the relative heart mass of this species also changes, scaling negatively with total body mass; a strong

shift is observed when the small juveniles begin to search for air (V. M. F. Almeida-Val and C. Moyes, unpublished data).

ASR is also considered to be affected by aquatic oxygen availability. All of the above-described factors that affect air-breathing behavior also affect ASR. Therefore, decreasing oxygen availability in the water induces ASR in most observed fish species, and the efficiency of this innate behavior in terms of oxygen blood loading was found to be enough to guarantee tambaqui survivorship during long episodes of oxygen depletion (Val, 1995). Juveniles of *Astronotus ocellatus*, a cichlid fish that, in adulthood, tolerates 6 hours under anoxia at 28 °C (Muusze *et al.*, 1998) are able to survive hypoxia indefinitely if allowed to practice ASR (S. C. Land, personal communication), but are not able to tolerate long-term hypoxia if denied access to the water surface (Almeida-Val, Paula-Silva *et al.*, 1999).

D. Cichlids: The Good “Strategists” against Environmental Hypoxia

The cichlids are advanced teleosts that belong to the Perciformes, super-order Acanthopterygii (Nelson, 1994). This family is a diversified group (*ca.* 1300 species – (Kullander, 1998) distributed across Africa, Madagascar, Central and South America, Mexico, southern India and Sri Lanka (Kullander and Nijssen, 1989; Kullander, 1998). The vast majority of species is found in African lakes (Lowe-McConnell, 1987; Kullander, 1998). Following the African species, the greatest diversity of cichlids is found in South America (Nelson, 1994), the home of nearly 300 species, which corresponds to 6–10% of the freshwater fish fauna. Approximately 150 species are found in the Amazon basin (Lowe-McConnell, 1991), representing the third most abundant family of fish in the Amazon (Géry, 1984). The plasticity of this group is noticeable in color patterns, shape, feeding behavior, reproduction, and ability to adapt to the most diverse environments. The geographic distribution and genetic-evolutionary characteristics of this family are well documented in both Africa and in South America (Kornfield and Smith, 1982; Kornfield, 1984; Greenwood, 1991; Lowe-McConnell, 1991; Ribbink, 1991; Stiassny, 1991).

In recent studies, Farias and co-workers suggested that neotropical cichlids constitute a monophyletic clade and show rapid rates of evolution, with a significantly higher genetic variability compared to their African counterparts (Farias *et al.*, 1999). Many authors attribute this ability of adaptation to heterogeneous habitats, as well as their rapid adaptive radiation as the causes of the many events of speciation (Fryer and Illes, 1972; Kornfield, 1979, 1984; Stiassny, 1991). Therefore, this group of fishes has been considered extremely plastic (Stiassny, 1991; Almeida-Val, Paula-Silva *et al.*, 1999; Almeida-Val, Val *et al.*, 1999).

Isozymes have proven to be excellent tools to understand the relationship between an animal and its habitat (Kettler and Whitt, 1986; Whitt, 1987). The preferential distribution of isozymes in different organs and tissues of fishes reflects the metabolic adjustments that occur during certain periods, such as stress, growth, migration, or sexual maturation. The choice for a specific isozyme system is normally based on the parameters and metabolic adjustments to be analyzed. The isozyme system of LDH (LDH; E.C. 1.1.1.27) is one of the most studied in vertebrates and has been considered one of the best tools with which to study metabolic adjustments to environmental changes or intrinsic adjustments of animals (Almeida-Val *et al.*, 1995; Almeida-Val, Paula-Silva *et al.*, 1999; Almeida-Val, Val *et al.*, 1999). In vertebrates, LDH is a tetramer composed of two subunits types, LDH-A and LDH-B, which are encoded by two genes. Subunit A is found primarily in skeletal muscle and is very efficient in the conversion of pyruvate to lactate under anaerobiosis. Subunit B is typical of cardiac muscle and is inhibited by high pyruvate concentrations, preventing accumulation of lactate in this organ. A random combination of these subunits results in five isozymes that retain different properties. These isozymes are expressed differentially in different tissues according to energetic needs, oxygen availability and physiological functions (Markert and Holmes, 1969; see also Almeida-Val and Val, 1993 for a review of this subject). An increase in isozyme LDH-A₄ (isoform predominant in vertebrate skeletal muscle) expression in tissues considered typically aerobic, such as the heart and brain, can be detected in animals exposed to hypoxia or anoxia (Hochachka and Storey, 1975). In studies of cichlids exposed to hypoxia, we found that a decrease in LDH-B expression in aerobic tissues may occur after hypoxia exposure, which is combined with an increase in LDH-A, resulting in increased anaerobic capacity (Almeida-Val *et al.*, 1995).

The isozyme systems, especially LDH, have been the subject of many studies on Amazon fishes (Almeida-Val *et al.*, 1992; Almeida-Val and Val, 1993). Two different models have been suggested for LDH metabolic distribution in fish species: (i) predominance of isozyme B₄ in the heart, indicating the maintenance of the aerobic metabolism at low rates during hypoxia episodes; and (ii) low expression of the LDH-B* gene combined with a strong expression of the LDH-A* gene in all tissues, suggesting activation of anaerobic metabolism during hypoxia (Almeida-Val *et al.*, 1993). Reduction in LDH-B* gene expression in the heart was first observed in wild flatfish (Markert and Holmes, 1969) and in sticklebacks (Rooney and Ferguson, 1985). Previous research on rainbow trout LDH (Moon and Hochachka, 1971) and other isozyme systems in fish (Hochachka, 1965; Baldwin and Hochachka, 1970; Schwantes and Schwantes, 1982a,b; De Luca *et al.*, 1983; Almeida-Val *et al.*, 1995; Farias *et al.*, 1997) indicate that

the expression of different enzyme variants depends on environmental parameters. We have shown that LDH tissue distribution in Amazon cichlids is related to the ability of these animals to tolerate hypoxic environments, and express some degree of phenotypic plasticity in the heart, therefore revealing their preferential habitats (Almeida-Val *et al.*, 1995). One good example is the presence of two models of LDH distribution in the heart of *Cichlasoma amazonarum*. Depending on the availability of oxygen in their habitat, these animals will show a predominance of isozyme B₄ or A₄ in the heart (Almeida-Val *et al.*, 1995). When this species is exposed to severe hypoxia (about 30 mmHg) for a long period of time (51 days), significant changes in LDH distribution may be observed. Isozyme A₄ expression increases in heart and brain, while isozyme B₄ increases in the liver and “disappears” in the skeletal muscle. However, the most significant change can be observed through enzyme assays in the brain, which adopted muscle-type kinetics that caused by the new LDH isozyme distribution (Almeida-Val *et al.*, 1995; Val *et al.*, 1998).

Other species of Amazon cichlids (*Astronotus ocellatus*, *Cichla monoculus*, *Satanoperca aff. jurupari*) show the same ability to change LDH distribution as described for *Cichlasoma amazonarum* exposed to hypoxia (Chippari Gomes *et al.*, 2003). On the other hand, species such as *Geophagus sp.*, *Pterophylum sp.*, *Acarichthys heckelli*, *Crenicichla sp.*, *Hypselecara sp.*, and *Symphysodon sp.* present the distribution considered common for all vertebrates: predominance of isozyme A₄ in skeletal muscle and predominance of isozyme B₄ in the heart. Reduction in cardiac muscle LDH-B* expression is evident in *Astronotus crassipinnis*, *Heros sp.*, *Heros severum*, *Acaronia nassa* and *Geophagus cff. harreri*.

VIII. THE LDH GENE FAMILY AS A STUDY MODEL: REGULATORY AND STRUCTURAL CHANGES AND THEIR EVOLUTIONARY ADAPTIVE ROLES

The radiation of fishes into the richest vertebrate group in terms of number of species is thought to be an evolutionary success that began approximately 500 MYA. Successive genome duplication events during the first radiation episode in vertebrate evolution gave rise to many and multiple new types of proteins and, therefore, new metabolic possibilities and adaptive opportunities. Gene duplication occurred and continues to occur in restricted regions of the DNA, resulting in the appearance of new proteins or new ways of regulating their transcription. During evolution, duplicated genes may remain similar to the original gene, without any specialization, or they may differentiate from the original gene, producing proteins for a specialized new metabolic function. These duplicated and differentiated genes are homologous, and the proteins produced are termed isoforms, or

isozymes if they are related to enzymes. A third potential fate of duplicated genes is that they may become silenced, i.e., they remain in the genome but are not translated. The occurrence of true novelties, i.e., the appearance of completely new genes, is an exceptional event since there is a strong trend for the preservation of critical sequences of both structural and regulatory genes. Thus, structural and functional properties of single enzymes are helpful in evaluating evolutionary aspects of a given animal group.

Isoforms, or isozymes, are different forms of a single enzyme, which show exactly the same specificity and catalyze the same reaction. No other isozyme system or gene family has been so much investigated as extensively as LDH, and studies have elucidated many metabolic adaptations to different environments as well as many mechanisms that have occurred during evolutionary history of fish groups (Almeida-Val and Val, 1993). The current distribution of LDH isozymes among teleost fishes reflects the presence of three duplicated genes that originated at different times. Recent studies show that an ancient gene (LDH-C), first thought to be the most recent one, gave rise to LDH-A gene, which is highly specialized in anaerobic metabolism, at the beginning of vertebrate evolution (*ca.* 500 MYA). After successive gene duplications, LDH-A gave rise to LDH-B, which is adapted to recovering periods of anaerobiosis, as it has the ability to convert lactate to pyruvate during recovery from periods of hypoxia or estivation (Hochachka, 1980; Whitt, 1984; Crawford *et al.*, 1989).

The distribution of LDH genes in fish is tissue-specific and varies with phylogeny, and the analysis of homologies between them may elucidate several adaptive processes that fish have undergone. Genes generated by evolutionary duplication are referred to as paralogs and are studied from the functional point of view in order to identify preferential metabolism in different organs of the same species. Genes generated by speciation events during evolution of taxonomic groups are referred to as orthologs (same gene in different species) and these are used in comparative metabolic studies (Powers *et al.*, 1983; Powers and Schulte, 1998; Hochachka and Somero, 2002). The following sections summarize most of the investigations on fishes of the Amazon regarding this enzyme family, which has shown to be an excellent tool for evolutionary studies as well as for analysis of metabolic adaptations to environmental changes. The regulation of its genes is described as one of the best processes with which to deal with short-term oscillations in oxygen availability in aquatic environments.

A. Increase in Anaerobic Power during Development

The plasticity in regulating the expression of genes LDH-A* and LDH-B* found in Amazon cichlids indicates the capacity of these animals to base their metabolism on anaerobic glycolysis and in the expression of LDH-A*

when oxygen availability is low. Therefore, such plasticity offers these animals the ability to visit localities with low oxygen concentration for feeding or breeding. Cichlids are territorial fish with very aggressive behavior and strong parental care (Chellapa *et al.*, 1999). Therefore, burst swimming is common, and a strong anaerobic power is useful in their tissues. Although the hearts are relatively smaller in cichlids compared to other species (personal observations), it is possible that anaerobic glycolysis takes place during limited oxygen availability and that heart work is sustained for short periods at glucose expenses. The LDH-A isozyme will be more helpful in such situations than LDH-B isozyme. In fact, species in the genus *Astronotus* do tolerate anoxia as adults, and their increased hypoxia tolerance with fish growth may be explained as an increase in their anaerobic power (represented by absolute LDH levels in their tissues and organs) rather than a decrease in specific metabolic rates (Almeida-Val, Paula-Silva *et al.*, 1999; Almeida-Val *et al.*, 2000).

Studies conducted in our laboratory have demonstrated that both LDH enzyme levels and hypoxia survivorship are a function of body mass (Figure 10.6; Almeida-Val, Paula-Silva *et al.*, 1999; Almeida-Val *et al.*, 2000). As fish size increases, the ability to survive under severe hypoxia increases as well. The total amount of time required to reach disequilibrium (i.e., loss of orientation preceding death) increases as the animal increases in size, suggesting that *Astronotus ocellatus* shows an increase in hypoxia tolerance with age (Figure 10.6; Almeida-Val *et al.*, 2000).

Scaling effects on hypoxia tolerance in fishes have not been described for any other fish species, and it is interesting to explore the physiological

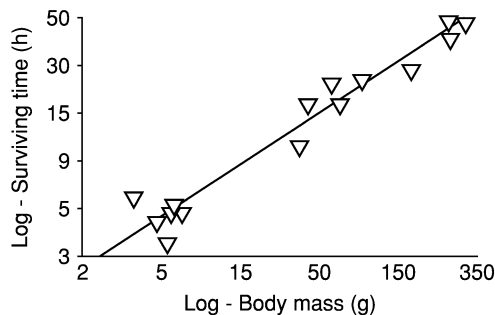


Fig. 10.6 Relationship between body mass (g) of *Astronotus ocellatus* and its ability to survive hypoxia (hours). The log-log regression shows a close relationship ($r = 0.98$). (Redrawn from Almeida-Val *et al.*, 1999, using the following regression coefficients: $a = 1.74$ and $b = 0.59$, derived from $Y = 1.74W^{0.59}$, where Y is the time (h) required for each fish to reach the disequilibrium that immediately precedes death. This value was interpreted as hypoxia survivorship. W = weight in grams.

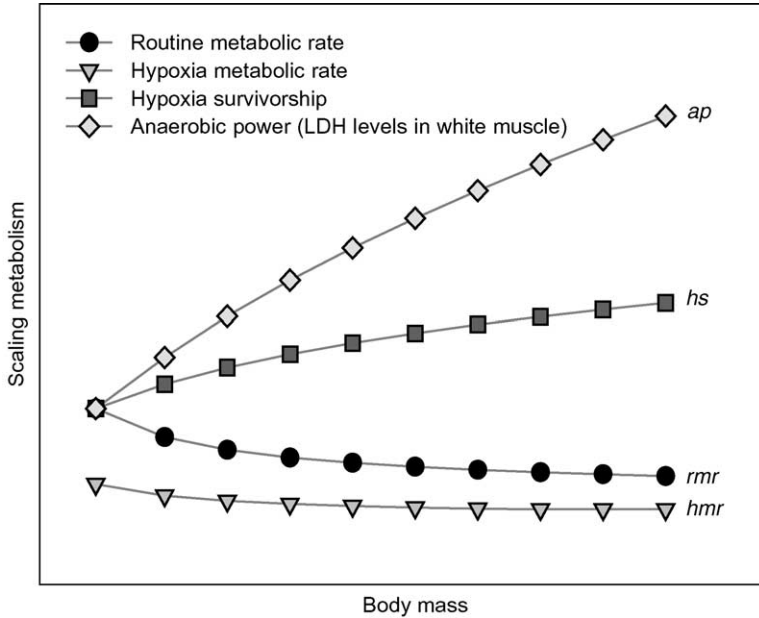


Fig. 10.7 Diagrammatic representation of the relationships between body mass (g) and mass-specific metabolic rate (*rmr*); hypoxia suppressed metabolic rate (*hmr*); hypoxia survivorship (*hs*) and mass-specific anaerobic power (*ap*) obtained from white muscle LDH levels (U.gwt^{-1}) of different-sized *Astronotus ocellatus*. Values for each variable (*Y*) were analyzed in relation to body mass (*X*) using exponential equations where *a* and *b* values were obtained using Sigma Plot software (first described in Almeida-Val, Paula-Silva *et al.*, 1999 and Almeida-Val *et al.*, 2000). As the contribution of anaerobic power increases, there is a decrease in the contribution of suppression of metabolic rate to facilitate survival of hypoxia. These results can be interpreted as an increase in the white muscle somatic index related to liver and heart somatic indices, which remain lower for this species with increased growth.

meaning of these results considering hypoxia defense mechanisms in these groups. Three key factors determine the hypoxia tolerance of any given fish species: absolute *rmr* (routine metabolic rate), hypoxia-mediated suppression rate *hmr* (hypoxia suppressed metabolic rate), and back-up anaerobic mechanisms *ap* (anaerobic power – mainly making up any remaining energy deficit by anaerobic glycolysis). If we assume that *hmr* scales in parallel with *rmr* and that LDH activity *vs.* log *M* (body mass) is an expression of glycolytic scaling or *ap*, then we can illustrate the relationships of all three above parameters with body mass in a diagram, as in Figure 10.7. These results were first presented in 2000, and since then, we have been investigating other parameters regarding gene expression in different-sized cichlids, mainly *Astronotus* species (Almeida-Val and co-workers, unpublished data).

B. Thermal Properties of Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is a glycolytic enzyme that catalyzes the interconversion of pyruvate and lactate, using the cofactors NADH and NAD⁺, respectively. The temperature-adaptive interspecific variations in its kinetic properties reflect species thermal acclimation in the same way temperature affects enzyme levels. According to [Hochachka and Somero \(2002\)](#) there is a temperature-compensatory modification of enzymatic function that can be observed considering how the catalytic performance of orthologs of A₄-LDH varies among species that have evolved at widely different temperatures. Reviewing data from 18 different species of vertebrates (fish, amphibian, reptilian, mammals, and avian), these authors concluded that interspecific differences in kinetic properties are a reflection of evolution under different thermal conditions, rather than a consequence of the evolutionary lineage to which a species belongs. The relationship between adaptation temperature and catalytic rate constant (k_{cat}) is found in all branches of the evolutionary tree ([Hochachka and Somero, 2002](#)). These data are in accordance with the idea that there is a relationship between acclimation temperature and enzyme activity levels, which ultimately reflects metabolic profiles and whole-body metabolic rates of vertebrate species.

Comparisons of K_m (apparent Michaelis–Menten constant) values for orthologs of enzymes from differently adapted species also reveal a common pattern: increases in measurement temperature cause increases in K_m for all orthologs, but at the normal acclimation temperature of differently adapted species, K_m values are highly conserved. Apparent K_m can be used as an approximate index of the affinity between enzyme and substrate or cofactor. A low K_m indicates high affinity for the ligand in question; a high value denotes weak binding. The comparison among different fish species of apparent K_m values of pyruvate for orthologs of LDH-A₄ reveals that higher enzyme affinity occurs at lower temperatures and is distributed in accordance with their thermal ranges (reviewed in [Hochachka and Somero, 2002](#)). Studies of A₄-LDH orthologs of congeneric fishes are useful to demonstrate that small differences in maximal habitat temperature are sufficient to favor adaptive changes. [Holland et al. \(1997\)](#) studied LDH orthologs of barracudas (genus *Sphyraena*) and suggested that differences in apparent K_m (pyruvate) values are adaptive and related to the thermal regimes to which they are acclimated, i.e., temperate barracuda species show higher K_m values than subtropical and tropical congeners. The number of sequence changes that underlie the different kinetic properties of A₄-LDH orthologs of barracuda congeners is small, since only a single difference at position 8 distinguishes the orthologs of the south temperate species and the subtropical species (reviewed by [Hochachka and Somero, 2002](#)).

Another extremely interesting study has been performed by Powers and co-workers, and relates LDH-B* alleles to the temperature gradient in which the killifish *Fundulus heteroclitus* is distributed. Powers *et al.* (1993) showed that in this species only one or two amino acid substitutions are sufficient to explain the differences in kinetics and thermal stability of LDH-B* alleles in populations occurring in the north and south of the north American Atlantic coast. Further studies, based in molecular tools, indicated that a limited number of mutations in the regulatory sequence of LDH-B* gene from *Fundulus heteroclitus* resulted in changes in its expression. Schulte (2001) suggested that increased LDH activity and changes in LDH-B* regulatory sequences, respectively phenotypic and genotypic differences between populations, have been affected by natural selection, rather than genetic drift, in response to the impact of environmental thermal adaptation. Thus, studies of differently adapted confamilial or congeneric and conspecific organisms have shown that temperature differences of a few degrees have sufficient effects on proteins to favor adaptive change (Hochachka and Somero, 2002).

Thermal stability of proteins is linked to conformational flexibility, which in turn is linked to protein function. Because proteins cannot become too rigid, mainly in those regions of the molecule involved in recognizing ligands, thermal stability properties are an important issue from evolutionary point of view. Also, a relative maintenance of kinetic properties regardless temperature acclimation should be advantageous from ecological point of view, particularly in fishes living at high thermal regimes, since adaptations to higher temperatures may induce increases in structural stability of proteins (Hochachka and Somero, 1984). One of the consequences of increasing structural stability by increasing the number of weak bonds is the decrease of catalytic efficiency, increasing the energy of activation. Furthermore, there is a loss in catalytic efficiency as protein stability is increased. If body temperature is related to heat stability, experiments with fish exposed to different thermal regimes should provide some idea about this relationship. For fish of the Amazon, life at a higher temperature range may have selected more stable proteins. Optimal K_m values for fish of the Amazon were suggested to fit between 25 and 30 °C. In fact, the initial measurements by Hochachka and Somero (1968) showed that lungfish LDH has optimal K_m values between 30 and 35 °C. Apparent K_m for pyruvate from muscle LDH of fish species acclimated to extreme thermal regimes showed that optimal K_m values were found to occur in the range of environmental temperatures (reviewed by Val and Almeida-Val, 1995).

In summary, there are at least five different processes by which fish can compensate for temperature changes: (i) changes in substrate concentrations and products in a single pathway; (ii) changes in modulator concentrations affecting the enzymatic reactions; (iii) changes in the enzyme

conformations that affects its substrate affinity (K_m) and its velocity (V_{max}); (iv) quantitative changes in the enzyme synthesis by gene regulation; and (v) qualitative changes in isoforms (isozymes). Thus, adaptation may be achieved in different metabolic steps, from transcriptional to post-transcriptional phases. In general, all these mechanisms strongly influence thermal acclimatization or acclimation. Long-term temperature changes are not observed in the current climate of the Amazon region and temperature effectiveness in inducing metabolic changes is probably reduced. Pronounced temperature drops may occur annually, but tend to last no longer than 3 days. Short-term oscillations are mainly compensated for by behavioral responses, e.g., avoiding daily oscillations by migrating to different microhabitats. Long-term climatic changes, however, provide enough time for phenotypic adjustments via transcriptional adjustments, i.e., gene regulation, which result in changes in quantities or types of enzymes (or isozymes). The enzyme properties currently observed in nature are thus the result of genetic modifications over the course of evolution, i.e., in the course of many generations through geological time. These changes include structural changes in enzymes and, consequently, changes in physical and chemical catalytic properties. Current thermal stability of many enzymes should then result from adjustments developed through their past experiences regarding environmental changes and, particularly, thermal regimes.

1. LDH THERMAL SENSIBILITY FOLLOWS THE PHYLOGENY OF MAJOR GROUPS OF AMAZON FISHES

Most enzymes can be characterized by their differential properties of thermal stability. Different isozymes show different thermal stabilities, which may be used to characterize their origins. For more than three decades, these thermal properties were used as powerful tools in identifying gene orthology, i.e., homology between two genes generated by speciation (Wilson *et al.*, 1964; Goldberg and Wuntch, 1967; Hauss, 1975; D'Ávila-Limeira, 1989). Thermal stability properties differ for isozymes encoded at MDH genes, LDH genes, PGI genes, for example. An interesting picture has emerged from the studies by D'Ávila-Limeira (1989) while searching for properties of homology among LDH orthologs from fish of the Amazon. The differences obtained for thermal stability of LDH-B₄ isozyme from 27 different fish species indicate a phylogenetic relationship between maximal thermal stability and species/orders phylogeny. LDH-B₄ thermal stability was found to be lower in non-specialized groups compared to highly specialized groups, such as the advanced teleosts. Further studies have completed this picture, and we have found that among these two extremes, some orders, such as the Curimatidae, that have originated prior to the Andes uplift and have

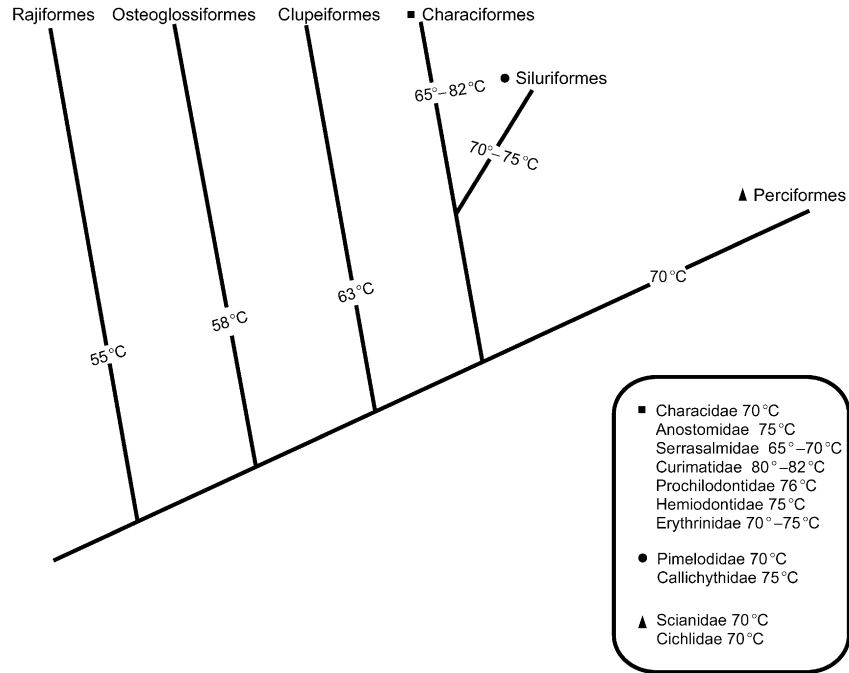


Fig. 10.8 Simplified phylogenetic diagram of the main groups of fish, modified from [Val and Almeida-Val \(1995\)](#), showing the relationship between the thermal stability of LDH-B* orthologs and the phylogenetic positions of specific fish orders. The box details the amplitude of the range of values obtained for the thermal stability of LDH-B* orthologs for each studied family. These results can be related to thermal history of each group (see text for a detailed explanation).

probably experienced a variety of thermal regimes, show exceptionally high thermal stability temperatures ([Figure 10.8](#); [Val and Almeida-Val, 1995](#)).

The main role of enzymes is to decrease the activation energy of a chemical reaction, enabling it to occur at moderate temperatures. The flexibility of the enzyme structure probably occurs during binding events; this modification is known as “induced fit” ([Koshland, 1973](#)) or “hand to glove” model ([Hochachka and Somero, 1984](#)). Changes in enzyme conformation will be accompanied by changes in the energy inputs or outputs that are associated with conformational changes and are probably responsible for changes in the activation energy. [Wilson *et al.* \(1964\)](#) investigated the thermal stability of LDH-B₄ isozymes in 55 species of vertebrates (from lower to higher taxa, including mammals) and suggested that the increases in heat stability of this isozyme are related to the phylogenetic status of the species. Thus, mammals will be found in one branch of the phylogenetic tree and reptiles and birds on the other branch, the latter having LDHs with 20 °C

difference in resistance compared to fish group. Thermal history is thus the most probable pressure determining thermal stability in protein structure, mainly in enzymes that play important roles in metabolism. Endotherms such as mammals and birds, which maintain their body temperature at 37 °C and 39 °C, respectively, may then present proteins with higher heat stability. According to [Hochachka and Somero \(1984\)](#), adaptations to higher temperature regimes induce increases in structural stability of proteins. One of the consequences of such increase is a higher structural stability, which increases the number of weak bonds and reduces catalytic efficiency. There is, however, a loss of catalytic efficiency as protein stability increases. However, for fish of the Amazon acclimated to different thermal ranges, we have found that as acclimation temperature increases, LDH thermal resistance decreases, indicating a compensation for obtaining more efficient catalytic properties ([Val and Almeida-Val, 1995](#)). It is clear that the acclimation process affects thermal resistance and that LDH-B₄ orthologs lose their thermal stability at higher temperatures and tend to behave as a thermal independent isozyme.

As stated above, for fish of the Amazon, life at higher temperatures may have imposed compensatory adjustments in their proteins. The catalytic efficiency of many enzymes may be investigated more effectively through their functional properties rather than through their structural properties. Such considerations lead us back to kinetic properties such as K_m , which can be thermally modulated and reflect thermal regimes of species. For Amazonian fishes, optimal K_m for enzymes should lie within the range of 25–30 °C. The comparison of lungfish and tuna muscle LDH suggests the importance of acclimation temperature for metabolic functions. In fact, it is important to keep K_m values close to the substrate concentrations in the tissues; otherwise, the enzyme will not be able to work properly or will require higher activation energy to reach its optimum rates.

2. WHY DOES TAMBAQUI LDH NOT RESPOND TO TEMPERATURE OSCILLATIONS?

Intracellular pH varies according to temperature changes ([Reeves, 1977; Somero, 1981](#)). The combination of pH and temperature has a significant effect on the post-transcriptional regulation of enzyme activity levels in all organisms. According to [Hochachka and Lewis \(1971\)](#), LDH temperature modulation in trout liver may be severely affected in alkaline pHs. Changes in intracellular pH can be harmful to the organism, and metabolic regulation decreases the effects of temperature on physiological pH changes and therefore decreases the effects of temperature on enzyme K_m .

The LDH of Amazonian fishes is similarly affected by temperature and pH, despite the small oscillations in environmental temperatures in the

Amazon basin (Val and Almeida-Val, 1995). Comparative studies of temperature and pH effects on LDH in tambaqui and pacu, *Mylossoma duriventris*, showed that the heart muscle LDH is differently affected by temperature (Almeida-Val *et al.*, 1991) and is minimized when pH is close to the physiological norm. The heart of tambaqui shows no variation in K_m values and substrate saturation values under different assay temperatures when pH is 7.5. Keeping K_m values constant at physiological pH is almost mandatory in this species, since the combination of high temperatures and low oxygen availability requires it to increase anaerobic glycolysis and go to the water surface, which is richer in oxygen but retains higher temperatures. Very low Q_{10} values are evident in heart LDH of tambaqui at different temperature ranges, varying from 0.9 to 1.5, compared to 1.0–2.7 in *Mylossoma duriventris* LDH (Val and Almeida-Val, 1995). Similar results were found after thermal acclimation by tambaqui (Farias, 1992). However, temperate fish species living in different thermal regimes keep their LDH K_m values for pyruvate practically constant when facing a large temperature range (eurythermic), while species facing a narrow temperature range (stenothermic) exhibit compensatory responses to thermal acclimation (Coppes and Somero, 1990). This thermal independence of LDH in tambaqui reflects a reduced change in metabolism and, consequently, a compensatory response to short-term daily oscillations in water surface temperatures. These preliminary results therefore warrant further investigation.

C. Control of LDH and Other Genes Under Hypoxia – Fine Adjustments

As indicated earlier, the ability 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 better 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, Val *et al.*, 1999). As far as morphology and anatomy are concerned, changes in the structure of DNA and proteins may be tolerated without phenotypic effects, i.e., they may 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), or the result of homeostatic reactions (gene regulation via negative feedback at the level of transcription and translation, physiological homeostasis, pH-buffering). Wilson (1976) called attention to the importance of gene regulation events during evolution of plants and animals. This author stated that

“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 environmental changes and the investigations about the kind of genetic (or metabolic) control over phenotypes under different environmental conditions have revealed that some genes are turned on or off accordingly (Walker, 1979; Smith, 1990; De Jong, 1995; Land and Hochachka, 1995; Hochachka, 1996; Walker, 1997; Hochachka *et al.*, 1998). As we have seen in the previous sections, long-term adaptational responses to the low-oxygen environments involved oxidative metabolic suppression in fish of the Amazon, as first suggested by Hochachka and Randall (1978) and corroborated by Driedzic and Almeida-Val (1996) and West *et al.* (1999). However, the immediate hypoxia responses from fish of the Amazon have been poorly studied from the evolutionary point of view (reviewed by Almeida-Val, Val *et al.*, 1999).

Oxygen sensing and its physiological and biochemical consequences in cells are not fully understood yet, despite the fact that some mechanisms have been extensively studied in isolated cell models, e.g., rat cardiac myocytes (Webster *et al.*, 1994), rat liver hepatocytes (Keitzmann *et al.*, 1992, 1993), or aquatic turtle hepatocytes (Land and Hochachka, 1995). The best studied system is the type I cell of the carotid body of mammals. All these studies suggest that some DNA sites are suppressed and some are activated when cells are exposed to hypoxia. Hochachka (1996) summarized these data and suggested that up- or down-regulation of certain genes or group of genes is dependent on the intensity of hypoxia constraint and the ability of the model to tolerate this constraint. According to his review, a series of messengers (first and second) will be activated by an oxygen-sensing mechanism that will affect several hundred 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 to aerobic pathways. Thus, up-regulation of glycolytic rates is considered to be certain in most hypoxia-responsive tissues. In the last decade, studies on mammalian cells have described 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; Wang *et al.*, 1995; Ebert *et al.*, 1996; Jiang *et al.*, 1996). These studies were summarized by Hochachka *et al.*

(1998) and showed that most glycolytic enzymes are induced, in a second round of gene expression, by HIF 1. The activation of PFK, PGK (phosphoglycerate kinase), and LDH-A is induced by HIF 1, which in turn is synthesized after a signal transduction pathway is activated by oxygen-sensing mechanisms. In a recent review, Nikinmaa (2002) summarized the most important developments in the field of hypoxia adaptation in fish; more than 120 genes are hypoxia-regulated (Gracey *et al.*, 2001), and up to 40 genes are known to be induced by hypoxia in mammals (Semenza, 1999). A comparison between studies conducted in fish and mammals shows that at least some genes are commonly up-regulated after hypoxia exposure, e.g. glycolytic enzymes such as LDH, enolase, and triosephosphate isomerase. Only recently, the putative O₂ sensor molecule was identified in mammals as a protein belonging to the family of the prolylhydroxylases, which catalyze the hydroxylation of proline 564 of HIF1 α , promoting its stabilization in hypoxic cells (Bruick and McKnight, 2001; Yu *et al.*, 2001). Another protein, asparaginyl hydroxylase, may also be involved in HIF1 α stabilization and oxygen sensing (Lando *et al.*, 2002).

Studies conducted with cDNA microarrays in the mudsucker *Gillichthys mirabilis* by Gracey and co-workers (2001) showed that surviving hypoxia involves three molecular strategies: (i) down-regulating genes for protein synthesis and locomotion to reduce energy consumption; (ii) up-regulating genes for anaerobic ATP production and gluconeogenesis, and (iii) suppressing cell growth and channeling energy to essential metabolic processes. Observed gene expression was tissue-specific and reflected different metabolic roles when the animals experienced hypoxia. Reviewing the evolution of the coordinated regulation of glycolytic enzyme genes by hypoxia, Webster (2003) suggested that the regulation of these genes by hypoxia in insects, fish, reptiles, birds and mammals and possibly all mobile multicellular species is multifactorial, with clear origins in the prokaryotic and fungal regulatory systems. These genes are coordinately and individually regulated by a variety of hypoxia-responsive transcription factors including HIF-1 α . HIF-1 α is probably the main component and is largely responsible for coordinating the induction. HIF-1 α binding sites have been reported in at least eight glycolytic enzyme genes, including PFK, aldolase, PK, PGK, enolase, LDH, HK, and glyceraldehyde phosphate dehydrogenase (GAPDH). Webster (2003) also mentioned that HIF-1 pathway has been described in fish, and it is possible that this pathway developed in the Silurian period about 500 MYA, when highly mobile sea and land species were evolving. In fact, this evolutionary period coincides with high DNA duplication rates due to polyploidy events, which induced the radiation of vertebrate groups and the appearance of new duplicated genes, which gave rise to most gene families currently known to exist.

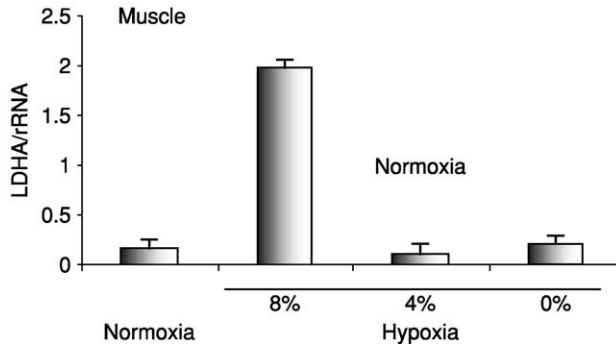


Fig. 10.9 Diagram showing the ability of juvenile *Astronotus* to regulate LDH synthesis in skeletal muscle to differentially respond to hypoxia and anoxia. Experiments were performed over 8 hours ($n = 5$). Results are obtained after RT-PCR, followed by northern-blotting detection. The ability to depress metabolism and, consequently, suppress LDH-A expression is related to the time fish is allowed to acclimate to the new condition. (Redrawn from [Oliveira et al., 2002](#).)

Recent experiments conducted in our laboratory have revealed that, at least for the gene LDH-A*, hypoxia may induce or suppress its expression, according to animal size, and time and intensity of exposure to oxygen depletion. Juveniles *Astronotus* show differential responses, which are related to their ability to tolerate hypoxia and anoxia. Furthermore, the ability to depress metabolism and, consequently, suppress LDH-A expression is related to the time a fish is allowed to acclimate to the new hypoxic condition ([Figure 10.9](#)).

In summary, the diversity of strategies adopted by fish during their evolution to cope with hypoxic environments has added to species diversity, making this subject one of the most interesting in the field of comparative physiology. In addition, our knowledge about the mechanisms involved in metabolic adaptations to tolerate hypoxia in fish is far from being complete; as more sophisticated tools become available, the more we realize the size of our knowledge gap regarding adaptations by fish to hypoxia.

IX. WHAT THE FUTURE HOLDS

Two major concerns are worth mentioning in this section. The first is the occurrence of global warming caused by the effects of increased CO₂ release in the atmosphere that is a result of fossil fuel burning and deforestation. The second is the speed with which hypoxia is spreading globally throughout water bodies as a result of pollution. Both problems are related to the main issues discussed in this chapter: temperature and oxygen availability. According to [Hochachka and Somero \(2002\)](#), global warming will certainly

affect the strong role of temperature in governing the distribution patterns of organisms in the planet. Thus, understanding the effects of temperature changes in metabolism assumes a practical and economical significance. Some of the questions that may be important to investigate are: (i) what is the minimum amount of change in temperature that will perturb a system enough to induce adaptive responses in a population or species?; (ii) what are the tolerance limits of extant species?; and (iii) at what temperature does a system (whole body or specific metabolic pathway) show optimum performance? These questions remain unanswered for most vertebrate groups, including fishes. Amazonian fishes are already subject to changes in their environment, not only in terms of temperature *per se*, but also in terms of the effects that global warming has on the annual rhythms of flood pulses (e.g., effects of El Niño and of La Niña), which are strongly related to all biological rhythms and processes of organisms living in the Amazon.

On the other hand, hypoxia is a natural phenomenon in the Amazon basin and has imposed so many pressures throughout evolution that most fish groups have become dependent on surface waters, either during their entire life span or during the first part of it. However, healthy environments have been transformed into poorly oxygenated waters due to excessive anthropogenic input of nutrients and organic material, particularly in poorly circulated waters, and this will soon become a problem in the Amazon region. Hypoxia and anoxia currently affect thousands of square kilometers of marine and freshwater environments of North and South America, Africa, Europe, India, Southeast Asia, Australia, Japan, and China.

Although it is a typically hypoxic environment, the Amazon basin has its own rhythms, and any changes in oxygen content of the system will disturb the balance between fish and their environment, just as in any other ecosystem. In addition, some chemical pollutants associated with our modern way of life, including polymers, metals, petroleum, aquaculture nutrients, pesticides, and herbicides, will continuously present new metabolic challenges to fishes of the Amazon. In the past few decades, increases in nutrient levels have become evident in coastal and inland waters, and such increases are caused primarily by intensive farming, application of fertilizers, deforestation, and discharge of domestic wastewaters coincident with human population growth. Thus, hypoxia caused by eutrophication and organic pollution is now considered to be among the most pressing water pollution problems in the world (Wu, 2002).

With regard to promising progress in fish metabolic adaptation, future work will include molecular approaches to investigate gene regulation of proteins involved in adapting to environmental change. Vis-à-vis the above-mentioned review of glycolytic gene regulation (Webster, 2003), it is worth mentioning that HIF-1 α pathway evolved concurrently with most of the

genes it regulates. The same recognition sequence is essential (although not sufficient) for gene activation by HIF-1, and it is known that this same sequence is required for hypothermia, dehydration, and the UV response of *Arabidopsis* genes involved in carbohydrate metabolism. According to Webster (2003), it is possible that this recognition sequence and the protein that binds it are related in plants and animals, and this may provide the link between gene regulation in hypoxic root tips and the HIF-1 pathway of insects, fish, birds, and mammals. At this point, we can still recommend studies on LDH gene family, which continues to be one of the best studied enzyme systems and one of the most responsive enzymes to several environmental challenges. Changes in protein sequence and expression of LDH-B in *Fundulus heteroclitus*, which are correlated with populations adapted to different thermal regimes, show that there are many ways to approach the role of evolution and natural selection on fish metabolic adaptation: quantitative genetics, comparative methods, and molecular population genetics (Schulte, 2001).

In fact, the question is the same as that posed by Darwin and rephrased by Peter Hochachka in the 1960s: "How have living systems, which are based on a common set of biochemical structures and processes and subject to a common set of physical-chemical laws, been able to adapt to the enormously wide spectrum of environmental conditions found in the biosphere?" Some answers to this question are found in the directions for research on biochemical adaptation registered in the literature produced by Peter Hochachka and his co-workers. In particular, his earlier research insights about Amazon fish adaptations was already very clear in his mind when he wrote the book *Living without Oxygen* in 1980. In his preface to this book he states: "the seed for this book was first planted in my mind during the 1976 expedition to the Amazon, where oxygen is one of the most vicarious of wild environmental parameters faced by aquatic organisms." One of the main legacies Peter Hochachka leaves is his suggestion to follow and pursue the elucidation of mechanisms and their adaptive significance, and to understand the evolution of such mechanisms and adaptations. According to Mangum and Hochachka (1998), what we have missed in early attempts to analyze the evolution of physiological systems was hard evidence, including ways how to test, that the physiological adaptations arose from and were maintained by natural selection, thereby enhancing species fitness. Currently, the most penetrating studies in evolutionary physiology and biochemistry appear to be those that successfully integrate mechanistic/adaptational knowledge with larger evolutionary issues, often using molecular biology techniques to clarify process and phylogeny. Thus, it is important to keep in mind that while the tools do matter, what matters most is the problem to be addressed and the integrative approach to solve it, without

neglecting the local issues regarding the evolutionary costs of metabolic and genetic adaptations when facing new environmental challenges.

In conclusion, we suggest that future approaches should take all the above advice into account, but should also consider both the social and economic consequences of human activities before planning the direction of new investigations regarding metabolic adaptations of fish.

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