Am J Physiol Regul Integr Comp Physiol 288: R1079–R1090, 2005;
doi:10.1152/ajpregu.00626.2004.

Invited Review

Oxygen-dependent gene expression in fishes

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Nikinmaa, Mikko, and Bernard B. Rees. Oxygen-dependent gene expression in fishes. *Am J Physiol Regul Integr Comp Physiol* 288: R1079–R1090, 2005; doi:10.1152/ajpregu.00626.2004.—The role of oxygen in regulating patterns of gene expression in mammalian development, physiology, and pathology has received increasing attention, especially after the discovery of the hypoxia-inducible factor (HIF), a transcription factor that has been likened to a "master switch" in the transcriptional response of mammalian cells and tissues to low oxygen. At present, considerably less is known about the molecular responses of nonmammalian vertebrates and invertebrates to hypoxic exposure. Because many animals live in aquatic habitats that are variable in oxygen tension, it is relevant to study oxygen-dependent gene expression in these animals. The purpose of this review is to discuss hypoxia-induced gene expression in fishes from an evolutionary and ecological context. Recent studies have described homologs of HIF in fish and have begun to evaluate their function. A number of physiological processes are known to be altered by hypoxic exposure of fish, although the evidence linking them to HIF is less well developed. The diversity of fish presents many opportunities to evaluate if inter- and intraspecific variation in HIF structure and function correlate with hypoxia tolerance. Furthermore, as an aquatic group, fish offer the opportunity to examine the interactions between hypoxia and other stressors, including pollutants, common in aquatic environments. It is possible, if not likely, that results obtained by studying the molecular responses of fish to hypoxia will find parallels in the oxygen-dependent responses of mammals, including humans. Moreover, novel responses to hypoxia could be discovered through studies of this diverse and species-rich group.

hypoxia-inducible factor; teleost fish; hypoxia; transcription factor; hypoxia response element

THE ROLE OF OXYGEN in development, physiology, and pathology is an area of long-standing biological interest (30). Considerable recent attention has focused on the molecular links between changes in oxygen tension and its consequences on cellular or organismal function. Most of our understanding about the role of oxygen in mechanisms and patterns of gene expression in animals comes from studies on mammals or a few, well-characterized model organisms, such as *Caenorhabditis elegans* (40), *Drosophila* (2, 68), and zebrafish, *Danio rerio* (75, 105). The context for many of these studies has been human health and disease, where changes in oxygen tension contribute to both normal physiological function and several important pathologies (3, 29, 47, 64).

In contrast, ecologically meaningful fluctuations in oxygen tensions and their effects on oxygen-dependent gene expression have been little studied (although see 22 and 95). Indeed, studies on nonhuman mammals and other model systems rarely take into account the organism's natural environment in the design of experiments and interpretation of results. For example, although *Caenorhabditis elegans* lives in soil with rotting vegetation and can thus be expected to regularly experience hypoxic conditions, very few studies have investigated the possible role of oxygen in the evolution and present biology of the species. Similarly, studies on zebrafish seldom consider its natural tropical habitat and good hypoxia tolerance: it survives 2 kPa oxygen tension as adult (86) and can tolerate full anoxia for 24 h as embryo (76).

Our purpose in this review is to discuss oxygen-dependent gene expression in fishes from ecological and evolutionary contexts. First, we introduce the aquatic environment with its marked variation in the oxygen levels. Then, we present the characteristics of fish that make them particularly suitable for studying oxygen-dependent gene expression. Among these characteristics are the pronounced differences in hypoxia tolerance and the diversity of morphological, behavioral, and physiological responses to hypoxia among extant species. The diversity suggests that variation in oxygen levels has acted as an important selective agent during the long evolutionary history of the group. This is followed by the major part of the review, in which we summarize the paradigm of the hypoxia-inducible factor (HIF) and oxygen-dependent gene expression in mammalian cells and present evidence for the presence and possible roles of this transcription factor in fish. Finally, we suggest possible future directions for studies of oxygen-dependent gene expression in this diverse and species-rich group.

OXYGEN IN THE AQUATIC ENVIRONMENT

The inherent properties of water, in combination with variable rates of oxygen consumption and production, result in

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marked temporal and spatial heterogeneity in the oxygen content of the aquatic environment. The amount of oxygen contained in a given volume of water is only 1/30th of the amount contained in the same volume of air (11). At 20°C, for example, 1 liter of pure water in equilibrium with air contains ~0.31 mmol, whereas 1 liter of air contains 9.35 mmol oxygen. As temperature or the concentration of salts increases, the amount of oxygen that can be dissolved in water decreases. Moreover, the rate of diffusion of oxygen in water is 10,000 times slower than the rate in air. Consequently, even modest oxygen consumption by biological or nonbiological processes can rapidly decrease the oxygen tension in the aquatic environment. The situation is exacerbated when the surface of the water column is covered by ice or vegetation, limiting the diffusion of oxygen from the atmosphere, or when the water column is vertically stratified due to thermal or salinity gradients, limiting mixing of surface and deep water masses.

Therefore, areas of episodic or chronic hypoxia, and even anoxia, occur in a variety of marine, estuarine, and freshwater habitats. Generally, oceans are stable environments with relatively high oxygen tensions. Frequently, however, oxygen minimum zones occur between depths of 200 and 1,000 m because oxygen consumption in this low-light habitat exceeds photosynthetic oxygen production and diffusion of oxygen from the surface (56). Marine benthic habitats are also commonly low in oxygen, due to high rates of biological and nonbiological oxidation and limited oxygen diffusion from the surface (12). Hypoxia is common in coastal areas, bays, and estuaries. In many cases, eutrophication caused by human impact increases the length or severity of hypoxia as in the Baltic Sea (10), in the Gulf of Mexico (84) and Chesapeake Bay (126). However, bouts of hypoxia also occur naturally for example in areas where tidal conditions cause the generation of shallow pools with high oxygen consumption by organisms such as corals (72). In the freshwater environment, tropical waters are especially prone to hypoxia due to the high respiration rates, elevated levels of organic matter, and, in many cases, presence of thickly vegetated surface (87). In most of these cases, large diurnal fluctuations in oxygen tension occur. In temperate areas, winter ice cover can lead to seasonal hypoxia, especially in eutrophied waters with significant oxygen consumption even at the prevailing low temperatures (Fig. 1).

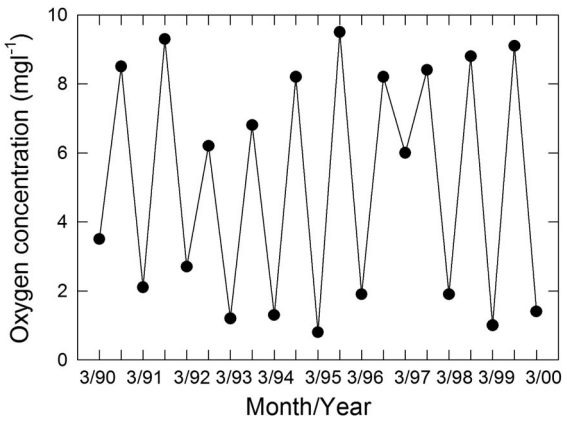

Fig. 1. Seasonal variation in the oxygen concentration at the bottom of a temperate lake (Lake Herunen, Nurmijärvi, Southern Finland). The oxygen concentration in March and September from 1990 to March 2000 is given. Data are from a report of Nurmijärvi Environmental Board on the Water Quality of Lakes in Nurmijärvi.

WHY FISHES ARE PARTICULARLY SUITABLE TO STUDY OXYGEN-DEPENDENT PHENOMENA?

With more than 25,000 species, occupying a variety of habitats, fishes are the most diverse and species-rich group of vertebrates (111). Fishes arose in the early Cambrian period, ~500 million years ago (98), and teleost fish, the largest group of fishes, have evolved independently from the tetrapod lineage of vertebrates for the last 350–400 million years (24). After the split from tetrapods, teleost evolution has probably included at least one genomewide duplication, as well as additional large-scale gene duplications (125). Polyploidy, an extreme case of gene duplication, is common among extant fishes (55). Consequently, the genetic diversity in teleost fish is greater than that in other vertebrate groups. This diversity constitutes the raw material of evolutionary adaptation, including adaptation to changes in environmental oxygen tension.

That variation in environmental oxygen availability has played an important role in the evolution of fishes is suggested by the variety of anatomic, behavioral, and physiological strategies used by fishes to acquire oxygen and deliver it to tissues. For example, air breathing has evolved several times during fish evolution, presumably as an adaptation to stagnant, often hypoxic, tropical waters. At present, 400–500 species of fishes from various families show air breathing (24). In addition, several water-breathing fish species engage in aquatic surface respiration, a behavior in which fish respire water in the upper, well-oxygenated layer of the water column, or hold air bubbles in their buccal cavity to aerate water that is passing over the gills (50, 107). In terms of oxygen transport to the tissues, fish display a remarkable diversity of hemoglobin structure and function (35, 38, 82, 115). Compared with fish from well-oxygenated habitats, the hemoglobins from several hypoxia-tolerant species display amino acid substitutions in functionally critical places that confer higher intrinsic oxygen affinity upon the hemoglobin tetramer (35, 38). Fish have also adapted to extreme hypoxia or anoxia by employing alternate metabolic pathways for anaerobic energy production. Members of the cyprinid genus, *Carassius*, are the only vertebrates that are known to produce energy by fermentation of glucose to ethanol and carbon dioxide (32, 97, 108).

In addition to diversity in the ambient oxygen tension experienced by fish, their internal oxygen tensions (i.e., oxygen levels experienced by the cells) vary more than in other vertebrates. For example, while salmonids and other active fish ventilate their gills continuously with arterial oxygen tensions approaching that of the environment (ca. 15 kPa; 101), species such as hypoxia-tolerant cyprinids breathe periodically in normoxic water (33) and have low mean arterial P_{O_2} values (e.g., 3 kPa in resting goldfish; 6). While cyprinid cells are often confronted with low oxygen tensions, hyperbaric oxygen tensions, up to 100 kPa, can be reached in the vicinity of the eye of salmonids and swim bladder of the eel (77). Thus, although hyperoxia is not considered further in this review, studies with fish might also yield valuable information about cellular and molecular responses to high oxygen tensions.

The diversity of fishes and their habitats indicates that fishes have solved the problem of hypoxia tolerance in various

interesting ways, and probably display a range of molecular adaptations to hypoxia that is not paralleled in other vertebrate groups. With regard to oxygen-regulated gene expression, reviewed below, it is likely that the current state of knowledge only scratches the surface.

MECHANISM OF OXYGEN-DEPENDENT GENE EXPRESSION

Oxygen-Dependent Gene Expression and the HIF in Mammals

In mammals, research over the past decade points to a central role of the hypoxia-inducible factor (HIF) in the regulation of gene expression during hypoxia. HIF was discovered in studies of the regulation of erythropoietin (EPO) expression in the mammalian Hep3B cell line (94). It is a heterodimeric transcription factor comprised of HIF-α and HIF-β subunits, both of which are members of the PAS-domain family of transcription factors (named for the first members of the family, Per, ARNT, Sim). Three forms of α-subunit have been described, HIF-1α, HIF-2α (123), and HIF-3α (26). The β-subunit is the same as the previously described aryl hydrocarbon receptor nuclear translocator (ARNT). In addition to its role in hypoxic signaling, ARNT plays other roles in gene regulation. For example, it dimerizes with the aryl hydrocarbon receptor during xenobiotically induced gene expression, thus playing a role in the response of cells to dioxins and other hydrocarbon pollutants (27, 28). After the discovery of HIF and its role in the hypoxic induction of EPO, HIF was found to be expressed in a variety of cell types and to be involved in the hypoxic regulation of a variety of genes (5, 83, 92, 118, 119). Because of the widespread expression of HIF and the diverse roles of HIF targets, this transcription factor has been likened to a "master switch" of the molecular response to low oxygen in mammals (92, 118).

The oxygen sensitivity of HIF-mediated gene expression is conferred, in part, by the oxygen dependence of HIF-α protein level: although the protein is made continuously, during normoxia it is rapidly degraded. Degradation of HIF-α is mediated by an oxygen-dependent degradation (ODD) domain, in which specific conserved proline residues are covalently modified by prolyl hydroxylase enzymes. When hydoxylated, HIF-α is recognized by the von-Hippel-Lindau protein (pVHL), ubiquitylated, and degraded via the proteasomal pathway. In hypoxia, prolyl hydroxylation does not occur. The onset of hypoxia thus leads to essentially instantaneous stabilization and accumulation of HIF-α (39). HIF-α then travels to nucleus, dimerizes with ARNT, and binds to hypoxia response elements (HREs) in the promoter or enhancer region of hypoxia-inducible genes. Together with the general transcriptional activator CBP/p300, and possibly other accessory factors, HIF stimulates gene transcription. Transactivation of gene expression also depends upon oxygen tension because the interaction between HIF and CBP/p300 is blocked by the oxygen-dependent hydroxylation of a specific asparagine residue in the COOH terminus of HIF-α. This hydroxylation event is catalyzed by an asparaginyl hydroxylase, also known as factor inhibiting HIF-1 (FIH-1) (59). Similar to prolyl hydroxylation, this modification requires oxygen and is inhibited during hypoxia. Thus low oxygen tension is permissive for the interaction between HIF and CBP/p300. The sequence of events leading to increased gene expression during hypoxia is summarized in Fig. 2. Further details of HIF function (in mammals) can be found in a number of recent reviews (19, 44, 63, 78, 91, 93, 118, 122).

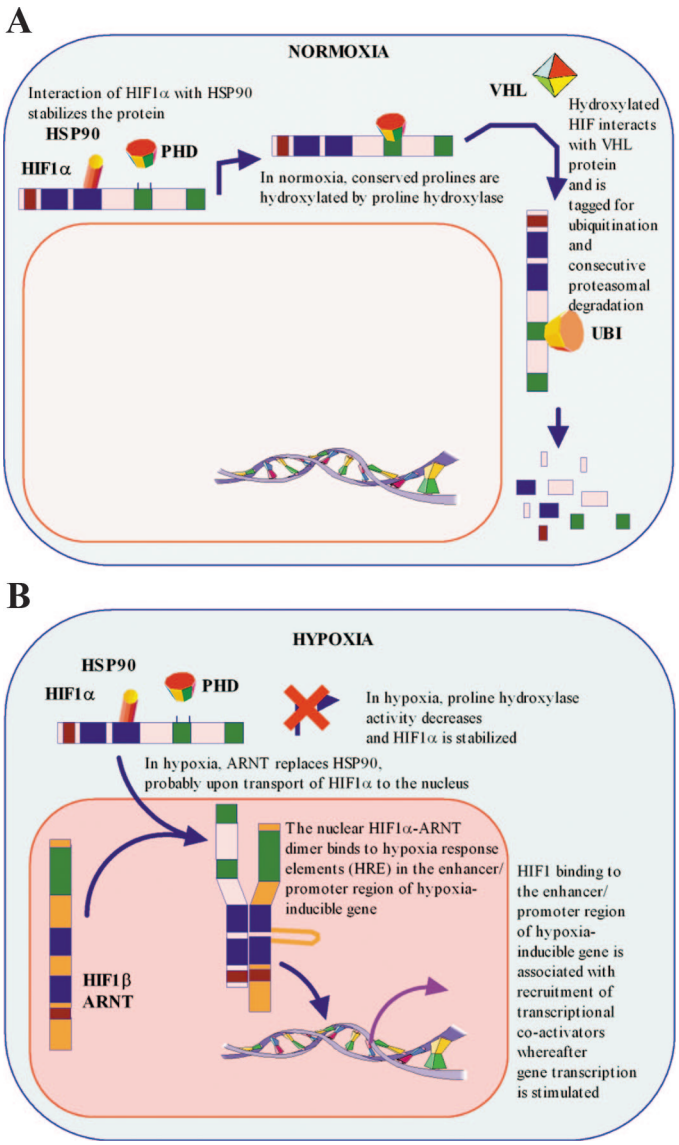

Fig. 2. Schematic representation of hypoxia-inducible factor (HIF) function. *A*: normoxia. *B*: hypoxia. HIF-1α protein is produced both during normoxic and hypoxic conditions. Interaction of the protein with heat shock protein 90 (HSP90) at PAS-B domain exerts a stabilizing influence on the protein. *A*: in normoxia, conserved proline residues of HIF-1α &rtf-space;are hydroxylated by proline hydroxylase (PHD) enzymes, which enables interaction between HIF-1α and von Hippel-Lindau (VHL) protein. As a result of this interaction, HIF-1α protein is tagged for ubiquitination (UBI = ubiquitin) and consecutive degradation via the proteasomal pathway. *B*: in hypoxia, the activity of PHDs is decreased, and HIF-1α is stabilized. HIF-1α is then transported from cytoplasm to nucleus, where it forms a dimer with aryl hydrocarbon receptor nuclear translocator (ARNT). The dimer recruits further transcriptional activators and binds to the hypoxia response elements in the promoter or enhancer regions of genes inducible genes. Ultimately, the production of mRNA for hypoxia-induced genes is stimulated.

HIFs in Fish

It now appears that fish possess homologs of HIF-α and -β, which may play similar roles as those in mammals in hypoxic gene expression. While ARNT had been characterized in fish

in the context of pollutant stress in the 1990s (80), the sequence of first fish HIF-α was reported from rainbow trout in 2001 (100). The cDNA encodes a protein of 766 amino acids and includes regions recognizable as basic-helix-loop-helix, PAS, and ODD domains of the α-subunits of HIF. Moreover, proline and asparagine residues, which are the hydroxylation targets in mammals, are conserved in the rainbow trout protein. Similarity searches of the deduced amino acid sequence revealed this protein to be most similar to HIF-1α from other vertebrates. In the subsequent year, a second HIF-α was cloned and sequenced from the killifish *Fundulus heteroclitus* (81). The deduced protein is 873 amino acids long, contains domains and specific amino acid residues thought to be functionally important, and is most similar to HIF-2α from other vertebrates.

At the time of this writing, there are six fish HIFs available in the Swiss-Prot and TrEMBL databases (accession numbers Q98SW2, Q6STN7, Q8QGM4, Q6STN6, Q6EHI4 and Q6EGR9). Phylogenetic analyses show that these proteins fall into three discrete groups (Fig. 3). In addition to the deduced rainbow trout protein (Q98SW2), putative HIF-1αs have been sequenced in grass carp, *Ctenopharyngodon idella* (Q6STN7), and zebrafish (Q6EHI4). The only fish HIF-2α described to date is from killifish (Q8QGM4). The two remaining putative HIF proteins are from grass carp (Q6STN6), and zebrafish (Q6EGR9). These proteins are equally distantly related to HIF-1α and HIF-2α, and they form a distinct group in phylogenetic analyses. Interestingly, they are not very similar to mammalian HIF-3α, either, making their placement in a phylogenetic tree uncertain. From the limited data available, it is unclear whether this last group of fish HIF-α subunits are orthologs of mammalian HIF-3α or if they represent a distinct gene, perhaps specific to fish. Indeed, although the zebrafish protein has been named as HIF-3α, the grass carp protein has tentatively been named HIF-4α. More robust analyses, which include a larger number of HIF-3α and potential HIF-4α sequences from a broader distribution of fish and nonmammalian tetrapod vertebrates, will be necessary to resolve the relationship of these enigmatic HIF-α subunits.

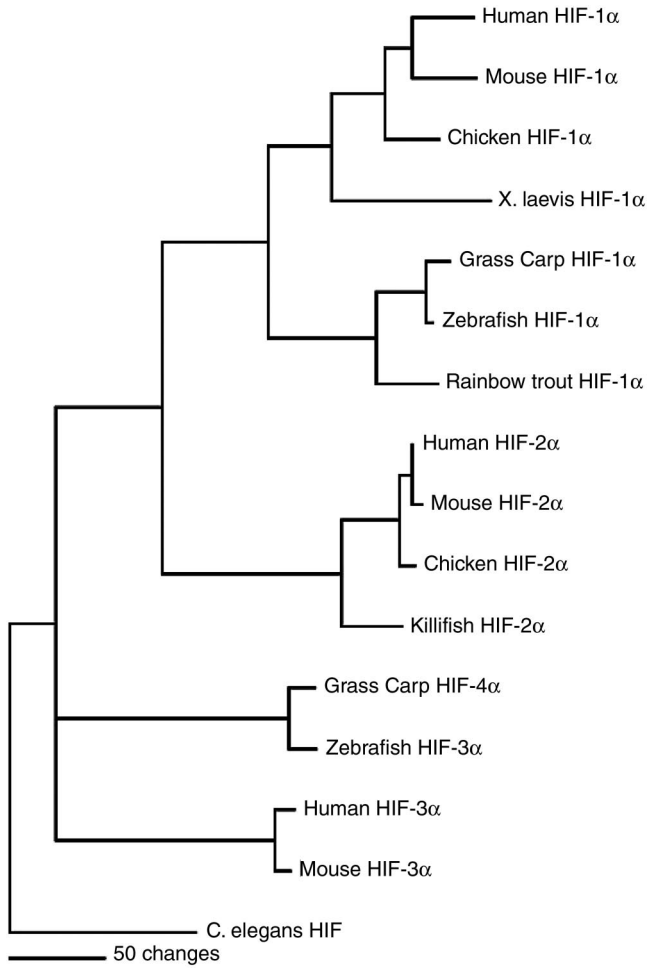

Fig. 3. A phylogram of predicted HIF-α amino acid sequences from fish and representative other vertebrates. The SwissProt/TrEMBL accession numbers of the sequences used are human HIF-1α, Q16665; mouse HIF-1α, Q61221; chicken HIF-1α, Q9YIB9; *Xenopus* HIF-1α, Q9I8A9; trout HIF-1α, Q98SW2; grass carp HIF-1α, Q6STN7; zebrafish HIF-1α, Q6EHI4; human HIF-2α, Q99814; mouse HIF-2α, P97481; chicken HIF-2α, Q9W7C6; killifish HIF-2α, Q8QGM4; grass carp HIF-4α, Q6STN6; zebrafish HIF-3α, Q6EGR9; human HIF-3α, Q9Y2N7; mouse HIF-3α, Q9Z2I5. Deduced amino acid sequences were aligned with ClustalX, version 1.83, excluding positions with gaps. Because of poor sequence alignments in the COOH-terminal half of the proteins, only the NH_2-terminal 376 amino acids were used for this analysis (numbered according to human HIF-1α). The tree shown was the result of a parsimony analysis of phylogenetic relatedness of the aligned HIF-α sequences using PAUP 4.0. The horizontal lengths are proportional to the number of amino acid differences among HIF-α sequences, with the scale bar indicating 50 amino acid changes. Concordant results were obtained from phylogenetic analyses based upon distance methods.

HIF Stability, Function, and Expression in Fish

With regard to the oxygen dependence of fish HIF stability and function, the only published studies to date have been done on HIF-1α from salmonids (70, 100). The initial characterization of this protein in primary cultures of rainbow trout hepatocytes or cell lines derived from rainbow trout (RTG-2) or chinook salmon (CHSE-214) showed that, although it was often observed in normoxic conditions, both the level of HIF-1α and its DNA binding increase markedly during hypoxic exposure (100). Interestingly, in both the RTG-2 and the CHSE-214 cell lines (derived from rainbow trout gonadal fibroblasts and chinook salmon embryonic epithelia, respectively), maximum HIF-1α protein expression occurred at 5% oxygen. Such oxygen levels commonly occur in venous blood of salmonid fish kept in normoxic conditions (101). Typically, the oxygen tension in tissues is similar to or lower than the venous oxygen tension, suggesting that tissue oxygen tension may be low enough to allow substantial accumulation of HIF-1α protein even in normoxia (see *Linking HIF, Oxygen, and Gene Expression in Fish*). However, when RTG-2 cells were transiently transfected with a plasmid bearing an HRE, reporter gene expression was maximal at 0.5% oxygen (B. B. Rees, Y. I. Figueroa, B. Beckman, P. M. Schulte, unpublished observations). In addition, whereas HIF-1α protein expression occurs within 1–4 h (100), reporter gene expression peaks at 48 h of hypoxic exposure. These temporal and oxygen-level-dependent differences between HIF-1α protein levels and reporter gene expression suggest that other steps in the pathway between HIF-1α accumulation and gene expression may also be oxygen dependent. These steps could include posttranslational modifications, nuclear localization, dimerization, DNA binding or transactivation of HIF-1α.

Along these lines, the stability, DNA-binding ability, and phosphorylation state of salmonid HIF-1α are influenced by reagents that alter the reduction-oxidation (redox) status of cells (70). Reagents that foster a reducing environment under normoxic conditions lead to the accumulation of and DNA-binding by HIF-1α in RTG-2 and CHSE-214 cells. Conversely, oxidizing agents blunt the stabilization and activity of HIF-1α normally associated with hypoxic exposure. Notably, mammalian HIF-2α but not HIF-1α is similarly modulated by redox reagents (52). This effect has been attributed to an amino acid difference in the NH_2-terminal portion of the protein: mammalian HIF-2α has a cysteine at position 25, which aligns with a serine at position 28 in mammalian HIF-1α. In support of a role for this amino acid in redox regulation, site-directed mutagenesis of Ser28 of mammalian HIF-1α to Cys28 conferred redox sensitivity to DNA binding (52). These observations are relevant in the present context, since rainbow trout HIF-1α has a cysteine at position 28. Both serine and cysteine are found among fish HIF-1αs in the position aligning with Ser28 of mammalian HIF-1α, suggesting species differences in the redox sensitivity of DNA binding by HIF-1α in fish.

In addition to a potential role for Cys28, rainbow trout HIF-1α contains several cysteine residues in the ODD domain. It is possible that the reduction or oxidation of these residues may also play a role in the redox sensitivity of salmonid HIF-1α stability and DNA binding (70). Notably, grass carp and zebrafish HIF-1αs lack the numerous cysteines present in the ODD of rainbow tout HIF-1α. Consequently, in these species redox-sensitive sulfhydryl modification cannot occur in the vicinity of the conserved proline residues, which are the substrates of the prolyl hydroxylases, determining the stability of HIF-1α. Thus the HIF-1α stability of these hypoxia-tolerant cyprinids may lack the redox sensitivity shown by rainbow trout HIF-1α. It would be of considerable interest to determine if these amino acid differences are correlated with interspecific differences in HIF function and gene expression during hypoxia. While speculative, such a correlation could explain, in part, the difference in hypoxia tolerance of these species: rainbow trout is relatively hypoxia intolerant, whereas grass carp and zebrafish are relatively hypoxia tolerant. Such structure-function comparisons highlight the insights that can be gained by studying a group of vertebrates as diverse as fish.

The few data that exist suggest that HIF-α subunits are expressed in a broad range of fish tissues. Western blot analyses indicate that HIF-1α protein is expressed in salmonid cells derived from liver, gonad, and embryonic tissues (100). During development of Baltic salmon (*Salmo salar*), HIF-1α protein levels in embryonic tissues increase (Fig. 4). In adult mummichog, HIF-2α mRNA is present during normoxia in liver, spleen, heart, brain, gonads, intestine, gill, and kidney (81).

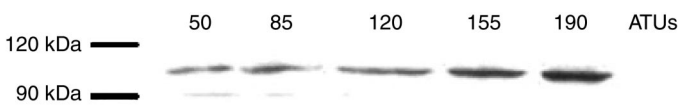

Fig. 4. An increase in the HIF-1α protein level during development of Baltic salmon (*Salmo salar*) as detected by an antibody against rainbow trout HIF-1α (100) at 50, 85, 120, 155 and 190 accumulated temperature units (ATUs = °C × days). Because the development of poikilothermic animals such as fish depends both on the temperature and time, similarity of ATUs indicates that embryos are in the same stage of development. Data from Vuori et al. (113).

There are no published reports of the tissue expression patterns, oxygen dependence, or function of other fish HIF-αs, including the novel HIF-3/4α subunits. In this regard, it would be very interesting if fish HIF-3/4α subunits play an analogous role to mammalian HIF-3α, certain forms of which act as negative regulators of HIF-induced gene expression (60).

Evidence for HIF-Modifying Enzymes in Fish

As discussed above, the oxygen sensitivity of HIF-α stability and activity requires specific residues, notably proline and asparagine residues (which are conserved in fish HIF-α), and the proteins that modify and interact with them. Several lines of evidence point to the existence and functionality of these other proteins in fish. First, HIF-α accumulates when salmonid cells are incubated in the presence of proteasome inhibitors, implying that degradation of fish HIF-α normally follows the same pathway as in mammals (100). Second, although prolyl hydroxylases have not been characterized in fish, multiple open reading frames similar to human prolyl hydroxylases are present in the genomes of zebrafish and the pufferfish. Furthermore, an asparaginyl hydroxylase (FIH-1) homolog has been sequenced from zebrafish (Swiss-Prot P59723). Finally, and perhaps most convincingly, RTG-2 cells transiently transfected with a reporter gene bearing an HRE show hypoxia-inducible reporter gene expression (see above). These observations suggest that fish cells have all the components needed for the reversible stabilization of HIF-α and the activation of gene expression under hypoxia. The extent to which these components vary among cells, tissues, and species is, at present, unknown.

TARGETS OF OXYGEN-DEPENDENT GENE EXPRESSION

In mammals, several HIF target genes have been characterized, including genes involved in red blood cell production, vascularization, apoptosis, and iron, catecholamine, and carbohydrate metabolism (5, 83, 92, 118, 119). Numerous studies suggest that oxygen-dependent gene expression is similarly widespread among fishes. Below, we review *1*) studies that focus on specific target genes or physiological processes, for which there was an a priori expectation of a hypoxic response (Table 1), and *2*) recent studies that seek to elucidate broad-scale, or global, patterns of gene expression under hypoxia. Notably, most of these studies do not address the mechanism of the observed effects of low oxygen. While it seems likely that HIF is involved, the possibility that other mechanisms are operative cannot be discounted. We conclude this section with a brief consideration of the one fish gene for which a functional HRE has been characterized.

Red Blood Cell Formation and Oxygen Transport

One of the hallmark responses of fishes to low oxygen is an increase in hematocrit value (36). The possible mechanisms that have been forwarded to account for this increase include erythrocyte swelling, release of preformed red blood cells, changes in plasma volume, plasma skimming, and new red blood cell formation (18, 69, 71). In mammals exposed to hypoxia, the hematocrit value is primarily regulated by formation of new red blood cells, signaled for by increased levels of EPO, which in turn is transcriptionally regulated by HIF. In fish, there is also evidence suggesting that red blood cell

Table 1. *Oxygen-dependent responses and evidence for the role of HIF in fish*

Erythropoiesis	Fish homologs of EPO exist and the expression of EPO has been measured in tissues of pufferfish (8). Although hypoxia can stimulate erythropoiesis in fish, the role of HIF and EPO in this process has not been characterized.
Hemoglobin synthesis	Reduced globin gene expression coincides with aberrant HIF regulation in the early mortality syndrome of Baltic salmon.
Angiogenesis	There is a correlation between reduced DNA binding of HIF-1α, reduced VEGF protein level and decreased capillary density in Baltic salmon embryos suffering from early mortality syndrome (113).
Changes in gill surface area	Hypoxic fish are characterized by a larger surface area than normoxic ones (7, 90, 102). Apoptosis has been suggested as a mechanism (102), but so far HIF has not been conclusively linked to the process.
Glycolysis	Several studies have demonstrated changes in glycolytic enzyme activities or mRNAs during hypoxia. A putative hypoxia response element has been described in the LDH-B gene of *Fundulus heteroclitus* (85).
Glucose transport	Hypoxia-sensitive glucose transporter from grass carp *Ctenopharyngoden idella* has been characterized (124).
Growth suppression	cDNA microarray data on *Gillichthys mirabilis* and *G. seta* indicate hypoxic upregulation of genes involved in growth suppression (22). In hypoxic zebrafish embryos, genes involved in translation and cell cycle progression were inhibited (105).

HIF, hypoxia-inducible factor; EPO, erythropoietin; VEGF, vascular endothelial growth factor.

production is EPO sensitive. Injection of human EPO stimulates red blood cell production in fish (69, 103). Proteins (121) and mRNA (96) from rainbow trout cross-react with probes against mammalian EPO. Furthermore, a search of the SwissProt and TrEMBL databases reveals three putative EPO sequences from fish, two from the Japanese pufferfish, *Takifugu rubripes* (Q6JV22, Q6JV23) and one from the green puffer, *Tetraodon nigriviridis* (Q6UAM1). Studies of the regulation of *Takifugu rubripes* EPO in cell culture found that the promoter of this gene could not confer hypoxic regulation of reporter gene expression in cell culture, although mRNA splicing appeared to be sensitive to hypoxia (8). Thus fish appear to possess homologs of EPO, and EPO can influence red blood cell production, but the role of hypoxia and HIF in the expression and function of EPO in fish remain virtually unexplored. Interestingly, the EPO gene of pufferfish does not have a flanking HRE (8).

Hemoglobin is the circulating oxygen-transport protein in fishes as in other vertebrates. Teleost fish display a greater diversity of different hemoglobin components within the erythrocytes of one individual than other vertebrate groups (35, 114), presumably due to selection pressures, including low oxygen concentration. The effects of acute or chronic exposure to hypoxia on globin gene expression at the individual level, however, are less clear. Some studies suggest that the globin chain expression pattern of erythrocytic hemoglobin is influenced by hypoxic exposure of the fish (61), whereas other studies have failed to see a response (37). Naturally, it is possible that the responses are species specific. However, even if the pattern of erythrocytic hemoglobin expression were influenced by hypoxia, at present the mechanisms and pathways for the hypoxia responses are completely unknown. Interestingly, developmental disturbances and death of offspring from naturally spawning Baltic Sea salmon are associated with reduced expression of globin genes involved in the formation of erythrocytic hemoglobin (K. A. M. Vuori and M. Nikinmaa, unpublished data). The same developmental anomalies are associated with aberrant HIF regulation (113), suggesting a possible link between globin gene expression and HIF.

Development of Circulatory and Respiratory Structures

In mammals, HIF signals for an increase in vascular endothelial growth factor (VEGF), which in turn stimulates the growth of blood vessels. VEGF has been sequenced from zebrafish (SwissProt O73682), and multiple splice variants have been characterized (20). VEGF and its receptors play a role in the vascular development in zebrafish (54, 74). Furthermore, a recent study on developmental defects in Baltic salmon demonstrates a correlation between HIF function, VEGF expression, and vascular development (113). Briefly, a large proportion of Baltic salmon suffer from a syndrome known as M74, characterized by high levels of mortality in yolk-sac stage fry. Normal development is associated with a marked increase in HIF-1α DNA binding, which remains constant or decreases in M74 affected individuals (113). There is a corresponding decrease in VEGF protein levels and capillary density in tissues from M74 fry. On the basis of these correlations, it is reasonable to speculate that HIF regulates vascular development during normal development, presumably by modulating VEGF levels, as in mammals. Mammalian fetuses lacking HIF-1α die at midgestation (34), and disturbances in the development of vasculature, heart, and neural tube are observed (9, 48, 89).

Oxygen uptake is facilitated both by effective circulation and by an increase in the area of respiratory surfaces. Hypoxia appears to regulate the functional area of fish gills. The total respiratory area of sea bass (*Dicentrarchus labrax*) gills was inversely proportional to the oxygen concentration during 3-mo cultivation (90). In the African cichlid *Pseudocrenilabrus multicolor victoriae*, total gill area is greater in individuals from source populations that regularly encounter hypoxia in their environment than in those from populations seldom experiencing hypoxia (7). Similarly, in laboratory experiments, specimens exposed to hypoxia (1 mg oxygen/l, 5 mo) displayed larger gill surface areas than fish raised in normoxic (7.5 mg oxygen/l) conditions, although the morphological components of the increase differed in natural vs. laboratory populations (7). In crucian carp (*Carassius carassius*), 1 wk of hypoxic exposure causes a dramatic increase in gill surface area, mainly because secondary lamellae develop (Fig. 5). The appearance of secondary lamellae is associated with a twofold decrease in the number of cells between lamellae, possibly as a result of programmed cell death (apoptosis) of interlamellar cells (102). If apoptosis is under the control of HIF as it is in hypoxia-exposed mammalian cells (19), this would provide a molecular link between hypoxia and gill development.

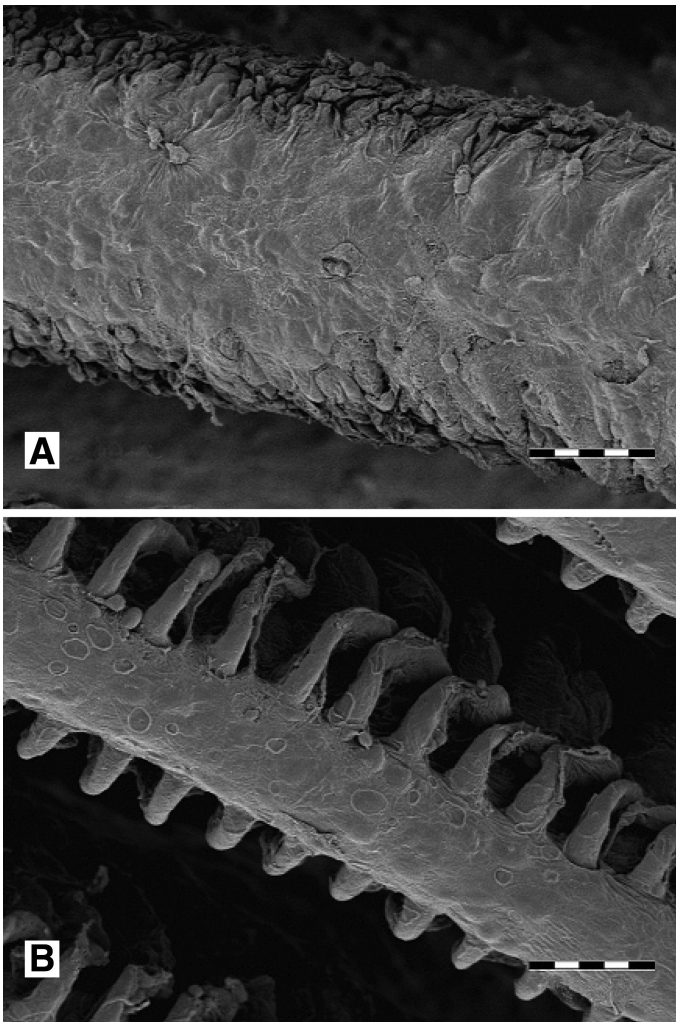

Fig. 5. Scanning electron micrographs of crucian carp (*Carassius carassius*) gills in normoxia (*A*; >10 mg oxygen/l) and after 7-day hypoxia (*B*; 0.75 mg oxygen/l). Reproduced from Sollid et al. (102) by permission of the authors and Company of Biologists Ltd.

Energy Metabolism

During severe hypoxia, even the above adjustments to increase oxygen delivery may not be sufficient to meet the tissue needs for energy production via aerobic metabolism, and a variety of fish rely upon increased anaerobic carbohydrate metabolism for energy production (14, 25, 51, 112). One way to increase the rates of uptake and utilization of carbohydrates is to increase the amounts and activities of transport and enzyme proteins catalyzing these processes. In mammalian systems, the transcription of glucose transporters and a number of glycolytic enzymes increase during hypoxia (16, 92, 118, 119), leading to speculation that such coordinated regulation is widely distributed among animals (117).

Among fish, few studies have measured transcriptional regulation of glycolytic enzymes during hypoxia, although there are many reports of enzyme activities in tissues from fish exposed to low oxygen. Results from these studies, however, are mixed: glycolytic enzyme activities can increase, decrease, or remain unchanged during hypoxia (1, 13, 25, 57, 109). In cases where multiple tissues and enzymes were analyzed, the effects of hypoxia were invariably tissue specific and were restricted to a subset of the enzymes analyzed. Data supporting a concerted upregulation of glycolytic enzyme gene expression come from studies of "global" gene expression in fish exposed to hypoxia (22, 105). As described below, mRNA levels for multiple glycolytic enzymes increased in both studies, even though different species and developmental stages were examined. As observed for measures of enzyme activity, however, the changes in transcript levels were tissue specific and not observed for all glycolytic enzymes. Consequently, while hypoxia might induce certain glycolytic enzymes, the effects are not uniformly observed among species, tissues, or enzymes. Differences may reflect, in part, differences in experimental conditions (severity and duration of hypoxia) but could also reflect diverse strategies used by fish to deal with hypoxia. Notably, many species suppress metabolism during hypoxia rather than maintain high rates of energy metabolism (see below).

Another response to hypoxia documented in tissues of fish is a change in the isozyme pattern of lactate dehydrogenase (LDH), the terminal enzyme of anaerobic carbohydrate metabolism. The two predominant isozymes, LDH-A and LDH-B, differ in their kinetics in the forward and reverse reactions. LDH-A is better suited for converting pyruvate into lactate, whereas LDH-B is more efficient in converting lactate to pyruvate (62). The proportions of LDH-A and LDH-B vary between tissues, but the LDH-A/LDH-B ratio also correlates with environmental oxygen availability (1). In the South American cichlid, *Cichlasoma amazonarum*, LDH-A is expressed in hearts from fish from hypoxic habitats, whereas LDH-B is the predominant isozyme in hearts from fish sampled from normoxic habitats. In the same species, laboratory acclimation to low oxygen led to a decrease in LDH-B expression in muscle and brain, but an increase in liver. Hypoxic exposure of the gobies, *Gillichthys mirabilis* and *G. seta*, led to increased expression of LDH-A mRNA in liver, although the level of this transcript remained unchanged in skeletal and cardiac muscle (22). These results suggest that oxygen tension may be involved in regulating the expression of the genes encoding these isozymes, although tissue-dependent and perhaps species-dependent factors appear to influence the effects of oxygen.

In addition to glucose utilization, there is evidence that the uptake of glucose by fish tissues is influenced by hypoxia. Recently, the sequence and hypoxia sensitivity of a class I glucose transporter has been reported from grass carp (124). Both short-term (4 h) and long-term (4–7 days) hypoxic exposure of live fish resulted in an increased expression of mRNA for this transporter in kidney, eye, and gill. Expression in other tissues was unaffected by the degree and duration of hypoxia studied. These results demonstrate that, at least in certain tissues, the capacity for glucose uptake may be enhanced during hypoxia, as observed in certain mammalian cell lines and tissues (16).

Global Measures of Gene Expression

Recent technological developments have enabled the measurement of broad-scale or "global" patterns of gene expression (21). Using these approaches, some investigations have examined the gene expression pattern in fish exposed to hypoxia. Gracey et al. (22) used cDNA microarray technology to

examine gene expression in liver, brain, skeletal muscle and heart from adult gobies (*G. mirabilis* and *G. seta*) exposed to hypoxia for 6 days. In liver, genes involved in glycolysis, iron metabolism, amino acid metabolism, and growth suppression were upregulated. In both skeletal muscle and heart, the predominant effect was a downregulated expression of some genes involved in protein translation and muscle contraction, although fewer genes appeared to be affected in the heart than skeletal muscle. Ton et al. (105) used similar approach to assess the effects of 24-h hypoxic exposure on gene expression patterns of zebrafish embryos. In whole embryos, hypoxia increased the expression of some glycolytic genes, whereas genes involved in oxidative carbohydrate metabolism, muscle contraction, translation, and cell cycle progression were suppressed. Despite the use of distinctly different species and developmental stages, both studies support the idea of a metabolic reorganization in hypoxia that reduces energetically expensive processes and augments anaerobic ATP production. Moreover, HIF-1α mRNA was induced by hypoxia in zebrafish embryos (105), and although it was not identified in hypoxic gobies, many of the genes affected are targets of HIF regulation in other systems (22). Another important similarity was that both studies measured increases in the expression of unidentified genes, suggesting additional, potentially novel, responses to hypoxia in these fish.

The application of cDNA microarray technology provides extremely valuable information regarding the transcriptional control of a wide range of genes. However, the response of the cell and organism depends upon the expression and the post-translational modifications of proteins. Therefore, measures of broad-scale patterns of protein expression can complement and extend results from cDNA microarrays. Using 2-dimensional gel electrophoresis, Bosworth et al. (4) found only minor changes in patterns of protein expression in white skeletal muscle from normoxic and hypoxic adult zebrafish. The effects of hypoxia were restricted to a small number of low-abundance proteins, one of which, observed only in tissues from hypoxic zebrafish, had a molecular weight and isoelectric point similar to those predicted for fish HIF-α subunits (81, 100). Under the conditions of this study, however, there were no indications of a broad reorganization of metabolism. The difference between results obtained from cDNA arrays and techniques based upon proteins illustrates the need to integrate both approaches for a more complete understanding of the effects of hypoxia on patterns of gene expression in fish.

Definition of a Functional Fish HRE

Although evidence is accumulating that hypoxia can affect the expression of a wide array of fish genes, a functional HRE has been characterized only for the LDH-B gene from *Fundulus heteroclitus*. In mammals, the minimal HRE corresponds to (A/G)CGTG and has been found in the promoter or enhancer regions of a variety of genes (46, 119). Based upon sequence similarity to the mammalian HRE, a putative HRE was identified in intron 2 of the LDH-B gene from *F. heteroclitus* (85). Functional studies, however, indicated that the promoter of the LDH-B, rather than intron 2, conferred oxygen sensitivity on reporter gene expression (B. B. Rees, Y. G. Figueroa, B. S. Beckman, P. M. Schulte, unpublished observations). Furthermore, the putative HRE in this promoter is different from the canonical mammalian HRE, but identical to that found in mammalian aldolase-A (46) . Given the diversity of HIF in fish and other vertebrates (see above), it will be important to determine the ability of other forms of HIF to interact with this HRE and those which will undoubtedly be identified in other fish genes.

FUTURE DIRECTIONS

In the preceding pages, we have attempted to lay out the current state knowledge on the effects of hypoxia on gene expression in fishes, both in terms of HIF-based mechanisms and targets of oxygen-dependent gene expression. We close by calling attention to a number of unanswered questions that may serve as a foundation of several areas of potentially fruitful future research.

Linking HIF, Oxygen, and Gene Expression in Fish

Clearly, a more complete description of HIF and its targets of regulation in fishes are necessary. The first fish homolog of HIF-1α was described in 2001. Since then, there has been one published report of another HIF-α, along with a number of unpublished and partial HIF-α sequences. Future research with fish will help to resolve the number of HIF-α forms and their relationships to one another and to other vertebrate HIFs. Research to date suggests an important role not only for oxygen, but also for cellular redox state, in HIF stability and function. Whether the different forms of HIF-α show different sensitivities to oxygen and cellular redox state remains unexplored. Answers to these questions may help illuminate why fish species differ in their hypoxia tolerances. In addition, HIF-1α protein levels, as assessed by Western blot analyses of fish tissue, are often considerable in normoxic conditions (H. Numminen, E. Rissanen, and M. Nikinmaa, unpublished data), suggesting that HIF-1α may have oxygen-independent roles in the physiology of fish. The normoxic levels, furthermore, show marked individual differences (H. Numminen, E. Rissanen, and M. Nikinmaa, unpublished data). Whether interindividual variation in normoxic levels of HIF is correlated with other aspects of the life history or physiology of fish is another fascinating question.

A number of studies suggest a diversity of potential targets of low-oxygen mediated gene regulation in fish. These potential targets include several which are known to be affected by hypoxia in mammals, such as vascularization and carbohydrate metabolism. However, although there are a number potential genes regulated by hypoxia in fish, data in support of a direct role of HIF in the hypoxia-induced changes are extremely limited. Accordingly, it will be important to identify HREs in these genes and demonstrate a functional interaction with HIF in vitro and, ultimately, in vivo. It is possible that subtle variation in the sequence of HREs from target genes could lead to large differences in the effects of hypoxia on gene expression in different species of fish. Indeed, changes in the regulatory region of genes may be a more important force in the evolution of gene expression than changes in the coding region (104). Because of the diversity of fish, with closely related species, or populations within a given species, occurring in habitats of differing oxygen availability, there are numerous opportunities for "natural experiments" evaluating the relation-

ships between gene regulation, hypoxia tolerance, and ecological distribution.

More research is also needed on "traditional" oxygen-regulated processes for which the possible link to HIF has not been investigated. For example, the role of catecholamines in hypoxia responses in fishes has been intensively studied. Catecholamines regulate ventilation (73), influence red blood cell pH and hematocrit value (69), and play a role in the activation of gluconeogenesis (110) in hypoxia. Given that there is an interaction between the transcriptional activity of HIF and cAMP, the second messenger involved in β-adrenergic responses (53), and that the expression of α-adrenergic receptors is HIF regulated (65) in mammals, it would not be surprising to find HIF-dependent regulation of adrenergic responses in fish.

With respect to novel oxygen-regulated processes, studies of global patterns of gene expression in fish under hypoxia hold much promise (22, 105). Targets of oxygen-dependent gene expression revealed in such studies may reflect specializations of fish to aquatic habitats. Alternatively, these genes may be important also in the hypoxic response of mammals but have simply been overlooked because of their tissue, oxygen, or temporal dependence.

One understudied area in the hypoxic responses of fish is the mechanism of gene downregulation. Many fish demonstrate reduced rates of metabolism, protein synthesis, and growth when facing reduced oxygen availability (99). However, the mechanism of this suppression, its oxygen dependency, and its possible links to HIF are unknown.

Ultimately, measurements of HIF, its activity, and the expression of its targets need to be made in fish exposed to levels of oxygen and lengths of hypoxia that are environmentally relevant. Since fish occur in habitats ranging widely in their oxygen concentrations, information from field measurements of oxygen should be incorporated into the design of laboratory experiments. Naturally occurring hypoxia is often cyclical, varying diurnally, tidally, or seasonally. Thus studies with hypoxia should include both cyclical and continuous hypoxia, which may cause different responses, as described for carp erythrocyte function (58, 116). In addition, ecologically relevant hypoxia normally occurs in concert with elevated carbon dioxide tension in freshwater (37) or with elevated hydrostatic pressures in the oxygen minimum zones of oceans. Thus studies of HIF function should take into account these additional environmental variables.

Potential Interactions Between Hypoxia and Other Environmental Stressors

Aquatic hypoxia is frequently associated with changes in temperature, food availability, or pollutant exposure, each of which may interact with oxygen-dependent responses in fish. Hitherto, temperature effects on HIF function have been little studied, since most of the work on HIF relates to homeothermic organisms. However, HIF is required for heat acclimation in *Caenorhabditis elegans* (106), indicating that in poikilothermic animals HIF function may also be related to temperature. The molecular details of the effect of temperature on HIF are presently unknown. Notably, however, a heat shock protein, HSP90, interacts with HIF-α and affects its function (66). It appears that HSP90 binds to PAS-B domain of HIF-α and exerts a stabilizing influence on the protein (43).

Fish exposed to hypoxia grow more slowly than fish in well oxygenated environments, primarily due to decreased intake of food (17). Two observations suggest that the nutritional status and HIF-dependent responses may interact. First, glucose levels affect HIF-mediated gene expression in cell culture (45), and, second, the effects of insulin may be transmitted via a pathway that shares elements of the HIF-dependent pathway of gene expression (88).

Human activity may affect both temperature and food cycles, but, in addition, causes pollution of the aquatic environment. In addition to their direct effects on biological function, several pollutants could also potentially interact with HIF-mediated processes. Pollutants such as dioxin and other halogenated hydrocarbons affect gene expression via binding to the aryl hydrocarbon receptor (AHR), which dimerizes with ARNT to affect gene expression (23). Because HIF-α subunits also dimerize with ARNT, it is possible that hypoxia-induced gene expression might be lessened due to competition for this transcription factor or accessory factors (although see Ref. 79). In rats, Hofer et al. (31) observed interaction between dioxin and carbon monoxide exposure, the latter being a treatment that induces physiological hypoxia. Kraemer and Schulte (49) showed that prior exposure of *Fundulus heteroclitus* to 3,3′,4,4′-tetrachlorobiphenyl suppressed the induction of glycolytic enzymes during hypoxia. Another current concern is the presence of estrogenic substances in aquatic environments (41, 42, 120). Recent reports suggest that estrogen analogs interfere with the function of HIF (15, 67). Finally, HIF function in fish may be sensitive to the redox state of the cells (70), whereby anthropogenic influences on the environment that cause oxidative stresses, such as increased ultraviolet radiation and metal contamination, may disturb HIF function. Thus oxygen-dependent gene expression, and possibly hypoxia tolerance, in fish may be impacted by all the above forms of pollution.

Fish are ideally suited for investigating the interactions between low oxygen and these other environmental stressors. Results from such studies may indicate unexpected ramifications of these stressors on fish physiology and ecology. In addition, results with fish may find parallels in the oxygen-dependent responses of mammals, including humans. By studying the molecular responses of fish to hypoxia, therefore, we can better understand this diverse and important group of vertebrates, and in the process, we can learn more about ourselves.

ACKNOWLEDGMENTS

B. B. Rees acknowledges support from the U.S. National Science Foundation (IBN-0236494) and M. Nikinmaa from the Academy of Finland (8202426).

REFERENCES

1. **Almeida-Val VMF, Farias IP, Silva MNP, Duncan WP, and Val AL.** Biochemical adjustments to hypoxia by Amazon cichlids. *Braz J Med Biol Res* 28: 1257–1263, 1995.
2. **Bacon-Neil CM, Wappner P, O'Rourke JF, Bartlett SM, Shilo B, Pugh CW, and Ratcliffe PJ.** Regulation of the *Drosophila* bHLH-PAS protein Sima by hypoxia: functional evidence for homology with mammalian HIF-1α. *Biochem Biophys Res Commun* 249: 811–816, 1998.
3. **Bing OH, Apstein CS, and Brooks WW.** Factors influencing tolerance of cardiac muscle to hypoxia. *Recent Adv Stud Card Struct Metab* 10: 343–354, 1975.

4. **Bosworth CA, Chou CW, Cole RB, and Rees BB.** Protein expression patterns in zebrafish skeletal muscle: initial characterization and the effects of hypoxic exposure. *Proteomics* In press. 2005.
5. **Bracken CP, Whitelaw ML, and Peet DJ.** The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cell Mol Life Sci* 60: 1376–1393, 2003.
6. **Burggren WW.** "Air gulping" improves blood oxygen transport during aquatic hypoxia in the goldfish *Carassius auratus*. *Physiol Zool* 55: 327–334, 1982.
7. **Chapman LJ, Galis F, and Shinn J.** Phenotypic plasticity and the possible role of genetic assimilation: hypoxia-induced trade-offs in the morphological traits of an African cichlid. *Ecol Lett* 3: 387–393, 2000.
8. **Chou CF, Tohari S, Brenner S, and Venkatesh B.** Erythropoietin gene from a teleost fish, *Fugu rubripes*. *Blood* 104: 1498–1503, 2004.
9. **Compernolle V, Brusselmans K, Franco D, Moorman A, Dewerchin M, Collen D, and Carmeliet P.** Cardia bifida, defective heart development and abnormal neural crest migration in embryos lacking hypoxia-inducible factor-1α. *Cardiovasc Res* 60: 569–579, 2003.
10. **Conley DJ, Humborg C, Rahm L, Savchuk OP, and Wulff F.** Hypoxia in the Baltic Sea and basin-scale changes in phosphorus biogeochemistry. *Environ Sci Technol* 36: 5315–5320, 2002.
11. **Dejours P.** *Principles of Comparative Respiratory Physiology*. Amsterdam: North-Holland, 1975.
12. **Diaz RJ.** Overview of hypoxia around the world. *J Environ Qual* 30: 275–281, 2001.
13. **Driedzic WR and Almeida-Val VMF.** Enzymes of cardiac energy metabolism in Amazonian teleosts and the fresh-water stingray (*Potamotrygon hystrix*). *J Exp Zool* 274: 327–333, 1996.
14. **Dunn JF and Hochachka PW.** Turnover rates of glucose and lactate in rainbow trout during acute hypoxia. *Can J Zool* 65: 1144–1148, 1987.
15. **Earley S and Resta TC.** Estradiol attenuates hypoxia-induced pulmonary endothelin-1 gene expression. *Am J Physiol Lung Cell Mol Physiol* 283: L86–L93, 2002.
16. **Ebert BL, Firth JD, and Ratcliffe PJ.** Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct cis-acting sequences. *J Biol Chem* 270: 29083–29089, 1995.
17. **Foss A, Evensen TH, and Oiestad V.** Effects of hypoxia and hyperoxia on growth and food conversion efficiency in the spotted wolffish *Anarhichas minor* (Olafsen). *Aquaculture Res* 33: 437–444, 2002.
18. **Gallaugher P and Farrell AP.** Hematocrit and blood oxygen-carrying capacity. In: *Fish Respiration*, edited by Perry SF and Tufts BL. San Diego: Academic, 1998, p. 185–227.
19. **Goda N, Dozier SJ, and Johnson RS.** HIF-1 in cell cycle regulation, apoptosis, and tumor progression. *Antioxid Redox Signal* 5: 467–473, 2003.
20. **Gong BW, Liang D, Chew TG, and Ge RW.** Characterization of the zebrafish vascular endothelial growth factor A gene: comparison with VEGF-A genes in mammals and Fugu. *Biochim Biophys Acta-Gene Struct Expr* 1676: 33–40, 2004.
21. **Gracey AY and Cossins AR.** Application of microarray technology in environmental and comparative physiology. *Annu Rev Physiol* 65: 231–259, 2003.
22. **Gracey AY, Troll JV, and Somero GN.** Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. *Proc Natl Acad Sci USA* 98: 1993–1998, 2001.
23. **Gradin K, Mcguire J, Wenger RH, Kvietikova I, Whitelaw ML, Toftgard R, Tora L, Gassmann M, and Poellinger L.** Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the ARNT transcription factor. *Mol Cell Biol* 16: 5221–5231, 1996.
24. **Graham JB.** *Air-Breathing Fishes*. San Diego, CA: Academic, 1997.
25. **Greaney GS, Place AR, Cashon RE, Smith G, and Powers DA.** Time course of changes in enzyme activities and blood respiratory properties of killifish during long-term acclimation to hypoxia. *Physiol Zool* 53: 136–144, 1980.
26. **Gu YZ, Moran SM, Hogenesch JB, Wartman L, and Bradfield CA.** Molecular characterization and chromosomal localization of a third α-class hypoxia inducible factor subunit, HIF3α. *Gene Expr* 7: 205–213, 1998.
27. **Hahn ME.** Dioxin toxicology and the aryl hydrocarbon receptor: insights from fish and other non-traditional models. *Mar Biotechnol* 3: S224–S238, 2001.
28. **Hankinson O.** The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* 35: 307–340, 1995.
29. **Hess ML and Manson NH.** Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. *J Mol Cell Cardiol* 16: 969–985, 1984.
30. **Hochachka PW.** Defence strategies against hypoxia and hypothermia. *Science* 231: 234–241, 1986.
31. **Hofer T, Pohjanvirta R, Spielmann P, Viluksela M, Buchmann DP, Wenger RH, and Gassmann M.** Simultaneous exposure of rats to dioxin and carbon monoxide reduces the xenobiotic but not the hypoxic response. *Biol Chem* 385: 291–294, 2004.
32. **Holopainen IJ, Hyvärinen H, and Piiroinen J.** Anaerobic wintering of crucian carp (*Carassius carassius* L.). II. Metabolic products. *Comp Biochem Physiol* 83A: 239–242, 1986.
33. **Hughes GM.** Effects of low oxygen and pollution on the respiratory systems of fish. In: *Stress and Fish*, edited by Pickering AD. London: Academic, 1981, p. 121–146.
34. **Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, and Semenza GL.** Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. *Genes Dev* 12: 149–162, 1998.
35. **Jensen FB, Fago A, and Weber RE.** Hemoglobin structure and function. In: *Fish Respiration*, edited by Perry SF and Tufts BL. San Diego: Academic, 1998, p. 1–40.
36. **Jensen FB, Nikinmaa M, and Weber RE.** Environmental perturbations of oxygen transport in teleost fishes: causes, consequences and compensations. In: *Fish Ecophysiology*, edited by Jensen FB and Rankin JC. London: Chapman and Hall, 1993, p. 161–179.
37. **Jensen FB and Weber RE.** Respiratory properties of tench blood and hemoglobin. Adaptation to hypoxic-hypercapnic water. *Mol Physiol* 2: 235–250, 1982.
38. **Jensen FB and Weber RE.** Proton and oxygen equilibria, their anion sensitivities and interrelationships in tench hemoglobin. *Mol Physiol* 7: 41–50, 1985.
39. **Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, and Gassmann M.** Induction of HIF-1α in response to hypoxia is instantaneous. *FASEB J* express article 10.1096, 2001.
40. **Jiang H, Guo R and Powell-Coffman JA.** The *Caenorhabditis elegans* hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc Natl Acad Sci USA* 98: 7916–7921, 2001.
41. **Jobling S, Reynolds T, White R, Parker MG, and Sumpter JP.** A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 103: 582–587, 1995.
42. **Jobling S and Sumpter JP.** Detergent components in sewage effluent are weakly estrogenic to fish—an in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat Toxicol* 27: 361–372, 1993.
43. **Katschinski DM, Le L, Schindler SG, Thomas T, Voss AK, and Wenger RH.** Interaction of the PAS B domain with HSP90 accelerates hypoxia-inducible factor-1 α stabilization. *Cell Physiol Biochem* 14: 351–360, 2004.
44. **Kewley RJ, Whitelaw ML, and Chapman-Smith A.** The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int J Biochem Cell Biol* 36: 189–204, 2004.
45. **Kietzmann T, Krones-Herzig A, and Jungermann K.** Signaling crosstalk between hypoxia and glucose via hypoxia- inducible factor 1 and glucose response elements. *Biochem Pharmacol* 64: 903–911, 2002.
46. **Kimura H, Weisz A, Ogura T, Hitomi Y, Kurashima Y, Hashimoto K, D'Acquisto F, Makuuchi M, and Esumi H.** Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. *J Biol Chem* 276: 2292–2298, 2001.
47. **Koch CJ.** Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. *Methods Enzymol* 352: 3–31, 2002.
48. **Kotch LE, Iyer NV, Laughner E, and Semenza GL.** Defective vascularization of HIF-1α-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Dev Biol* 209: 254–267, 1999.
49. **Kraemer LD and Schulte PM.** Prior PCB exposure suppresses hypoxia-induced up-regulation of glycolytic enzymes in Fundulus heteroclitus. *Comp Biochem Physiol C Toxicol Pharmacol* 139: 23–29, 2004.
50. **Kramer DL and McClure M.** Aquatic surface respiration, a widespread adaptation to hypoxia in tropical fresh-water fishes. *Environ Biol Fishes* 7: 47–55, 1982.

51. **Krumschnabel G, Schwarzbaum PJ, Lisch J, Biasi C, and Wieser W.** Oxygen-dependent energetics of anoxia-tolerant and anoxia-intolerant hepatocytes. *J Exp Biol* 203: 951–959, 2000.
52. **Lando D, Pongratz I, Poellinger L, and Whitelaw ML.** A redox mechanism controls differential DNA binding activities of hypoxia-inducible factor (HIF) 1α and the HIF-like factor. *J Biol Chem* 275: 4618–4627, 2000.
53. **Lee M, Hwang JT, Lee HJ, Jung SN, Kang IS, Chi SG, Kim SS, and Ha JH.** AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *J Biol Chem* 278: 39653–39661, 2003.
54. **Lee P, Goishi K, Davidson AJ, Mannix R, Zon L, and Klagsbrun M.** Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish. *Proc Natl Acad Sci USA* 99: 10470–10475, 2002.
55. **Leggatt RA and Iwama GK.** Occurrence of polyploidy in the fishes. *Rev Fish Biol Fisheries* 13: 237–246, 2003.
56. **Levin LA.** Oxygen minimum zone benthos: Adaptation and community response to hypoxia. *Oceanogr Mar Biol* 41: 1–45, 2003.
57. **Lushchak VI, Bahnjukova TV, and Storey KB.** Effect of hypoxia on the activity and binding of glycolytic and associated enzymes in sea scorpion tissues. *Braz J Med Biol Res* 31: 1059–1067, 1998.
58. **Lykkeboe G and Weber RE.** Changes in the respiratory properties of the blood in the carp, *Cyprinus carpio*, induced by diurnal variation in ambient oxygen tension. *J Comp Physiol* 128: 117–125, 1978.
59. **Mahon PC, Hirota K, and Semenza GL.** FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 15: 2675–2686, 2001.
60. **Makino Y, Kanopka A, Wilson WJ, Tanaka H, and Poellinger L.** Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3 α locus. *J Biol Chem* 277: 32405–32408, 2002.
61. **Marinsky CA, Houston AH, and Murad A.** Effect of hypoxia on hemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. *Can J Zool* 68: 884–888, 1990.
62. **Markert CL.** Biology of isoenzymes. *Bioscience* 25: 365–368, 1975.
63. **Maxwell P and Salnikow K.** HIF-1—an oxygen and metal responsive transcription factor. *Cancer Biol Ther* 3: 29–35, 2004.
64. **McDonough KH and Spitzer JJ.** Effects of hypoxia and reoxygenation on adult rat heart cell metabolism. *Proc Soc Exp Biol Med* 173: 519–526, 1983.
65. **Michelotti GA, Bauman MJ, Smith MP, and Schwinn DA.** Cloning and characterization of the rat α_{1a}-adrenergic receptor gene promoter—demonstration of cell specificity and regulation by hypoxia. *J Biol Chem* 278: 8693–8705, 2003.
66. **Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J, and Michiels C.** Hypoxia-induced activation of HIF-1, role of HIF-1 α-Hsp90 interaction. *FEBS Lett* 460: 251–256, 1999.
67. **Mukundan H, Kanagy NL, and Resta TC.** 17-β estradiol attenuates hypoxic induction of HIF-1α and erythropoietin in Hep3B cells. *J Cardiovasc Pharmacol* 44: 93–100, 2004.
68. **Nagao M, Ebert BL, Ratcliffe PJ, and Pugh CW.** Drosophila melanogaster SL2 cells contain a hypoxically inducible DNA binding complex which recognizes mammalian HIF-1 binding sites. *FEBS Lett* 387: 161–166, 1996.
69. **Nikinmaa M.** *Vertebrate Red Blood Cells*. Heidelberg: Springer 1990.
70. **Nikinmaa M, Pursiheimo S, and Soitamo A.** Redox state regulates HIF-1α protein level, its DNA binding and phosphorylation in salmonid cells. *J Cell Sci* 117: 3201–3206, 2004.
71. **Nikinmaa M and Tervonen V.** Regulation of blood haemoglobin concentration in hypoxic fish. In: *Proceedings of the 7th International Symposium on Fish Physiology, Toxicology, and Water Quality*, edited by Rupp GL and White MD. Athens, GA: U.S. Environmental Protection Agency, Ecosystems Research Division ,2004, p. 243–252.
72. **Nilsson GE and Ostlund-Nilsson S.** Hypoxia in paradise: widespread hypoxia tolerance in coral reef fishes. *Proc Royal Soc London B-Biol Sci* 271: S30–S33, 2004.
73. **Nilsson S.** *Autonomic Nerve Function in the Vertebrates*. Heidelberg: Springer, 1983.
74. **Ober EA, Olofsson B, Makinen T, Jin SW, Shoji W, Koh GY, Alitalo K, and Stainier DYR.** VEGFc is required for vascular development and endoderm morphogenesis in zebrafish. *EMBO Rep* 5: 78–84, 2004.
75. **Orkin SH and Zon LI.** Genetics of erythropoiesis: induced mutations in mice and zebrafish. *Annu Rev Genet* 31: 33–60, 1997.
76. **Padilla PA and Roth MB.** Oxygen deprivation causes suspended animation in the zebrafish embryo. *Proc Natl Acad Sci USA* 98: 7331–7335, 2001.
77. **Pelster B and Randall DJ.** The physiology of the Root effect. In: *Fish Respiration*, edited by Perry SF and Tufts BL. San Diego, CA: Academic, 1998, p. 113–139.
78. **Poellinger L and Johnson RS.** HIF-1 and hypoxic response: the plot thickens. *Curr Opin Genet Dev* 14: 81–85, 2004.
79. **Pollenz RS, Davarinos NA, and Shearer TP.** Analysis of aryl hydrocarbon receptor-mediated signaling during physiological hypoxia reveals lack of competition for the aryl hydrocarbon nuclear translocator transcription factor. *Mol Pharmacol* 56: 1127–1137, 1999.
80. **Pollenz RS, Sullivan HR, Holmes J, Necela B, and Peterson RE.** Isolation and expression of cDNAs from rainbow trout (*Oncorhynchus mykiss*) that encode two novel basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) proteins with distinct functions in the presence of the aryl hydrocarbon receptor—evidence for alternative mRNA splicing and dominant negative activity in the bHLH/PAS family. *J Biol Chem* 271: 30886–30896, 1996.
81. **Powell WH and Hahn ME.** Identification and functional characterization of hypoxia-inducible factor 2α from the estuarine teleost, *Fundulus heteroclitus*: interaction of HIF-2α with two ARNT2 splice variants. *J Exp Zool* 294: 17–29, 2002.
82. **Powers DA.** Structure, function, and molecular ecology of fish hemoglobins. *Ann NY Acad Sci* 241: 472–490, 1977.
83. **Pugh CW, Maxwell PH, and Ratcliffe PJ.** Oxygen mediated gene regulation. *Nephrol* 7: S21–S25, 2002.
84. **Rabalais NN, Turner RE, and Wiseman WJ.** Gulf of Mexico hypoxia, aka "The dead zone". *Annu Rev Ecol Syst* 33: 235–263, 2002.
85. **Rees BB, Bowman JA, and Schulte PM.** Structure and sequence conservation of a putative hypoxia response element in the lactate dehydrogenase-B gene of *Fundulus*. *Biol Bull* 200: 247–251, 2001.
86. **Rees BB, Sudradjat FA, and Love JW.** Acclimation to hypoxia increases survival time of zebrafish, *Danio rerio*, during lethal hypoxia. *J Exp Zool* 289: 266–272, 2001.
87. **Rosenberger AE and Chapman LJ.** Hypoxic wetland tributaries as faunal refugia from an introduced predator. *Ecol Freshwater Fish* 8: 22–34, 1999.
88. **Roth U, Curth K, Unterman TG, and Kietzmann T.** The transcription factors HIF-1 and HNF-4 and the coactivator p300 are involved in insulin-regulated glucokinase gene expression via the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 279: 2623–2631, 2004.
89. **Ryan HE, Lo J, and Johnson RS.** HIF-1 α is required for solid tumor formation and embryonic vascularization. *EMBO J* 17: 3005–3015, 1998.
90. **Saroglia M, Terova G, De Stradis A, and Caputo A.** Morphometric adaptations of sea bass gills to different dissolved oxygen partial pressures. *J Fish Biol* 60: 1423–1430, 2002.
91. **Schofield CJ and Ratcliffe PJ.** Oxygen sensing by HIF hydroxylases. *Nature Rev Mol Cell Biol* 5: 343–354, 2004.
92. **Semenza GL.** HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 88: 1474–1480, 2000.
93. **Semenza GL.** O_2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1. *J Appl Physiol* 96: 1173–1177, 2004.
94. **Semenza GL and Wang GL.** A nuclear factor induced by hypoxia via de novo protein-synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 12: 5447–5454, 1992.
95. **Shams I, Avivi A, and Nevo E.** Hypoxic stress tolerance of the blind subterranean mole rat: expression of erythropoietin and hypoxia-inducible factor 1α. *Proc Natl Acad Sci USA* 101: 9698–9703, 2004.
96. **Shiels A and Wickramasinghe SN.** Expression of an erythropoietin-like gene in the trout. *Br J Haematol* 90: 219–221, 1995.
97. **Shoubridge EA and Hochachka PW.** The origin and significance of metabolic carbon dioxide production in anoxic goldfish. *Mol Physiol* 1: 315–338, 1981.
98. **Shu DG, Luo HL, Morris SC, Zhang XL, Hu SX, Chen L, Han J, Zhu M, Li Y, and Chen LZ.** Lower Cambrian vertebrates from South China. *Nature* 402: 42–46, 1999.
99. **Smith RW, Houlihan DF, Nilsson GE, and Brechin JG.** Tissue-specific changes in protein synthesis rates in vivo during anoxia in

crucian carp. *Am J Physiol Regul Integr Comp Physiol* 271: R897–R904, 1996.

100. **Soitamo AJ, Rabergh CMI, Gassmann M, Sistonen L, and Nikinmaa M.** Characterization of a hypoxia-inducible factor (HIF-1α) from rainbow trout. Accumulation of protein occurs at normal venous oxygen tension. *J Biol Chem* 276: 19699–19705, 2001.
101. **Soivio A, Nikinmaa M, Nyholm K, and Westman K.** The role of gills in the responses of Salmo gairdneri during moderate hypoxia. *Comp Biochem Physiol* 70A: 133–139, 1981.
102. **Sollid J, De Angelis P, Gundersen K, and Nilsson GE.** Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J Exp Biol* 206: 3667–3673, 2003.
103. **Taglialatela R and Della Corte F.** Human and recombinant erythropoietin stimulate erythropoiesis in the goldfish *Carassius auratus*. *Eur J Histochem* 41: 301–304, 1997.
104. **Tautz D.** Evolution of transcriptional regulation. *Curr Opin Genet Dev* 10: 575–579, 2000.
105. **Ton C, Stamatiou D, and Liew CC.** Gene expression profile of zebrafish exposed to hypoxia during development. *Physiol Genomics* 13: 97–106, 2003.
106. **Treinin M, Shliar J, Jiang HQ, Powell-Coffman JA, Bromberg Z, and Horowitz M.** HIF-1 is required for heat acclimation in the nematode *Caenorhabditis elegans*. *Physiol Genomics* 14: 17–24, 2003.
107. **Val AL.** Oxygen transfer in fish: morphological and molecular adjustments. *Braz J Med Biol Res* 28: 1119–1127, 1995.
108. **Van den Thillart G.** Adaptations of fish energy metabolism to hypoxia and anoxia. *Mol Physiol* 2: 49–61, 1982.
109. **Van den Thillart G and van Waarde A.** Teleosts in hypoxia: aspects of anaerobic metabolism. *Mol Physiol* 8: 393–407, 1985.
110. **Van den Thillart G, Vianen G, Ponce MC, Lelieveld H, Nieveen M, Van Raaij M, Steffens A, and Zaagsma J.** Differential role of adrenoceptors in control of plasma glucose and fatty acids in carp, *Cyprinus carpio* (L.). *Am J Physiol Regul Integr Comp Physiol* 281: R615–R624, 2001.
111. **Venkatesh B.** Evolution and diversity of fish genomes. *Curr Opin Genet Dev* 13: 588–592, 2003.
112. **Virani NA and Rees BB.** Oxygen consumption, blood lactate and inter-individual variation in the gulf killifish, *Fundulus grandis*, during hypoxia and recovery. *Comp Biochem Physiol A Mol Integr Physiol* 126: 397–405, 2000.
113. **Vuori KAM, Soitamo A, Vuorinen PJ, and Nikinmaa M.** Baltic salmon (*Salmo salar*) yolk-sac fry mortality is associated with disturbances in the function of hypoxia-inducible transcription factor (HIF-1α) and consecutive gene expression. *Aquat Toxicol* 68: 301–313, 2004.
114. **Weber RE.** Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. In: *Animal Nutrition and Transport Processes 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects*, edited by Truchot J-P and Lahlou B. Basel: Karger, 1990, p. 58–75.
115. **Weber RE and Jensen FB.** Functional adaptations in hemoglobins from ectothermic vertebrates. *Annu Rev Physiol* 50: 161–179, 1988.
116. **Weber RE and Lykkeboe G.** Respiratory adaptations in carp blood. Influences of hypoxia, red cell organic phosphates, divalent cations and CO_2 on hemoglobin-oxygen affinity. *J Comp Physiol* 128: 127–137, 1978.
117. **Webster KA.** Evolution of the coordinate regulation of glycolytic enzyme genes by hypoxia. *J Exp Biol* 206: 2911–2922, 2003.
118. **Wenger RH.** Mammalian oxygen sensing, signalling and gene regulation. *J Exp Biol* 203: 1253–1263, 2000.
119. **Wenger RH and Gassmann M.** Oxygen(es) and the hypoxia-inducible factor-1. *Biol Chem* 378: 609–616, 1997.
120. **White R, Jobling S, Hoare SA, Sumpter JP, and Parker MG.** Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135: 175–182, 1994.
121. **Wickramasinghe SN.** Erythropoietin and the human kidney—evidence for an evolutionary link from studies of Salmo gairdneri. *Comp Biochem Physiol A-Physiol* 104: 63–65, 1993.
122. **Wiesener MS and Maxwell PH.** HIF and oxygen sensing; as important to life as the air we breathe? *Ann Med* 35: 183–190, 2003.
123. **Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, and Maxwell PH.** Induction of endothelial PAS domain protein-1 by hypoxia: Characterization and comparison with hypoxia-inducible factor-1α. *Blood* 92: 2260–2268, 1998.
124. **Zhang ZP, Wu RSS, Mok HOL, Wang YL, Poon WWL, Cheng SH, and Kong RYC.** Isolation, characterization and expression analysis of a hypoxia-responsive glucose transporter gene from the grass carp, *Ctenopharyngodon idellus*. *Eur J Biochem* 270: 3010–3017, 2003.
125. **Zhou RJ, Cheng HH, and Tiersch TR.** Differential genome duplication and fish diversity. *Rev Fish Biol Fisheries* 11: 331–337, 2001.
126. **Zimmerman AR and Canuel EA.** A geochemical record of eutrophication and anoxia in Chesapeake Bay sediments: anthropogenic influence on organic matter composition. *Mar Chem* 69: 117–137, 2000.