Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish, *Danio rerio*

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In recent years, natural and anthropogenic factors have increased aquatic hypoxia the world over. In most organisms, the cellular response to hypoxia is mediated by the master regulator hypoxia-inducible factor-1 (HIF-1). HIF-1 also plays a critical role in the normal development of the cardiovascular system of vertebrates. We tested the hypothesis that hypoxia exposures which resulted in HIF-1 induction during embryogenesis would be associated with enhanced hypoxia tolerance in subsequent developmental stages. We exposed zebrafish (*Danio rerio*) embryos to just 4 h of severe hypoxia or total anoxia at 18, 24 and 36 h post-fertilization (hpf). Of these, exposure to hypoxia at 24 and 36 hpf as well as anoxia at 36 hpf activated the HIF-1 cellular pathway. Zebrafish embryos that acutely upregulated the HIF-1 pathway had an increased hypoxia tolerance as larvae. The critical window for hypoxia sensitivity and HIF-1 signalling was 24 hpf. Adult male fish had a lower critical oxygen tension (*P*<sub>crit</sub>) compared with females. Early induction of HIF-1 correlated directly with an increased proportion of males in the population. We conclude that mounting a HIF-1 response during embryogenesis is associated with long-term impacts on the phenotype of later stages which could influence both individual hypoxia tolerance and population dynamics.

1. Introduction

Low environmental oxygen (hypoxia) represents a major selective pressure in vertebrate evolution, particularly in aquatic ecosystems, driving morphological, physiological and behavioural adaptations [1]. In recent years, the prevalence and intensity of aquatic hypoxic episodes has increased worldwide [2]. Many systems that are particularly prone to both chronic and diurnal hypoxia (i.e. slow-moving, shallow and warm waters) are also ideal nursery habitats for aquatic vertebrates [3]. Developing organisms use their early environment to predict the environmental conditions they will most likely encounter as adults and adjust their developmental trajectory accordingly to produce traits best suited for those environments [4]. For example, there is some evidence that early exposure to low oxygen (O<sub>2</sub>) may increase hypoxia tolerance. When parental zebrafish (*Danio rerio*) are exposed to two to six weeks of low O<sub>2</sub>, their offspring maintain equilibrium longer in severe hypoxia [5]. Additionally, zebrafish reared chronically in mild hypoxia maintain resting metabolic rates at a lower level of dissolved O<sub>2</sub> (DO; [6]). It appears that early exposure to hypoxia may prime aquatic animals to better cope with hypoxic environments they may face as adults.

Given the natural variation in both the frequency and duration of hypoxic episodes in aquatic ecosystems, developing fishes are likely to experience fluctuating levels of low DO [3]. It is well known that the level of low O<sub>2</sub> that fishes can tolerate changes throughout embryogenesis [7–9]. Additionally, in mammals, birds and invertebrates, the downstream phenotypic effects of early hypoxia are developmental stage-specific [10,11]. However, the importance of the timing of early hypoxic events and the mechanisms driving developmental
plasticity of hypoxia tolerance in fish embryos are poorly understood. It is particularly important to understand this dimension of hypoxia stress because frequent, acute bouts of hypoxia are thought to be more stressful and cause greater physiological impairment than chronic low O2 environments [3].

One potential mechanism that could mediate developmental plasticity of hypoxia tolerance lies in the highly conserved hypoxia stress response. In all animals, the cellular response to low O2 is thought to be mediated by hypoxia-inducible factors (HIFs; [12,13]). HIFs are heterodimeric compounds with hypoxia sensitive α-subunits [12]. HIF-1 is a master regulator transcription factor that controls the expression of many genes that act downstream to increase O2 uptake and decrease O2 demand. These include the growth-suppressing insulin-like growth-factor-binding protein-1 (igfbp-1; [14]), the haematopoietic regulating hormone erythropoietin (epo; [15]) and the angiogenesis-promoting cytokine vascular endothelial growth factor (végf; [16]). In addition to its role in the cellular hypoxia response, HIF-1 is required for the regulation of many of its target genes during ontogeny [10,17] and is essential for proper development of vertebrate cardiovascular systems [17,18]. It is therefore likely that an induction of HIF-1 in response to hypoxia during development could have numerous long-term consequences.

In this study, we tested the hypothesis that zebrafish embryos exposed to acute low O2 during critical stages of development would exhibit enhanced hypoxia tolerance in subsequent developmental stages. Furthermore, we hypothesized that this enhanced tolerance would be associated with an induction of HIF-1 in response to low O2 during embryogenesis. To test these hypotheses, we exposed embryos to acute (4 h) episodes of severe hypoxia (5% DO) or total anoxia (less than 0.5% DO). We targeted three specific stages of embryogenesis (18, 24 and 36 h post-fertilization; hpf). At 18 hpf, zebrafish embryos are highly anoxia tolerant though their cardiovascular and nervous systems are just beginning to form [19]. By 24 hpf, the heart tube is developed and heart rate is initiated [20]. At this stage, anoxia tolerance declines and embryos are known to upregulate 148 genes in response to severe hypoxia (10% DO; [21]). Finally, by 36 hpf, the cardiovascular system is relatively well developed with circulating red blood cells [22], anoxia tolerance is diminished and embryos are hunting competent, though they generally will not hatch until 48 hpf [19].

2. Material and methods

(a) Animals

Fertilized eggs were collected from divided chamber breeding baskets with four adult males and four adult females from a breeding colony of zebrafish, housed at the University of Guelph, Hagen Aqualab. For each experiment, eggs from four to five breeding baskets were pooled to increase genetic diversity. Fish raised to adulthood originated from two to three separate breeding events. Adult fish were maintained at 26°C with a 14L:10D photoperiod. Developmental staging was performed as previously described [23].

(b) Experimental design

Embryos at 18, 24 or 36 hpf were exposed to one of three O2 regimes for 4 h: a control treatment (normoxia; DO: 95% or approx. 7.2 mg l−1), hypoxic treatment (DO: 5% or 0.4 mg l−1) and anoxic treatment (DO: less than 0.5% or 0.04 mg l−1). Preliminary experiments showed that low O2 treatments caused 2–4 h developmental delays (electronic supplementary material, figure S1) so an additional developmentally matched control group (DO: 95%) was included. Water O2 was controlled by regulating delivery of N2 gas or air through ceramic air stones into 20 l tanks and monitored using an O2 electrode (Liquid Dissolved O2 Field Probe, Hach, Loveland, CO, USA). Embryos were sampled immediately after exposure or allowed to recover in normoxia for an additional 4 h and stored at −80°C for later analysis. Finally, embryos exposed to the same O2 conditions described above were reared in normoxia to 4 dpf (larvae) or sexual maturity (six months post-fertilization), when their hypoxia tolerance was determined. Briefly, larvae were transferred to 1 l flow-through tanks at 4 dpf. At 5 dpf, larvae were fed 30 μm rotifer replacement supplements (Argent Chemical Laboratories, Redmond, WA, USA) 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, USA). Animal density was maintained between tanks.

(c) Protein extraction

To quantify whole embryo levels of HIF-1α protein, groups of 20 embryos (n = 6) were thawed in radio immune-precipitation assay lysis buffer with protease inhibitors (1 mM PMSF, 2 mM EDTA) and dispersed with a 1 ml Pasteur pipette to disrupt the chorion and yolk. Samples were briefly centrifuged (8000 × g for 2 min) to separate the yolk proteins and protein concentration was determined using a Bradford assay (BioRad Protein Assay Dye Reagent, BioRad, Hercules, CA, USA).

(d) Hypoxia-inducible factor-1α western blots

Forty micrograms of total protein was separated by 12% SDS-poly-acrylamide gel electrophoresis and transferred to PVDF membranes. Blots were probed with an affinity-purified polyclonal antibody generated in rabbits against full-length zebrafish HIF-1α (1:500; [24]) and HRP-conjugated goat anti-rabbit IgG secondary antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were imaged colorimetrically with the ChemiDocXRS + system (BioRad) ensuring that only non-saturated bands were quantified. Band density was determined via densitometry with ImageJ software (US National Institute of Health, Bethesda, MD, USA) and was expressed relative to total protein which was determined by subsequently staining blots with GelCode Blue Safe Stain Reagent (Thermo Scientific, Waltham, MA, USA; [25]). To compare between blots, all samples were normalized to a single sample (hypoxia exposed embryos at 24 hpf) ran on every gel. Western blot analysis of zebrafish fibroblast cells [26], 36 hpf zebrafish larvae and full-length recombinant zebrafish HIF-1α using anti-zebrafish HIF-1α antibody revealed specific binding at the expected molecular mass of 86 kDa as well as cross reaction to non-specific proteins (electronic supplementary material, figure S2). Densitometric analysis of the non-specific bands showed that the expression of these proteins was not affected by the low O2 treatments. Similarly, inclusion of a 100 kDa non-specific band (figures 1–3) with the 86 kDa HIF-1α band in a separate densitometric analysis did not affect the overall differences in HIF-1α signal between the different treatments. Therefore, only the 86 kDa band was used for quantification of HIF-1α levels at all developmental stages. The increase in HIF-1α band density and width in response to low O2 (figures 1–3; electronic supplementary material, figure S2) is probably a result of post-translational modifications of the stabilized protein [27,28].

(e) Quantification of mRNA levels of hypoxia-inducible factor-1 target genes

Total RNA was extracted from pools of 20 embryos using QIAzol Lysis Reagent (QIAGEN, Toronto, Ontario, Canada). Whole
embryo mRNA levels of igfbp-1, epo, hif-1α, ef1 α and the embryonic isoform of vegf (vegf-165; [24]) were quantified using quantitative real-time PCR as previously described [29]. For epo, 2× TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) was used. For cycling conditions, see the electronic supplementary material, figure S3. Gene expression was normalized to the housekeeping gene ef1-α and quantified as previously described. All primer sets had an amplification efficiency more than 80% (electronic supplementary material, table S1).

(f) L-lactate concentrations

Lactate concentrations (pools of 10 embryos) were determined spectrophotometrically [30].
(g) Routine metabolic rate, regulation index, O2 uptake limit and \( P_{\text{crit}} \)

Flow-through glass respirometry chambers were used to measure routine metabolic rate (\( M_r \)), regulation index (RI; [31]) and the O2 treatment in 4 dpf larvae, and to measure \( M_t \) and the critical O2 tension (\( P_{\text{crit}} \)) in single adult fish. Chambers were sealed following a 1 h (larvae) or 2 h (adults) acclimatization period, and O2 consumption was recorded using a 25 \( \mu \)m tip DO microelectrode (larvae; Unisense, Aarhus, Denmark) or a Clark-type DO probe and LOGGER PRO recorded using a 25

(h) Loss of equilibrium

Individual adult zebrafish were placed in a 1 l airtight, plastic container, filled with hypoxic water (5% DO). This insured that fish were unable to perform aquatic surface respiration. Time to loss of equilibrium (LOE) was determined as previously described [5].

(i) Sex determination

All adult fish were sexed based on external morphology. The accuracy of this method was confirmed by dissecting a random subset of fish (n = 133–140 embryonic stage \(-1\)) fixed in 4% paraformaldehyde in phosphate buffered saline (0.2% Tween20).

(j) Statistical analysis

All data are presented as mean \( \pm \) s.e.m. Significant differences between O2 treatments within a developmental stage were analysed by one-way ANOVA for HIF-1\( \alpha \) protein, lactate, larval \( M_r \), RI and O2 uptake limit. Larval O2 consumption versus environmental DO was analysed with two-way repeated measures ANOVAs. Two-way ANOVAs were used to determine the effects of time and O2 treatment on mRNA expression. At 18 and 36 hpf, \( \text{igfbp-1} \) gene expression data were log-transformed to meet assumptions of normality. Two-way ANOVAs were also used to determine the effects of embryonic O2 treatment and sex on adult \( M_r \), \( P_{\text{crit}} \) and time to LOE. Adult population sex ratios were compared against an expected outcome of 50% male using \( \chi^2 \) analysis. If significant main effects were determined, then Tukey’s post hoc test was used for further analysis.

3. Results

(a) Cellular response

At 18 hpf, both hypoxia and anoxia treatments caused a small non-significant induction of HIF-1\( \alpha \) protein (figure 1a; \( p = 0.054 \)) with no changes in hif-1\( \alpha \) mRNA (figure 1c). Only \( \text{igfbp-1} \) 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 1d; \( p = 0.048, p = 0.027 \)). Note that treated groups differed from both a time-matched (normoxia) and developmental control (control), and the p-values are given in that order.

At 24 hpf, both hypoxia and anoxia caused a twofold increase in HIF-1\( \alpha \) protein relative to controls (figure 2a; 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 2c). Hypoxia exposure caused a fivefold induction of \( \text{igfbp-1} \) (figure 2d, \( p < 0.001, p < 0.001 \)) and \( \text{epo} \) (figure 2e, \( p < 0.001, p < 0.001 \)), both of which returned to control levels after 4 h of recovery.

Embryos exposed to both hypoxia and anoxia at 36 hpf accumulated significant levels of HIF-1\( \alpha \) protein, 1.5- to 2.5-fold of both controls (figure 3a; hypoxia, \( p < 0.001, p = 0.024 \); anoxia, \( p < 0.001, p < 0.001 \)). Again, neither of these low O2 treatments caused hif-1\( \alpha \) expression levels to change (figure 3c). At 36 hpf,
both hypoxia and anoxia exposure resulted in an increased expression of multiple HIF-1 target genes (figure 3d–f). Hypoxia exposure caused a two- to fourfold induction of *igfbp-1*, *epo* and *vegf-165* (*p < 0.001, *p < 0.001; *p = 0.005, *p = 0.034; *p = 0.003, *p = 0.006*), while embryos exposed to anoxia upregulated *igfbp-1* and *vegf-165* (*p = 0.018, *p = 0.034; *p = 0.044, *p = 0.027*) by twofold. Anoxia exposure also resulted in a significant twofold accumulation of tissue lactate (table 1; *p = 0.010, *p = 0.013*).

(b) Larval hypoxia tolerance

The *M*ₚ of 4 dpf larvae was similar across all groups regardless of prior low O₂ treatment as embryos and they were able to regulate *M*ₚ to a certain degree until approximately 40–50% DO (figure 4a–c). However, those larvae that had been previously exposed to hypoxia at 24 hpf had greater rates of O₂ consumption at 15% DO than either anoxia or control (figure 4d) and were able to maintain O₂ uptake at significantly lower levels of DO (figure 4f; *p = 0.047*). Both anoxia and hypoxia exposure at 36 hpf resulted in higher rates of O₂ consumption than the control at both 20 and 15% DO (figure 4g) and these larvae were able to maintain O₂ uptake at significantly lower environmental O₂ levels than controls (figure 4i, *p < 0.001*). Anoxia exposure at 36 hpf also resulted in a significantly higher RI (better O₂ regulator; *p = 0.028*) than controls (figure 4h).

(c) Adult hypoxia tolerance

Survival rates were similar across all groups (40.1% ± 3.3%, data not shown) and mortalities primarily occurred during larval development in all treatments. Adult fish, exposed as embryos to hypoxia or anoxia, had similar *M*ₚ to control fish regardless of the developmental stage of their exposure.

Table 1. Whole zebrafish embryo lactate concentration (μmol g⁻¹) in response to 4 h of normoxia (control), hypoxia (5% DO) or anoxia (less than 0.5% DO). (Within a developmental stage, treatments that do not share a common letter are statistically different as determined by one-way ANOVA and Tukey’s post hoc test (*p < 0.05*). Data are mean ± s.e.m. (*n* = 8.)

<table>
<thead>
<tr>
<th>developmental stage (hpf)</th>
<th>normoxia (N)</th>
<th>hypoxia (H)</th>
<th>anoxia (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>11.85 ± 1.70⁰</td>
<td>14.97 ± 1.59⁰</td>
<td>12.72 ± 2.01⁰</td>
</tr>
<tr>
<td>24</td>
<td>10.40 ± 2.15⁴</td>
<td>12.53 ± 2.11⁴</td>
<td>17.42 ± 1.57⁴</td>
</tr>
<tr>
<td>36</td>
<td>9.09 ± 2.12⁴</td>
<td>11.09 ± 1.43⁴</td>
<td>15.95 ± 1.24⁴</td>
</tr>
</tbody>
</table>

Figure 4. O₂ consumption, RI and O₂ uptake limit of zebrafish (*D. rerio*) larvae (4 dpf) exposed as embryos at 18 (a–c), 24 (d–f) or 36 (g–i) hpf to 4 h of normoxia (N), hypoxia (5% DO; H), or anoxia (less than 0.5% DO; A). Values are mean ± s.e.m. (*n* = 6) and are representative of measurements from pools of 20 larvae. The entire O₂ consumption curves were used to calculate the RI values. Patterns were analysed with two-way RM ANOVA. Asterisks and dagger symbols indicate that hypoxia and anoxia treatments differs from normoxia, respectively. For RI and O₂ uptake limit values, significant differences are indicated by dissimilar letters (one-way ANOVA and Tukey post hoc test, *p < 0.05*).
As with the larvae, adult *P. crit* was not affected by exposure to low O2 at 18 hpf (figure 5b). In contrast to larvae, there was no effect of hypoxia or anoxia exposure on *P. crit* of adult fish exposed at 24 or 36 hpf (figure 5d, f). There was a significant effect of sex on hypoxia tolerance where, regardless of embryonic O2 treatment, female fish always had a higher *P. crit* than males (figure 5b–f; 18 hpf, *p* = 0.022; 24 hpf, *p* = 0.003; 36 hpf, *p* = 0.017). There was no effect of embryonic exposure to low O2 on LOE regardless of the timing of the exposure (electronic supplementary material, table S2).

(d) Sex ratios

Fish that had been previously exposed to hypoxia at 24 hpf were 72.5% male (*p* < 0.01) compared with control or anoxia-exposed fish which were 47% and 55% male, respectively (table 2). Fish exposed to anoxia at 36 hpf were also male-biased at 71% compared with 41% males in the control group (*p* < 0.05). There were no changes in sex ratios of adult fish treated at 18 hpf.

### Table 2. Population sex ratios of adult zebrafish exposed as embryos to 4 h of normoxia (control), hypoxia (5% DO) or anoxia (less than 5% DO). (Asterisk (*) indicates significantly different from an expected χ² value of 50% (*p* < 0.05).)

<table>
<thead>
<tr>
<th>Embryonic stage when treated (hpf)</th>
<th>Control</th>
<th>Hypoxia</th>
<th>Anoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>Female (%)</td>
<td>Male (%)</td>
<td>Female (%)</td>
</tr>
<tr>
<td>18 (N = 140)</td>
<td>47</td>
<td>53</td>
<td>50</td>
</tr>
<tr>
<td>24 (N = 133)</td>
<td>47</td>
<td>53</td>
<td>73*</td>
</tr>
<tr>
<td>36 (N = 133)</td>
<td>41</td>
<td>59</td>
<td>61</td>
</tr>
</tbody>
</table>

(figure 5a, c, e, f). As with the larvae, adult *P. crit* was not affected by exposure to low O2 at 18 hpf (figure 5b). In contrast to larvae, there was no effect of hypoxia or anoxia exposure on *P. crit* of adult fish exposed at 24 or 36 hpf (figure 5d, f). There was a significant effect of sex on hypoxia tolerance where, regardless of embryonic O2 treatment, female fish always had a higher *P. crit* than males (figure 5b–f; 18 hpf, *p* = 0.022; 24 hpf, *p* = 0.003; 36 hpf, *p* = 0.017). There was no effect of embryonic exposure to low O2 on LOE regardless of the timing of the exposure (electronic supplementary material, table S2).

### Figure 5. O2 consumption and critical O2 tension (*P. crit*) of zebrafish (*D. rerio*) adults exposed as embryos at 18 (a, b), 24 (c, d) or 36 (e, f) hpf to 4 h of hypoxia (5% DO) or anoxia (less than 0.5% DO). Values are mean ± s.e.m. (*O2 consumption, n = 8, P. crit, n = 4*). Significant differences are indicated by dissimilar letters (one-way ANOVA and Tukey post hoc test, *p* < 0.05).

4. Discussion

We have shown that acute hypoxia exposure causes zebrafish embryos to mount differential cellular responses based on developmental stage and severity of O2 deprivation. Early in development (18 hpf), exposure to low O2 did not elicit a HIF-1 response, however as zebrafish age and become increasingly more sensitive (36 hpf), they are HIF-1 responsive to low O2. Our study has identified 24 hpf as the start of the critical window for hypoxia sensitivity as embryos at this stage initiate HIF-1 signalling to hypoxia (5% DO) but not anoxia (less than 0.5% DO). These HIF-1 responsive fish display marked increases in hypoxia tolerance as free-swimming larvae and had a greater propensity to become males. To our knowledge, this is the only study to directly compare the effects of HIF-1 induction at different developmental stages in any vertebrate. These data also indicate that activation of HIF-1 during early development corresponds with altered larval and adult phenotypes of zebrafish.
(a) Embryonic cellular hypoxia response
In order for a stressor to alter the developmental trajectory of an organism, it must: (i) occur within a specific critical window of development, and (ii) meet the innate response threshold of a system [35]. We have identified both the critical developmental window and the response threshold for HIF-1 by exposing zebrafish embryos at different developmental stages to different severities of low O2. We have strong evidence of HIF-1 activity in response to hypoxia at 24 hpf and both hypoxia and anoxia at 36 hpf. These embryos accumulated significant amounts of HIF-1α protein which corresponded with two- to fivefold increases in the expression of multiple HIF-1 target genes. Twenty-four hours post-fertilization occurs just prior to the onset of the first heart beat [20]. In mammalian systems, HIF-1 is required for the vascularization of the heart and inhibition of HIF-1 during cardiac hypertrophy leads to heart failure [36]. The onset of HIF-1 responsiveness at this stage could be a cardio-protective mechanism.

We have also demonstrated that the response threshold for HIF-1 activation changes with development. At 24 hpf, anoxia exposure does not appear to result in a HIF-1-mediated cellular response, although there is a clear response by 36 hpf. We did observe accumulation of HIF-1α protein in anoxia-exposed embryos at 24 hpf but there were no corresponding changes in any of the HIF-1 target genes measured. Interestingly, roughly half of the zebrafish embryos exposed to anoxia at 36 hpf hatched during the treatment (electronic supplementary material, figure S4). At 36 hpf, embryos exposed to anoxia also accumulate significant amounts of lactate, probably as a result of their active efforts to hatch. Embryos at 24 hpf are not hatching competent and therefore cannot use this strategy to avoid anoxia. These data suggest that until hatching competency is reached, there is an energetic trade-off below a DO limit where mounting a HIF-1 response is not beneficial. Mechanistically, this trade-off could be mediated by other low O2-sensitive transcription factors such as HIF-2 or p53 which can both out-compete HIF-1 for its required cofactors and targets [37–39].

Differential hypoxia dosage thresholds within a critical window have been previously identified in chicken embryos [40] where different levels of hypoxia were required to alter different physiological traits. This is particularly noteworthy as our results suggest that HIF-1 regulates different genes based on the level of DO and developmental timing.

(b) Plasticity of larval hypoxia tolerance
In general, zebrafish larvae show a higher degree of O2 conformation than adults within their tolerable range of environmental DO. This is particularly true below 40–50% DO; however, we have shown that there is a level of plasticity in the degree to which they conform under severely hypoxic conditions. Regardless of O2 level or developmental stage of treatment, acute induction of the HIF-1 pathway was correlated with an increased ability to regulate and maintain O2 uptake in hypoxia in larvae at 4 dpf. Conversely, larvae that did not have a HIF-1 response to low O2 during development had similar capacity to regulate O2 uptake to normoxic controls. These findings support our hypothesis that the plasticity of hypoxia tolerance is associated with the induction of HIF-1 during critical developmental windows.

There are several factors that can influence hypoxia tolerance in fishes which could explain the differences observed in our study. Mandic et al. [41] found that 70% of variation in \(P_{\text{crit}}\) in intertidal sculpin can be explained by differences in \(M_b\), haemoglobin–O2 (Hb–O2) binding affinity or gill-surface area. We found that all previously exposed larvae had similar routine \(M_b\) suggesting that routine \(M_b\) is not a driving factor in this case. Alternatively, HIF-1 regulates the expression of Hb isoforms. However, young larvae may not use Hb to transport O2 and instead rely on passive diffusion [42]. Therefore, while altered Hb–O2 binding may contribute to increased hypoxia tolerance at 4 dpf, it seems unlikely to be the sole mechanism.

Finally, respiratory surface area may be a factor in increased hypoxia tolerance in larvae. The skin is the main respiratory surface for larval zebrafish before 14 dpf [43], and an increase in the capacity for epidermal O2 uptake would be analogous to increased gill-surface area in adult fish. Hypoxia exposure remodels larval amphibian skin to be thinner and more highly vascularized, increasing gas exchange efficiency [44]. It is possible that increased hypoxia tolerance in zebrafish larvae is partially a result of HIF-1-mediated skin remodelling. These changes could confer a competitive advantage for larvae that forage for food and actively avoid predators in hypoxic waters.

(c) Adult hypoxia tolerance
Although we have shown that larval differences in hypoxia tolerance were not sustained to adulthood, we were surprised to find a sex difference in adult \(P_{\text{crit}}\). To our knowledge, this is the first study to report that male zebrafish have a higher hypoxia tolerance (i.e. lower \(P_{\text{crit}}\)) than females. Rees et al. [45] and colleagues found that female zebrafish had slightly higher survival in lethal hypoxia than males, however this reversed following acclimatization to mild hypoxia. Reardon & Chapman found similar sex differences in \(P_{\text{crit}}\) of Pseudocrenilabrus multicolor that corresponded with differences in \(M_b\), whereas exposure to chronic hypoxia during development eliminated this sex effect [46]. In our study, differences in \(P_{\text{crit}}\) were not driven by resting \(M_b\) which was similar between sexes. Correspondingly, we found that populations of fish that mounted a HIF-1 response to low O2 during development had a higher proportion of males as adults. Therefore, induction of the cellular hypoxia response during early development results in an improved hypoxia tolerance at the population level.

Hypoxia-induced, male-biased populations of fishes have been observed in laboratory and wild populations of fishes living in chronic hypoxic dead-zones [47–50]. In every case, this has been linked to increased testosterone production as a result of impaired aromatase activity which has been observed [47–50]. Interestingly, aromatase activity is regulated by HIF-1β, therefore it is possible that the activation of HIF-1 could indirectly disrupt steroid hormone production during embryogenesis [51]. Although the low O2 exposures in this study occurred long before sexual differentiation in zebrafish (approx. 10 dpf, [52]), in rainbow trout differences in brain aromatase activity are present in males and females long before gonadal development [53]. Exposure to environmental oestrogens prior to hatching is also known to affect zebrafish sex determination [54]. Therefore, it is possible that even acute disruption of oestrogen production during embryogenesis could alter sex determination. Alternatively, differential survivorship between sexes during larval development may have resulted in the biased sex ratios observed in this study, but this is unknown.
Given that males had a lower \( P_{\text{crit}} \), a male-biased zebrafish population would have an advantage in acute hypoxic conditions. In this respect, hypoxia-induced sex ratio imbalances could be adaptive in fluctuating \( O_2 \) environments. However, reproductive output of a population is limited by the number of females. Therefore, male-biased populations may prove mal-adaptive for long-term reproductive fitness in chronically hypoxic dead-zones caused by eutrophication [46].

5. Conclusion

The embryonic cellular response to low \( O_2 \) can be influenced by both developmental stage and the severity of \( O_2 \) deprivation that they encounter. We have shown evidence for the induction of the HIF-1 pathway in embryos exposed to low \( O_2 \) at around the timing of cardiac maturation. Our data suggest that very acute induction of the HIF-1 pathway during embryogenesis alters larval hypoxia tolerance. Additionally, our data indicate that a secondary effect of HIF-1 may alter sex determination. These gender biases could improve population hypoxia tolerance while limiting reproductive output, only adding to the importance of understanding the many aspects of hypoxia-induced developmental plasticity.

All animal care and experimentation was conducted according to the University of Guelph animal care protocol no. 1256.

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Data accessibility. All data used in this paper have been archived in the dryad repository (doi:10.5061/dryad.3ks4h).

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