

EVOLUTIONARY FEATURES OF HYPOXIA TOLERANCE
IN FISH OF THE AMAZON:
FROM MOLECULAR TO BEHAVIORAL ASPECTS

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Abstract

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

Introduction

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

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

Revisiting hypoxia adaptation in fish of the Amazon

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

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

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

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

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

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

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

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

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

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

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

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

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

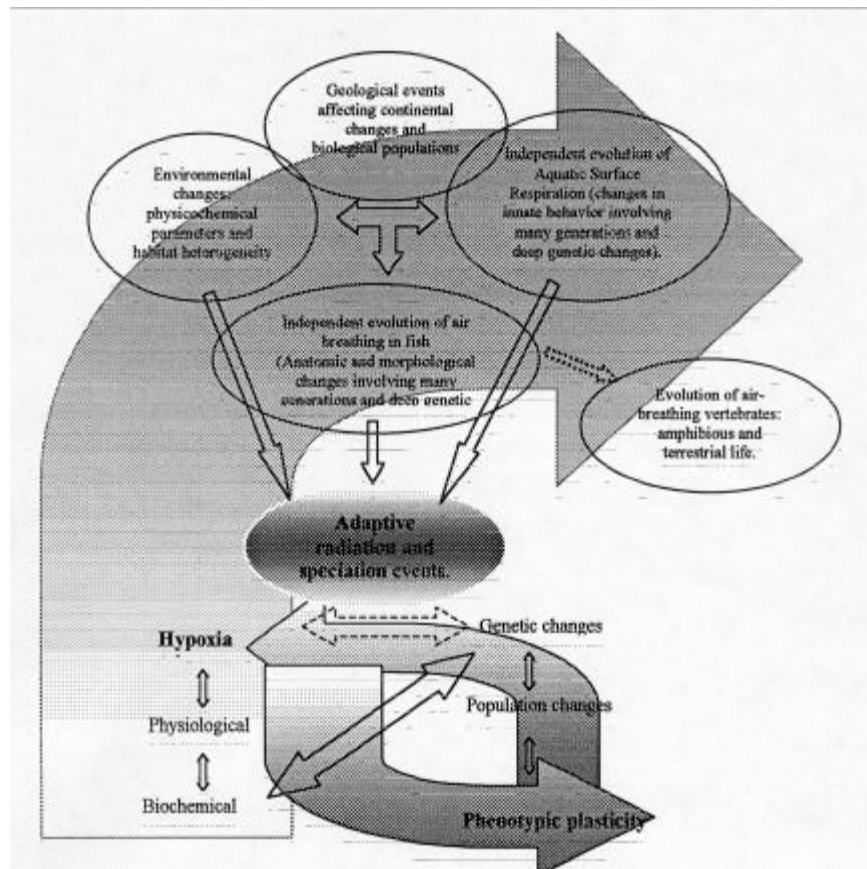


Figure 1. Possible relationships between physico-chemical and biological parameters in the origin of adaptive responses fish developed to survive low oxygen levels and its effects on the diversification and adaptive radiation. The bottom of the figure describes the different ways such adaptive radiation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

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

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

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

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

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

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

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

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

- a) Convergence of LDH-B to LDH-A function, as occurs in turtles and hagfish, both subunits presenting A-type like kinetics. This is typical of good anaerobes (hypoxia tolerant vertebrates);
- b) Widespread restriction of LDH B* gene expression, leaving the tissues only with A-type isozymes; a different way to reach the same result as above. It occurs in advanced teleosts, which live in oxygen limiting situations;
- c) Non-divergence between A and B subunits. Some fish present this pattern and their metabolism is predominantly aerobic. This trend is supposed to occur in fish that present aquatic surface respiration; and
- d) LDH C* widespread distribution in non specialized teleosts as Osteoglossomorpha, Elopomorpha, and Clupeomorpha groups, followed by its possible gene silencing in some fish groups (absence of LDH C isozyme in all Ostariophysan groups), and a strong restriction typically occurring in advanced teleosts (Acanthopterygian groups) (Almeida-Val and Val, 1993).

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

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

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

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