Developmental Plasticity of the Cellular Hypoxia Response in Zebrafish, 
*Danio rerio*

by

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Abstract
Developmental plasticity of the cellular hypoxia response in zebrafish, Danio rerio

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In most organisms the cellular response to hypoxia is mediated by the master regulator hypoxia-inducible factor-1 (HIF-1). Zebrafish embryos can also arrest development (suspended animation) to tolerate low oxygen. I tested the hypothesis that induction of HIF-1 and associated target genes (e.g., erythropoietin) during embryonic development would alter the hypoxia tolerance phenotype of larval and adult fish. I exposed zebrafish embryos at 3 developmental stages to acute (4 h) bouts of hypoxia (5% dissolved oxygen, DO) or anoxia (<0.5% DO). I found that embryos that mount a HIF-1 response have a greater hypoxia tolerance as larvae. Additionally, populations that experienced embryonic HIF-1 induction show an increase in the proportion of males (~70% male), that are more hypoxia tolerant than female fish, compared to control populations (~45% male). Overall, induction of HIF-1 during ontogeny alters the larval and adult zebrafish phenotype to better tolerate future hypoxic bouts.
# Table of Contents

Abstract .................................................................................................................................................. ii  
Table of Contents .................................................................................................................................. iii  
Acknowledgements .............................................................................................................................. iv  
List of Figures ......................................................................................................................................... v  
Introduction ........................................................................................................................................... 1  
Materials and Methods ....................................................................................................................... 10  
Results .................................................................................................................................................. 19  
Discussion ............................................................................................................................................ 40  
General Discussion .............................................................................................................................. 51  
  Literature Cited .................................................................................................................................... 57  
Supplementary Material ....................................................................................................................... 69
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Finally, I would like to thank all of my friends and family who have supported me throughout this (often sleep deprived) experience. I love you all.
List of Figures

Figure 1  Effects of acute O\textsubscript{2} deprivation on the growth and development of zebrafish \textit{(Danio rerio)} embryos. At 18 hpf, 24 hpf or 36 hpf embryos were exposed to 4 h of \textit{normoxia} (95\% dissolved oxygen; DO), \textit{hypoxia} (5\% DO) or \textit{anoxia} (<0.5\% DO). Embryos were then recovered in 95\% DO and both growth (A, C and E) and hatch (B, D and F) rate were recorded throughout recovery. The symbols * and † indicate that hypoxia or anoxia treated embryos are significantly delayed ($P<0.05$) compared to the normoxia control, using size (18 hpf, A; 24 hpf, C) or otic vesicle length (OVL; 36 hpf, E inset) as a measure of development. Embryo length (EL) and OVL data are mean ± SEM ($N=15$) and were analyzed using 2-Way ANOVA and Tukey’s post hoc test.................................................................p. 24

Figure 2  HIF-1 cellular response of zebrafish \textit{(Danio rerio)} embryos exposed to 4 h of hypoxia (5\% DO) or anoxia (<0.5\% DO) at 18 hpf. A representative Western blot of the HIF-1\textalpha protein band at 86 kDa (A). HIF-1\textalpha levels, relative to total protein of 20 pooled embryos ($N=6$), immediately following low O\textsubscript{2} treatment (B, $P=0.054$). Expression levels of \textit{hif-1\alpha} (C) and HIF-1 target genes \textit{(igfbp-1 D; epo E; vegf-165 F)} mRNA immediately following treatment and after 4 h of recovery in 95\% DO. To account for developmental delays caused by low O\textsubscript{2} treatments
both developmentally matched (control) and time matched (normoxia) control groups are included. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA or 2-Way ANOVA and Tukey’s post hoc test ($P<0.05$).

Figure 3  **HIF-1 cellular response of zebrafish** (*Danio rerio*) **embryos exposed to 4 h of hypoxia (5% DO) or anoxia (<0.5% DO) at 24 hpf.** A representative Western blot of the HIF-1α protein band at 86 kDa (A). HIF-1α levels, relative to total protein of 20 pooled embryos ($N=6$), immediately following low O$_2$ treatment (B). Expression levels of hif-1α (C) and HIF-1 target genes (*igfbp-1* D; *epo* E; *vegf-165* F) mRNA immediately following treatment and after 4 h of recovery in 95% DO. To account for developmental delays caused by low O$_2$ treatments both developmentally matched (control) and time matched (normoxia) control groups are included. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA or 2-Way ANOVA and Tukey’s post hoc test ($P<0.05$).

Figure 4  **HIF-1 cellular response of zebrafish** (*Danio rerio*) **embryos exposed to 4 h of hypoxia (5% DO) or anoxia (<0.5% DO) at 36 hpf.** A representative Western blot of the HIF-1α protein band at 86 kDa (A). HIF-1α levels, relative to total protein of 20 pooled embryos ($N=6$), immediately following low O$_2$ treatment (B). Expression levels of hif-1α (C) and HIF-1 target genes (*igfbp-1* D; *epo* E; *vegf-165* F) mRNA immediately following treatment and after 4 h of recovery in
95% DO. To account for developmental delays caused by low O\textsubscript{2} treatments both developmentally matched (control) and time matched (normoxia) control groups are included. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA or 2-Way ANOVA and Tukey’s post hoc test ($P<0.05$). .........................................................p. 30

**Figure 5**  
O\textsubscript{2} consumption and critical oxygen tension ($P_{\text{crit}}$) of zebrafish (*Danio rerio*) larvae (4 dpf) exposed as embryos at 18 hpf (A, B), 24 hpf (C, D) or 36 hpf (E,F) to 4 h of normoxia (95% DO), hypoxia (5% DO) or anoxia (<0.5\% DO). Values are mean ± SEM ($N=6$) and represent the O\textsubscript{2} consumption curves and $P_{\text{crit}}$ of pools of 20 larvae. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA and Tukey’s post hoc test ($P<0.05$). .................................................................p. 32

**Figure 6**  
O\textsubscript{2} consumption and critical oxygen tension ($P_{\text{crit}}$) of male and female adult zebrafish (*Danio rerio*) exposed as embryos at 18 hpf (A, B), 24 hpf (C, D) or 36 hpf (E,F) to 4 h of normoxia (95% DO), hypoxia (5% DO) or anoxia (<0.5\% DO). All data are mean ± SEM (O\textsubscript{2} consumption, $N=8$; $P_{\text{crit}}$, $N=4$). Groups that do not share a common letter are significantly different as determined by 2-Way ANOVA and Tukey’s post hoc test ($P<0.05$). ........................................................................................................p. 34

**Figure 7**  
Time to loss of equilibrium (LOE) in severe hypoxia (5\% DO) of adult
zebrafish (*Danio rerio*) exposed as embryos at 18 hpf (A), 24 hpf (B) or 36 hpf (C) to 4 h of normoxia (100% DO), hypoxia (5% DO) or anoxia (<0.5% DO). Values are mean ± SEM (N=8) and include equal numbers of males and females. Analysis with 2-Way ANOVA failed to detect an effect of sex or embryonic treatment on LOE at 18 hpf (P=0.585; P=0.503), 24 hpf (P=0.522; P=0.205) or 36 hpf (P=0.783; P=0.907) with an α level of 0.05.

**Figure 8**  Population sex ratios of adult zebrafish exposed as embryos at 18 hpf (A), 24 hpf (B) or 36 hpf (C) to 4 h of normoxia (100% DO) hypoxia (5% DO) or anoxia (<0.5% DO). Values indicated by * are significantly different from the expected proportions of 50% male, 50% female ($\chi^2$, $P<0.05$). p. 38

**Figure 9**  Summary of cellular and phenotypic changes caused by acute oxygen deprivation exposure during development. Zebrafish (*Danio rerio*) embryos were exposed to acute (4 h) anoxia (<0.5% DO) or hypoxia (5% DO) at 18 hpf, 24 hpf or 36 hpf. The cellular response to low oxygen was measured immediately following treatment while hypoxia tolerance was measured at 4 dpf and adulthood... p. 50
Introduction

Variations in water quality have been key driving factors in the evolution of fish and other aquatic organisms (Romer, 1958). Of these, low dissolved oxygen (DO) or hypoxia has been one of the most prevalent and influential factors (Diaz and Rosenberg, 1995). Temperature, depth, salinity and cover by vegetation and ice all effect the concentration of oxygen in water; and these levels can vary daily or seasonally (Truchot and Duhamel-Jove, 1980; Nikinmaa and Rees, 2005). Human activity also leads to hypoxia, as nutrient run off and pollution cause algal blooms which decrease oxygen levels (Diaz and Rosenburg, 1995). In recent years the frequency and severity of these hypoxic occurrences has increased drastically in all aquatic ecosystems, with slow moving, warm, shallow, fresh water systems being among the most susceptible to oxygen depletion (Diaz and Breitburg, 2009).

Aquatic organisms have evolved a variety of coping strategies for dealing with fluctuating oxygen levels including avoidance, aerial surface respiration and finally metabolic depression (Chapman and Mackenzie, 2009). Exposure to severe hypoxia is especially challenging for fish embryos and young larvae, as they most often lack mobility and cannot escape adverse environmental conditions or respire in different layers of the water column. Additionally larval fishes often inhabit those areas that are most prone to hypoxic episodes. The overall goal of my MSc. research was to assess the short and long term impacts of hypoxia exposure during early development in teleosts, specifically in zebrafish (*Danio rerio*).

Developmental plasticity of hypoxia tolerance

Developing in a hypoxic environment can permanently alter many aspects of adult fish
behaviour (e.g. aggression, swimming capacity), gross morphology (e.g. gill surface area, body size) as well as sex ratios in a population (Chapman et al, 2000; Marks et al, 2005; Shang et al, 2006; Widmer et al, 2006; Miller et al, 2008). These changes are examples of developmental plasticity, a common phenomenon across the animal kingdom (West-Eberhard, 2005). For the purpose of this study, developmental plasticity refers to the ability of a single genotype to produce separate larval or adult phenotypes in response to early rearing environment (Pelster, 2003). For example, African cichlids (Pseudocredilabrus mutlicolor victoriae) reared in hypoxia exhibit increased gill surface area and filament length as adults (Chapman et al, 2000). When adult fish are acclimated to hypoxia they exhibit an increased tolerance when challenged with a second bout of low oxygen (Rees et al, 2001; Shimps et al, 2005; Fu et al, 2011). While developing in chronic hypoxia has many downstream effects on adult phenotype it is still not fully understood if these changes are as adaptive in the face of subsequent hypoxia challenges as adult acclimation.

The response to neonatal hypoxia exposure has been shown to be extremely maladaptive in mammals. Exposure to high altitudes or chronic hypoxia during development results in an impaired hypoxic ventilatory response in adult rats, sheep and humans (Bavis et al, 2004). Similar studies found no effect of chronic hypoxia during development on the adult ventilatory response in zebrafish (Danio rerio; Vulesevic and Perry, 2006). However, zebrafish reared in mild hypoxia (~55% DO) can maintain their metabolic rate at much lower levels of DO and exhibit lower lactate levels following exercise than their normoxia-reared counterparts (Wildmer et al, 2006; Barrionuevo et al, 2010). There is also evidence that when parent zebrafish are exposed to chronic hypoxia their offspring can tolerate longer bouts of hypoxia as larvae than control (Ho and Burggren, 2012). All of these findings suggest that hypoxia tolerance is an
inherently plastic trait, which can be altered by acclimation to low oxygen in both mammals and fish. Although developmental plasticity may play a key role in adapting the physiology of an organism to its environment, little is known about the molecular mechanisms responsible for this process (West-Eberhard, 2005).

**Epigenetics and developmental plasticity**

Recently, the study of epigenetics has begun to elucidate these mechanisms. Epigenetics encompasses all factors that change gene expression in response to environmental stimuli without changing the underlying sequence of the gene itself (Ho and Burggren, 2010). Examples of these processes include DNA methylation and histone modification, both of which alter the accessibility of genes to transcription factors. These alterations are induced by the environment and fundamentally change if and when a gene is expressed (Ho and Burggren, 2010). Genome wide epigenetic programming is a crucial part of normal development (Dean et al, 2003) and perturbations to these patterns can alter the adult phenotype of an organism (for review see Murgatroyd and Spengler, 2011). It is therefore possible that cellular adaptations to hypoxia may have long-lasting effects on overall gene expression and phenotype.

**Cellular hypoxia response**

One likely mediator of the plastic hypoxia response is the master-regulator transcription factor, hypoxia inducible factor -1 (HIF-1), which controls the cellular hypoxia response in all animals (Soitamo et al, 2001; Fedele, et al, 2002; Rytkonen et al, 2007; Shen et al, 2010; Loenarz et al, 2011). HIF-1 regulates a host of genes which suppress major energy-dependent processes such as growth and protein synthesis, and increase oxygen delivery to tissues (Semenza and
Wang, 1992). HIF-1 is a heterodimeric compound with an α and a β subunit (Semenza et al, 1997). Although both subunits are constitutively expressed, the activity and turnover of the HIF-1α protein is hypoxia-dependent (Wang et al, 1995). During normoxia, HIF-1α is actively degraded by the ubiquitin/proteasome system. This process is mediated by the von Hippel-Lindau protein (pVHL), an ubiquitin ligase. During normoxia, the pVHL binds to the oxygen-dependent degradation domain (ODD) of HIF-1α. The ODD contains two proline residues that are hydroxylated in normoxic conditions. Hypoxia stabilizes HIF-1α by inhibiting pVHL binding. The stabilized HIF-1α then translocates to the nucleus and binds to its counterpart HIF-1β (Xia et al, 2009). Once assembled, HIF-1 regulates the transcription of its target genes by binding specific hypoxia-responsive elements (HREs) in their promoter regions (Fedele et al, 2002). Important HIF-1 targets include erythropoietin (epo) and vascular endothelial growth factor (vegf), which increase red blood cell formation and angiogenesis, respectively (Semenza and Wang, 1992). Additionally, HIF-1 is a known regulator of insulin-like growth factor binding protein-1 (IGFBP-1) which arrests growth and development (Kajimura et al, 2006). Together these HIF-1 targets act to increase oxygen uptake and decrease energy demand. The regulation of these genes is central to the organism’s whole body adaptive response to hypoxia.

Role of HIF-1 during development

During development HIF-1 is required for the basal expression of many of its target genes, as evidenced by HIF-1α knockout mice embryos that fail to develop organized vasculature or neurons (Ryan et al, 1998). This phenomenon also occurs in fish. Zebrafish embryos express hif-1α mRNA differentially throughout development of the notochord, cardio-vasculature and brain (Rojas et al, 2007). It is HIF-1 targets, such as VEGF that mediate the
formation and differentiation of these tissues (Liang et al, 2001). Interestingly, hypoxia exposure alters the order of cardiovascular developmental events in zebrafish and rainbow trout (Onchohynchus mykiss), with the onset of adrenergic responses and vasodilatation occurring earlier in hypoxia (Bagatto, 2005; Miller et al, 2011). A further example of the inherent connection between cardiovascular development and the HIF-1 pathway was recently discovered by Kopp et al (2010). They found that mutant zebrafish larvae with compromised blood flow and cardiac activity elicited a cellular hypoxia response. Clearly the development of the cardiovascular system is intimately linked to the HIF-1 pathway.

Various members of the HIF-1 pathway are associated with epigenetic mechanisms. In order to function as a transcription factor HIF-1 requires the presence of a variety of co-factors which form a complex that binds DNA (Kallio et al, 1988). Several of these co-factors and HIF-1 targets are enzymes that de-acetylate histone proteins (Kato et al, 2004; Kim et al, 2007). HIF-1 targets are also sensitive to epigenetic alteration. For example, hypermethylation of epo has been shown to block the binding of HIF-1 in vitro, in mammalian cells (Yin and Blanchard, 2000). Interestingly, in vitro work in human cell lines has also shown that chronic hypoxia causes widespread changes in both DNA methylation and histone acetylation (Watson et al, 2009). This reprogramming of the genome could easily explain the plasticity of hypoxia tolerance phenotypes, however this has never been tested.

Critical developmental windows

The sensitivity of organisms to environmental stressors often varies throughout ontogeny. Developmental stages that are particularly susceptible to an environmental factor are known as
critical periods (Penfireld and Roberts 1959; McKim, 1977). Given the role of HIF-1 in normal ontogeny it is likely that certain stages of development are more sensitive to hypoxic bouts than others. Exposure to low oxygen during these critical periods could have a greater impact on shaping the future hypoxia tolerance phenotype of an animal. Recently a detailed study of critical developmental windows of hypoxia sensitivity and growth was carried out in *Drosophila melanogaster* (Heinrich et al, 2011). Flies exposed to acute bouts of hypoxia (10% O₂) during development were much smaller as adults than those maintained at normoxia. This was exacerbated when the exposure took place during the late larval and pupal stages. Essentially all of the previous work investigating the long term effects of early hypoxia exposure in vertebrates has focused on chronic exposures. This approach does not allow us to differentiate between the effects on specific developmental stages.

*Zebrfish as a model for hypoxia and development*

Zebrfish with their short generation time are an ideal model for studying developmental processes and as such their ontogeny is well characterized (Kimmel et al, 1995). They are also relatively hypoxia tolerant compared to other fish and have been used extensively as model organisms to investigate the role of cardiovascular development and hypoxia in human diseases (Briggs, 2002). Studying the ultimate mechanisms that have evolved in hypoxia tolerant species helps elucidate the proximate mechanisms for coping with low oxygen. In the wild, zebrafish spawn in low oxygen environments such as shallow pools, rice paddies and slow moving streams (Engeszer et al, 2007). As such, their eggs are routinely exposed to hypoxic conditions.

To deal with fluctuating oxygen levels in the environment zebrafish embryos employ one of two strategies, which may depend on the severity of oxygen depletion. During the earliest
stages of development embryos can tolerate up to 24 hours of total oxygen depletion (i.e. anoxia; Padilla and Roth, 2001). To do so they enter a state of developmental arrest known as suspended animation. Once embryos are returned to normoxia development to adulthood continues unhindered (Bagatto, 2005).

Interestingly, zebrafish can only use the suspended animation strategy during early development and key aspects of zebrafish cardiovascular development correlate with an abrupt change in hypoxia tolerance (Padilla and Roth, 2001). At 24 hours post fertilization (hpf), the heart begins to beat and red blood cells containing haemoglobin (Hb) are present in circulation. Up until this stage, embryos can tolerate severe hypoxia and anoxia. They can also tolerate repeated bouts of acute hypoxia with no adverse effects on survival (Mendelsohn et al, 2008). Around 24 hpf anoxia tolerance begins to rapidly decrease. Once fish have hatched (~48 hpf), 24 hours of anoxia results in 100% mortality (Padilla and Roth, 2001). Anoxia is lethal, regardless of exposure duration, by 52 hpf (Mendelsohn et al, 2008). The connection between HIF-1α and cardiovascular development along with the correlated reduction in hypoxia tolerance by 24 hpf is intriguing. It is reasonable to speculate that an induction of the HIF-1 pathway during these critical windows of sensitivity could impact the development of the cardiovascular system.

Hypoxia may also cause long term alterations in gene expression and development independent of HIF-1 (Johnson et al, 2008). Suspended animation is characterized by a shutdown of transcription and protein synthesis, while the HIF-1 mediated cellular response is thought to involve the transcription and translation of up to 148 target genes in zebrafish (Ton et al, 2003). Therefore, it has been suggested that suspended animation does not involve HIF-1 (Mendelsohn et al, 2008). This process therefore provides us with a unique tool to separate the
effects of HIF-1 from oxygen deprivation.

*Hypotheses and Predictions*

I designed my experiments under the assumption that there is an oxygen level dependent trade-off between suspended animation and a HIF-1 response. I hypothesized that if the two strategies do not occur simultaneously then by treating embryos with either total anoxia and severe hypoxia (5% DO) a differential HIF-1 response should be elicited based on severity of oxygen depletion. Furthermore, I hypothesized that if complex organ systems are more sensitive to hypoxia then more developed embryos will require a more robust cytoprotective mechanism than earlier stages. To test these hypotheses I exposed zebrafish at 3 different points in embryonic development (18, 24 and 36 hpf) to low oxygen. These stages track the loss of ability to use suspended animation along with the loss of anoxia tolerance which occurs through ontogeny. I predicted the following outcomes:

1. The expression of HIF-1α protein and *epo*, *vegf*, and *igfbp-1* mRNA will increase more in response to acute hypoxia (i.e. 5% DO) than total anoxia (<0.5% DO).
2. As embryos age and can no longer use suspended animation, the magnitude of their HIF-1 response should increase.

I further tested two competing hypotheses pertaining to the long term impacts of early HIF-1 induction. First, I hypothesized that if the plasticity of hypoxia tolerance is driven by HIF-1 and its downstream targets then larval and adult zebrafish that mounted a HIF-1 mediated cellular response during development will express an altered hypoxia tolerance phenotype. Therefore, I predicted the following outcomes:
1. If the changes caused by HIF-1 are adaptive, larval and adult zebrafish who mount a HIF-1 response as embryos will have an increased hypoxia tolerance relative to naive fish.

2. If the changes caused by HIF-1 are maladaptive, embryos that elicit a HIF-1 response will have a decreased hypoxia tolerance as larvae and adults.

3. The alternate larval and adult phenotypes should be most apparent in fish that mounted larger HIF-1 responses as embryos.

Alternatively, I hypothesized that if hypoxia itself causes changes that are independent of the HIF-1 pathway then all zebrafish exposed to low oxygen (i.e. both hypoxia and anoxia) during development will have an altered adult phenotype.

1. Embryos exposed to low oxygen during development will have an altered hypoxia tolerance as adults independent of their cellular strategy.

2. The alternate larval and adult phenotypes will be most apparent in fish exposed as embryos to total anoxia compared to hypoxia.
Materials and Methods

Animals
A breeding colony of zebrafish (*Danio rerio*), housed at the University of Guelph Hagan Aqualab, was maintained at 26°C with a 14:10 h light-dark photoperiod. Fish were spawned at 9:00 a.m. in divided chamber breeding baskets and eggs were collected 1 h following spawning. For each experiment, eggs from several breeding baskets were pooled to increase genetic diversity. Once collected, fertilized eggs were sorted into mesh (100 μm) bottom cups (150 mL) and incubated in 47 mm diameter microbiological dishes (Millipore, Billerica, MA) containing egg water (60 μg L⁻¹ Instant Ocean Sea Salts, United Pet Group, Cincinnati, OH) with 0.0002 % methylene blue at 28°C. As developmental staging of zebrafish embryos has been previously characterized in great detail at 28°C experiments were performed at 28°C for embryos and 26°C for adult fish (Kimmel et al, 1995).

Experimental Design

Experiment 1: Effects of acute hypoxia and anoxia exposure on growth and development

Groups of 20 embryos at 18, 24 or 36 hour post fertilization (hpf) were exposed to one of three O₂ regimes for 4 h: a control treatment (normoxia; dissolved oxygen saturation, DO= 95% or ~7.2 mg/L), hypoxic treatment (DO= 5% or 0.4 mg/L) and anoxic treatment (DO <0.5% or 0.04 mg/L). Water O₂ levels were controlled by regulating delivery of N₂ gas or air through ceramic air stones into 20 L treatment tanks. O₂ levels were allowed to stabilize for 8 h prior to the addition of embryos and were continually monitored using an O₂ electrode (Liquid Dissolved Oxygen Field Probe, Hach, Loveland, CO). Following treatment, embryos were either terminally...
anesthetized with an overdose of 2-phenoxyethanol (2mL L$^{-1}$) or allowed to recover in normoxia at 28°C for 4, 8, 24 or 48 h prior to sampling. Sampled embryos were dechorinated using 25G 5/8 needles (Becton Dickinson and Co, Franklin Lakes, NJ) and photographed under a stereomicroscope (SMZ 1500, Nikon Instruments Inc, Melville, NY) with a Retiga 1300 camera (QImaging, Surey, BC) for later determination of growth and development. To analyze hatch rate an additional group of embryos was allowed to recover to 6 days post fertilization (dpf). Percent hatch was recorded each morning.

**Experiment 2: Cellular response to acute hypoxia or anoxia exposure.**

Groups of 60 embryos were exposed to the same O$_2$ treatments as described in experiment 1. Since both hypoxia and anoxia exposure were found to cause 2-4 h developmental delays in experiment 1 an additional developmental control group was maintained at 95% DO for the requisite period of time so as to match the delayed developmental stage of the hypoxia and anoxia exposed embryos after 4 h of treatment. Following exposure, embryos were immediately flash frozen on dry ice, thawed briefly at 1°C, sorted into groups (lactate n=10; mRNA and protein n=20) and stored at -80°C prior to analysis. An additional group of embryos were allowed to recover in normoxia at 28°C for 4h prior to sampling for mRNA analysis.

**Experiment 3: Long term effects of early O$_2$ deprivation on hypoxia tolerance**

Embryos exposed to the same initial O$_2$ treatments as described in experiment 1 were reared to 4 dpf (larvae) or adulthood (6 months post fertilization). Briefly, following exposure embryos were transferred to 200 mL beakers with 100 mL of egg water (Westerfield, 2007) and maintained at 28°C until 4 dpf. One group was immediately used for critical oxygen tension
measurements, whereas a second group was transferred to 1 L flow through tanks (26°C). At 5dpf larvae were fed 30 μm, rotifer replacement supplements (Argent Chemical Laboratories, Redmond, WA) twice a day and maintained on this diet until 20 dpf when they were fed a mixture of newly hatched Artemia and TetraMin fish flakes (Tetra, Blacksburg, VA) until they reached sexual maturity at 6 months.

Analysis

**Developmental staging and Growth**

Total embryo length (EL) and otic vesicle length (OVL), as defined in Kimmel et al. (1995) were measured using Open Lab 5.5 software (Perkin Elmer, Waltham, MA). Growth rates under normoxic conditions were similar to published values for D. rerio embryos raised at 28°C (Kimmel et al, 1995). Exposure to anoxia caused some embryos to hatch prematurely which confounded the EL measurements. Hence, the OVL ratio, the migration of the otic vesicle towards the eye during development, was employed. The OVL ratio refers to the number of additional otic vesicles that would fit between the actual otic vesicle and the eye (Supplementary Figure 1). The EL and OVL methods for developmental staging provided similar results in fish treated at 24 hpf.

**HIF-1α Protein Levels**

To quantify whole embryo levels of HIF-1α protein (experiment 2) groups of 20 embryos (N=6) were thawed in radio immuno precipitation assay (RIPA) lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.1% SDS, 50 mM Tris pH 8.0, 0.5% sodium deoxycholate) with protease inhibitors (1mM phenylmethylsulfonyl fluoride, PMSF; and 2mM
ethylenediaminetetraacetic acid, EDTA) and dispersed with a glass 1 mL Pasteur pipette to disrupt the chorion and yolk. Samples were briefly centrifuged (8000 g x 2 min) to separate the yolk proteins. Total protein was extracted from the remaining tissue which was homogenized in additional RIPA buffer and spun (16000 g x 10 min, 4°C) to separate soluble protein from chromatin. Protein concentration was determined using a Bradford assay (BioRad Protein Assay Dye Reagent, BioRad, Hercules, CA) measured at 595nm. Samples were diluted so that equal volumes with 40 μg of total protein were loaded on 12% poly-acrylamide mini gels and separated by molecular weight using SDS-PAGE. Proteins were transferred to PVDF membranes and blocked in 5% milk protein (Carnation, Smucker Foods of Canada Co, Markham, ON) in PBS-T (0.1% Tween-20). Blots were probed with a custom made anti-zebrafish HIF-1α polyclonal 1°Ab (1:500; Kopp et al, 2010) and HRP-conjugated goat anti-rabbit IgG 2°Ab (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA).

The antibody signals were detected using enhanced chemiluminescence reagents (Pierce ECL, Thermo Scientific, Rockford, IL) and imaged with the ChemiDoc™XRS+ system (Bio Rad) and Image Lab™ software (Bio Rad). Blots were subsequently stained with GelCode Blue Safe Stain Reagent (Thermo Scientific) which utilizes Coomasie G-250 dye to detect total protein. Prior to re-imaging, blots were destained with 50% methanol and 1% acetic acid (3 x 3 min). Blots were imaged colorimetrically with the ChemiDoc™XRS+ system. HIF-1α band density and total protein for each lane were determined via densitometry using Image J software (U.S. National Institute of Health, Bethesda, MD). To account for variation in gel loading and protein transfer, band density was expressed relative to total protein (Aldridge et al, 2008). To compare between blots, concentration of samples were normalized to a single sample that was run on every gel.
mRNA levels of HIF-1α target genes

Whole embryo mRNA levels of insulin-like growth factor binding protein-1 (igfbp-1), erythropoietin (epo), hif-1α, elongation factor -1 alpha (ef1-a) and the embryonic isoform of vascular endothelial growth factor (vegf-165) were quantified (experiment 2). Igfbp-1 is a confirmed HIF-1 target in zebrafish (Kajimura et al, 2006); while epo and vegf-165 have been confirmed as HIF-1 targets in other organisms (Semenza, 1994; Tsuzuki et al, 2000). I also attempted to quantify the adult vegf isoform (vegf-121) but the mRNA levels were too low to be detected at early developmental stages. Briefly, total RNA was extracted from pools of 20 embryos using QIAzol Lysis Reagent (QIAGEN, Toronto, ON) as per the manufacturer’s instruction. Additionally, glycogen (RNA grade, Thermo Scientific) was added to help precipitate RNA from these small samples. The concentration of total RNA was quantified using the NanoDrop 8000 (NanoDrop Technologies, Wilmington, DE) and RNA quality was assessed via electrophoresis using the 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Following extraction, 1 μg of total RNA was DNase I treated (Ambion DNase I, RNase free, Life Technologies Inc., Burlington, ON) and reverse transcribed to cDNA using Superscript II RNaseH Reverse Transcriptase (Invitrogen, Life Technologies Inc.). A subset of samples was prepared without the RT enzyme to serve as negative controls (RT-). The cDNA products were amplified using either PerfeC Ta SYBR Green Fast Mix (ef1-a, igfbp-1, hif-1α and vegf-165; Quanta Biosciences, Gaithersburg, MD) or 2X TaqMan Gene Expression Master Mix (epo; Applied BioSystems, Foster City, CA) and the ABI 7000 sequence detection system (Applied Biosystems). Default cycling conditions were used with the TaqMan reagents: 95°C for 10min,
40 cycles of 15s at 95°C and 1min at 60°C. The amplified products were run on agarose gels to confirm their specificity (Supplementary Figure 2). Cycling conditions with SYBR reagents were optimized as follows: 95°C for 5min followed by 40 cycles of 1s at 95°C and 30s at 60°C. Amplification was followed by a dissociation melt curve protocol at 60°C to confirm the specificity of the PCR products. RT- and water-only negative controls were run on each plate and all samples were run in triplicate. To account for differences in sample dilutions and amplification efficiency of different primer sets, serial dilutions of cDNA were used to create a standard curve for each target gene and fit the threshold cycle of each sample to the curve (Bernier and Craig, 2005). Each sample was normalized to the expression level of the housekeeping gene ef1-a to account for differences in sample loading or cDNA synthesis. Primers and probes (epo only) were designed using Primer Express 3.0 software (Applied Biosystems). In zebrafish the epo transcript has 3 unique splice variants (Chu et al, 2007; Paffett-Lugassy et al, 2007). In this case primers and a FAM-Tamra tagged probe were designed to amplify a conserved region between the 3 sequences so that only a single product was produced. All primer sets had an amplification efficiency >80% (Table 1).

**L-Lactate Concentrations**

Pools of 10 embryos (N=9, experiment 2) were blotted dry, weighed, homogenized in 8% perchloric acid and centrifuged (15 000 g x 10 min). Lactate concentrations of the supernatant were determined spectrophotometrically (Bergmeyer and Bernt, 1974). Concentration was expressed as μmol.g⁻¹ dry mass.

**Routine Metabolic Rate and Pcrit of 4 dpf Larvae and Adults**
The routine metabolic rate ($M_r$) and critical oxygen tension ($P_{\text{crit}}$) were measured in 4 dpf larvae and adult zebrafish previously exposed to 4 h of hypoxia or anoxia at 18, 24 or 36 hpf. Measurements were done between 9 a.m. and 6 p.m. Order of measurement between the treatment groups was randomly assigned each day and there was no effect of time of day on either measurement. Approximately 20 larvae or a single adult fish were placed in custom built, glass respirometry chambers at 26°C. After a 1 h (larvae) or 2 h (adults) acclimation period the water flow was shut off. For larvae, a 25 μm tip dissolved oxygen microelectrode (Unisense, Aarhus, Denmark) was inserted into the chamber using a micro-manipulator and the chamber was sealed. O$_2$ levels were recorded using Lab Chart software (AD Instruments, Spechbach, Germany) over a 2 h period. Adult O$_2$ consumption was measured with a Clark-type dissolved oxygen probe and Logger Pro software (Vernier, Beaverton, OR) for 3 h. Electrode O$_2$ consumption was measured daily and subtracted from the total O$_2$ consumption. $M_r$ was calculated using the slope of the oxygen consumption curve between 100% and 70% saturation (mg L$^{-1}$ h$^{-1}$) and was expressed as μmol O$_2$·larvae$^{-1}$·h$^{-1}$ or μL O$_2$·g$^{-1}$·h$^{-1}$. $P_{\text{crit}}$ was calculated using Physiological Regulation and Conformation REGRESS software (Wake Forest University). Briefly, two regression lines were drawn through the oxygen consumption curve, the first starting at 100% saturation the second starting at 0%. $P_{\text{crit}}$ was calculated as the intercept of the two lines (Reager and Ultsch, 1989). A random subset of adult fish (35 out of 72) were euthanized and fixed overnight in 4% PFA in PBS-T. They were later dissected to confirm sex.

**Time to Loss of Equilibrium (LOE)**

Individual adult zebrafish were placed in a 1 L airtight, plastic container, filled with hypoxic water (5% DO). This insured that fish were denied access to the air/water interface and
were therefore unable to perform aquatic surface respiration (ASR). Timing began immediately after the fish was added. Time to LOE was determined when a fish was unable to right itself for 3 seconds after losing equilibrium (Ho and Burggren, 2012). Fish were then recovered in fully oxygenated water and later weighed.

**Statistical Analysis**

One-way ANOVAs were performed to determine the effect of oxygen treatment, within an embryonic developmental stage, on OVL, HIF-1α protein levels, whole animal lactate levels, larval metabolic rate and larval $P_{crit}$. With measurements taken during recovery, embryo length and mRNA expression, two-way ANOVAs were used to determine the effects of time and oxygen treatment on the measured variable. At the 18 and 36 hpf stage *igfbp-1* gene expression data failed to meet assumptions of normality. Those data were therefore log-transformed prior to analysis. Two-way ANOVAs were also used to determine the effects of embryonic oxygen treatment and sex on adult $P_{crit}$, routine metabolic rate and time to LOE. One-way repeated measures ANOVA was used to analyze $O_2$ consumption patterns with embryonic treatment as a between subjects factor and %DO level as the repeated measure. Adult population sex ratios were compared against an expected outcome of 50% male using Chi-Squared analysis. If significant main effects were determined using ANOVAs then Tukey’s post hoc test was used to further analyze trends. Sigma Stat 3.0 software (Systat Software Inc, Chicago, IL) was used for all analysis and the alpha level for all tests was 0.05.
Table 1  Sequences of primer pairs used in real-time reverse transcriptase-polymerase chain reaction assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Accession No</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ef1α</td>
<td>F-GGG CAA GGG CTC CTT CAA R-CGC TCG GCC TTC AGT TTG</td>
<td>NM_131263</td>
<td>100.3</td>
</tr>
<tr>
<td>igfbp-1</td>
<td>F-AGC CGT GCT TTG GGA TGA R-TAG AAT GCT GCC ACC CAG AA</td>
<td>AB_181657</td>
<td>93.8</td>
</tr>
<tr>
<td>vegf-165*</td>
<td>F-GAA AAC CAC TGT GAG CCT TG R-GCA GGA TTT ACA GGT GA</td>
<td>AF_016244</td>
<td>100.7</td>
</tr>
<tr>
<td>vegf-121*</td>
<td>F-AAA GGC AGA AGT CAA AGC AAA GAA AG R-GCG TTC GCT CGA TCA TCA TCT TGG CTT TTC</td>
<td>AF_059661</td>
<td>83.7</td>
</tr>
<tr>
<td>hif-1α</td>
<td>F-AAG TCT GCG ACG TGG AAT R-CCT CGT GCA CCC GAA CA</td>
<td>AY_326951</td>
<td>84.4</td>
</tr>
<tr>
<td>GPO</td>
<td>F-CTC GCC AAT GCA CCT GTC T R-GCC TTT GCC CTG AAT TGT TTA T P-[6′-FAM] TCG ACA GGG TGT CAG C[Tamra~Q]</td>
<td>EF_426725</td>
<td>95.4</td>
</tr>
</tbody>
</table>

ef1α, elongation factor 1α; GPO, erythropoietin; F, forward primer; hif-1α, hypoxia inducible factor 1α; R, reverse primer; P, probe; vegf-121, adult vascular endothelial growth factor; vegf-165, embryonic vascular endothelial growth factor; *sequences from Kopp et al, 2010
Results

Growth and Development

Both hypoxia and anoxia treatments caused 18 hpf embryos to delay growth (hypoxia, \( P<0.001 \); anoxia, \( P=0.023 \)). They remained significantly delayed relative to control embryos for an additional 4 h into recovery (22-26 hpf; hypoxia, \( P<0.001 \); anoxia, \( P<0.001 \)). However, after 24 h of recovery (42 hpf) these embryos were the same size as fish that had never been exposed to low oxygen and therefore exhibited compensatory growth (Figure 1A). Neither the hypoxia nor anoxia treatment affected time to hatch (Figure 1B). As with the 18 hpf treated embryos, both hypoxia and anoxia treatments at 24 hpf caused significant developmental delays (hypoxia, \( P=0.040 \); anoxia, \( P<0.001 \)). These embryos also demonstrated compensatory growth and had reached the size of control embryos within 48 h of recovery (72 hpf; Figure 1C). Additionally, the time to hatch was similar across all treatments (Figure 1D). At 36 hpf both hypoxia and anoxia treatments caused a significant proportion of embryos to immediately hatch (Figure 1F; hypoxia, 26%; anoxia, 43%). Therefore, measurements of embryo length as a measure of developmental delay were not valid (Figure 1E). However, both hypoxia and anoxia treatments caused significant developmental delays (\( P<0.001 \)), as confirmed by OVL measurements (Figure 1E inset).

Cellular Response

At 18 hpf anoxia treatment caused a small, statistically non-significant induction of HIF-1\( \alpha \) protein (Figure 2B; \( P=0.054 \)). There was also no change in the expression levels of hif-1\( \alpha \) mRNA with either low O\(_2\) treatment (Figure 2C; hif-1\( \alpha \) \( P=0.493 \)). Of the HIF-1 target genes measured, only igfbp-1 was induced during hypoxia exposure (\( P=0.002, P<0.001 \)) and was
lower than either of the control levels following 4 h of recovery (Figure 2D; \( P=0.048 \), \( P=0.027 \)). When treated groups are different from both the time matched and developmental control the P values are given in that order. Anoxia exposure had no effect on the expression of any of the measured genes (Figure 2; anoxia, \( igfbp-1, P=0.774, P=0.307 \); overall, \( epo P=0.142, vegf-165 P=0.196 \)). Also, neither hypoxia nor anoxia exposure resulted in a significant accumulation of tissue lactate at this stage (Table 2; \( P=0.291 \)).

At 24 hpf both hypoxia and anoxia treatments caused a twofold increase in HIF-1\( \alpha \) protein relative to both time matched and developmentally matched controls (Figure 3B; hypoxia, \( P=0.013, P=0.040 \); anoxia, \( P<0.001, P=0.002 \)), however there was no change in hif-1\( \alpha \) mRNA expression (Figure 3C, \( P=0.169 \)). Additionally, only embryos exposed to hypoxia showed an increased expression of any of the measured HIF-1 target genes. Hypoxia exposure caused a five-fold induction of \( igfbp-1 \) (Figure 3C, \( P<0.001, P<0.001 \)) and \( epo \) (Figure 3D, \( P<0.001, P<0.001 \)), both of which returned to control levels after 4h of recovery. Anoxia exposure had no effect on the expression of any of the HIF-1 target genes (anoxia, \( igfbp-1 P=0.945, P=0.401, epo P=0.951, P=1.000 \); overall, \( vegf-165 P=0.170 \)). Neither low oxygen treatment resulted in a significant accumulation of lactate (Table 2, \( P=0.412 \)).

Embryos exposed to both hypoxia and anoxia at 36 hpf accumulated 1.5-2.5 fold more HIF-1\( \alpha \) protein, compared to either the time matched or developmentally matched controls (Figure 4B; hypoxia, \( P<0.001, P=0.024 \); anoxia, \( P<0.001, P<0.001 \)). Again, neither of these low O\(_2\) treatments caused hif-1\( \alpha \) mRNA expression levels to change (Figure 4C, \( P=0.105 \)). Unlike embryos treated at earlier developmental stages, at 36 hpf both hypoxia and anoxia exposure resulted in an increased expression of multiple HIF-1 target genes (Figure 4D, E, F). Hypoxia exposure caused a 2-4 fold induction of \( igfbp-1, epo \) and \( vegf-165 (P<0.001, P<0.001; \)
P=0.005, P=0.034; P=0.003, 0.006) while embryos exposed to anoxia up-regulated igfbp-1 and vegf-165 (P=0.018, P=0.034; P=0.044, P=0.027) by 2 fold. Anoxia exposure also resulted in a significant 2 fold accumulation of tissue lactate (Table 2; P=0.010, P=0.013), whereas there were no significant changes in embryos exposed to hypoxia.

**Larval P\text{crit} and Metabolic Rate:**

The routine metabolic rate (O\textsubscript{2} consumption at 100\% DO) of 4 dpf larvae was similar across all groups regardless of prior low oxygen treatment as embryos (Figure 5A,C,E). Larvae exposed to 4h of hypoxia or anoxia at 18hpf, had similar P\text{crit} levels to control larvae (Figure 5B, P=0.634). However, those larvae that had been previously exposed to hypoxia at 24 hpf had a significantly lower P\text{crit} than control fish (Figure 5D, P= 0.029), whereas anoxia at 24 hpf had no effect (P=0.067). Both anoxia and hypoxia exposure at 36 hpf resulted in a lower larval P\text{crit} than control (Figure 5F; hypoxia, P=0.011; anoxia, P=0.007).

**Adult Hypoxia Tolerance**

Adult fish exposed as embryos to hypoxia or anoxia had similar routine metabolic rates compared to control fish that were never exposed to low oxygen, regardless of the developmental stage of their exposure (18 hpf, Figure 6A, P=0.968; 24 hpf, Figure 6C, P=0.190; 36 hpf, Figure 6E, P=0.926). As with the larvae, neither exposure to hypoxia nor anoxia at 18 hpf had any effect on P\text{crit} of adult fish (Figure 6B, P=879). In contrast to larvae, there was no effect of hypoxia or anoxia exposure on P\text{crit} of adult fish exposed at 24 hpf or 36 hpf (Figure 6D, P=0.264; Figure 6F, P=0.848). There was a significant effect of sex on hypoxia tolerance where, regardless of embryonic oxygen treatment, female fish always had a higher P\text{crit} than
males (Figure 6; 18hpf, $P=0.022$; 24hpf, $P=0.003$; 36 hpf, $P=0.017$). There was no effect of embryonic exposure to hypoxia or anoxia on LOE regardless of the timing of the exposure (18hpf, Figure 7A, $P=0.503$; 24hpf, Figure 7B, $P=0.205$; 36 hpf, Figure 7C, $P=0.907$).

Sex Ratios

Low oxygen-exposure at 18 hpf had no effect on sex ratios in the adult population (Figure 8A). Proportions of male and female fish that had been hypoxia-exposed (50% male), anoxia-exposed (45% male) or reared in normoxia (47% male) were similar to the expected outcome of 50:50 males to females (control, $P=0.9$, hypoxia, $P=0.5$, anoxia, $P=0.95$). However, groups of fish that had been previously exposed to hypoxia at 24 hpf were 73% male (Figure 8B; $P<0.01$) compared to control or anoxia exposed fish which were 47% and 55% male respectively ($P=0.8$, $P=0.2$). Groups of fish exposed to hypoxia and anoxia at 36 hpf were also male-biased at 62% and 71% respectively, compared to 41% males in control groups (Figure 8C). Only the proportions of anoxia treated fish were significantly different from the expected outcome ($P<0.05$).
Figure 1  Effects of acute O₂ deprivation on the growth and development of zebrafish (Danio rerio) embryos. At 18 hpf, 24 hpf or 36 hpf embryos were exposed to 4 h of normoxia (95% dissolved oxygen; DO), hypoxia (5% DO) or anoxia (<0.5% DO). Embryos were then recovered in 95% DO and both growth (A, C and E) and hatch (B, D and F) rate were recorded throughout recovery. The symbols * and † indicate that hypoxia or anoxia treated embryos are significantly delayed ($P<0.05$) compared to the normoxia control, using size (18 hpf, A; 24 hpf, C) or otic vesicle length (OVL; 36 hpf, E inset) as a measure of development. Embryo length (EL) and OVL data are mean ± SEM ($N=15$) and were analyzed using 2-Way ANOVA and Tukey’s post hoc test.
Figure 1
Figure 2  HIF-1 cellular response of zebrafish (Danio rerio) embryos exposed to 4 h of hypoxia (5% DO) or anoxia (<0.5% DO) at 18 hpf. A representative Western blot of the HIF-1α protein band at 86 kDa (A). HIF-1α levels, relative to total protein of 20 pooled embryos (N=6), immediately following low O₂ treatment (B, P=0.054). Expression levels of hif-1α (C) and HIF-1 target genes (igfbp-1 D; epo E; vegf-165 F) mRNA immediately following treatment and after 4 h of recovery in 95% DO. To account for developmental delays caused by low O₂ treatments both developmentally matched (control) and time matched (normoxia) control groups are included. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA or 2-Way ANOVA and Tukey’s post hoc test (P<0.05)
Figure 2
Figure 3  HIF-1 cellular response of zebrafish (*Danio rerio*) embryos exposed to 4 h of hypoxia (5% DO) or anoxia (<0.5% DO) at 24 hpf. A representative Western blot of the HIF-1α protein band at 86k Da (A). HIF-1α levels, relative to total protein of 20 pooled embryos (N=6), immediately following low O₂ treatment (B). Expression levels of *hif-1a* (C) and HIF-1 target genes (*igfbp-1* D; *epo* E; *vegf-165* F) mRNA immediately following treatment and after 4 h of recovery in 95% DO. To account for developmental delays caused by low O₂ treatments both developmentally matched (control) and time matched (normoxia) control groups are included. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA or 2-Way ANOVA and Tukey’s post hoc test (P<0.05).
Figure 3
Figure 4  HIF-1 cellular response of zebrafish (Danio rerio) embryos exposed to 4 h of hypoxia (5% DO) or anoxia (<0.5% DO) at 36 hpf. A representative Western blot of the HIF-1α protein band at 86 kDa (A). HIF-1α levels, relative to total protein of 20 pooled embryos (N=6) immediately following low O₂ treatment (B). Expression levels of hif-1α (C) and HIF-1 target genes (igfbp-1 D; epo E; vegf-165 F) mRNA immediately following treatment and after 4 h of recovery in 95% DO. To account for developmental delays caused by low O₂ treatments both developmentally matched (control) and time matched (normoxia) control groups are included. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA or 2-Way ANOVA and Tukey’s post hoc test (P<0.05).
Figure 4
Figure 5  

$O_2$ consumption and critical oxygen tension ($P_{\text{crit}}$) of zebrafish ($Danio rerio$) larvae (4 dpf) exposed as embryos at 18 hpf (A, B), 24 hpf (C, D) or 36 hpf (E,F) to 4 h of normoxia (95% DO), hypoxia (5% DO) or anoxia (<0.5% DO). Values are mean ± SEM ($N=6$) and represent the $O_2$ consumption curves and $P_{\text{crit}}$ of pools of 20 larvae. Groups that do not share a common letter are significantly different as determined by 1-Way ANOVA and Tukey’s post hoc test ($P<0.05$).
Figure 5

A 18 hpf

O$_2$ consumption (μmol·larvae$^{-1}·h^{-1}$)

B 18 hpf

Larval $P_c$ (μmol·larvae$^{-1}·h^{-1}$)

C 24 hpf

D 24 hpf

E 36 hpf

F 36 hpf

Larval $P_c$ (μmol·larvae$^{-1}·h^{-1}$)

Embryonic Treatment

Normoxia  Hypoxia  Anoxia

a  a  a

a  b  a

a  b  b
Figure 6  

O₂ consumption and critical oxygen tension (P_{crit}) of male and female adult zebrafish exposed as embryos at 18 hpf (A, B), 24 hpf (C, D) or 36 hpf (E,F) to 4 h of normoxia (95% DO), hypoxia (5% DO) or anoxia (<0.5% DO). All data are mean ± SEM (O₂ consumption, N=8; P_{crit}, N=4). Groups that do not share a common letter are significantly different as determined by 2-Way ANOVA and Tukey’s post hoc test (P<0.05).
Figure 6
Figure 7    Time to loss of equilibrium (LOE) in severe hypoxia (5% DO) of adult zebrafish (*Danio rerio*) exposed as embryos at 18 hpf (A), 24 hpf (B) or 36 hpf (C) to 4 h of normoxia (100% DO), hypoxia (5% DO) or anoxia (<0.5% DO). Values are mean ± SEM (N=8) and include equal numbers of males and females. Analysis with 2-Way ANOVA failed to detect an effect of sex or embryonic treatment on LOE at 18 hpf (*P*=0.585; *P*=0.503), 24 hpf (*P*=0.522; *P*=0.205) or 36 hpf (*P*=0.783; *P*=0.907) with an α level of 0.05.
Figure 7
Figure 8  Population sex ratios of adult zebrafish (*Danio rerio*) exposed as embryos at 18 hpf (A), 24 hpf (B) or 36 hpf (C) to 4 h of normoxia (100% DO) hypoxia (<5% DO) or anoxia (0.5% DO). Values indicated by * are significantly different from the expected proportions of 50% male, 50% female ($X^2$, $P<0.05$).
Figure 8
Table 2 Whole zebrafish embryo lactate concentrations (μmol·g⁻¹) in response to normoxic, hypoxic and anoxic conditions.

<table>
<thead>
<tr>
<th>Stage of Treatment</th>
<th>Developmental Control (95% DO)</th>
<th>Time matched Control (95% DO)</th>
<th>4 h Hypoxia (5% DO)</th>
<th>4 h Anoxia (&lt;0.5% DO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 hpf</td>
<td>11.85 ± 1.72 a</td>
<td>14.97 ± 1.59 a</td>
<td>12.72 ± 2.01 a</td>
<td>12.60 ± 1.65 a</td>
</tr>
<tr>
<td>24 hpf</td>
<td>10.40 ± 2.15 a</td>
<td>12.53 ± 2.11 a</td>
<td>17.42 ± 1.57 a</td>
<td>13.75 ± 2.48 a</td>
</tr>
<tr>
<td>36 hpf</td>
<td>9.09 ± 2.12 a</td>
<td>11.09 ± 1.43 a</td>
<td>15.95 ± 1.24 ab</td>
<td>18.42 ± 1.83 b</td>
</tr>
</tbody>
</table>

Within a developmental stage groups that do not share a common letter are statistically different as determined with 1-Way ANOVA and Tukey’s post hoc test (P<0.05).
Discussion

Since its discovery the HIF-1 response has been well characterized in many organisms, however this is one of the few studies to examine the long term physiological consequences of mounting this response during development. I have shown that acute hypoxia exposure causes zebrafish embryos to mount different cellular responses based on developmental stage and severity of O₂ deprivation. These data also suggest that activation of the HIF-1 pathway during early development alters both the larval and adult phenotype of zebrafish (Figure 9).

Embryonic O₂ deprivation coping strategy

In this study I used embryo length as a proxy for development and I have shown that exposure to both hypoxia and anoxia causes zebrafish embryos at all 3 tested developmental stages (18, 24 and 36 hpf) to slow or halt growth. Gain and loss of function studies have shown that when IGFBP-1 accumulates in hypoxia it impairs growth by inhibiting the activity of growth promoting factors (IGFs; Kajimura et al, 2005). Zebarfish igfbp-1 is also the only fish gene confirmed by functional assays to be regulated by HIF-1 (Kajimura et al, 2006). I have shown a 3-5 fold increase in igfbp-1 expression relative to controls in embryos exposed to hypoxia at each developmental stage, and anoxia at 36 hpf. The developmental delays seen in these 4 treatment groups are likely a result of elevated IGFBP-1 levels, possibly mediated by HIF-1.

There is further evidence of HIF-1 activity in 3 of the low O₂ exposed groups. Embryos exposed to hypoxia at 24 hpf and both hypoxia and anoxia at 36 hpf accumulated significant amounts of HIF-1α protein. These changes in transcription factor protein levels corresponded with 3-5 fold increases in the expression of multiple HIF-1 target genes. Interestingly, all 3 groups showed different gene expression patterns. Clearly the HIF-1 pathway is not an all or
nothing response and may be altered based on the developmental timing and the severity of O$_2$
deprivation. It is also noteworthy that the accumulation of HIF-α protein never corresponded
with an induction of $hif$-$1\alpha$ mRNA. The regulation of $hif$-$1\alpha$ gene expression is poorly
understood even in mammals as it often does not change and sometimes decreases with hypoxia
(Wenger et al, 1997). In fish the induction of $hif$-$1\alpha$ mRNA may depend on the duration of the
hypoxic stress (Law et al, 2006; Terova et al, 2008) and it has been previously shown that much
longer hypoxia exposures than those used in this study (24 h) at 24 hpf do increase $hif$-$1\alpha$ mRNA
levels in zebrafish (Ton et al, 2003). Despite increasing $igfbp$-$1$ expression, hypoxia exposure at
18 hpf did not correlate with an increase in HIF-1α protein or expression changes in any of the
other genes measured. Therefore, these embryos likely did not mount a HIF-1 mediated cellular
hypoxia response, and a regulatory mechanism outside the scope of this study, for example HIF-
2, could be at work (Rojas et al, 2007).

Interestingly, anoxia exposure at 18 hpf and 24 hpf also resulted in significant
developmental delays. However, embryos in these groups did not increase the expression of
$igfbp$-$1$ or any of the other measured genes. It is possible that instead these embryos are entering
a state of suspended animation which is characterized by a severe depression of both metabolism
and cellular activity, corresponding with the cessation of the cell cycle. While measuring these
parameters was not an objective of this study, it has been well documented that anoxia exposure
this early in development results in complete arrest of the cell cycle (Padilla and Roth, 2001;
Mendelsohn et al, 2008; 2009). It has been hypothesized that suspended animation is not
mediated by the HIF-1 cellular pathway (Padilla et al, 2002). The first evidence for this in
zebrafish was provided by Mendelsohn and colleagues (2008) who showed that HIF-1α
knockdown embryos could survive anoxia exposure. This would explain why there was no up
regulation of any hypoxia responsive genes in either of these groups.

I found a significant accumulation of HIF-1α protein in anoxia exposed embryos at 24 hpf despite no corresponding changes in any of the HIF-1 target genes measured. It is important to note that the accumulation of HIF-1α is a passive process as it involves a decrease in protein degradation rather than an increase of protein synthesis. Therefore, accumulating HIF-1α protein under low O₂ conditions does not imply that transcription and translation are actively occurring within a cell. These data suggest that at 24 hpf the mechanism mediating the response to anoxia is inhibiting HIF-1 mediated transcriptional activity.

HIF-1 requires a variety of stabilizing co-factors to bind to its target genes. Several of these co-factors, including the histone acetyltransferase p300, are also required by the tumor suppressor transcription factor p53 (Chan and La Thangue, 2001; Freedman et al, 2002). Cancer researchers have shown that p53 accumulates in vitro during severe or prolonged hypoxia and outcompetes HIF-1 for their shared cofactors, inhibiting HIF-1 transcriptional activity (Graeber et al, 1994; Alarcon et al, 1999; Blagosklonny et al, 1998; Schmid, 2004; Sermeus and Michiels, 2011). In normal vertebrate development p53 regulates the cell cycle (Wallingford et al, 1997; Langheinrich et al, 2002) and regulates glycolysis and oxidative phosphorylation during metabolic stress (for review see Maddocks and Vousden, 2011). Inhibiting any step of oxidative phosphorylation causes zebrafish embryos to arrest, making energy status the likely proximate mechanism for suspended animation (Mendelsohn et al, 2008; 2009). It is possible that the anoxia response during early development is regulated by this pathway. An accumulation of p53 would explain why I found an increase in HIF-1α protein but no HIF-1-mediated transcriptional activity in anoxia-exposed embryos at 24 hpf. Clearly this mechanism must be developmentally dependent as HIF-1 is not inhibited under anoxic conditions at 36 hpf.
Roughly half of zebrafish embryos exposed to anoxia at 36 hpf also hatched during the treatment. Precocious hatching in response to low O₂ is seen in many fish and amphibian species (Alderice et al., 1958; Warkentin, 2007). Hatching is a very active process that involves the production of chorinase enzymes as well as the physical disruption of the chorion by the embryo (Yammamoto and Yamagami, 1975; Yamagami, 1981). Hatching is particularly challenging earlier in development when the chorion is thickest (Czerkies et al., 2001; Warkentin, 2007). The chorion itself also acts as a barrier to O₂. In rainbow trout (*Onchorynchus mykiss*) embryos DO levels can be >90% lower within the chorion than the surrounding water (Ciuhandu et al., 2007). These levels decrease even further when embryos are exposed to hypoxia (Miller et al., 2008). At 36 hpf, embryos exposed to anoxia also accumulate significant amounts of lactate, which is likely a result of their active efforts to hatch (Ninness et al., 2006). Embryos at 18 and 24 hpf are not hatching competent and therefore cannot use this strategy to avoid anoxia. If bouts of low O₂ are prolonged it may be that hatching is more effective protection than entering a state of dormancy.

The cellular response to low O₂ can be influenced by both developmental stage of the embryo and the severity of O₂ deprivation that they encounter. I have shown evidence for the induction of the HIF-1 pathway in embryos exposed to hypoxia at 24 and 36 hpf as well as anoxia at 36 hpf. Embryos exposed to hypoxia and anoxia at 18 hpf, and anoxia at 24 hpf are clearly using alternate strategies, not mediated by HIF-1, for coping with low O₂.

*Plasticity of larval hypoxia tolerance*

Regardless of O₂ level or developmental stage of treatment, acute induction of the HIF-1 pathway was correlated with an increased hypoxia tolerance (lower P<sub>crit</sub> than control) in larvae at
Conversely, larvae that did not have a HIF-1 response to low O\textsubscript{2} during development had a similar P\textsubscript{crit} to normoxic controls. These findings support the hypothesis that the plasticity of hypoxia tolerance is a result of HIF-1 and its downstream targets. In the wild, zebrafish spawn in low O\textsubscript{2} environments such as shallow pools, rice paddies and slow moving streams (Engeszer et al, 2007). These changes to P\textsubscript{crit} could confer a competitive advantage for larvae that forage for food in hypoxic waters.

Using a group of closely related and widely distributed intertidal sculpin species Mandic et al (2009) determined that independent of phylogeny, differences in hypoxia tolerance could be attributed to three specific traits. They found that 70\% of variation in P\textsubscript{crit} can be explained by differences in routine metabolic rate (M\textsubscript{r}), hemoglobin-oxygen (Hb-O\textsubscript{2}) binding affinity or gill-surface area. The differences in P\textsubscript{crit} observed in this study cannot be attributed to M\textsubscript{r} as larvae at 4 dpf consume O\textsubscript{2} at similar rates under normoxic conditions regardless of any previous low O\textsubscript{2} exposure. In contrast, it is possible that increased hypoxia tolerance in zebrafish larvae is a result of an increased Hb-O\textsubscript{2} binding affinity or respiratory surface area.

Hb isoform expression is regulated by HIF-1 in many species (Gorr et al, 2004). Overall O\textsubscript{2}-binding affinity can be altered by changing the ratio of Hb isoforms that are expressed. Many fish species, including rainbow trout (Oncorhynchus mykiss), African cichlids (Astatoreochromis alluaudi) and gilt-head seabream (Sparus aurata) use Hb isoform switching to cope with chronic hypoxia (Marinsky et al, 1990; Rutjes et al, 2007; Campo et al, 2008). In general embryonic and larval hemoglobin isoforms have a higher O\textsubscript{2} binding affinity than adult isoforms (Rombough, 1997). Zebrafish have at least 13 different globin genes, each with different O\textsubscript{2}-binding affinities (Ganis et al, 2012). Between 24 and 36 hpf they switch from embryonic to larval Hb and switch again to adult isoforms around 22 dpf (Ganis et al, 2012). Although the results are equivocal.
(Ton et al, 2003; Rombough and Drader, 2008) there is evidence that both Hb concentration and affinity can affect the hypoxia tolerance of 7 dpf zebrafish larvae (Schwerte et al, 2005; Guan et al, 2010). The effects of Hb on hypoxia tolerance vary considerably with development. Therefore, while altered Hb isoforms may contribute to increased hypoxia tolerance at 4 dpf, it seems unlikely to be the sole mechanism.

At 4 dpf zebrafish possess primordial gill filaments however, their gills do not fully develop until 28 dpf (Rombough, 2002). Ablation studies suggest that by 7 dpf zebrafish rely on their developing gills primarily for ion-exchange and instead use their skin for O2 uptake (Rombough, 2002). Therefore, it is possible that 4 dpf larvae that demonstrate higher hypoxia tolerance have an increased gill surface area. However, it is unlikely that this would impact $P_{crit}$ at this early larval stage. It is more likely that early O2 exposure causes an increase in the vascularisation of the skin in these larvae. As the skin is the main respiratory surface for larval zebrafish before 14 dpf (Rombough, 2002) an increase in the capacity for epidermal O2 uptake would be analogous to increased gill surface area in adult fish (Mandic et al, 2009). It is well known that HIF-1 up-regulates many factors involved in angiogenesis, including embryonic VEGF as shown in this study (for review see Hirota and Semenza, 2005). Additionally, larvae that over express HIF-1α have significantly increased vascularisation of the skin compared to wild-type zebrafish (van Rooijen et al, 2010). Hypoxia exposure can remodel larval amphibian skin to be thinner and more highly vascularised, increasing gas exchange efficiency (Krogh, 1904; Feder and Burggren, 1985, Burggren and Mwalukoma, 1983). It would be interesting to determine if hypoxia-exposed zebrafish larvae have thinner skin than their normoxic controls. It is possible that increased hypoxia tolerance in zebrafish larvae is partially a result of HIF-1 mediated skin remodeling, which allows them to extract O2 more efficiently from the
Adult hypoxia tolerance

I have shown that larval increases in hypoxia tolerance are not sustained through adulthood. However, to my knowledge this is the first study to report that male zebrafish have a higher hypoxia tolerance (i.e. lower $P_{\text{crit}}$) than female zebrafish regardless of embryonic treatment. When $O_2$ availability decreases, vertebrates shunt energy away from non-essential functions such as growth, digestion and reproduction to maintain energy homeostasis (for review see Wang et al, 2009). Compared to the costs of maintaining basal metabolic rate, gamete production in males is very inexpensive (0.1% of energy), while the energetic cost of gamete production in females is consistently 3.5 orders of magnitude higher than males across all vertebrate taxa (Hayward and Gillooly, 2011). Therefore, male fish, with their superior energy reserve, maintain metabolic rate at lower $O_2$ levels ($<P_{\text{crit}}$) than females.

Adult sex ratios

My results demonstrate that populations of fish that mounted a HIF-1 response to low $O_2$ during development had a higher proportion of males as adults. The sex determination mechanism in zebrafish is currently unknown though it is thought to be primarily genetic but influenced by environmental conditions (Liew et al, 2012). Zebrafish larvae pass through a hermaphroditic stage around 10 dpf and they are fully differentiated into males and females around 40 dpf (Takahashi, 1977). Though embryos in this study were exposed to low $O_2$ well before sexual differentiation took place, exposure to other endocrine disrupting compounds prior to hatch are known to affect zebrafish sex determination (Andersen et al, 2002). Additionally,
chronic hypoxia throughout ontogeny also results in male-biased zebrafish populations (Shang et al, 2006). This is likely caused by increased testosterone production, observed in hypoxia treated embryos as early as 48 hpf, the earliest stage tested to date (Shang and Wu, 2004; Shang et al, 2006). It is therefore possible that the male-biases observed in this study are a result of sex hormone production downstream of the HIF-1 pathway.

**Summary and Conclusions**

My data provide evidence that mounting a robust cellular hypoxia response during development alters several aspects of the zebrafish phenotype. Early induction of the HIF-1 pathway corresponded with adaptive plasticity of hypoxia tolerance which is likely a result of improved O$_2$ uptake in larvae (**Figure 9**). Further studies are needed to assess if these changes occur at the level of the skin as the primary respiratory surface or the Hb molecules themselves. While improved hypoxia tolerance is lost by adulthood it seems that one downstream effect of a hypoxia cellular response early in ontogeny is the altered production of sex hormones, resulting in a higher proportion of males. Given that reproductive output of a population is limited by the number of females, Shang et al. (2006) previously suggested that a male-biased population of fish will have a decreased fitness level and that hypoxia exposure could decimate wild populations (Wu, 2009). However, given that males had a lower P$_{crit}$, a male-biased population would have a competitive advantage in hypoxic conditions. In this respect hypoxia-induced sex ratio imbalances could be adaptive.

To my knowledge this study is the first to provide mechanistic insight into the plasticity of the hypoxia response. The HIF-1 cellular hypoxia response involves the decrease of aerobic metabolism and a suppression of energy requiring processes such as growth. It also promotes the
transcription and translation of many target genes as well as the formation of new blood cells and vessels (Nikinma and Rees, 2005). However beyond these immediate benefits in O₂ uptake and utilization efficiency the HIF-1 pathway appears to alter the phenotype of individual fish, better enabling them to cope with fluctuating O₂ environments as both larvae and adults. This is possibly achieved by alteration of HIF-1 target gene expression causing remodeling of the skin and sex hormone production. The trade-off between HIF-1 and other low O₂ coping strategies, such as suspended animation, may be regulated by hypoxia responsive factors such as p53. Zebrafish embryos therefore have an arsenal of mechanisms to cope with low O₂ bouts of varying severity and duration. Further studies are needed to elucidate these mechanisms, their tradeoffs and their potential downstream effects.
Figure 9. Summary of cellular and phenotypic changes caused by acute oxygen deprivation exposure during development. Zebrafish embryos were exposed to acute (4 h) anoxia (<0.5% DO) or hypoxia (5% DO) at A) 18 hpf, B) 24 hpf or C) 36 hpf. The cellular response to low oxygen was measured immediately following treatment while hypoxia tolerance was measured at 4 dpf and adulthood.
Fertilization

Time: 0 hpf
18 hpf
24 hpf
36 hpf
48 hpf
4 dpf
Adult

O₂ deprivation coping strategy

Hypoxia → Anoxia

No HIF-1 Response

HIF-1 response

Phenotypic changes associated with embryonic O₂ response

Male Hypoxia Tolerance > Female

Anoxia Tolerance High

Anoxia Tolerance Decreases

Anoxia = Lethal

Hypoxia Tolerance

70% Male vs 45% Male

Pre-1st Feeding Event

Hatch

1st Heart Beat

Male

Female

O₂ deprivation coping strategy

HIF-1 response

Phenotypic changes associated with embryonic O₂ response

Male Hypoxia Tolerance > Female

Anoxia Tolerance High

Anoxia Tolerance Decreases

Anoxia = Lethal
General Discussion

I have shown that mounting a cellular hypoxia response during embryonic development, pre-disposes zebrafish to an increased hypoxia tolerance as larvae and adults. I have argued that these altered phenotypes are mediated by the induction of the HIF-1 pathway. The strongest evidence for this argument is that embryos exposed to low $O_2$ levels that fail to mount a HIF-1 response (18 hpf; 24 hpf anoxia) do not appear any different from control fish, maintained in normoxia throughout development. The fact that these groups all respond to low $O_2$ as embryos with slightly different cellular strategies adds more support to the idea that HIF-1 is responsible for the altered phenotypes that they fail to express. However, there are additional possibilities that warrant consideration.

Perspectives

It is important to remember that the cellular hypoxia response involves much more than the induction of HIF-1. Using microarray technology Ton et al (2003) found that zebrafish embryos at 24 hpf exposed to severe hypoxia up-regulated the expression of 148 different genes. Many homologues of putative HIF-1 gene targets in fish contain HREs, the HIF-1 promoter regions (Rees et al, 2009). The first of which was discovered in the gene coding for lactate dehydrogenase in the killifish (*Fundulus heteroclitus*; Rees et al, 2001). However, other than *igfbp-1* none of these genes are confirmed HIF-1 targets in zebrafish (Kajimura et al 2005; 2006). Therefore, other factors may be involved in the regulation of hypoxia responsive genes in fish. For example, at 18 hpf I have shown a hypoxia sensitive induction of *igfbp-1* expression in the absence of significant HIF-1α accumulation.

There are two additional hypoxia responsive HIF-α isoforms that have been identified to
date (Ema et al, 1997; Gu et al, 1998). HIF-2α appears to either up regulate or inhibit the expression of some HIF-1 target genes (Raval et al, 2005; Rankin et al, 2007) and is most prevalent early in zebrafish development (Rojas et al, 2007). In mammals HIF-3α can negatively regulate HIF-1 under hypoxic conditions by dimerizing with HIF-1α (Makino et al, 2002). HIF-3α sequences have been identified in seven fish species to date (Law et al, 2006; Richards, 2009). However, the functional similarities of these of these proteins to mammalian HIF-3α have yet to be confirmed (Nikitma and Rees, 2005). It is possible that one or both of these factors are involved in regulating the cellular hypoxia response of zebrafish embryos. This could explain why I observed an up-regulation of igfbp-1 without HIF-1 at 18 hpf and 3 different gene expression patterns among the 3 treated groups that mounted a HIF-1 response.

Given that HIF-1 plays a role in normal development there must be developmentally specific factors regulating its activity even in normoxia. Additionally, I suspect that hypoxia responsive factors such as HIF-2 and HIF-3 are selectively inhibiting aspects of the HIF-1 response, based on the energetic demands of embryos at different stages. For example, at 36 hpf hypoxia-exposed embryos up regulate both vegf and epo, while at the same developmental stage anoxia exposure does not result in epo induction. Unlike adult fish that use circulating red blood cells and Hb for O₂ transport, embryos at 36 hpf get all of their O₂ through passive diffusion (Rombough and Drader, 2009). Producing unnecessary red blood cells, filled with large Hb proteins, comes at a metabolic cost. Anoxia exposure may be enough of an energetic stressor to necessitate the inhibition of costly red blood cell production. Instead, it is possible that by altering the expression of certain HIF-1 activated genes, anoxia exposed embryos at this stage can focus the majority of their energy on attempting to hatch. Conversely, hypoxia exposure at 36 hpf may not represent a severe enough metabolic stress to necessitate shutting down the
expression of genes like *epo*. It is possible that embryos that lacked these regulatory mechanisms would mount a full adult HIF-1 response any time they encountered any degree of hypoxia.

*Future Studies*

In addition to characterizing the full scope of HIF-1 regulation during development, future studies are needed to confirm that the downstream phenotypic changes I observed are directly mediated by the HIF-1 pathway. Ideally, my experiments would be repeated on embryos that lack HIF-1. These embryos should not display altered larval and adult phenotypes. The other way to identify HIF-1 specific effects is by inducing HIF-1 under normoxic conditions. If embryos that undergo HIF-1 induction in the absence of low O\(_2\) also exhibit altered hypoxia tolerance phenotypes it would confirm the conclusions of this study.

The largest challenge associated with altering HIF-1 expression of embryos is being able to do so over controlled, acute periods of time. As previously discussed, HIF-1 plays an integral role in the development of the vertebrate cardiovascular system. Null mutations in *hif-1α* or *hif-1β* are lethal and therefore cannot be used in long term studies (Maltepe et al, 1997; Ryan et al, 1998). It is also possible to knock down the expression of a specific gene by injecting embryos with small strands of antisense RNA, known as morpholinos, which bind to mRNA, preventing translation. Unfortunately, *hif-1α* morpholino knockdown *Xenopus* embryos have extensive cardiac and vasculature mutations (Nagao et al, 2008). A zebrafish morpholino has been designed against *hif-1α* (Mohamed, 2007). However, the efficiency of the knockout has not been confirmed and the downstream effects have never been examined past 40 hpf. While *hif-1* knockouts could be useful for further characterizing embryonic cellular responses to low O\(_2\), the effects of knocking down *hif-1* expression on normal development make it impossible to use
these methods to study downstream phenotypic effects of hypoxia exposure.

Transgenic over expression of genes is a very popular technique in zebrafish developmental biology. This process involves the injection of specific DNA sequences into the 1-2 cell stage of the embryo, which then inserts into the chromosomes. However, the efficiency of this technique is low and generally results in a mosaic-like expression of the target gene throughout the fish (Xu, et al, 1999). Similarly, over expressing HIF-1 by knocking out the enzymes that degrade HIF-1α protein cause zebrafish embryos to chronically mount a hypoxia response. These embryos develop polycythemia and other cardiovascular disease-like states (van Rooijen et al, 2010). Chronically over-expressing HIF-1 presents many of the same challenges as knocking it out. Any changes to the hypoxia tolerance phenotype of larvae or adults would be confounded by the developmental defects associated with these techniques. On the other hand injecting specific RNA strands into early embryos results in the acute over expression of the desired gene. The technique is similar to that described above except that the RNA is translated into protein and then degraded. Micro-injection of hif-1α mRNA has been used in zebrafish embryos successfully (Kajimura et al, 2006; Chu et al, 2010). However, it is impossible to regulate the duration of this over expression as it depends entirely on how quickly the injected RNA and resulting proteins are degraded by the individual cells (Xu, 1997). It is unknown how long into development injected hif-1α would persist in zebrafish embryos. However HIF-1α protein is degraded very quickly in normoxia and over expression of HIF-1 has never been used past 12 hpf (Kajimura et al, 2006; Chu et al, 2010). It would be very difficult to target later developmental stages using this technique.

In order to control the timing of a transgenic gene it is possible to add a heat inducible promoter to the sequence. This technique has been used in zebrafish embryos, and allows
researchers to activate their gene of interest by exposing embryos to 38°C. Returning embryos to 28°C then inhibits the expression of the gene (Shoji and Sato-Maeda, 2008). This could be a very useful tool for studying the acute effects of HIF-1 induction. Interestingly, in some fish HIF-1 is induced by increased temperatures (Rissanen et al, 2006). Given that increased temperatures result in decreased oxygen saturation, thermal sensitivity may act as a cue for hypoxic or anoxic episodes in fish (Stenslokken, 2010). It could be possible to compare the downstream effects of acute thermal stress during development with this study in order to further elucidate the role of HIF-1. However, the cellular heat stress response is also very complex, and can alter development on its own (Schaefer and Ryan, 2006). It may be difficult to tease apart the specific effects of HIF-1 using this method.

The final possibility is to chemically induce HIF-1 with iron chelators, such as CoCl$_2$. These chemicals stabilize HIF-1α protein under normoxic conditions (Yuan et al, 2003). Treatment with CoCl$_2$ for 24 h has been shown to induce leptin (a suspected HIF target) expression in 24 hpf zebrafish embryos, without any developmental toxicity (Chu et al, 2010). In my study I attempted to induce HIF-1 using CoCl$_2$. However, the short 4 h exposures were not long enough to induce the HIF-1 pathway. Longer exposures of CoCl$_2$ could be effective for future work. However, in order to identify the specific effects at the critical developmental periods in this study, it was important to maintain the same acute treatment windows.

Conclusions

The hypoxia responsive aspect of the HIF-1 pathway is highly conserved and was probably fully functional in the common ancestor of all metazoans (Rytkonen et al, 2011). As such, even the simplest living animal, Tricoplax adherans, appears to have multiple regulatory mechanisms to control the expression of HIF-1 (Loenarz et al, 2010). Given that the HIF-1
pathway acts as a mediator of both normal vertebrate development and the hypoxia stress response, isolating the effects of early hypoxia exposure at specific developmental windows is not trivial. However, it also emphasizes the importance of understanding the downstream effects of this pathway. Hypoxic dead zones in all aquatic environments are becoming more prevalent and it is increasingly important to understand how even brief hypoxia exposure during development will influence the adult phenotypes of wild fish the world over. My study is the first step in understanding the mechanistic role that HIF-1 plays in this process. Using a combination of the techniques described above it will be possible to tease apart the many roles of this universal master-regulator.
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Supplementary Material

S. Figure 1 Developmental staging measurements on a zebrafish embryo (24hpf); modified from Kimmel et al. (1995). **A.** Embryo length (EL) is measured from the tip of the tail to the flattest part of the head. **B.** The otic vesicle length (OVL) is the number of additional otic vesicles that would fit before the eye and is calculated as the length between the otic vesicle and the eye (L) divided by the horizontal diameter of the otic vesicle (OV). OVL = L/OV.
S. Figure 2. Primer and probe specificity for *erythropoietin* qPCR amplification. Lane 1 shows the 1kb DNA ladder (Invitrogen), lanes 2-5 are the 59 bp qPCR amplification products using the *epo* primers and probe. The qPCR reactions loaded in lanes 2-4 used cDNA samples from control embryos at 20 hpf while lane 5 used a negative control no reverse transcriptase cDNA sample.