

Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia

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Summary

Hypoxia survival in fish requires a well-coordinated response to either secure more O₂ from the hypoxic environment or to limit the metabolic consequences of an O₂ restriction at the mitochondria. Although there is a considerable amount of information available on the physiological, behavioral, biochemical and molecular responses of fish to hypoxia, very little research has attempted to determine the adaptive value of these responses. This article will review current attempts to use the phylogenetically corrected comparative method to define physiological and behavioral adaptations to hypoxia in intertidal fish and further identify putatively adaptive biochemical traits that should be investigated in the future. In a group of marine fishes known as sculpins, from the family Cottidae, variation in hypoxia tolerance, measured as a critical O₂ tension (P_{crit}), is primarily explained by variation in mass-specific gill surface area, red blood cell hemoglobin–O₂ binding affinity, and to a lesser extent variation in routine O₂ consumption rate (\dot{M}_{O_2}). The most hypoxia-tolerant sculpins consistently show aquatic surface respiration (ASR) and aerial emergence behavior during hypoxia exposure, but no phylogenetically independent relationship has been found between the thresholds for initiating these behaviors and P_{crit} . At O₂ levels below P_{crit} , hypoxia survival requires a rapid reorganization of cellular metabolism to suppress ATP consumption to match the limited capacity for O₂-independent ATP production. Thus, it is reasonable to speculate that the degree of metabolic rate suppression and the quantity of stored fermentable fuel is strongly selected for in hypoxia-tolerant fishes; however, these assertions have not been tested in a phylogenetic comparative model.

Key words: metabolic rate suppression, critical oxygen tensions, sculpins.

Introduction

Environmental hypoxia is a common, naturally occurring phenomenon in many aquatic ecosystems, the prevalence of which is increasing as a result of eutrophication (Diaz and Breitburg, 2009). Aquatic hypoxia occurs when the rate of oxygen consumption (\dot{M}_{O_2}) by organisms (e.g. bacteria, animals and plants during their dark cycle) exceeds O₂ production rates or the capacity of O₂ to diffuse in from the air (Graham, 1990). Naturally occurring hypoxia is therefore prevalent in stagnant, stratified or poorly mixed waters where it can occur for prolonged periods, such as those seen in oceanic O₂ minimum zones (OMZ) (see Seibel, 2011) or in waters with a high biomass, where O₂ levels tend to fluctuate on a daily basis. Eutrophication enhances bacterial and algal growth, exacerbating O₂ fluctuations. In light of the natural fluctuations of O₂ in the aquatic environment, it is perhaps not surprising that within the vertebrates, fish have the largest number of hypoxia-tolerant species. Hypoxia has clearly been a potent evolutionary force driving the natural selection of many unique adaptive traits.

There is a large body of literature describing the responses of fish to hypoxia (e.g. Nikinmaa and Rees, 2005; Nilsson, 1990; Richards et al., 2009). In general, fish respond to hypoxia with a suite of physiological, behavioral, biochemical and molecular responses that either serve to enhance O₂ uptake from the O₂-depleted environment or limit the potentially devastating consequences of an O₂ limitation at the tissue. Fish enhance O₂ uptake by altering, among other things, respiration patterns (Gilmour and Perry, 2007), behavior (Sloman et al., 2008; Sloman et al., 2006), gill structure (Sollid et al., 2003; Sollid et al., 2005) and hemoglobin (Hb)–O₂ binding affinities (Wells, 2009). When responses aimed at maximizing O₂ uptake fail, other physiological and biochemical responses occur that serve to

alter the pathways of metabolic energy production and/or limit metabolic energy use (Martinez et al., 2006; Richards, 2009; Richards et al., 2008). Although there is a general picture of how fish respond to hypoxia to enhance survival, few studies have attempted to elucidate the mechanisms of hypoxia tolerance from an evolutionary perspective by identifying the adaptive traits that define and differentiate hypoxia tolerance in fish species.

The goal of this article is to outline recent advances in identification of adaptive traits involved in hypoxia tolerance in fish. To accomplish this task, I will use and refine models developed by others (Connett et al., 1990; Pörtner and Grieshaber, 1993; Richards, 2009) and use modern comparative analysis to explain the variation in hypoxia tolerance seen among fish species. The modern comparative approach aims to identify natural-selection-driven changes in mechanistic traits in species of known lineages that inhabit different environments. With the emergence and popularization of phylogenetically independent contrasts (Felsenstein, 1985; Garland et al., 1992), comparative physiologists and biochemists now have the tools necessary to isolate natural-selection-based differences in a character of interest from simple inheritance based relationships (Mangum and Hochachka, 1998). In order to study environmental adaptation to hypoxia using the comparative approach, it is essential to identify a group of closely related organisms that inhabit an environmental gradient that varies in the prevalence or duration of hypoxia. The marine intertidal zone provides just such an environment.

Using the nearshore marine environment to understand the evolution of hypoxia tolerance

The nearshore marine environment is heavily influenced by the daily or, more usually, twice daily ebb and flow of tides, which

varies depending on lunar cycle and time of year. Within the intertidal zone, many different habitats exist including tidepools, sandy beaches, barren rock and mudflats. Tidepools, also known as rock pools, are pools of seawater that are left behind by tidal ebb and occur at different levels within the intertidal zone, typically referred to as low, middle and high on the basis of the vertical height at low tide. The low intertidal zone is located next to the subtidal area and is isolated from the ocean for only short periods of time during each tidal cycle. The high intertidal zone, by contrast, is isolated from the bulk ocean for much longer (many hours to sometimes days) and is only submerged by the highest tides. The mid-intertidal zone lies between these two extremes and is often exposed for hours. The vertical distance between a high and low tidepool can, in some cases, be only a meter, but because of the influence of the tide on these pools, the intertidal zone is characterized by steep spatial and temporal gradients in O_2 , temperature, salinity and pH (Truchot and Duhamel-Jouve, 1980). Tidepools located high in the intertidal zone can experience enormous fluctuations in O_2 , temperature and pH (Fig. 1), with O_2 falling to near zero at night. Only 6–12 h later however, if still exposed or newly emerged, these same pools can reach O_2 tensions up to 300–400% air saturation during daylight as a result of high algal content and photosynthesis. Lower tidepools experience, typically, much less variation in these environmental parameters. In the subtidal environment immediately below the shore, there can also be fluctuations in O_2 and temperature because of the reduced mixing associated with being near shore, but the variation observed in this environment is typically far less than observed elsewhere in the intertidal zone. Species located far from the shore or in mid-

depth waters are less likely to experience hypoxia on a regular basis; however, deep dwelling organisms such as those found in O_2 minimum zones can experience hypoxia for prolonged periods and can show extreme levels of hypoxia tolerance (Childress and Seibel, 1998; Diaz and Breitburg, 2009).

The gradient of environmental variation seen along the marine nearshore is well known to strongly influence species distribution (Doty, 1946; Stillman and Somero, 1996), and some physiological and biochemical correlates with environmental variation and species distribution have been examined. Organisms that live high in the intertidal zone must be able to withstand large variation in environmental condition, whereas those living lower in the intertidal zone or subtidally experience much less environmental variation and therefore do not necessarily require the capacity to survive harsh conditions. Of all the environmental variables that are known to fluctuate in the intertidal zone, temperature, and the role it plays in species distribution, has been the most studied. In particular, the vertical zonation patterns of porcelain crabs (genus *Petrolisthes*) along the nearshore has been shown to be positively associated with upper thermal tolerance limits, with the most warm-temperature-tolerant crabs being located higher in the intertidal zone, where water temperatures can be much higher than lower in the intertidal zone (Stillman and Somero, 2000). Stillman and Somero further demonstrated through the application of phylogenetically independent contrasts that the upper thermal tolerance limits of individual species evolved in response to maximal microhabitat temperatures (Stillman and Somero, 2000). Few studies have used the marine nearshore environment to understand adaptation to other environmental variables.

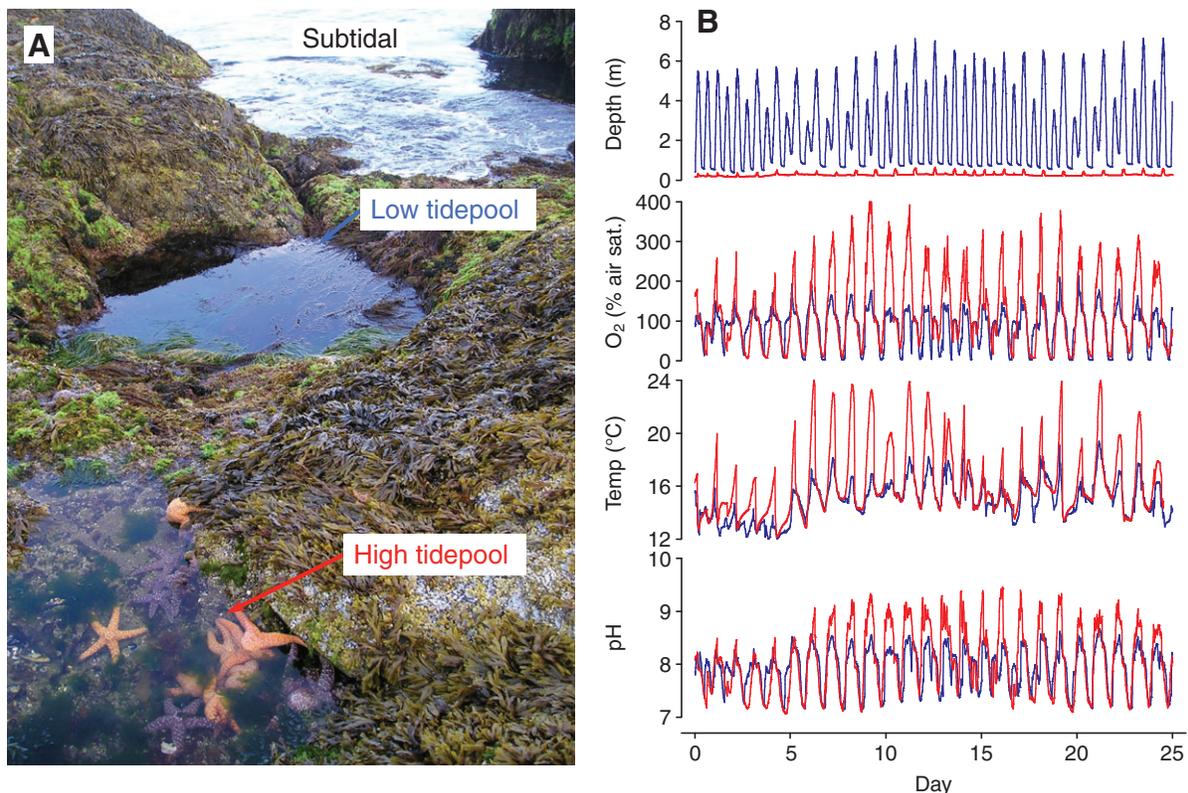


Fig. 1. (A) Marine intertidal zone, showing subtidal region, low and high tidepools at Wizard Island near the Bamfield Marine Sciences Centre, Bamfield, British Columbia, Canada. (B) The environment within tidepools over 25 days. Pools located high in the intertidal zone (red lines) have a very variable environment, with large fluctuations in O_2 , temperature and pH. Lower tidepools (blue lines) are much less variable. Photo courtesy of Milica Mandic.

Fish of the family Cottidae (commonly known as sculpins) are a highly specious group of marine fish that inhabit a wide variety of environments including the nearshore, where there is a distinct vertical zonation pattern, with different species of sculpin inhabiting different portions of the nearshore environment (Eschmeyer and Herald, 1983; Froese and Pauly, 2007). Within marine sculpins a phylogenetically independent correlation has been demonstrated between hypoxia tolerance, quantified as the critical O₂ tension (P_{crit}), and species' depth ranges as indicated by maximum recorded depth (a proxy for habitat breadth in the near shore; Fig. 2) (Mandic et al., 2009b). It must be noted that the deeper-dwelling species used by Mandic et al. were all collected within 10m depth and typically from O₂ stable environments (Mandic et al., 2009b). Critical O₂ tension is the environmental partial pressure of oxygen (P_{O_2}) at which an organism's O₂ uptake rate transitions from being independent of environmental O₂ to being dependent on environmental O₂ (see Fig. 3). Hypoxia-sensitive species, those with high P_{crit} values, are confined to the more O₂-stable environments (deeper water and subtidal environments), whereas the more hypoxia-tolerant species with low P_{crit} values are located in the more variable intertidal zone. The large variation in P_{crit} seen among these species provides a tractable system in which to understand the mechanisms and evolution of hypoxia tolerance. In the remaining portion of this article, I will use the recent advances made with this comparative system to refine models describing hypoxia tolerance and attempt to identify physiological and behavioral features that account of inherent variation in hypoxia tolerance seen among sculpins.

Defining hypoxia and hypoxia tolerance

Simply put, hypoxia is a shortage of O₂. Simple definitions of this nature have long been applied to the aquatic environment and it is generally held that dissolved O₂ concentrations below 2–3 mg O₂ l⁻¹ in marine and estuarine environments and below 5–6 mg O₂ l⁻¹ in freshwater environments is hypoxic. Normal is considered 7–8 and 9–10 mg O₂ l⁻¹ for marine and freshwater environments, respectively, depending on temperature (Diaz and Breitburg, 2009). These simple and somewhat arbitrary thresholds are attractive to government regulators because they represent an easily quantified value and, for the most part, protect the most sensitive fish species in the environment. However, these thresholds do not take into account natural variation in hypoxia tolerance among fish species. Indeed, Vaquer-Sunyer and Duarte showed that the environmental thresholds for sublethal responses to hypoxia ranged from 2 to 10 mg O₂ l⁻¹ among numerous fish species (Vaquer-Sunyer and Duarte, 2008), and few studies have attempted to understand the mechanistic basis for this variation. As a result, using a single environmental O₂ concentration or partial pressure to define what is hypoxic is a gross oversimplification and provides no context for comparative analysis.

Defining hypoxia from an organismal perspective is far from simple. The complexity of the definition emerges from two interrelated factors. First, an O₂ limitation at one level of biological organization may not represent an O₂ limitation at another level because of physiological and biochemical compensations. For example, a relatively minor reduction in inspired P_{O_2} may elicit changes in ventilation frequency and amplitude, clearly illustrating that the animal is detecting hypoxia, but compensating to prevent changes in arterial or tissue P_{O_2} . Thus the level of biological

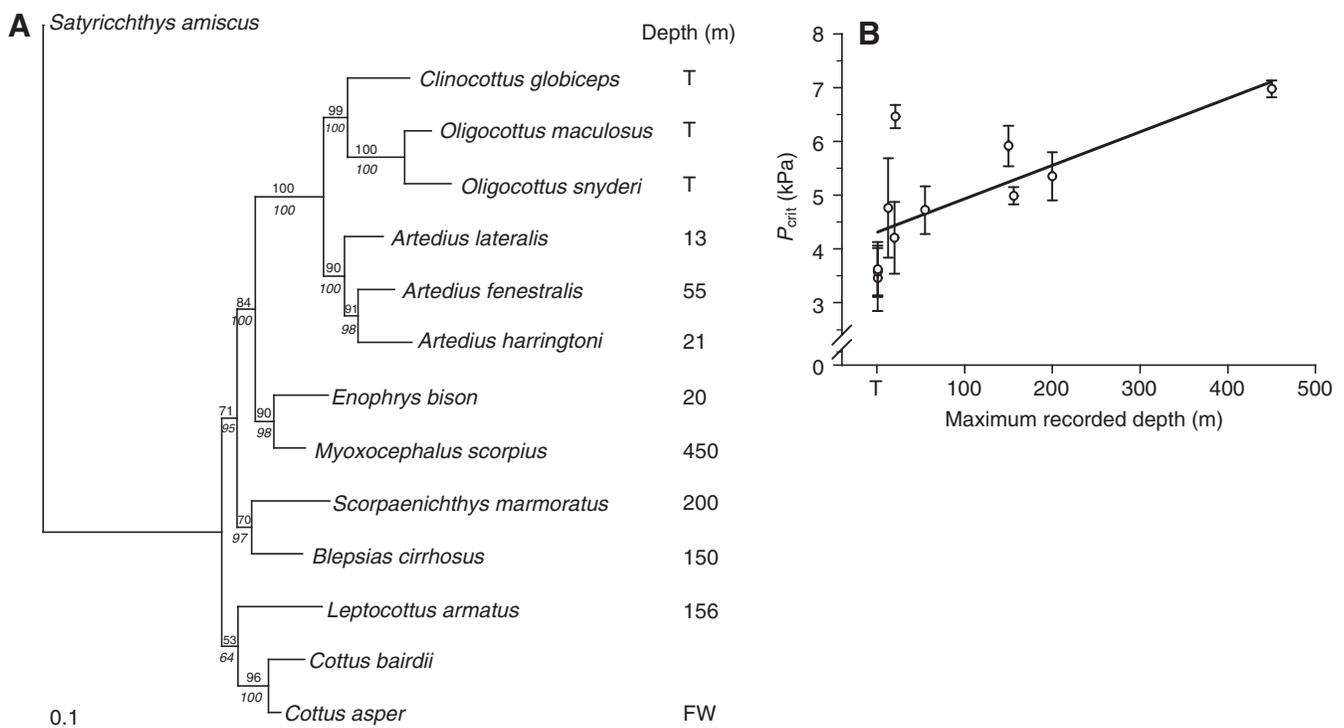


Fig. 2. (A) Phylogenetic relationship of 13 species of marine sculpin from the family Cottidae, based on a maximum likelihood tree using cytochrome *b* sequences. Numbers above lines show the percentage of 100 maximum-likelihood bootstrap replicates and italicized numbers below lines show posterior probability from Bayesian analysis. Maximum-recorded depths (in meters) are shown to right of the phylogenetic tree, with T indicating tidepool and FW indicating freshwater. Taken from Mandic et al. (Mandic et al., 2009b). (B) Conventional correlation ($r^2=0.52$, $P<0.01$) between critical O₂ tension (P_{crit}) and maximum recorded depths for the species in the phylogenetic tree.

organization must be considered when defining hypoxia. Second, fish species are well known to differ in their hypoxia tolerance, thus what is hypoxic for one species may not elicit a hypoxic stress response in another species.

In order to be able to define O_2 limitation from an organismal perspective, Connett et al. suggested defining O_2 limitations at four separate yet interrelated levels of biological organization: one environmental and three physiological–biochemical, each defined by its own term (Connett et al., 1990). They proposed that the term hypoxia be used only to mean an inspired environmental P_{O_2} less than normal. This definition, as pointed out by the authors, relies somewhat tenuously on ascertaining what is ‘normal’, which from a comparative perspective is problematic. Connett et al. further described physiological and biochemical levels of hypoxia, including: (1) hypoxemia, which denotes an arterial P_{O_2} less than normal; (2) adapted cell hypoxia, which was described as an intracellular P_{O_2} less than in the absence of stress but sufficient to maintain O_2 and ATP turnover; and (3) dysoxia, which is a resurrected term defined as an O_2 limitation at the mitochondrial cytochrome oxidase leading to a limitation in cytochrome turnover and therefore reduced oxidative phosphorylation (Connett et al., 1990). Although these definitions are useful as a framework to illustrate that there are many sites of regulation in the O_2 cascade, these definitions do not provide a workable framework for comparative analysis, which is the focus of this article.

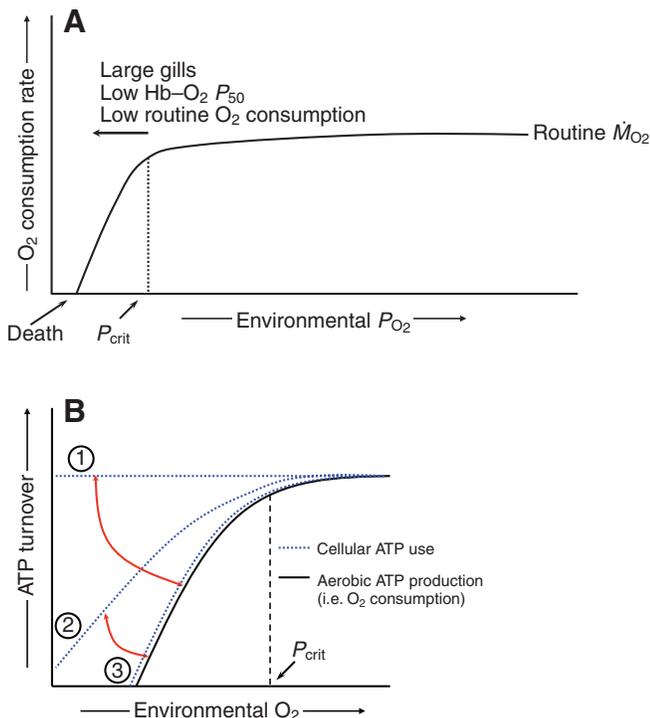


Fig. 3. Metabolic responses of fish to O_2 changes. (A) Critical O_2 tension (P_{crit}) is the point at which O_2 consumption rate transitions from being independent of environmental O_2 levels (often referred to as O_2 regulation) to being dependent on environmental O_2 (often referred to as O_2 conforming). Hypoxia-tolerant fish have low P_{crit} values, in part, as a result of a large gill surface area, high Hb– O_2 binding affinity, and a low routine O_2 consumption rate. (B) At O_2 levels below P_{crit} , survival is dependent upon the ability of an animal to suppress metabolic energy use to limit the extent of the activation of O_2 -independent pathways of ATP production. See text for more detail.

For the purpose of this article, environmental hypoxia will be defined as the water P_{O_2} (P_{wO_2}) when physiological function is first compromised and metabolic rate cannot be maintained (i.e. P_{crit}). At a P_{wO_2} below P_{crit} , the fundamental issue is the maintenance of metabolic energy balance and efficient use of the available O_2 . If environmental hypoxia leads to hypoxemia and dysoxia, then mitochondrial ATP production will be compromised and the fish has only a limited time before anaerobic substrates are exhausted and death occurs.

In order to provide a framework for comparative analysis, we must first elucidate the factors that define the point at which environmental hypoxia is first detected as a metabolic response. It has long been recognized [and described in detail (e.g. Pörtner and Grieshaber, 1993)] that different organisms have different patterns of routine \dot{M}_{O_2} when exposed to decreasing environmental P_{O_2} . Many fish species maintain a relatively constant routine \dot{M}_{O_2} over a wide range of environmental P_{O_2} values (termed O_2 regulation) until O_2 uptake is compromised, after which routine \dot{M}_{O_2} declines as ambient P_{O_2} declines (termed O_2 conforming). The transition point between the O_2 -independent (regulating) portion of respiration and the O_2 -dependent portion (conforming) of respiration is termed the P_{crit} . An alternate strategy observed in some fish species or individuals within a species, is an aerobic oxy-conforming strategy, where \dot{M}_{O_2} is highly dependent on environmental P_{O_2} across a broad range of O_2 tension starting at levels where changes in environmental P_{O_2} are unlikely to elicit hypoxemia. However, even these so-called aerobic O_2 -conforming species will still often display a transition in O_2 consumption rate that is indicative of environmental hypoxia being transduced to a deficiency in cellular O_2 and this can be quantified as a P_{crit} (although, experimentally, P_{crit} can be difficult to define in these species).

The ability of an animal to acquire O_2 from its environment has long been considered an important determinant of hypoxia tolerance (Hughes, 1973). Organisms that possess a greater capacity for O_2 extraction are able to maintain a routine \dot{M}_{O_2} at lower O_2 tensions and exploit more O_2 variable environments. Critical O_2 tensions are known to differ widely among species (Chapman et al., 2002) and there is general consensus that hypoxia-tolerant fish have lower P_{crit} values than more active hypoxia-sensitive fish (Chapman and McKenzie, 2009). Thus, modifications to the respiratory cascade that enhances O_2 uptake from the environment will lead to an increase in hypoxia tolerance and be a potential target of natural selection in organisms living in environments characterized by periods of hypoxia. Many physiological modifications can affect P_{crit} , including any alternation that increases O_2 extraction from the environment (e.g. gill surface area, ventilation rate and volume), O_2 transport in the body (e.g. hematocrit, Hb– O_2 binding affinity), as well as potential differences in metabolic demands. Of the many steps of the respiratory cascade that can be altered to enhance O_2 uptake during hypoxia, the greatest attention has been afforded to the O_2 -binding characteristics of haemoglobin. Interspecific variation in Hb– O_2 binding affinity (measured as P_{50}) can be attributed to differences in Hb multiplicity, Hb subunit and/or isoform heterogeneity, as well as variation in the intracellular concentrations of allosteric modulators (Brix et al., 1999; Perutz, 1983).

Although there is a general acceptance that an enhanced O_2 uptake is associated with hypoxia tolerance, few studies have attempted to understand the correlated evolution of traits involved in hypoxia tolerance in a phylogenetically rigorous fashion. Using marine sculpins, Mandic et al. used a phylogenetically corrected

multiple linear regression model to demonstrate that in normoxia-acclimated sculpins roughly 75% of the variation in P_{crit} could be explained by variation in mass-specific gill surface area, whole blood Hb-O₂ binding affinity and routine \dot{M}_{O_2} (Mandic et al., 2009b). Of these three variables, variation in mass-specific gill surface area and whole blood Hb-O₂ binding affinity were the two major contributors to variation in P_{crit} whereas variation in metabolic rate only explained a small, albeit significant portion of the variation in P_{crit} . The relatively minor contribution of metabolic rate to the variation in P_{crit} is consistent with the comparative analysis performed by Childress, who argued that low metabolic rates are not directly associated with low O₂ conditions (Childress, 1995). Overall, hypoxia-tolerant species, even when acclimated to fully aerated water have a low P_{crit} , in part because they have a large gill surface area and a high affinity Hb, allowing for greater O₂ uptake from the environment across the gill surface, and they have low metabolic demands as indicated by low routine \dot{M}_{O_2} . These traits, and the low inherent P_{crit} , are present in sculpins that routinely experience hypoxia. The study by Mandic et al. is the first to show a clear phylogenetically independent relationship between hypoxia tolerance and variation in gill surface area, high Hb-O₂ binding affinity and routine \dot{M}_{O_2} (Mandic et al., 2009b) (Fig. 4). Sculpins that are unable to tolerate hypoxia have a lower Hb-O₂ binding affinity, generally smaller gills and higher routine metabolic rates. Sculpins with higher Hb-O₂ binding affinity and large gills have an increased capacity to extract O₂ from the environment to supply a lower routine \dot{M}_{O_2} , thereby these species can tolerate more hypoxic conditions such as the ones that are routinely encountered in isolated tidepools at night.

Although the correlative analysis employed by Mandic et al. provides a generalized picture of the main factors that results in a low P_{crit} (Mandic et al., 2009b), not all hypoxia-tolerant species achieve a low P_{crit} in the same fashion. For example, the tidepool sculpin (*Oligocottus maculosus*; species 1 in Fig. 4) has remarkably small gills (Fig. 4B) relative to other hypoxia-tolerant species (cf. species 2, *Chaetopsylla globiceps* and species 3, *Oligocottus snyderi*; Fig. 4B), but the tidepool sculpin has the lowest of all Hb-O₂ P_{50} values (Fig. 4C), which probably compensates for the small gill to maintain O₂ extraction and yield a low P_{crit} . Thus, although the precise mechanisms for achieving a low P_{crit} in hypoxia-tolerant animals may differ, a low P_{crit} is still indicative of hypoxia tolerance, and enhanced O₂ extraction remains paramount for hypoxia tolerance.

Although the factors presented by Mandic et al. outline some of the important factors that are present in normoxia and help with hypoxia survival (Mandic et al., 2009b), this study did not assess the compensatory responses of these fish to hypoxia exposure. Fish are able to compensate for decreasing environmental O₂ by increasing gill ventilation (Graham, 1997), increasing gill perfusion (Russell et al., 2008; Smith et al., 2001), increasing gill surface area (Sollid et al., 2005; Sollid and Nilsson, 2006), altering cardiac output (Speers-Roesch et al., 2010), increasing the blood Hb concentration or Hb-O₂ binding affinity (Wells, 2009) or even potentially increasing tissue O₂ extraction. In sculpins, there is evidence to suggest that the more hypoxia-tolerant species have a greater capacity to adjust their Hb-O₂ binding affinity than the hypoxia-intolerant species, which may be advantageous for living in a highly O₂-variable environment. Allosteric modification of Hb can quickly affect Hb-O₂ binding affinity, and in fish, red blood [ATP], [GTP] and [H⁺] are the main allosteric modulators that affect Hb-O₂ binding affinity to bring about short-term improvements in O₂ uptake from the environment (Hochachka and

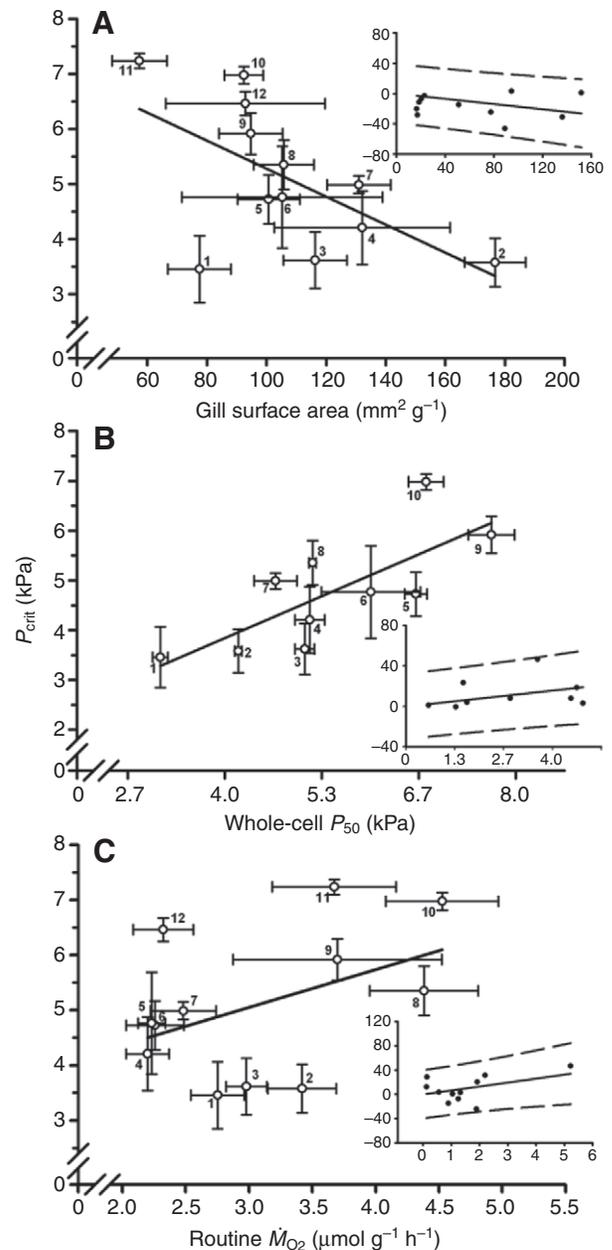


Fig. 4. Relationships between P_{crit} and mass-specific gill surface area (A), P_{crit} and whole blood Hb-O₂ P_{50} (B), and P_{crit} and routine oxygen consumption rate (\dot{M}_{O_2} ; C) in marine sculpins. Conventional correlations are shown in A ($r^2=0.35$, $P=0.04$), B ($r^2=0.57$, $P=0.01$) and C ($r^2=0.20$, $P=0.15$) and phylogenetically independent contrasts are shown in the inset in each panel. Correlation coefficients for the phylogenetically independent contrasts in A, B and C are $r^2=0.38$, $P=0.03$, $r^2=0.45$, $P=0.03$, and $r^2=0.35$, $P=0.04$, respectively. All data are means \pm s.e.m. in the main panels and 95% prediction intervals are shown in the insets. Numbers 1–12 represent different species: (1) *Oligocottus maculosus*, (2) *Chaetopsylla globiceps*, (3) *Oligocottus snyderi*, (4) *Enophrys bison*, (5) *Artedius fenestralis*, (6) *Artedius lateralis*, (7) *Leptocottus armatus*, (8) *Scorpaenichthys marmoratus*, (9) *Blepsias cirrhosus*, (10) *Myoxocephalus scorpius*, (11) *Cottus asper* and (12) *Artedius harringtoni*. Data modified from Mandic et al. (Mandic et al., 2009b).

Somero, 2002; Wells, 2009). Decreases in [ATP], [GTP] and [H⁺] results in an increase in Hb-O₂ binding affinity (Wells, 2009). Hypoxia-tolerant sculpins had higher [ATP] than the more

hypoxia-sensitive species (Mandic et al., 2009b). Higher levels of ATP coupled with high intrinsic Hb–O₂ binding affinity in hypoxia-tolerant sculpins result in a significant capacity to endure not only severe hypoxia, but also large fluctuations in O₂. This plasticity is essential for hypoxia-tolerant sculpins as they are typically found in tidepools with highly variable O₂ (anoxia to supersaturation levels within hours; Fig. 1). This finding is similar to what was observed in hypoxia-tolerant cichlids from Lake Victoria, which have higher [ATP] and [GTP] compared with the relatively hypoxia-intolerant salmonids (Rutjes et al., 2007). Overall, sculpins exhibit a remarkable breadth in physiological capacity to tolerate hypoxia exposure, which contributes to their ability to exploit a diverse array of ‘O₂ habitats’ within the nearshore environment.

Behavioral responses to hypoxia

In response to hypoxia, many fish species employ a suite of behavioral responses to facilitate O₂ uptake and enhance survival (Chapman and McKenzie, 2009). In intertidal fish, the two most common behavioral responses to hypoxia are aquatic surface respiration (ASR) and aerial emergence (Martin, 1995; Yoshiyama et al., 1995; Watters and Cech, 2003), both of which allow a fish to access more O₂ rich environments when the bulk water is hypoxic. ASR involves the selective skimming of the well-oxygenated water at the water–air interface whereas aerial emergence involves moving out of the water to respire in air. Because both of these behavioral responses facilitate O₂ uptake, there is a long-standing assumption in the literature that there must be a link between hypoxia tolerance, the frequency and threshold for displaying a behavioral response, and distribution, particularly in sculpins distributed along the nearshore environment (Yoshiyama et al., 1995; Martin, 1996; Watters and Cech, 2002; Sloman et al., 2008). Among 11 different species of sculpin examined by Mandic et al. (Mandic et al., 2009a) only nine species performed ASR in response to exposure to hypoxia, and among those that did, there was no relationship between the percentage of individuals of each species that perform ASR and P_{crit} . Of the nine species that showed ASR, at least periodically, the O₂ threshold for initiating ASR (P_{ASR}) was negatively correlated with hypoxia tolerance (Fig. 5A), with the most tolerant species engaging in ASR at higher O₂ tensions than the less hypoxia-tolerant species. This negative relationship between P_{ASR} and P_{crit} , however, was not independent of phylogeny, primarily because the most hypoxia-tolerant species were clustered on a single phylogenetic clade (*Globiceps*, *O. maculosus* and *O. snyderi*).

Aerial emergence is common in intertidal fishes and it has been postulated that hypoxia-tolerant fishes will utilize aerial emergence to a greater extent than hypoxia-sensitive fishes. Indeed, among five species of sculpin (*Ascelichtys rhodorus*, *O. maculosus*, *Jordania zonope*, *Chitonotus pugetensis* and *Icelinus borealis*) those inhabiting the intertidal zone were able to emerge and respire in air, whereas those located in the subtidal or deeper waters did not survive past a 2-h aerial exposure (Martin, 1996). It has also been proposed that differences in the threshold O₂ tension for initiating aerial emergence are related to differences in hypoxia tolerance among sculpins (Yoshiyama et al., 1995). In agreement with the work of Martin, Mandic et al. demonstrated that sculpins located in the intertidal zone consistently displayed aerial emergence behavior but species from the subtidal zone were more variable in their behavior; however, among the species that showed aerial emergence, there was no relationship between the O₂ threshold for emergence (P_{OUT}) and hypoxia tolerance (P_{crit} ; Fig. 5B) (Martin, 1996; Mandic et al., 2009). These data indicate

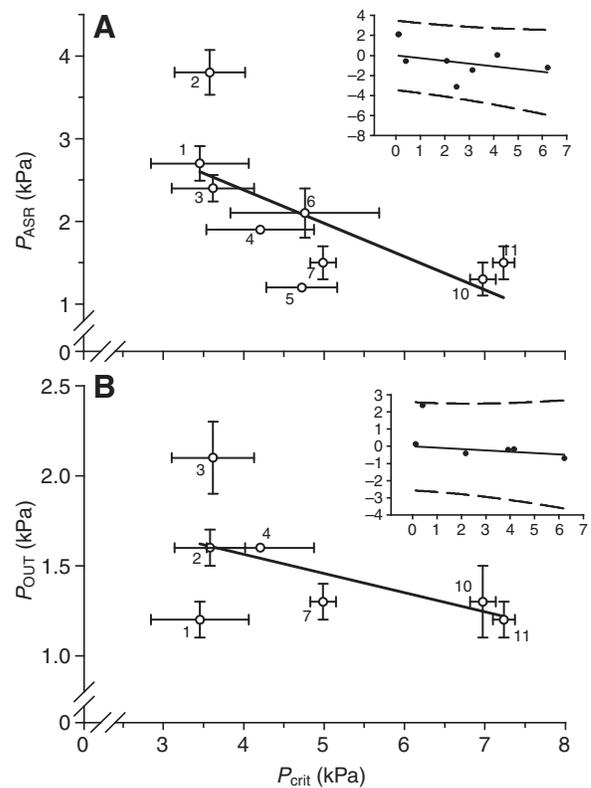


Fig. 5. Relationships between P_{crit} and O₂ threshold for aquatic surface respiration (P_{ASR} ; A) and the O₂ tension at which the fish emerge (P_{OUT} ; B) in marine sculpins. Conventional correlations are shown in A ($r^2=0.45$, $P=0.04$) and B ($r^2=0.23$, $P=0.28$) and phylogenetically independent contrasts are shown in the inset in each panel. Correlation coefficients for the phylogenetically independent contrasts in A and B are $r^2=0.25$, $P=0.20$ and $r^2=0.07$, $P=0.59$, respectively. All data are means \pm s.e.m. in the main panels and 95% prediction intervals are shown in the insets. Numbers 1–12 represent different species: (1) *O. maculosus*, (2) *C. globiceps*, (3) *O. snyderi*, (4) *E. bison*, (5) *A. fenestralis*, (6) *A. lateralis*, (7) *L. armatus*, (8) *S. marmoratus*, (9) *B. cirrhosus*, (10) *M. scorpius*, (11) *C. asper* and (12) *A. harringtoni*. Data modified from Mandic et al. (Mandic et al., 2009b).

that hypoxia-tolerant species do not delay emergence relative to intolerant species. Although there is some discrepancy between studies, fish that actively emerge are generally able to maintain O₂ consumption rates in air that are similar to, or only slightly reduced, compared with those measured in water (Wright and Raymond, 1978; Yoshiyama and Cech, 1994; Martin, 1996; Sloman et al., 2008). In fact, when tidepool sculpins were placed on a moist substrate for up to 72 h, there were no measurable increases in whole body lactate levels, suggesting that the fish are able to extract sufficient O₂ to maintain an aerobic metabolism (Sloman et al., 2008). If, however, fish are prevented from accessing the water surface or performing emergence behavior during hypoxia exposure, they will often adopt a quiescent state, which probably serves to limit metabolic energy use (Yoshiyama et al., 1995).

Although there is a great deal of evidence that behavioral responses to hypoxia are an important means of extending survival in some intertidal fishes, the comparative analysis performed by Mandic et al. did not demonstrate a phylogenetically independent relationship between hypoxia tolerance and behavioural responses to hypoxia (Mandic et al., 2009a). As with all correlative analysis, increasing sample size and expanding the phylogeny to encompass

a greater number of intertidal species may increase the power to detect the adaptation by showing a relationship between hypoxia tolerance and thresholds for ASR and emergence.

The metabolic challenge of surviving O₂ tensions below P_{crit}

At O₂ tensions below P_{crit}, the fundamental challenge to survival is the maintenance of the cellular energy balance. It has long been known that P_{crit} is associated with an activation of anaerobic metabolism as indicated by the increases in whole animal lactate measured at O₂ tensions around P_{crit} (Pörtner and Grieshaber, 1993; Pörtner, 2010). These data suggest that at O₂ tensions below P_{crit} there is a dramatic shift from aerobic to anaerobic metabolism where ATP can only be generated by O₂-independent mechanisms such as glycolysis or other metabolic pathways that rely on substrate-level phosphorylation. Although anaerobic metabolism can generate ATP during hypoxia, the total ATP yield is roughly 15 times lower than from mitochondrial processes when the same substrate is used (Hochachka and Somero, 2002). The switch from aerobic to anaerobic metabolism during hypoxia exposure imposes two main metabolic stressors. First, the lower ATP yield from anaerobic metabolism places a substrate-limited cap on the duration of hypoxic survival. Without O₂, the only metabolic substrates available for ATP production are glycogen and high-energy phosphates and because of the lower ATP yield of anaerobic metabolism, these fuels can be used up quickly. The second issue of relying on anaerobic metabolism is that it produces metabolic wastes such as lactate (plus an associated proton) and its accumulation can have devastating effects on tissues. Overall, the lower ATP yield and accumulation of metabolic wastes during hypoxia exposure presents a problem for fish, particularly hypoxia-sensitive fish, which quickly succumb to hypoxia because of an inability to maintain cellular energy balance (Boutilier, 2001).

Although it is reasonably well documented that fish activate anaerobic glycolysis at O₂ tensions below P_{crit}, the duration of survival at these O₂ tensions will be dictated by two interrelated factors: (1) the availability of substrates and the biochemical capacity for O₂-independent ATP production, and (2) the capacity to reduce metabolic demands through a controlled metabolic rate suppression. As a result, it is reasonable to predict that the ability to store ample anaerobic substrates and utilize metabolic rate suppression should have been selected for in fishes that experience hypoxia; however, no in-depth comparative analysis has been performed to validate this prediction. Thus, the remainder of this article will focus on the interactions between these variables, to provide a basic framework for future comparative analysis. Fig. 3B illustrates the relationship between the capacity for O₂-independent ATP generation and metabolic rate suppression in extending hypoxia survival at environmental O₂ tensions below P_{crit}. The line labeled 1 in Fig. 3B represents the situation in an hypoxia-sensitive fish that at O₂ tensions below P_{crit} attempts to maintain metabolic rate through a large activation of anaerobic metabolism (indicated by long red arrow), which results in the rapid depletion of glycogen and accumulation of lactate and H⁺. In this case, the length of survival will primarily be dependent on the amount of anaerobic substrate available and the capacity of the organism to deal with the metabolic waste products. It must be noted at this point that fish and other animals are limited in their capacity to store fermentable fuels such as glucose and glycogen because, unlike aerobic fuels such as fat, these fuels must be stored intracellularly and are hydrated, thus fermentable fuels occupy a greater space than an equivalent mass of fat. Therefore, without some reduction in metabolic rate, stored fermentable fuels will be quickly exhausted.

By contrast, lines 2 and 3 in Fig. 3 represent the situation with increasing levels of hypoxia tolerance, where decreases in metabolic rate limit the need to activate anaerobic metabolism (shorter red arrows in Fig. 3B), extending the time that a set quantity of glycogen or other fermentable fuel can support metabolism. The more a fish can reduce its metabolic rate in response to hypoxia exposure, the longer it will live.

The ability to suppress ATP demand and reduce metabolic rate to match the limited capacity for O₂-independent ATP production has emerged as the unifying adaptive theory explaining hypoxia survival at environmental O₂ levels below P_{crit} (Hochachka et al., 1996). However, experimental evidence supporting this theory is limited. Part of the reason that there is very little evidence to support this theory is that ATP turnover cannot be measured directly and therefore surrogate measures must be used. The most accurate surrogate method for measuring ATP turnover (or total metabolic rate) is to monitor metabolic heat production because a decrease in heat production signifies a decrease in ATP turnover and metabolic rate. Decreases in heat production in response to hypoxia exposure have been measured in many fish species including goldfish (van Waversveld et al., 1989a; van Waversveld et al., 1989b; van Waversveld et al., 1988), crucian carp (Johansson et al., 1995), tilapia (*Oreochromis mossambicus*) (van Ginneken et al., 1999; van Ginneken et al., 1997) and European eel (*Anguilla anguilla*) (van Ginneken et al., 2001), and in isolated liver cells from rainbow trout (*Oncorhynchus mykiss*) (Rissanen et al., 2006). Interestingly, across this very broad range of fish species that includes species considered to be extremely hypoxia tolerant (crucian carp, goldfish, and tilapia) and tissues from fish considered to be very hypoxia sensitive (rainbow trout), all species show the capacity to decrease metabolic rate in response to hypoxia exposure.

The precise O₂ conditions that elicit metabolic rate suppression differ between species, but with the limited number of measurements available it is impossible to draw any broad, meaningful conclusions about the relationship between hypoxia tolerance and the capacity to suppress metabolic rate. However, the most impressive reductions in metabolic rate occurred in the goldfish, tilapia and European eel with an approximate 70% decrease in metabolic rate during hypoxia. Theoretically, and with all other things being equal, this reduction in metabolic rate could extend survival time by many fold. The degree of metabolic rate suppression in response to hypoxia exposure in other species appears to be much less. These data support the notion that metabolic rate suppression is enhanced in hypoxia-tolerant animals compared with hypoxia-sensitive species, but there is a pressing need to carefully examine the relationship between the ability to utilize metabolic rate suppression and hypoxia tolerance in a larger number of species, where phylogeny can be taken into account.

Any process that reduces whole animal or cellular energy expenditure during hypoxia exposure will conserve precious ATP and prolong life. At the cellular level, the most energetically expensive processes are protein turnover and maintenance of transmembrane ion gradients. Indeed, in an 'average' cell, protein turnover (included synthesis and breakdown) can account for up to 55% of the total ATP demand and maintenance of ion gradients can account for up to 25% of the total ATP demand (Buck et al., 1993a; Buck et al., 1993b). Other processes that contribute require ATP include gluconeogenesis, urea synthesis, RNA turnover, substrate cycling and myosine ATPase for intracellular transport (Hochachka and Somero, 2002). The precise cellular breakdown of the important contributors to cellular metabolic rate will depend on

the cell under examination. Hypoxia-induced reductions in metabolic rate are achieved through the dramatic downregulation of Na^+ pumping, protein turnover, urea synthesis and gluconeogenesis (Richards, 2009). It is now well accepted that the cellular mechanisms underlying metabolic rate suppression are similar across broad taxonomic groups and the mechanisms involved in metabolic rate suppression in carp are very similar to those observed in other species, such as the anoxia-tolerant turtle (*Chrysemys picta*), which can reduce cellular metabolic rate by up to 94% during anoxia exposure (Buck and Hochachka, 1993; Buck et al., 1993a). However, there are tissue-specific differences in the use of metabolic rate suppression among species. For example, in the heart of turtles, anoxia exposure induces metabolic rate suppression resulting in a reduction of cardiac performance to roughly 5% of its normal level (Stecyk and Farrell, 2007), whereas in the anoxic crucian carp (Stecyk et al., 2009), heart performance remained close to routine levels because of a large capacity for anaerobic ATP generation. Despite these tissue-specific differences, whole animal metabolic rate is still suppressed in both the crucian carp and turtle. Nevertheless, different tissues contribute differently to the overall decrease.

Hypoxia exposure elicits a strong activation of substrate-level phosphorylation *via* glycolysis. Endogenous glycogen serves as the primary source of fermentable fuel in fish during hypoxia exposure, thus tissue concentrations of glycogen generally indicate the capacity of that tissue to support ATP turnover *via* glycolysis. Although no thorough comparative analysis of the relationship between substrate availability and hypoxia tolerance have been performed, there is evidence in the available literature that hypoxia-tolerant species have much higher glycogen levels than hypoxia-sensitive species. For example, hypoxia-tolerant animals such as carp, goldfish, killifish and oscar typically have liver glycogen levels in the range of 300–2000 μmol glucosyl units g^{-1} wet mass tissue whereas hypoxia-sensitive fish, such as the rainbow trout, has only ~ 100 μmol glucosyl units g^{-1} wet mass tissue (Vornanen et al., 2009). Thus, it seems reasonable to conclude that across broad taxonomic groups of fish, those animals with more glycogen, will be able to produce more ATP for longer periods of time at lower O_2 levels (see Fig. 3B); however, without the use of metabolic rate suppression, even large amounts of stored fuels could be quickly exhausted.

Conclusions

It has long been assumed that the prevalence of environmental hypoxia in the aquatic environment has been a powerful evolutionary force resulting in the selection of the traits that either enhance O_2 uptake from the environment or allow hypoxia-tolerant fish to defend against the metabolic consequences of an O_2 limitation. Indeed, fish represent the ideal model system to understand the selection of traits underlying hypoxia tolerance because of the extremely O_2 -diverse environments they inhabit. With the advent of the phylogenetic comparative method, comparative physiologists can now identify the repeated evolution of traits correlated with one or more putatively selective variables while factoring out the possible confounding effects of shared ancestry among species (Felsenstein, 1985). Variation in hypoxia tolerance among species, as measured by P_{crit} is primarily explained by the variation in mass-specific gill surface area, red blood cell Hb– O_2 binding affinity, and routine $\dot{M}\text{O}_2$. Below P_{crit} , hypoxia survival requires a rapid reorganization of cellular metabolism to reduce ATP consumption through a regulated metabolic rate suppression to match the limited capacity for O_2 -independent ATP

production. Thus, it appears reasonable to speculate that the degree of metabolic rate suppression and the quantity of stored fermentable fuel are probably strongly selected for in hypoxia-tolerant fishes; however, rigorous testing of this hypothesis has not occurred. Despite the wealth of information available on the metabolic and molecular responses of a variety of fish species to hypoxia, we are still far from a unified concept of the important adaptations underlying hypoxia tolerance. However, fish provide an incredibly tractable system to understand the evolution of hypoxia tolerance because of their incredible diversity as well as their diverse O_2 habitats.

List of symbols and abbreviations

Hb	hemoglobin
$\dot{M}\text{O}_2$	rate of O_2 consumption
P_{ASR}	O_2 threshold for initiating ASR
P_{crit}	critical O_2 tension
P_{OUT}	O_2 threshold for emergence of fish
P_{O_2}	partial pressure of O_2
$P_{\text{W}\text{O}_2}$	water P_{O_2} when physiological function is first compromised
P_{50}	partial pressure of O_2 at which hemoglobin is 50% saturated

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