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REVIEW

The evolutionary and physiological significance of the Hif pathway in teleost fishes

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ABSTRACT

The hypoxia-inducible factor (HIF) pathway is a key regulator of cellular O_2 homeostasis and an important orchestrator of the physiological responses to hypoxia (low O_2) in vertebrates. Fish can be exposed to significant and frequent changes in environmental O_2, and increases in Hif-α (the hypoxia-sensitive subunit of the transcription factor Hif) have been documented in a number of species as a result of a decrease in O_2. Here, we discuss the impact of the Hif pathway on the hypoxic response and the contribution to hypoxia tolerance, particularly in fishes of the cyprinid lineage, which includes the zebrafish (*Danio rerio*). The cyprinids are of specific interest because, unlike in most other fishes, duplicated paralogs of the Hif-α isoforms arising from a teleost-specific genome duplication event have been retained. Positive selection has acted on the duplicated paralogs of the Hif-α isoforms in some cyprinid sub-families, pointing to adaptive evolutionary change in the paralogs. Thus, cyprinids are valuable models for exploring the evolutionary significance and physiological impact of the Hif pathway on the hypoxic response. Knockout in zebrafish of either paralog of Hif-1α greatly reduces hypoxia tolerance, indicating the importance of both paralogs to the hypoxic response. Here, with an emphasis on the cardiorespiratory system, we focus on the role of Hif-1α in the hypoxic ventilatory response and the regulation of cardiac function. We explore the effects of the duration of the hypoxic exposure (acute, sustained or intermittent) on the impact of Hif-1α on cardiorespiratory function and compare relevant data with those from mammalian systems.

KEY WORDS: Hypoxia inducible factor, Hypoxia tolerance, Fishes, Hypoxic ventilatory response, Cardiac function, Temperature

Introduction

When environmental O_2 levels fall, animals respond rapidly with a suite of physiological adjustments aimed at minimizing disruption to energy metabolism. At the cellular level, the coordination of the transcriptional regulation of the hypoxic response is largely mediated by the hypoxia-inducible factor (HIF), a highly conserved transcription factor that activates the expression of target genes involved in energy metabolism, angiogenic signaling, growth factor signaling, apoptosis and embryonic development (for reviews, see Semenza, 2000, 2012; Schofield and Ratcliffe, 2004). Given the importance of HIF to cellular function when energy supply is threatened by the lack of O_2, much effort across disciplines – from medicine (e.g. cancer; Pezzuto and Carico, 2018) to comparative physiology (e.g. Nikinmaa and Rees, 2005) – has gone into understanding the far-reaching potential of HIF signaling in cellular and organismal regulation.

A heterodimeric protein, HIF is composed of an O_2-regulated HIF-α subunit and a constitutively expressed HIF-β [aryl hydrocarbon nuclear translocator (ARNT)] subunit (Fig. 1). Both HIF-α and HIF-β subunits contain a basic helix-loop-helix (bHLH) domain as well as one or two Per/ARNT/Sim (PAS) domains, which allow the two subunits to dimerize (Wang et al., 1995). However, only the HIF-α subunit contains an O_2-dependent degradation (ODD) domain and a C-terminal-transactivation (c-TAD) domain. The protein stability and activity of HIF-α are determined by O_2 availability through independent actions of prolyl hydroxylase (PHD) and factor inhibiting HIF (FIH) proteins, which hydroxylate specific residues in the ODD and c-TAD domains, respectively (Bruick and McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001; Hewitson et al., 2002).

In the presence of sufficient O_2, PHDs hydroxylate the proline residues in the amino- and carboxy-terminal oxygen degradation domains (NODD and CODD) of HIF-α (Fig. 1; Bruick and McKnight, 2001, Ivan et al., 2001, Jaakkola et al., 2001). The hydroxylated HIF-α is recognized by von Hippel–Lindau tumor-suppressor protein (pVHL), which binds to HIF-α and marks the protein for ubiquitination via E3 ubiquitin protein ligase, leading to degradation by the 26*S* proteasome (Ciechanover, 1998; Maxwell et al., 1999; Tanimoto et al., 2000). As O_2 falls, a decrease in the O_2-dependent hydroxylation of HIF-α results in a failure of VHL to recognize HIF-α and target it for degradation. Thus, HIF-α accumulates during hypoxia and enters the nucleus, where it dimerizes with HIF-β (Fig. 1). Along with its transcription co-activators p300 and CBP [CREB (cAMP response element-binding protein)-binding protein], HIF heterodimer binds to the consensus sequence 5′-(A/G)CGTG-3′ present in hypoxia responsive elements (HREs) of target genes and stimulates gene transcription during hypoxia (Kvietikova et al., 1995; Ruas et al., 2005).

The O_2-dependent regulation of gene transcription via HIF-α is present in most metazoans (Mills et al., 2018), and forms an important basis for cellular O_2 sensing and a myriad of hypoxic responses. Teleost fishes, in particular, are susceptible to substantial changes in environmental O_2 levels, given the high frequency and severity of hypoxic events in the aquatic environment (Mandic and Regan, 2018). In response to these changes in O_2, Hif-α activation has been documented in a variety of fish species, using both direct (e.g. protein quantification) and indirect (e.g. expression levels of downstream target genes) methods (Table 1). In some fish, unlike in mammals, hypoxia increases *hif-α* mRNA levels, providing evidence of Hif signaling activation (for review, see Pelster and Egg, 2018). However, it should be noted that indirect assessments of Hif-α protein levels through measurements of *hif-α* mRNA or

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List of abbreviations

APD	action potential duration
AR	acute response
bHLH	basic helix-loop-helix domain
CBP	CREB (cAMP-response-element-binding protein) binding protein
CNS	central nervous system
CODD	carboxy-terminal oxygen degradation domain
c-TAD	C-terminal-transactivation domain
CT_{max}	upper critical temperature
dpf	days post-fertilization
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
EPO	erythropoietin
FIH	factor inhibiting HIF
HD	hypoxic desensitization
HIF	hypoxia-inducible factor
HRE	hypoxia responsive element
HSP	heat shock protein
HVD	hypoxic ventilatory decline
HVR	hypoxic ventilatory response
iNOS	inducible nitric oxide synthase
K_{ATP}	ATP-sensitive potassium channels
LOE	loss of equilibrium
LTF	long-term facilitation
NEC	neuroepithelial cell
nNOS	neuronal nitric oxide synthase
NODD	amino-terminal oxygen degradation domain
NO	nitric oxide
NOS	nitric oxide synthase
NTS	nucleus tractus solitarius
OCLTT	oxygen- and capacity-limited thermal tolerance
ODD	O_2-dependent degradation domain
PA	progressive augmentation
PAS	Per/ARNT/Sim domain
P_{crit}	critical oxygen tension
PHD	prolyl hydroxylase
pVHL	von Hippel–Lindau tumor-suppressor protein
STD	short-term depression
STP	short-term potentiation
VAH	ventilatory acclimation to hypoxia

Glossary

Critical O_2 tension (P_{crit})
The partial pressure of oxygen at which oxygen consumption begins to decline as a result of increasing hypoxic severity.

Genetic drift
Random changes in the frequencies of alleles over time within a population.

Cardiac hypoxic preconditioning
A phenomenon whereby the tolerance of the heart to severe hypoxia/ischemia is increased when it is preceded by brief periods of hypoxia exposure.

Isoform
Two or more functionally similar proteins that differ in primary sequence either because they are encoded by different genes or as a result of differential splicing.

Loss of equilibrium (LOE)
The point when a fish can no longer maintain an upright position.

Negative selection
Also know as purifying selection, this is the elimination of deleterious alleles from a population.

Ortholog
Corresponding genes in different species that evolved from a common ancestral gene.

Paralog
Genes that are the result of a gene (or genome) duplication event within a lineage.

Positive selection
Selection of an allele that increases fitness.

transcription levels of downstream target genes can be problematic and require cautious consideration in experimental design (see Box 1). Evidence of Hif-α activation has provided important information on the Hif-α-controlled hypoxic response across different fish species. Coupled with studies using genetic tools in genetically tractable species, most prominently the zebrafish (*Danio rerio*), substantial strides have been taken toward a greater understanding of the role of Hif-α in influencing whole-animal hypoxia tolerance (Joyce and Perry, 2020; Mandic et al., 2020) via systemic effects across the entire organism.

Genome-wide duplication events, both at the base of the vertebrate lineage and in the teleost-specific lineage, have created genetic diversity, the necessary raw material for evolutionary adaptation. Significant changes in the HIF pathway have occurred following these genome-wide duplication events (Loenarz et al., 2011; Rytkönen et al., 2011). Of particular interest are the cyprinid fishes, in which duplicate isoforms (see Glossary) of Hif-α have been retained following a teleost-specific genome duplication (Rytkönen et al., 2013). In this Review, we discuss the molecular evolution of the Hif pathway and the selective forces that have acted on the Hif-α isoforms, influencing the hypoxic response and adaptation to aquatic hypoxia, particularly in cyprinids. We describe the impact of the Hif pathway on hypoxia tolerance and survival, with a central focus on the zebrafish, both because it belongs to the Cyprinidae and because its genetic tractability has allowed for direct manipulation of several components of the Hif pathway. To date, the majority of research in fishes has focused on Hif-1α, rather than the Hif-2α or Hif-3α isoforms; as a result, the scope of this Review is limited primarily to a discussion of Hif-1α. In the second part of the Review, we discuss the recent advances pertaining to the role of Hif-1α in regulating cardiorespiratory function and draw parallels to relevant research in mammals. The influence of the Hif pathway goes beyond hypoxia, and Hif has been implicated in transcriptional regulation of ion homeostasis and the circadian clock (for review, see Pelster and Egg, 2018). Lastly, we discuss current knowledge on the role of Hif-1α during periods of thermal stress in fish. The involvement of Hif in protection against multiple disturbances to cellular homeostasis, including O_2, temperature and ionic fluctuations, signifies the broad mechanistic importance of this pathway to the defense against environmental change in teleost fishes.

Evolutionary change in the HIF pathway

The origin of the HIF pathway

Although at one time the HIF pathway was thought to be a universal O_2-sensing mechanism in the metazoans (Loenarz et al., 2011), evidence now suggests that it evolved once early in the metazoan history rather than in the last common ancestor of extant animals (Mills et al., 2018). Species in the phyla Porifera (sponges) and Ctenophora (ctenophores/comb jellies) lack the key HIF pathway machinery (Mills et al., 2018). As sponges and ctenophores are likely to be sister taxa to the Bilateria, Cnideria and Placozoa clade, it would appear that the HIF pathway evolved at the base of the latter clade following the split from sponges and ctenophores (Mills et al., 2018). The earliest metazoans, therefore, functioned in low-O_2 environments without HIF-controlled transcriptional responses.

All metazoans, with the exception of sponges and ctenophores, possess at least one copy of HIF-α and PHD, and a single ODD site

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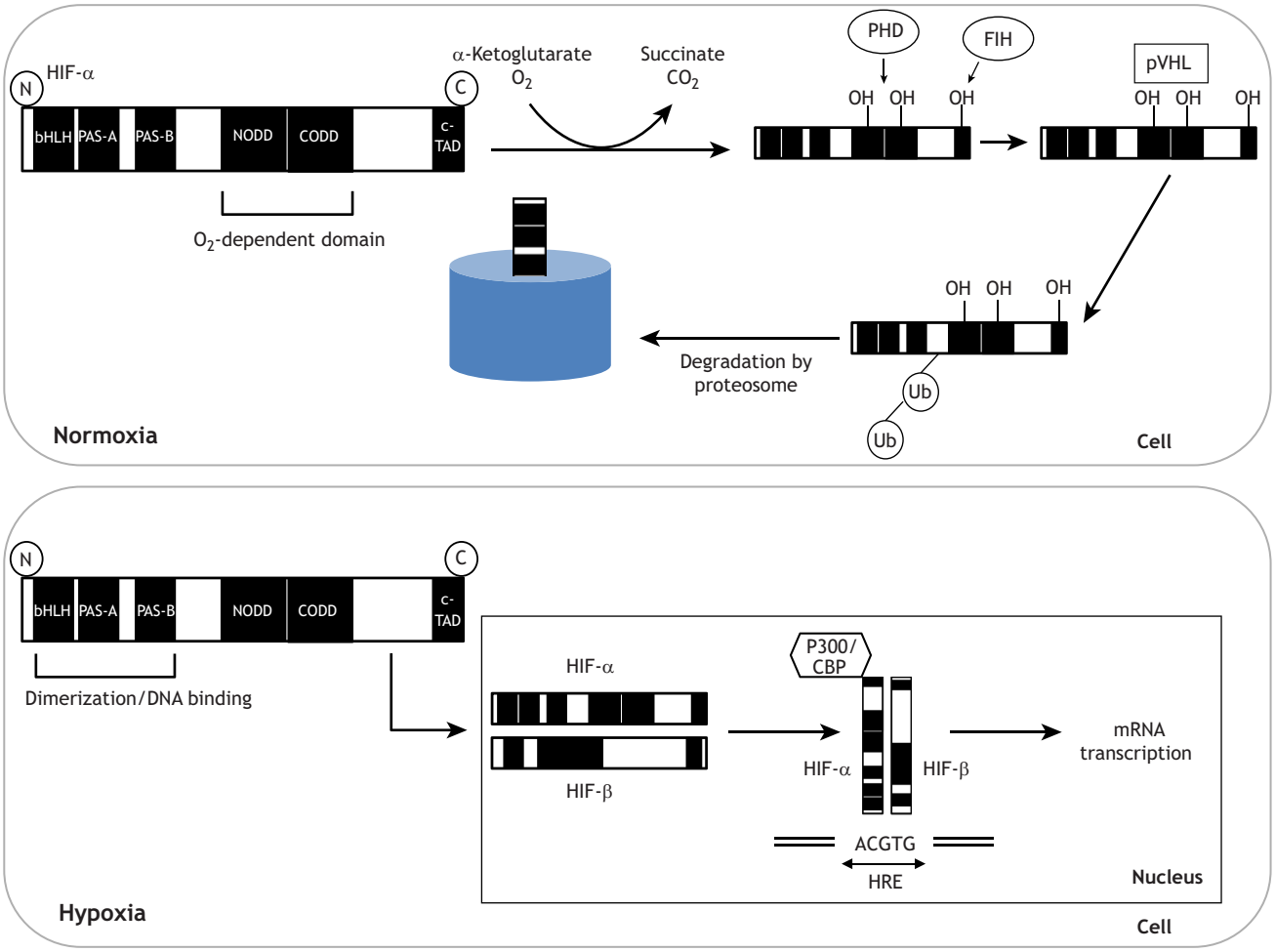

Fig. 1. Overview of the hypoxia-inducible factor (HIF) pathway. In normoxia, hydroxylation of HIF-1α by propyl hydroxylase (PHD) occurs in the amino- and carboxyl-terminal oxygen-dependent domains (NODD and CODD) and by factor inhibiting HIF (FIH) in the C-terminal-transactivation domain (c-TAD). Once NODD and CODD are hydroxylated, von Hippel–Lindau tumor-suppressor protein (pVHL) binds to HIF-1α, signaling the protein for ubiquitination (Ub). FIH-mediated hydroxylation of c-TAD prevents recruitment of transcriptional coactivators CBP and p300. The ubiquitinated HIF-α is then targeted for degradation by the proteasome. Under hypoxia, HIF-1α stabilizes, translocates into the nucleus, dimerizes with HIF-1β and – along with CBP and p300 – binds to hypoxia responsive elements (HREs) to alter the expression of hypoxia-inducible genes. PAS, Per/ARNT/Sim domain. For details, see text.

in the HIF-α protein (Loenarz et al., 2011). Non-bilaterian species and invertebrate bilaterians express one HIF-α (Graham and Presnell, 2017), with a single ODD site in the HIF-α of non-bilaterian species (Tarade et al., 2019). In contrast, HIF-α in most protostomes and deuterostomes has two ODD sites (Tarade et al., 2019), and in the vertebrate lineage there are three to four distinct HIF-α isoforms: HIF-1α, HIF-2α, HIF-3α and HIF-4α (Graham and Presnell, 2017; Townley and Rees, 2021 preprint). The increased number of HIF-α isoforms in vertebrates appears to be the result of two rounds of genome duplication in the ancestor of vertebrates, and further genome duplication events (e.g. teleost and salmonid specific) led to duplication of the HIF-1α and HIF-2α (*hif-1aa* and *hif-1ab*, *hif-2aa* and *hif-2ab*) isoforms that have been retained in only a few teleost lineages, e.g. cyprinids (see Box 2; Rytkönen et al., 2013; Townley and Rees, 2021 preprint).

Functional divergence of duplicates in the HIF pathway

Duplication of all or part of a genome is thought to be an effective mechanism in supplying raw genetic material to evolution (Ohno, 1970). Although the most likely outcome following a whole-genome duplication is the loss of the duplicated paralog (see Glossary) as a result of genetic drift (see Glossary), the duplicated genes can alternatively remain in the genome and evolve over time (Volff, 2005). Relaxed selective constraint on the copies of genes after a gene duplication event can lead to functional divergence and, in turn, greater functional scope. Following the vertebrate whole-genome duplication, the evolution of HIF-1α and HIF-2α isoforms has been similar across teleost and mammalian species with respect to exon structures, major regulatory domains and substitution rates (Rytkönen et al., 2011). However, relaxed selective constraint over evolutionary time has allowed for functional divergence (Zhang et al., 2010). The HIF-α isoforms differ in the timing of their signaling during hypoxia and in their regulation of the hypoxic response; HIF-1α typically governs the acute response to hypoxia, whereas HIF-2α may play a greater role during prolonged hypoxia (Holmquist-Mengelbier et al., 2006; Bartoszewski et al., 2019). Moreover, the two isoforms regulate the expression of unique gene targets (for review, see Loboda et al., 2012). The divergence of the roles of the two HIF-α isoforms in vertebrates over evolutionary time, coupled with a second ODD site in HIF-α and an increase in the number of PHD isoforms, allows for a more fine-tuned regulation of the HIF pathway and increasingly complex responses to hypoxia (Taylor and McElwain, 2010; Tarade et al., 2019).

Table 1. Summary of the results of studies that have documented increased levels of Hif-α isoforms in response to hypoxia in teleost fishes

Species	Level of hypoxia	Duration of hypoxia	Method	Citation
Astronotus crassipinnis	14 mmHg	1, 3, 5 h	mRNA of *hif-1α*	Heinrichs-Caldas et al., 2019
Astronotus ocellatus	10 mmHg	3 h	mRNA of *hif-1α*; mRNA of downstream genes	Baptista et al., 2016
Boleophthalmus pectinirostris	7 mmHg	3, 6, 12 and 24 h	Protein levels of Hif-1α; mRNA of *hif-1α*; mRNA of downstream genes	Guan et al., 2017
Callionymus valenciennei	41 mmHg	2 and 7 days	mRNA of *hif-1α*; mRNA of *hif-2α*	Kodama et al., 2012
Carassius carassius	9–12 mmHg	6, 24, 58 h	Protein levels of Hif-1α	Rissanen et al., 2006
Carassius carassius	9.4 mmHg	6, 24, 48 h	Protein levels of Hif-1α	Sollid et al., 2006
Chaenocephalus aceratus	68 mmHg	2 h	Protein levels of Hif-1α; mRNA of downstream genes	O'Brien et al., 2020
Clarias batrachus	17 mmHg	1 or 6 h	mRNA of *hif-1α*, *hif-2α*, *hif-3α*	Mohindra et al., 2013
Clupea pallasii	42 mmHg; 47 and 76 mmHg	0.5, 1, 2, 4, 8 and 16 h; 16 h	mRNA of *hif-1α*	Froehlich et al., 2015
Coregonus clupeaformis	24 and 54 mmHg; 36 and 60 mmHg	21, 38, 63, 83 and 103 dpf; 1, 2, 3 and 4 wph	mRNA of *hif-1α*; mRNA of downstream genes	Whitehouse and Manzon, 2019
Ctenopharyngod-on idellus	9 mmHg	4 and 96 h; 4 and 24 h	Protein levels of Hif-1α; mRNA of *hif-1α*;	Law et al., 2006
Danio rerio	16 mmHg	24 hpf, 48 hpf, 120 hpf, 10 dpf, 40 dpf (constant)	mRNA of *hif-1α*; mRNA of downstream genes	Ding et al., 2013
Danio rerio	22 mmHg	2 h	mRNA of downstream genes	Gerri et al., 2017
Danio rerio	12 mmHg	24 h	mRNA of *hif-1α*; mRNA of downstream genes	Kajimura et al., 2006
Danio rerio	38 mmHg	3 to 9 dpf (constant) 1 dpf, 2 dpf, 3 dpf (4 h)	Protein levels of Hif-1α, Hif-2α, Hif-3α	Köblitz et al., 2015
Danio rerio	75 mmHg 38 mmHg	9 to 15 dpf (constant)	Protein levels of Hif-1α and Hif-3α; mRNA of *hif-1α*, *hif-2α*, *hif-3α*; mRNA of downstream genes	Kopp et al., 2011
Danio rerio	36 mmHg	4 h	mRNA of *hif-1ab*; mRNA of downstream genes	Levesque et al., 2019
Danio rerio	0.76 mmHg 7.6 mmHg	18, 24, 36 hpf (4 h)	Protein levels of Hif-1α; mRNA of downstream genes	Robertson et al., 2014
Danio rerio	39 mmHg	6 h	mRNA of *hif-1aa*, *hif-1ab*, *hif-2aa*, *hif-2ab*	Rytkönen et al., 2013
Danio rerio	77 mmHg	8, 10, 12, 24 and 48 hpf	mRNA of *hif-1aa*, *hif-1ab*, *hif-2aa*, *hif-2ab*, *hif-3aa*, *hif-3ab*	Rytkönen et al., 2014
Dicentraarchus labrax	76 mmHg 34 mmHg	15 days (chronic) 4 h (acute)	mRNA of *hif-1α*	Terova et al., 2008
Fundulus heteroclitus	15 mmHg	28 days (constant) 28 days (intermittent) 12 h (acute)	Protein levels of Hif-1α	Borowiec et al., 2018
Gillichthys mirabilis	15 mmHg	6, 12, 24, 120 and 192 h	mRNA of *hif-1α*; mRNA of downstream genes	Gracey et al., 2011
Gobionotothen gibberifrons	68 mmHg 30 mmHg	2 h 12 h	mRNA of downstream genes	O'Brien et al., 2020
Hemiscyllium ocellatum	8 mmHg	2 h 12 h (intermittent)	mRNA of *hif-1α* and *hif-2α*	Rytkönen et al., 2012
Ictalurus punctatus	1 mg l^{-1}*	1.5 h 5 h	mRNA of *hif-1α*, *hif-2αa*, *hif-2αb*, *hif-3α*	Geng et al., 2014
Larimichthys crocea	97, 75 and 54 mmHg	3, 6, 12, 24, 48, 72 and 96 h	mRNA of *hif-1α*	Wang et al., 2017a,b
Lepisosteus oculatus	65 mmHg	71 days	mRNA of *hif-2α*	Rimoldi et al., 2016
Megalobrama amblycephala	18 mmHg	4 h	mRNA of *hif-1α*; mRNA of *hif-2α*	Shen et al., 2010
Megalobrama amblycephala	56 mmHg; continuous decrease to 65 mmHg; continuous decrease to 24 mmHg	4 and 12 h; 4 h; 10 h	mRNA of *hif-1α*	Wang et al., 2015a
Micropterus salmoides	17 mmHg	1, 2, 4, 8, 12 and 24 h	mRNA of *hif-1α*; mRNA of downstream genes	Yang et al., 2017

Continued

Table 1. Continued

Species	Level of hypoxia	Duration of hypoxia	Method	Citation
Micropogonias undulatus	39 mmHg; 39, 63 and 86 mmHg	1, 3 and 7 days; 3 weeks	mRNA of *hif-1α*; mRNA of *hif-2α*	Rahman and Thomas, 2007
Myxocyprinus asiaticus	40 mmHg 51 mmHg 59 mmHg	24 h	mRNA of *hif-1α*, *hif-2α*, *hif-3α*	Chen et al., 2012
Notothenia coriiceps	68 mmHg 30 mmHg	2 h 12 h	Protein levels of Hif-1α; mRNA of downstream genes	O'Brien et al., 2020
Oncorhynchus mykiss	7.5 mmHg 150-1.5 mmHg	2 and 4 h 1 h	mRNA of *hif-1α*; Protein levels of Hif-1α	Soitamo et al., 2001
Oncorhynchus tshawytscha	7.5 mmHg 150-1.5 mmHg	2 and 4 h 1 h	mRNA of *hif-1α*; Protein levels of Hif-1α	Soitamo et al., 2001
Oreochromis niloticus	119 mmHg	18 h	mRNA of *hif-1α*	Abarike et al., 2020
Oreochromis niloticus	13 mmHg	6, 12, and 24 h	mRNA of *hif-1α*	Li et al., 2017
Pelteobagrus vachelli	13 mmHg	6.5 h	mRNA of *hif-1α*, *hif-2α*, *hif-3α*	Zhang et al., 2017
Perca fluviatilis	46 mmHg	15 days	mRNA of *hif-1α*	Rimoldi et al., 2012

Increased levels of Hif-α were assessed directly by measuring changes in protein levels or indirectly through measurements of *hif-α* mRNA levels or mRNA of downstream Hif-α gene targets. hpf, hours post-fertilization; dpf, days post-fertilization; wph, weeks post-hatch. *No temperature reported.

In cyprinids, the significance of the retention of duplicated paralogs of HIF-α and PHD isoforms is not well understood. There is evidence of subfunctionalization, the process whereby the functions of the ancestral gene are divided among the duplicates (Rytkönen et al., 2013). In zebrafish, Hif-1aa evolved faster than Hif-1ab and under relaxed negative selection (see Glossary) in the ODD domain, suggesting that Hif-1aa is a less-sensitive O_2 regulator than its paralog (Rytkönen et al., 2013). Transcriptional patterns are consistent with this observation, with prominent expression changes occurring during development for the *hif-1aa* paralog and in response to changes in O_2 for *hif-1ab* (Rytkönen et al., 2013). In fishes of the cyprinid sub-family Schizothoracinae, which occupy the high-altitude lakes of the Tibetan plateau, *hif-1ab* also appears to be the more responsive paralog under hypoxic conditions (Guan et al., 2014). In addition to differences in transcription patterns, there is evidence of functional divergence of paralogs in their contribution to the control of breathing and overall hypoxia tolerance (Mandic et al., 2019, 2020), which will be discussed further below.

Molecular evolution of the HIF-α proteins

Computational methods have been used to identify the type of selective forces that have shaped the evolution of HIF-α proteins in vertebrates (Rytkönen et al., 2011; Graham and Presnell, 2017), particularly to determine whether there are signatures of selection in the HIF-α coding sequence that can be associated with variation in species' environmental O_2 profiles (Rytkönen et al., 2007, 2008). Broadly, across vertebrate phylogeny, crucial interaction domains of HIF-α have been under stringent negative or purifying selection (Rytkönen et al., 2008, 2011; Zhang et al., 2010; Graham and Presnell, 2017). It is perhaps not surprising that selection has acted against variants that would impact DNA-binding specificity (bHLH domain) or protein dimerization (PAS domain) of HIF-α. There is little general evidence for positive selection (see Glossary) in HIF-α isoforms (Rytkönen et al., 2008, 2011; Graham and Presnell, 2017), and no clear signatures of selection were detected in a comparison of teleost species with differences in hypoxia tolerance (Rytkönen et al., 2007). However, relaxation of negative selection is evident in less crucial domains of Hif-1α of teleost species as compared with HIF-1α of mammals, which was suggested to be a consequence of the greater O_2 variability in the aquatic environment (Rytkönen et al., 2008).

Studies of mammalian and teleost lineages living under high-altitude hypoxic conditions have detected signatures of selection on some HIF pathway genes. Relevant mammalian species include high-altitude human populations, Tibetan pigs, Tibetan mastiffs, Tibetan horses and high-altitude North American deer mice (Beall et al., 2010; Bigham et al., 2010; Hanaoka et al., 2012; Lorenzo et al., 2014; Scheinfeldt et al., 2012; Ma et al., 2019; Miao et al., 2016; Liu et al., 2019; Schweizer et al., 2019); high-altitude teleost lineages include the Schizothoracinae and members of the genus *Triplophysa* that are endemic to the Qinghai-Tibetan Plateau lakes and rivers. These aquatic habitats range in elevation from 700 to 5000 m and are marked by cold and hypoxic conditions (Wu and Wu, 1992). The Schizothoracinae and *Triplophysa* fishes are cyprinids, and like zebrafish, have retained duplicated paralogs of Hif-α (Guan et al., 2014; Chen et al., 2020). A specific mutation from LxxLAP to PxxLAP in the CODD of Hif-1ab was found in both lineages, and is thought to have functional consequences for the ODD (Guan et al., 2014; Wang et al., 2020). Significant evidence of positive selection was detected in Hif-1ab in schizothoracine fishes (Guan et al., 2014), whereas in *Triplophysa* fishes, Hif-1aa, Hif-1ab, Hif-2a and Hif-2ab were subject to positive selection (Wang et al., 2015b,c). Unlike in mammals, where positive selection predominantly affected one HIF-α isoform, it would appear that all paralogs of Hif-1α and Hif-2α have undergone selection in the fishes of the Tibetan Plateau, presumably contributing to their adaptation to the high-altitude hypoxic habitat.

Contribution of the HIF pathway to hypoxia tolerance and survival

The HIF pathway plays a pivotal role in the maintenance of cellular O_2 homeostasis; as such, there is significant interest in determining its role in hypoxia tolerance and survival. In mice, HIF-1α (Iyer et al., 1998; Compernolle et al., 2003), HIF-2α (Tian et al., 1998), PHD2 (Takeda et al., 2006) and pVHL (Kapitsinou and Haase, 2008) are all essential for normal development and survival; mice lacking the expression of these proteins die at the embryonic or fetal stage. The HIF pathway also contributes to increased tolerance and survival in mice exposed to hypoxia (Wang et al., 2017a,b; Kasiganesan et al., 2007; Aragones et al., 2008).

In zebrafish, it is possible to generate a viable mutant exhibiting complete loss of *hif-1α* (knockout of both *hif-1α* paralogs)

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Box 1. Challenges of assessing Hif-1α activation in fishes

Twenty years ago, a group of researchers led by Professor Mikko Nikinmaa published the first paper demonstrating a potential role for Hif-1α in promoting the physiological responses to hypoxia in fish (Soitamo et al., 2001). In many respects, this landmark publication has served as the gold standard with which to compare and guide further studies in this area. In particular, the study of Soitamo et al. (2001) was noteworthy for its development, validation and utilization of homologous polyclonal antibodies to quantify Hif-1α protein levels. The study demonstrated that increased levels of Hif-1α protein during hypoxia in trout cell cultures arise exclusively from its post-translational stabilization and occur in the absence of any changes in *hif-1α* mRNA levels. Thus, it cannot be assumed that measurements of *hif-1α* mRNA levels alone will provide evidence that Hif-1α protein levels are increasing during hypoxia. Indeed, the few studies that have measured both *hif-1α* mRNA and Hif-1α protein levels using homologous antibodies (e.g. Law et al., 2006; Kopp et al., 2011; Guan et al., 2017) demonstrated discrepancies between mRNA and protein levels that may vary with the duration and severity of hypoxia as well as the specific tissues being examined. A second important finding of the Soitamo et al. (2001) study was the observation that although Hif-1α protein increases in cultured trout cells exposed to hypoxia, peak levels are achieved with relatively mild exposure (38 mmHg) and actually decrease at the most severe levels of hypoxia employed. Therefore, selecting an appropriate level of hypoxia is crucial in studies designed to investigate Hif-1α function in fishes.

In light of the complexities associated with the interpretation of measurements of Hif-1α protein and/or mRNA levels, the determination of transcript abundance of Hif-1α downstream target genes is another common technique used to confirm activation of the Hif-α pathway (see Table 1). However, an elevation of Hif-1α protein is not always accompanied by increased mRNA levels of downstream target genes (e.g. O'Brien et al., 2020) or vice versa. In other cases, only a subset of classic hypoxia-responsive genes may be affected, with others remaining unchanged (e.g. Robertson et al., 2014).

Box 2. Rise of vertebrate HIF-α and PHD isoforms following multiple genome duplication events

Two rounds of gene duplication events prior to the divergence of cartilaginous fishes from tetrapods and teleosts led to the rise of the multiple HIF-α isoforms from a single ancestral copy (Rytkönen et al., 2011; Townley and Rees, 2021 preprint). Previously, there were thought to be three isoforms – HIF-1α, HIF-2α and HIF-3α – but recent evidence suggests that a fourth isoform, HIF-4α, also arose during this period (Graham and Presnell, 2017; Townley and Rees, 2021 preprint). However, unlike the other three isoforms, HIF-4α appears to be absent in Neoteleostei fishes (Townley and Rees, 2021 preprint) and in mammals (Law et al., 2006).

Additional whole-genome duplication led to a further increase in the number of HIF-α genes. Paralogs of two of the HIF-α genes (*hif-1aa* and *hif-1ab*, *hif-2aa* and *hif-2ab*) that arose during the teleost-specific genome-wide duplication have been retained in certain teleost lineages (Rytkönen et al., 2011, 2013). Some evidence suggests that both paralogs of HIF-3α have also been maintained (Rytkönen et al., 2013), although a loss of one of the paralogs of both HIF-3α and HIF-4α has been put forth as a different hypothesis for the evolution of HIF-α proteins (Townley and Rees, 2021 preprint). The duplicated paralogs were lost in all lineages with the exception of species in the cyprinid lineage (e.g. zebrafish) (Rytkönen et al., 2011) and, more broadly, species belonging to Otocephala (e.g. Atlantic herring) (Townley and Rees, 2021 preprint). Salmoniformes have also been found to have duplicated paralogs of HIF-1α, HIF-2α and HIF-3α that arose during the salmonid-specific genome duplication (Townley and Rees, 2021 preprint).

Similar patterns over evolutionary history are seen in the PHD isoforms. Most invertebrate species possess one PHD gene, with evidence of two PHD genes in chordate and cnidarian lineages, whereas, in vertebrates, there are three PHD isoforms (PHD1, PHD2 and PHD3) that arose from the ancestral invertebrate copy during the two successive genome duplication events (Loenarz et al., 2011; Rytkönen et al., 2011). The duplicates of PHD that arose during the teleost-specific genome-wide duplication were lost over time except in the cyprinids (Rytkönen et al., 2011).

(Gerri et al., 2017). Likewise, knockout of other components of the Hif pathway in zebrafish, such as *pvhl* (van Rooijen et al., 2009), *fih* (Cai et al., 2018) and *hif-3α* (Cai et al., 2020), also results in embryonically viable mutants. It is unknown why such knockouts do not cause early embryonic death as in mammals. Data suggest that, in mammals, loss of *hif-1α* results in developmental defects because the HIF pathway is integral to development itself, independent of its role in the hypoxic response (Compernolle et al., 2003). In fishes, however, there are likely to be species-specific differences in the involvement of the Hif pathway in development (Pelster and Egg, 2018). For example, in *Salmo salar*, reduced DNA-binding activity of Hif-1α is associated with high mortality at the yolk-sac fry stage (Vuori et al., 2004). In zebrafish, however, the development of viable embryos in knockouts (Gerri et al., 2017) suggests that Hif isoforms are not essential for development and that alternative mechanisms can compensate for functional disruption of the Hif pathway. Regardless of the underlying reason, the existence of viable mutants makes zebrafish a tractable system in which to test the contribution of the Hif pathway to the hypoxic response.

Loss of *hif-1α* (both paralogs) reduces hypoxia tolerance in adult zebrafish (Joyce and Perry, 2020; Mandic et al., 2020), as assessed by time to reach loss of equilibrium (LOE; see Glossary) when exposed to hypoxia. Individuals with a knockout of either *hif-1aa* or *hif-1ab* have significantly lower time to LOE as compared with wild-type individuals, while the shortest time to LOE occurs in the mutants with knockout of both paralogs (Fig. 2A), indicating the greatest impairment of hypoxia tolerance (Mandic et al., 2020). In larval zebrafish, loss of *hif-1α* increases critical O_2 tension (P_{crit}; see Glossary), indicating a decrease in hypoxia tolerance, an effect that is primarily attributable to the Hif-1aa and not Hif-1ab paralog (Fig. 2B; Mandic et al., 2020). The impact of prior hypoxia on a subsequent bout of hypoxia has been noted in larval zebrafish. Hypoxic exposure of larvae at 24 h post-fertilization leads to an increased ability to regulate and maintain O_2 uptake during subsequent hypoxia at 4 days post-fertilization (dpf), an effect that is associated with increased levels of Hif-1α (Robertson et al., 2014). A link between enhanced hypoxia tolerance and the Hif pathway after previous hypoxia exposure was confirmed in *hif-1α* knockout zebrafish larvae (Mandic et al., 2020). At 7 dpf, P_{crit} was significantly lower (indicative of enhanced hypoxia tolerance) in wild-type larvae that had been previously exposed to hypoxia (90 mmHg) for 3 days, whereas it was unaltered in *hif-1aa* knockouts (Fig. 2B). These results suggest that increases in Hif-1aa, but not Hif-1ab, during hypoxia confer increased tolerance during subsequent episodes of hypoxia in larval fish, pointing to functional divergence and possible subfunctionalization of the two paralogs (Mandic et al., 2020). A tentative link between the Hif-1α pathway and increased survival as a result of repeated hypoxic exposure has also been shown in adult zebrafish. Zebrafish previously exposed to a milder hypoxia (15 mmHg O_2) have a higher survival rate during more severe hypoxia (8 mmHg O_2) (Rees et al., 2001), and are found to have up-regulated glycolytic enzymes, which are downstream targets of Hif-1α, in skeletal muscle (Chen et al., 2013). The exact mechanism by which Hif-1α

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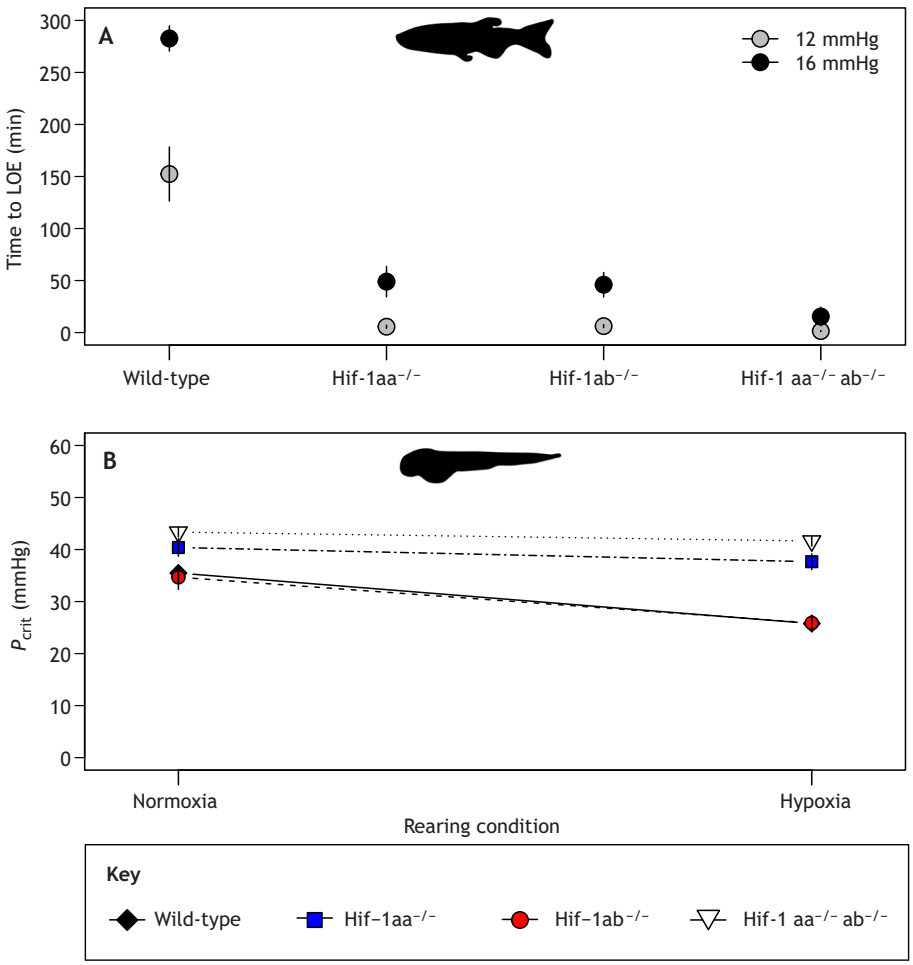

Fig. 2. Hypoxia tolerance in adult and larval zebrafish (*Danio rerio*) in wild-type and Hif-1α knockouts. (A) Time to loss of equilibrium (LOE) is significantly reduced in the single paralog knockouts, yielding a similar decrease in hypoxia tolerance as compared with wild-types in 12 or 16 mmHg hypoxia. The double knockout of Hif-1α has the lowest time to LOE, regardless of the level of hypoxia exposure. (B) Double knockout of Hif-1α results in a significantly higher critical O_2 tension (P_{crit}; lower hypoxia tolerance) in 7 days post-fertilization (dpf) larvae reared in normoxia (153 mmHg), and prior exposure of larvae to hypoxia (90 mmHg) from 4 to 7 dpf results in a decrease in P_{crit} in wild-types, an effect mediated by the Hif-1aa paralog. Data from Mandic et al. 2020.

contributes to both hypoxia tolerance and increased tolerance as a result of prior exposure is unknown, and continued effort is required to identify the exact role of each Hif-1α paralog in conferring hypoxia tolerance in fishes.

Loss of other components of the Hif pathway also affects hypoxia tolerance in zebrafish. For example, a decrease in hypoxia tolerance and survival occurs in adult zebrafish with knockout of *hif-3α*, probably due to reduced erythropoiesis via regulation of *gata1* expression (Cai et al., 2020). Similar to mice, knockout of *tet1* results in reduced survival in adult zebrafish (Wang et al., 2017a,b), and a deletion of the *fih* gene, an inhibitor of Hif-α transcriptional activity, increases hypoxic survival of both larval and adult zebrafish (Cai et al., 2018). Taken together, the data indicate a significant contribution of the Hif pathway to hypoxia tolerance in zebrafish. However, it is unknown whether there are functional differences in the Hif pathway among fish species that could explain the variation in hypoxia tolerance. Although there are no clear signatures of selection in the Hif-1α protein-coding sequence between hypoxia-tolerant and -intolerant fishes (Rytkönen et al., 2007), species-specific functional contribution of the Hif pathway to variation in hypoxia tolerance has not been assessed.

Hif-1α and the hypoxic ventilatory response

Fish rely on a well-coordinated cardiorespiratory response during exposure to hypoxia to minimize the consequences of O_2 limitation on physiological function. As P_{O_2} drops to a critical level in the environment, most fish initiate an increase in ventilation frequency and/or amplitude, a reflex known as the hypoxic ventilatory response (HVR; Perry et al., 2009). The onset of the HVR can be rapid, occurring within seconds of the onset of hypoxia, and it can be sustained indefinitely with chronic hypoxia. However, over the course of hypoxia, ventilation patterns can shift, creating discrete ‘time domains’ of ventilatory adjustment that reflect the changes in physiological mechanisms underlying the HVR (Powell et al., 1998; Porteus et al., 2011). Although there are a number of potential mechanisms and pathways underlying such time domains, significant attention has focused on the role of Hif-1α, particularly in mammals (Prabhakar and Jacono, 2005; Prabhakar and Semenza, 2012, 2016; Pamenter and Powell, 2016). The discrete time domains of the HVR depend on the duration and intensity of hypoxic exposure, falling into acute, sustained long-term or intermittent long-term patterns (Fig. 3; Powell et al., 1998). The contribution of Hif-1α to each of these patterns is discussed in more detail below.

Acute hypoxic time domains

Ventilatory responses are initiated by O_2 chemoreceptors, and in hypoxia, HIF-1α expression increases in the peripheral O_2 chemoreceptors, which in mammals are type I or glomus cells of the carotid body (Kline et al., 2002; Roux et al., 2005). This finding provided some of the earliest evidence of the possible involvement of HIF-1α in the sensory arm of the HVR. The acute time domain of the HVR begins within one to two breaths under hypoxia and, in mammals, is broken down into the acute response (AR) lasting seconds, and short-term potentiation (STP) and short-term depression (STD), lasting seconds to minutes (Fig. 3; Powell et al., 1998). During exposure to acute hypoxia (5 min), mice with

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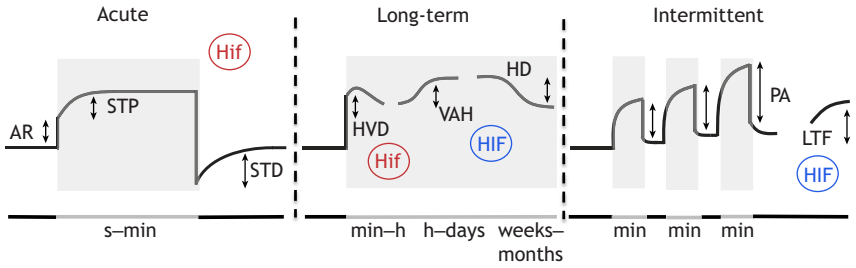

Fig. 3. Time domains of the hypoxic ventilatory response. The acute time domain consists of the acute response (AR), short-term potentiation (STP) and short-term depression. Hif-1α in fish (red) plays a role in the HVR during this phase, although the exact mechanism is unknown. During long-term hypoxia, the ventilatory time domains are hypoxic ventilatory decline (HVD), ventilatory acclimation to hypoxia (VAH) and hypoxic desensitization (HD). In fish, Hif-1α appears to have an impact on the HVD and in mammals, HIF-1α (blue) impacts the VAH. The intermittent time domain consists of progressive augmentation (PA) and long-term facilitation (LTF), with evidence of HIF-1α involvement in LTF in mammals. Gray-shaded boxes indicate the ventilatory response (ventilation volume) to hypoxia, with duration of exposure indicated on the *x*-axis. Schematic diagram adapted from Powell et al., 1998.

heterozygous loss of function of the *hif-1α* gene (*hif-1α*$^{+/-}$) have no impairment of the HVR (Kline et al., 2002; Peng et al., 2006). In both wild-type and *hif-1α*$^{+/-}$ mice, ventilation increases to a similar degree in response to hypoxia. However, the *hif-1α*$^{+/-}$ mice exhibit decreased carotid body sensitivity, suggesting that chemoreceptors elsewhere compensate for the loss of carotid body function, thus maintaining HVR (Kline et al., 2002). Considering the short interval of hypoxia exposure, mechanisms underlying HVR are unlikely to depend on changes in gene expression levels; thus, it is not surprising that evidence points to a lack of HIF-1α involvement in the control of breathing during the acute time domain in mammals.

In fish, little is known about patterns of ventilatory adjustment during the acute phase of the HVR (Porteus et al., 2011), although, as in mammals, acute adjustments of ventilation are unlikely to involve Hif-1α. However, if the acute phase of the HVR is considered on a longer time scale (several minutes to an hour), Hif-1α plays a role in the HVR in fish (Perry and Tzaneva, 2016; Mandic et al., 2019). The role of Hif-1α in the hypoxic control of breathing is particularly evident in zebrafish larvae. For example, when the Hif-1ab paralog is knocked down using a splice-blocking morpholino, larvae display a blunted HVR during exposure to acute hypoxia (40 mmHg for 30 min). Thus, in 4 dpf larvae, Hif-1α expression is required to mount a normal HVR (Perry and Tzaneva, 2016). Similarly, knockout of either the *hif-1aa* or *hif-1ab* paralog leads to significant blunting or abolishment of the HVR during acute hypoxia (55 mmHg for 30 min) in the early stages of zebrafish development (Mandic et al., 2019). As larvae age, the contribution of the Hif-1α paralogs lessens, and by 15 dpf, the HVR does not differ between wild-type and *hif-1α* knockout zebrafish. In adult zebrafish, a modest difference in the HVR is observed in *hif-1ab* knockouts during exposure to acute hypoxia (30 mmHg for 1 h), whereas there is no effect of *hif-1aa* knockout (Mandic et al., 2019). Thus, in zebrafish, there is a clear involvement of Hif-1α in regulating the magnitude of the HVR, playing a more important role in the early larval stages. The extent to which Hif-1α regulates the HVR during acute hypoxia across different species of fish is of interest, particularly given that many water-breathing species, unlike zebrafish, enhance ventilation by increasing ventilatory stroke volume rather than frequency.

The mechanisms whereby HIF-1α regulates the HVR are unknown. In mammals, a role for several HIF-1α target genes – including nitric oxide (NO), erythropoietin (EPO) and endothelin-1 (ET-1) – has been proposed. These candidates have been implicated in contributing to the plasticity of the HVR (Kuwaki et al., 1996; Soliz et al., 2005; Prabhakar et al., 1993). In fish, NO is an intriguing candidate, because an increase in NO following activation of Hif-1α-responsive neuronal nitric oxide synthase (nNOS) stimulates breathing in larval zebrafish (Porteus et al., 2015). Additionally, Porteus et al. (2015) reported the localization of nNOS to larval skin and adult zebrafish gill O_2-sensing neuroepithelial cells (NECs), which are the functional equivalent of the glomus cells of the carotid body (Zaccone et al., 1989; Milsom and Burleson, 2007; Porteus et al., 2012). In 10 dpf zebrafish larvae, loss of the *hif-1ab* paralog results in an attenuated HVR, which is not blunted further by exposure to the nitric oxide synthase (NOS) inhibitor L-NAME (N^{ω}-nitro-L-arginine methyl ester hydrochloride) during acute hypoxia. In contrast, wild-type larvae exhibit a decrease in the HVR upon exposure to L-NAME (Mandic et al., 2019). These results suggest that NO produced via nNOS contributes to the mechanism of action of the Hif-1ab paralog. To date, the links between NO, HVR and the Hif-1aa paralog have not been tested.

Long-term hypoxic time domain

During sustained long-term hypoxia, ventilatory time domain phases include hypoxic ventilatory decline (HVD; a decrease in ventilation relative to the initial increase), ventilatory acclimation to hypoxia (VAH; an increase in ventilatory O_2-sensitivity) and hypoxic desensitization (HD; a decrease in ventilatory O_2-sensitivity; Fig. 3) (Powell et al., 1998; Porteus et al., 2011). In mammals, one of the better understood long-term time domain responses is VAH, which involves remodeling both at the level of the carotid body and within the nucleus tractus solitarius (NTS) of the central nervous system (CNS; Pamenter and Powell, 2016). Although HIF-1α may not be essential for the acute phase of the HVR, it may play a role in fine-tuning VAH in mice during long-term hypoxia (Kline et al., 2002; Peng et al., 2006; Yuan et al., 2013). Specifically, during acute hypoxia that follows exposure to chronic hypoxia, wild-type mice increase ventilation, exhibiting greater ventilatory O_2-sensitivity. This increase in ventilation is reduced in heterozygous *hif-1α* mice (Kline et al., 2002), indicating an attenuated VAH response. Although the exact mechanism of action of *hif-1α* on VAH remains unknown, selective deletion of *hif-1α* in the CNS (Bavis et al., 2007), and specifically in the NTS (Moya et al., 2020), blunts the VAH in mice, suggesting that HIF-1α contributes to CNS-mediated control of the HVR. Together, these studies indicate that, in mammals, VAH is likely to be regulated by HIF-1α both at the level of the carotid body and within the NTS of the CNS.

Few studies have explicitly investigated time domains in fish exposed to prolonged hypoxia (e.g. in *Amia calva*; Porteus et al.,

2014) and, with the exception of a single study (Mandic et al., 2021), none have examined whether Hif-1α contributes to the ventilatory time domains. There was evidence of HVD but not VAH in adult zebrafish exposed to prolonged moderate hypoxia (72 h at 90 mmHg; Mandic et al., in revision). The pronounced HVD in zebrafish was not unexpected, given that a decrease in ventilation during prolonged hypoxia lowers the ventilatory convection requirement, thereby reducing the energetic costs of breathing, which are higher in water-breathers than in air-breathers (Dejours, 1976; Maina, 2000). Control of breathing during prolonged hypoxia in teleosts appears to be influenced by Hif-1α; zebrafish with knockout of either *hif-1aa* or *hif-1ab* exhibit a delay in the onset of HVD by 2–3 h as compared with wild-types (Mandic et al., in revision). During recovery from prolonged sustained hypoxia, wild-type zebrafish exhibit a pronounced undershoot in ventilation frequency or hypoventilation, a response that disappears in fish with combined knockout of both *hif-1α* paralogs. The recovery phase of the HVR is not well understood in fishes, but in zebrafish it appears to be regulated, at least in part, by Hif-1α.

Intermittent long-term time domains

Ventilatory responses during intermittent or episodic hypoxia are characterized by two time domains; progressive augmentation (PA), an increase in the magnitude of ventilation in successive identical cycles of hypoxia, and long-term facilitation (LTF), an increase in baseline ventilation (Fig. 3; Powell et al., 1998; Porteus et al., 2011). During chronic intermittent hypoxia, LTF was found to be a HIF-1α-dependent response in mice (Peng et al., 2006). Ventilation is significantly higher for 2 h following termination of chronic intermittent hypoxia in wild-type mice, whereas no significant change in baseline was noted in the heterozygous mice with partially deficient *hif-1α* (Peng et al., 2006), implicating HIF-1α in the control of LTF. Little is known of the time domains during intermittent hypoxia in fish and the contribution, if any, of Hif-1α.

Role of Hif-1α in the fish heart

The regulation of cardiac function is critical in fish during hypoxia exposure (Gamperl and Driedzic, 2009), and as HIF-1α is highly expressed within the myocardium (e.g. Rissanen et al., 2006), it is well positioned to play a leading role in regulating changes in cardiac metabolism and electrophysiology. As with the ventilatory responses to hypoxia, we will consider the functions of Hif-1α in the fish heart during acute and long-term hypoxia, as well as the central roles Hif-1α plays in cardiac hypoxic preconditioning (see Glossary) and cardiac regeneration.

Cardiac function in acute hypoxia

Within a matter of hours (acute exposure), hypoxia can increase Hif-1α protein expression in fish myocardium (Imbrogno et al., 2014; O'Brien et al., 2020). This is evident in excised hearts perfused with hypoxic saline (Imbrogno et al., 2014), and thus is intrinsic to the heart (i.e. it does not rely on extrinsic signals associated with oxygen sensing *in vivo*).

Like the Hif-1α-dependent regulation of ventilation (see above), the downstream effects are likely to be mediated, at least in part, by NO. In goldfish (a cyprinid) hearts, the increased Hif-1α expression during hypoxia coincides with increased phosphorylation of NOS (Imbrogno et al., 2014). The authors reported this to be endothelial NOS (eNOS), although no ortholog (see Glossary) for eNOS has yet been identified in fish (Syeda et al., 2013), so it seems more likely to represent another paralog (with cross-reactivity to the antibody employed), such as inducible NOS (iNOS). Phosphorylation of NOS is believed to promote its activity (Mount et al., 2007), and in mammalian cardiomyocytes it has also been shown that Hif-1α is necessary for increased iNOS expression during hypoxia (Jung et al., 2000). ATP-sensitive potassium (K_{ATP}) channels provide a mechanism to shorten action potential duration (APD) when ATP levels fall during hypoxia, enabling an energy-conserving mechanism to help match O_2 demand to a falling supply; these are a likely target of Hif-1-induced NO. In the goldfish heart, NO donors increase K_{ATP} channel activation and shorten APD, whereas NOS inhibition reduces hypoxia-induced K_{ATP} channel activation (Cameron et al., 2003). NO-dependent activation of K_{ATP} channels can thereby increase goldfish cardiomyocyte survival during hypoxia (Chen et al., 2005). High NO concentrations are able to stabilize HIF-1α (Mateo et al., 2003); thus, positive feedback between HIF and NO is likely to be important in regulating fish cardiac performance during hypoxia (Gattuso et al., 2018; Imbrogno et al., 2014; Leo et al., 2019).

Cardiac function during long-term hypoxia

Long-term hypoxia acclimation (i.e. exposure for days to weeks) also increases gene expression of Hif-1α and downstream target genes in zebrafish (21 days at P_{O_2} of 15 mmHg) (Marques et al., 2008) and goldfish (7 days at P_{O_2} of 46.5 mmHg) (Cameron et al., 2013) hearts. In goldfish, this is associated with shortened APD and increased K_{ATP} channel activity, as well as increased nitric oxide synthase (nos2; iNOS) gene expression (Cameron et al., 2013), suggesting that the same protective mechanisms invoked under acute hypoxia may be sustained for long-term acclimation. In a number of other fish species, hypoxia acclimation results in a suite of other changes in cardiac physiology, from reduced maximum cardiac output (Motyka et al., 2017; Petersen and Gamperl, 2010) and myocardial contractility (Carnevale et al., 2019) (possibly attributable to shortened APD), to altered mitochondrial form and function (Du et al., 2016; Lennard and Huddart, 1992). It may be fruitful for future studies to explore the potential role for different Hif isoforms in regulating each of these facets of cardiac physiology during long-term hypoxia acclimation.

Cardiac hypoxic preconditioning and regeneration

The effects of intermittent, fluctuating hypoxia may be particularly relevant in the heart, which is known to display hypoxic preconditioning (Kloner and Jennings, 2001; Murry et al., 1986). Although first described in mammalian hearts, preconditioning has since been shown in teleost species, including rainbow trout (*Oncorhynchus mykiss*) and cod (*Gadus morhua*) (Gamperl and Farrell, 2004; Gamperl et al., 2001; Overgaard et al., 2004). Thus, preconditioning may represent a common trait in the vertebrate heart.

In mammalian hearts, HIF-1α plays a critical role in preconditioning; inactivation of HIF-1α prevents preconditioning, whereas its pharmacological or genetic activation confers preconditioning-like protection from ischemia (Cai et al., 2008; Eckle et al., 2008). In the epaulette shark (*Hemiscyllium ocellatum*), *in vivo* exposure to repeated bouts of acute hypoxia activates Hif-1α gene expression in the heart, even when a single bout of hypoxia does not, providing molecular evidence for the induction of preconditioning (Rytkönen et al., 2012). In zebrafish hearts, Hif-dependent gene expression also increases during the period of reoxygenation following hypoxia (Parente et al., 2013). Unsurprisingly given its renowned importance in cardiac adaption to hypoxia, K_{ATP} channel activation appears to be important in hypoxic preconditioning of the mammalian heart (Ghosh et al., 2000; Gross and Peart, 2003), and is hypothesized to be involved in

the fish heart (Gamperl and Farrell, 2004), although this has yet to be substantiated.

Adenosine receptor activation may also be key to cardiac preconditioning in both mammals and fish. In the mammalian heart, the accumulation of adenosine and increased expression of adenosine receptors (specifically adenoreceptor 2B) – both of which are normally associated with preconditioning – are attenuated or abolished following Hif-1α inactivation (Eckle et al., 2008). Furthermore, HIF-activated cardioprotection is eliminated in adenoreceptor 2B knockout mice (Eckle et al., 2008). In *H. ocellatum*, adenosine receptor and transporter expression increase following preconditioning-like recurrent hypoxia exposure, but not after a single bout of hypoxia, strikingly mirroring the changes in Hif-1α gene expression (Rytkönen et al., 2012). Like K_{ATP} channel activation, adenosine is able to shorten the duration of contraction and increase K^+ current, as shown at least in atrial (but not ventricular) tissue of trout (Aho and Vornanen, 2002). Thus, it is likely that the probable effects of K_{ATP} channel and adenosine receptor activation are complementary. Future studies are required, however, to clarify the mechanistic link between HIF-1α activation and preconditioning in the fish heart.

In line with an outstanding ability for remodeling and growth in response to environmental changes (Gamperl and Farrell, 2004; Keen et al., 2016), the fish heart exhibits a remarkable capacity for regeneration following injury (Gillis and Johnston, 2017; Lien et al., 2012; Parente et al., 2013; Poss et al., 2002). Tissue damage, whether as a result of infarction or mechanical damage (i.e. resection), is associated with hypoxia, which stimulates cardiomyocyte proliferation (González–Rosa et al., 2017; Jopling et al., 2012). In the zebrafish heart, Jopling et al. (2012) established the critical role for Hif-1α in cardiac regeneration with the use of a transgenic line expressing dominant-negative Hif-1α (dnHif-1α), restricting Hif-1 activity. After ventricular amputation, dnHif1-α zebrafish exhibit impaired regeneration, which is similar to the effects of hyperoxia in wild-type zebrafish, suggesting that hypoxia is ordinarily required to induce Hif during cardiac regeneration (Jopling et al., 2012).

Beyond hypoxia: Hif-1α during periods of cooling and warming

It is well documented that Hif-1α is an important component underlying the myriad of responses to hypoxia in vertebrates, but there is also evidence linking Hif-1α to the regulation of other environmental disturbances, in particular, temperature change. Changes in body temperature affect energy demand and require metabolic reorganization, involving adjustments of gene expression (Gracey et al., 2004). One of the possible mechanisms of transcriptional control is thought to involve Hif-1α, as both Hif-1α protein levels and its binding activity increase during periods of cold or warm exposure (Heise et al., 2006a,b; Rissanen et al., 2006; Mladineo and Block, 2009; O'Brien et al., 2020).

Hif-1α and cooling environments

Several studies have documented changes in Hif expression or binding capacity in response to acute or long-term cold exposure. In crucian carp (*Carassius carassius*), cold acclimation at 8°C increases Hif-1α protein amounts in the liver, gills and heart, and increases Hif-1α DNA-binding activity in the gills, heart and kidney (Rissanen et al., 2006). Likewise, a 7 week cold acclimation at 15°C in juvenile Pacific bluefin tuna (*Thunnus orientalis*) increases Hif-1α protein levels in the gills and spleen (Mladineo and Block, 2009), and seasonal acclimation to winter cold in North Sea eelpout (*Zoarces viviparus*) results in significantly higher Hif-1α-binding activity in the liver (Heise et al., 2007). Exposure to acute cold also increases Hif-1α protein levels in the zebrafish brain (Tseng et al., 2011) and Hif-1α DNA-binding activity in the liver of the North Sea eelpout (Heise et al., 2006b). It would appear that there is stabilization of Hif-1α during cold exposure in some fish species, suggesting a role for Hif-1α in response to cold stress, but the downstream consequences of Hif-1α activation are unknown. It is yet to be determined whether the target genes of Hif-1α during normoxic cold exposure are the same as those induced by hypoxia (Rissanen et al., 2006).

The mechanism of Hif-1α stabilization during cold exposure is not well understood. One hypothesis is that the changes in cellular redox state alter the stability of Hif-1α (Huang et al., 1996; Ema et al., 1999). In *O. mykiss*, cellular reducing conditions affect different components of Hif-1α protein regulation, including its phosphorylation, stabilization and facilitation of Hif-1α DNA binding (Nikinmaa et al., 2004). A highly reduced cellular redox state occurs during acute cold exposure in the North Sea eelpout (Heise et al., 2006b), lending support to the redox mechanism of Hif-1α regulation in the cold. In apparent conflict, however, seasonally winter-acclimated North Sea eelpout exhibit a more oxidized cellular redox state than summer-acclimated individuals (Heise et al., 2007). It is possible that, depending on the time scale of exposure, different mechanisms affect Hif-1α stabilization. A possible alternative mechanism involves regulation by heat shock proteins (HSPs). In crucian carp, Rissanen et al. (2006), proposed that Hsp70 and Hsp90, both of which increase during cold exposure, stabilize the Hif-1α protein. HSPs, particularly Hsp90, appear to also be a mechanism of Hif-1α stabilization in fish exposed to warming temperatures (see below).

Hif-1α and warming environments

As during cold exposure, the Hif-1α signaling pathway may be activated in fish exposed to warming conditions. Acute exposure to mild heat stress results in an increase in DNA-binding activity of Hif-1α in North Sea eelpout (Heise et al., 2006a), and exposure to critical thermal maximum temperatures increases *hif-1α* mRNA levels in the heart of Antarctic *Notothenia coriiceps* (Beers and Sidell, 2011) and Hif-1α protein in the hearts of *N. coriiceps* and *Chaenocephalus aceratus* (O'Brien et al., 2020). Similarly, in mice, an increase in body temperature strongly induces HIF-1α accumulation by Hsp90-dependent mechanisms (Katschinski et al., 2002). Inhibition of chaperone activity of Hsp90 prevents the accumulation of HIF-1α, providing additional direct evidence for an involvement of HSPs in the regulation of HIF-1α, particularly during periods of warming (Katschinski et al., 2002). Using human microvascular endothelial cells, Minet et al. (1999) demonstrated that Hsp90 is a major regulator of HIF-1α and interacts with the transcription factor via the bHLH-PAS domain of HIF-1α.

The impact of HIF-1α activation during heat acclimation is considerable in nematodes; *hif-1α* loss-of-function strains exhibit a lack of acclimation to heat exposure, and strains with overexpression of HIF-1α exhibit greater heat stress tolerance than wild-type strains (Treinin et al., 2003). In fish, the significance of accumulation of Hif-1α during heat stress is less clear. One hypothesis is that the Hif-1α pathway is activated as a result of oxygen limitation at elevated temperatures (Heise et al., 2006a; O'Brien et al., 2020), an idea based on the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis (Pörtner, 2001, 2002), which proposes that exposure of animals, particularly aquatic ectotherms, to temperatures above or below an optimal range may result in an

imbalance of oxygen supply and demand that leads to tissue hypoxia and a decline in performance. From this perspective, Hif-1α signaling may not be a result of increasing temperature per se, but a consequence of a decrease in O_2 at high temperatures. If true, a loss in Hif-1α would ultimately affect organismal performance during heat stress. This raises an important question: does Hif-1α provide a link between hypoxia and thermal tolerance?

Although the universality of the OCLTT model has proven controversial (Jutfelt et al., 2018; Lefevre et al., 2021), there is convincing evidence that thermal tolerance and hypoxia tolerance may be correlated in at least some conditions or species (Anttila et al., 2013). A prediction of the model is that exposure to critical temperatures may result in the recruitment of Hifs (Pörtner, 2012; Pörtner et al., 2007), which is supported by evidence that exposure to upper critical temperatures in Antarctic fish increases Hif-1α protein expression in the heart (O'Brien et al., 2020).

If Hif-1α induction at high temperatures plays a functional role in mitigating the tissue hypoxia that could limit upper thermal tolerance, Joyce and Perry (2020) hypothesized that *hif-1α* (double paralog) knockout zebrafish would display reduced upper critical temperature (CT_{max}) in comparison to wild-type zebrafish. However, no difference in CT_{max} was observed between genotypes either during the initial warming trial or following a re-trial 48 h later, in which the two groups displayed a similar increase in CT_{max} ('heat hardening') (Joyce and Perry, 2020). Despite their clear reduction in hypoxia tolerance (Joyce and Perry, 2020; Mandic et al., 2020), the fact that *hif-1α* knockout zebrafish display unaltered acute thermal tolerance questions a fundamental link between hypoxia and thermal tolerance. However, because the study of Joyce and Perry (2020) investigated only the effects of acute (rapid) warming, it remains possible that Hif-1α is necessary for successful longer-term acclimation to warm temperatures (Pörtner, 2021), which could in itself have implications for hypoxia tolerance (Collins et al., 2021).

Conclusions and future directions

Since the pioneering study of Soitamo et al. (2001), there has been significant progress in elucidating the physiological roles of Hif in fish. With the development of increasingly diverse tools to manipulate Hif levels *in vivo* (Elks et al., 2015), researchers can more easily incorporate loss- and gain-of-function approaches into experiments addressing the physiology of Hif. Arguably, one of the most powerful approaches is to use of loss-of-function mutants (e.g. Gerri et al., 2017) obtained through CRISPR-Cas-9 gene editing, although currently this approach is limited to a few species including zebrafish (Zimmer et al., 2019). By using such a knockout approach, it was demonstrated that hypoxia tolerance, as measured by time to LOE, is decreased markedly in adult zebrafish lacking one or both of the Hif-1α paralogs. The mechanisms whereby Hif-1α extends the time to LOE are unknown, but appear to be unrelated to an enhancement of O_2 uptake based on the absence of any effect of Hif-1α knockout on P_{crit} (Mandic et al., 2020) and relatively minor effects on the HVR in adult zebrafish (Mandic et al., 2019).

Further research in this area should address two critical questions: (i) how is hypoxia tolerance as measured by two different metrics (time to LOE and P_{crit}) differentially affected by *hif-1α* knockout?; and (ii) what are the mechanisms underlying the effects of Hif-1α in extending the time to LOE during acute hypoxia? Previously, mechanisms of action of Hif-1α in conferring hypoxia tolerance have been inferred largely from studies on mammals. Clearly, future studies on fish should focus on elucidating the piscine-specific pathways regulated by the Hif isoforms.

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Competing interests
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