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RESEARCH ARTICLE

Hif-1α paralogs play a role in the hypoxic ventilatory response of larval and adult zebrafish (*Danio rerio*)

Milica Mandic*, Velislava Tzaneva, Vincent Careau and Steve F. Perry

ABSTRACT

Hypoxia-inducible factor (Hif) 1α, an extensively studied transcription factor, is involved in the regulation of many biological processes in hypoxia including the hypoxic ventilatory response. In zebrafish, there are two paralogs of Hif-1α (Hif-1A and Hif-1B), but little is known about the specific roles or potential sub-functionalization of the paralogs in response to hypoxia. Using knockout lines of Hif-1α paralogs, we examined their involvement in the hypoxic ventilatory response, measured as ventilation frequency (f_V) in larval and adult zebrafish (*Danio rerio*). In wild-type zebrafish, f_V increased across developmental time (4, 7, 10 and 15 days post-fertilization, dpf) in response to hypoxia (55 mmHg). In contrast, the Hif-1B knockout fish did not exhibit an increase in hypoxic f_V at 4 dpf. Similar to wild-type, as larvae of all knockout lines developed, the magnitude of f_V increased but to a lesser degree than in the wild-type larvae, until 15 dpf at which point there was no difference among the genotypes. In adult zebrafish, only in Hif-1B knockout fish was there an attenuation in f_V during sustained exposure to 30 mmHg for 1 h but there was no effect when fish were exposed for a shorter duration to progressive hypoxia. The mechanism of action of Hif-1α, in part, may be through its downstream target, nitric oxide synthase, and its product, nitric oxide. Overall, the effect of each Hif-1α paralog on the hypoxic ventilatory response of zebrafish varies over development and is dependent on the type of hypoxic stress.

KEY WORDS: Hypoxia-inducible factor 1α, Ventilation frequency, Hypoxia, Nitric oxide, Paralog, Gene duplication

INTRODUCTION

Most organisms have adapted to an aerobic lifestyle such that decreases in O_2 supply can limit their ability to produce sufficient ATP to meet cellular energetic demands. The transcription factor hypoxia-inducible factor (Hif), considered to be a master regulator of O_2 homeostasis at the molecular level, is found in species across the animal kingdom (Semenza, 2001). As O_2 becomes limiting in a cell, the O_2-regulated Hif-α subunit, which is expressed but degraded under normoxic conditions, begins to accumulate and travels to the nucleus. There, it dimerizes with the constitutively expressed Hif-β subunit. The Hif heterodimer then binds to hypoxia-responsive elements (HREs) in the genome and alters the expression of hypoxia-inducible genes involved in a number of physiological processes (Rolfs et al., 1997; Iyer et al., 1998; Semenza, 1998, 2003; Liu and Simon, 2004).

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The duplication of genes and entire genomes can be an effective mechanism for creating raw genetic material for evolution (Ohno, 1970), which is particularly important for species inhabiting variable environments. The most likely outcome of duplicated genes after whole-genome duplication or smaller gene duplication events is non-functionalization, or the loss of the duplicated paralog as a result of genetic drift (Volff, 2005). This is the case in most teleost fish species, where the duplicated paralogs of the three *hif-α* genes, *hif-1α*, *hif-2α* and *hif-3α*, were lost over time with the exception of the species in the cyprinid lineage, e.g. zebrafish (*Danio rerio*), which have retained the duplicate copies of each *hif-α* gene. Through phylogenetic analysis and the discovery of remnants of a non-functional second copy of *hif-2α* in the non-cyprinid stickleback (*Gasterosteus aculeatus*) and green puffer (*Tetraodon nigroviridis*) genomes, it is thought that the gene duplicates arose during the teleost-specific genome-wide duplication (Rytkönen et al., 2013). The underlying reason for the subsequent loss of *hif-α* duplicate copies in most teleost species with the exception of cyprinid fishes remains unknown. One hypothesis is that the duplication of *hif-α* provided greater evolutionary flexibility in adaptation to hypoxic environments in the hypoxia-tolerant species that typify the cyprinid lineage (Rytkönen et al., 2013). Elucidating the functional role of each of the Hif-1α paralogs in the hypoxic response is a critical step in gaining insight into the significance of the duplication of *hif-α* genes in hypoxia-tolerant species, such as zebrafish.

In most animals, the immediate response to hypoxia is an increase in ventilation volume achieved via increases in frequency and/or amplitude, typically referred to as the hypoxic ventilatory response (Perry et al., 2009). In mammals, peripheral O_2 chemoreception and the ensuing reflex increase in ventilation during hypoxia are initiated by type I or glomus cells of the carotid body that detect changes in arterial O_2 tension (Gonzalez et al., 1994). An increase in HIF-1α was documented in the carotid body (Kline et al., 2002) and specifically in the glomus cells (Roux et al., 2005) of wild-type mice during exposure to hypoxia. Studies in mice with a partial knockout of HIF-1α (heterozygous HIF-1α, i.e. HIF-1α$^{+/-}$) revealed a decrease in carotid body sensitivity (Kline et al., 2002) and a diminished ventilatory response to acute hypoxia following chronic exposure to hypoxia (Kline et al., 2002), and no increase in peripheral O_2 chemoreceptor sensitivity following chronic intermittent hypoxia (Peng et al., 2006). Moreover, increased levels of HIF-1α associated with a mutation in the von Hippel–Lindau (VHL) tumor suppressor protein (and thus the slowing of HIF-1α proteasomal degradation) caused an elevation of resting ventilation and an increased ventilatory response to hypoxia in humans (Smith et al., 2006). Taken together, evidence from mammalian studies points to the involvement of HIF-1α in the control of breathing during hypoxia.

In adult zebrafish, a decrease in ambient O_2 is sensed by the neuroepithelial cells (NECs) in the gill, which may be the functional equivalent of the glomus cells of the carotid body (Zaccone et al.,

Journal of Experimental Biology (2019) 222, jeb195198. doi:10.1242/jeb.195198

1989; Milsom and Burleson, 2007; Porteus et al., 2012). To date, Hif-1α has not been localized to NECs in fish, but Hif-1α was found to increase in the fish gill during hypoxia exposure (Sollid et al., 2006). Similar to non-functional VHL in humans, knockout of VHL in zebrafish caused larvae to hyperventilate even under normoxic conditions (van Rooijen et al., 2009). Additionally, zebrafish larvae with a morpholino knockdown of one of the Hif-1α paralogs (HIF-1B) at 4 days post-fertilization (dpf) showed an attenuated ventilatory response during exposure to acute hypoxia (V.T., J. Ho, H. Li and S.F.P., unpublished data). Similar to its function in mammals, Hif-1α appears to be involved in the cellular mechanisms underlying peripheral O_2 chemoreception and the subsequent downstream responses. However, almost nothing is known regarding the individual functions of the duplicated Hif-1α paralogs with regards to the hypoxic ventilatory response in larval and/or adult fish.

The primary goal of this study was to determine the extent of Hif-1α involvement in the hypoxic ventilatory response during early larval development and in adult zebrafish. We examined the hypoxic ventilatory response, specifically ventilation frequency (f_V), in wild-type, Hif-1A or Hif-1B knockout (Hif-1A$^{-/-}$ and HIF-1B$^{-/-}$, respectively) and double-knockout (Hif-1A$^{-/-}$B$^{-/-}$) mutant lines. We measured f_V in larvae at 4, 7, 10 and 15 dpf as well as in adult zebrafish in all four genotypes. We hypothesized that one or both paralogs are involved in regulating f_V in zebrafish during hypoxia and, accordingly, we predicted a diminished hyperventilatory response in either or both paralog knockout lines as well as the double-knockout line.

The mechanism by which Hif-1α contributes to the control of breathing in vertebrates is unknown, although several schemes have been proposed. For example, the positive feedback loop between reactive O_2 species (ROS) and HIF-1α is thought to lead to a sustained ventilatory response during chronic intermittent hypoxia in mice (see Peng et al., 2006). Other possibilities link Hif-1α with nitric oxide (NO) or hydrogen sulfide (H_2S) (Prabhakar and Semenza, 2012); H_2S is known to cause hyperventilation in adult rainbow trout (*Oncorhynchus mykiss*; Olson et al., 2008), while both gasotransmitters are known to cause hyperventilation in larval zebrafish (Porteus et al., 2014, 2015). In particular, NO is a likely candidate linking Hif-1α to the control of breathing as it has been found that the inducible nitric oxide synthase (iNOS, an enzyme that catalyzes the production of NO) gene in mammals contains the hypoxia response element (HRE) and is a putative binding site for Hif-1α (Melillo et al., 1995). Therefore, iNOS is a likely downstream target of Hif-1α and NO accumulation may be the mechanism whereby Hif-1α is involved in the hypoxic ventilatory response. Through the use of NOS inhibitors and NO donors, we investigated the proposed link between Hif-1α, NO and hypoxic ventilation in larval and adult zebrafish.

MATERIALS AND METHODS

Experimental animals

Adult zebrafish, *Danio rerio* (F. Hamilton 1822), were housed in 10 l polycarbonate tanks on a recirculating aquatic system (Aquatic Habitats, Apopka, FL, USA). Fish were maintained at 28°C under a 14 h:10 h light:dark cycle in dechloraminated city of Ottawa tap water and fed to satiation twice a day. Wild-type zebrafish were obtained from in-house stock at the University of Ottawa aquatic care facility. The Hif-1A$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$ mutant lines were generated originally via TALEN and CRISPR/Cas9 technologies (Gerri et al., 2017), while the Hif-1B$^{-/-}$ mutant line was originally generated via CRISPR/Cas9 (Joanna Yeh Lab, Harvard Medical School). Embryos from wild-type, Hif-1A$^{-/-}$, Hif-1B$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$ fish were obtained using standard protocols (Westerfield, 2000). Briefly, one male was kept separate from two females in a 2 l breeding tank and allowed to recover overnight. In the morning, the water was changed and fish were allowed to breed once the separator was lifted. Embryos were collected and reared in 50 ml Petri dishes containing dechloraminated city of Ottawa tap water and 0.05% Methylene Blue in an incubator maintained at 28.5°C. At 6 dpf, larvae were transferred to static 1 l tanks and fed twice daily. Water was changed daily in the Petri dishes and every alternate day in the static tanks. The larvae were raised in the static tanks until 15 dpf. All procedures for animal use and experimentation were carried out in compliance with the University of Ottawa Animal Care and Veterinary Service guidelines under protocol BL-226 and adhered to the recommendations for animal use provided by the Canadian Council for Animal Care.

Series 1: larval zebrafish

Hypoxia exposure

f_V was measured in wild-type and Hif knockout larvae at 4, 7, 10 and 15 dpf. An individual larva was placed in a carved SYLGARD® 184 silicone elastomer chamber that was irrigated with gravity-fed solutions maintained at 28.5°C. All solutions contained 0.05 mg ml^{-1} Tris-buffered MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma Aldrich Inc.) to anesthetize fish and minimize body movement. Each larva was left to recover in the chamber for 10 min in normoxic water (water O_2 tension, $P_{W_{O_2}}$=153 mmHg) prior to the start of the trial. To begin the trial, measurements of f_V were recorded for 5 min under normoxic conditions (baseline), followed by 30 min of hypoxia ($P_{W_{O_2}}$=55 mmHg) and 5 min after the return to normoxia. f_V was determined by counting either buccal or opercular movements captured by a high-speed camera (3CCD camera, Dage-MTI, Michigan City, IN, USA) mounted on a dissecting microscope (stereo trinocular microscope, AmScope). The selection of buccal versus opercular movements to quantify f_V depended on the fish orientation in the chamber and the visibility of the mouth and/or operculum.

NO donor and NOS inhibitor

Wild-type and Hif-1B$^{-/-}$ mutant zebrafish at 4 and 10 dpf were exposed to the NO donor *S*-nitroso-*N*-acetyl-DL-pencillamine (SNAP, Sigma Aldrich Inc.) and f_V was measured as described above. Individual larvae were exposed to 5 min of normoxic water, followed by 10 min of normoxic water containing 400 µmol l^{-1} of SNAP and then returned to control normoxic water for 5 min.

Wild-type and Hif-1B$^{-/-}$ mutant zebrafish were exposed to 1 mmol l^{-1} N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma Aldrich Inc.), a NOS inhibitor, from 8 to 10 dpf. At 10 dpf, larvae were placed in the chamber and f_V was measured while larvae were exposed to 5 min of normoxia, 30 min of hypoxia and 5 min of recovery at normoxia, with all solutions containing 1 mmol l^{-1} L-NAME. In parallel, f_V was also determined in larvae following the same protocol without exposure to L-NAME.

Series 2: adult zebrafish

Adult zebrafish were exposed to either progressive hypoxia or sustained hypoxia and f_V was measured using custom-built chambers constructed at the University of Ottawa (Vulesevic et al., 2006). An individual fish was placed into a cylindrical plastic chamber with two electrodes attached to the inside of the chamber at

either end. The electrodes were separated from the fish by coarse mesh and gravity-fed water was continuously supplied to the chambers. The electrodes detect bioelectric potentials generated by excitable cells controlling ventilation (principle outlined in Altimiras and Larsen, 2000). The signal detected by the electrodes was amplified (custom-made amplifier, University of Ottawa) and analog data were converted to digital output using AcqKnowledge™ data acquisition software (Biopac Systems Inc., Goleta, CA, USA). Analysis of f_V was performed as described by Vulesevic et al. (2006).

An individual zebrafish was placed into a chamber and allowed to recover for at least 1 h. For the sustained hypoxia experiment, f_V was measured for 15 min under normoxia, 1 h under hypoxia (Pw_{O_2}=30 mmHg) and 15 min under normoxia during recovery. For the progressive hypoxia experiment, f_V was measured for 15 min under normoxia and then in increasingly more severe hypoxia; 15 min at Pw_{O_2}=60 mmHg, 15 min at Pw_{O_2}=30 mmHg and 15 min at Pw_{O_2}=15 mmHg. Following the hypoxia exposures, fish were re-exposed to normoxia and f_V was measured for a further 15 min.

Statistical analysis

All statistical analyses were performed in R (https://www.R-project.org/). In the experiments using larvae, occasional periods of recording of ≤1 min were excluded from analysis when brief movement of the fish prevented buccal or opercular movements from being visible in the recording. To handle missing values in the dataset (1.5% data missing), a multivariate imputation by chained equations technique was used to impute values using the mice package (van Buuren and Groothuis-Oudshoorn, 2011). Data from the hypoxia and L-NAME experiments were analyzed using Markov chain Monte Carlo sampler for multivariate generalized linear models in MCMCglmm package (Hadfield, 2010). Multivariate generalized linear models were used because of increased power to detect significance in data where the f_V pattern was not consistent across exposure time (e.g. transient increase in hypoxic f_V in larval data), while modeling covariance among successive f_V measurements. Each model included all f_V measurements fitted as dependent variables and genotype as a fixed effect (fitted separately for each trait). The model included an unstructured (co)variance matrix at the residual level, using weakly informative inverse-Wishart priors with the scale parameter defined as a diagonal matrix containing values of one and distribution parameters set to 0.001 for the degrees of freedom (Hadfield, 2010). Posterior distributions were estimated from 13,000 MCMC iterations sampled at 10 iteration intervals following an initial burn-in period of 3000 iterations. This yielded effective samples sizes of 1000 for the parameters of interest. We inspected the 95% highest posterior density (HPD) associated with each fixed effect estimate to check whether they overlapped with zero. A 95% HPD interval contains most of the posterior distribution and is analogous to a confidence interval (CI) in the frequentist approach; a 95% HPD that overlaps 0 indicates that the effect does not differ significantly from zero. Thus, for each estimate associated with genotype we determined whether the 95% HPDs included or excluded zero.

The effects of SNAP on larval f_V and resting f_V in adult zebrafish were analyzed statistically using one-way analysis of variance (ANOVA). In the larval SNAP exposure experiment, data did not pass normality (Shapiro–Wilk test) or equal variance (Levene's test); thus, the Kruskal–Wallis test by ranks ANOVA was used followed by Dunn's *post hoc* test (PMCMR package, http://CRAN.R-project.org/package=PMCMR). For adult resting f_V data, normality and equal variance tests were passed; therefore, ANOVA was performed followed by Tukey's *post hoc* test. Significance was set at P<0.05.

The effects of genotype and oxygen levels on adult f_V during either sustained or progressive hypoxia were tested using repeated measures two-way ANOVA using the car package (Fox and Weisberg, 2011). If Mauchly's test for sphericity failed, the P-value was adjusted using the Greenhouse–Geisser correction. Tukey's *post hoc* test was used if a significant interaction was detected. Significance was set at P<0.05.

RESULTS

Series 1: larval zebrafish

Resting f_V

Early development stage larvae (4 and 7 dpf) of all genotypes had low resting f_V (<15 breaths min^{-1}). Resting f_V increased as development progressed, reaching 25–40 breaths min^{-1} by 15 dpf. There were no obvious effects of genotype on resting f_V (Fig. 1).

Hypoxia exposure

In wild-type larval zebrafish, there was a significant increase in f_V in response to hypoxia (Fig. 1; Fig. S1). However, the increase in hypoxic f_V was transient during the earlier stages of development; at 4 dpf, f_V was significantly elevated for the first 5 min of hypoxia (Fig. 1A; Fig. S1A) and at 7 dpf for the first 15 min of hypoxia (Fig. 1B; Fig. S1E). Once wild-type larvae developed to 10 dpf, the increase in f_V was sustained throughout the hypoxia exposure (Fig. 1C,D; Fig. S1I,M).

At 4 dpf, there was a significant difference in f_V during hypoxia between wild-type and Hif-1B$^{-/-}$ larvae (Figs 1A and 2A). The Hif-1B$^{-/-}$ larvae did not increase f_V in response to hypoxia (Fig. 1A), resulting in a significant difference between the knockout and wild-type fish during the first 5 min of hypoxia (Fig. 2A). In contrast, Hif-1A$^{-/-}$B$^{-/-}$ fish at 4 dpf had significantly higher f_V than wild-type fish after the initial 5 min of hypoxia (Figs 1A and 2C). There was no difference at 4 dpf between wild-type and Hif-1A$^{-/-}$ zebrafish (Figs 1A and 2B). With the exception of the first minute in hypoxia, there was no significant difference between wild-type and the Hif-1α knockout lines at 7 dpf (Figs 1B and 2D–F). However, wild-type zebrafish at 7 dpf maintained a significant increase in f_V for the first 15 min of hypoxia, whereas the Hif-1α single-paralog knockouts and the double knockout significantly increased f_V only for 5 min (Fig. S1E–H).

There was a significant difference in f_V throughout the hypoxia exposure between wild-type and Hif-1B$^{-/-}$ zebrafish and between wild-type and Hif-1A$^{-/-}$ zebrafish at 10 dpf (Figs 1C and 2G,H). Wild-type zebrafish exhibited consistently higher hypoxic f_V than either of the Hif-1α paralog knockouts. The Hif-1B$^{-/-}$ fish, unlike the wild-type and Hif-1A$^{-/-}$ fish, did not maintain elevated f_V during the entire length of the hypoxia exposure (Fig. S1J–K). In contrast to the single-paralog knockouts, the Hif-1A$^{-/-}$B$^{-/-}$ fish did not show a significant difference from the wild-type zebrafish for the first 9 min of hypoxia, although there was a significant difference for the remainder of the exposure during which the wild-type zebrafish had higher f_V (Figs 1C and 2I). At 15 dpf, there was no difference between the wild-type zebrafish and Hif-1α knockout lines (Figs 1D and 2J–L); all fish exhibited a sustained elevation in f_V during hypoxia (Fig. S1M–P).

Upon return to normoxia, the wild-type zebrafish displayed a significant increase in f_V at 4 and 7 dpf (Fig. 1; Fig. S1). By 10 dpf, f_V returned rapidly to normoxic resting levels in wild-type zebrafish upon re-oxygenation. In contrast, the Hif-1α knockout lines did not show a significant difference in f_V between resting and recovery at 4

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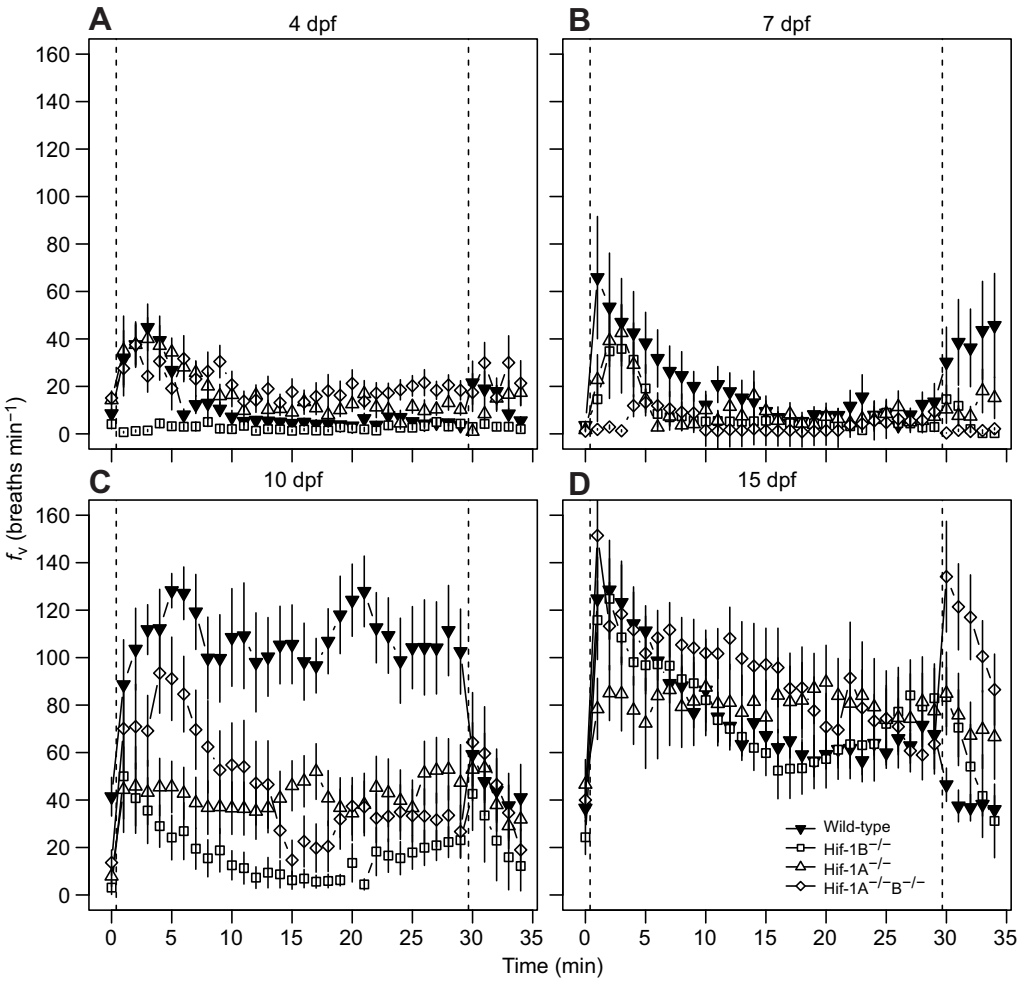

Fig. 1. The interactive effects of hypoxia and developmental age on ventilation frequency (f_V) in larval zebrafish. Wild-type (n=12 for 4 dpf, n=9 for 7 dpf and n=10 for 10 and 15 dpf), Hif-1B$^{-/-}$ (n=10), Hif-1A$^{-/-}$ (n=10) and Hif-1A$^{-/-}$B$^{-/-}$ (n=9 for 4 and 7 dpf, n=12 for 10 dpf and n=10 for 15 dpf) larvae at 4 days post-fertilization (dpf) (A), 7 dpf (B), 10 dpf (C) and 15 dpf (D) were exposed to hypoxia (55 mmHg) and subsequently returned to normoxia (153 mmHg). Vertical dashed lines represent delineations among normoxia, hypoxia and recovery. Data are presented as means±s.e.m.

and 7 dpf, but at 10 and 15 dpf, f_V remained significantly elevated during re-exposure to normoxia (Fig. S1).

Effects of NO and NOS inhibition

Wild-type and Hif-1B$^{-/-}$ zebrafish significantly increased f_V in response to 400 µmol l^{-1} SNAP at 4 and 10 dpf (Fig. 3). Wild-type zebrafish treated with L-NAME between 8 and 10 dpf exhibited lower f_V for the first 9 min of hypoxia in comparison to untreated fish (Fig. 4A,C). Peak f_V during hypoxia in untreated wild-type individuals was 155±17 breaths min^{-1}, while peak f_V in L-NAME-treated individuals was significantly diminished to 50±16 breaths min^{-1}. In contrast, L-NAME treatment was without effect on the ventilatory response to hypoxia in Hif-1B$^{-/-}$ zebrafish at 10 dpf (Fig. 4B,D). Peak f_V during hypoxia was similar between untreated (72±26 breaths min^{-1}) and L-NAME-treated (64±11 breaths min^{-1}) individuals.

Series 2: adult zebrafish

Resting f_V

Resting normoxic f_V values were calculated by pooling data from the sustained and progressive hypoxia experiments for each genotype (Fig. 5). Resting f_V was significantly higher in adult Hif-1B$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$ zebrafish (201±9 breaths min^{-1} and 192±9 breaths min^{-1}, respectively) than in adult wild-type zebrafish (150±9 breaths min^{-1}). Resting f_V in Hif-1A$^{-/-}$ was not statistically different from that of wild-type zebrafish or the other Hif-1α knockout lines.

Sustained hypoxia

All lines of zebrafish exposed to hypoxia increased and maintained elevated f_V throughout the period of exposure (Fig. 6A). Data at each O_2 tension averaged and normalized to normoxic f_V showed that the hypoxic ventilatory response was attenuated in the Hif-1B$^{-/-}$ zebrafish (Fig. 6B). Specifically, the average increase in f_V between normoxia and hypoxia was 170 breaths min^{-1} for wild-type fish, similar to that of Hif-1A$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$ fish (148 and 159 breaths min^{-1}, respectively), and unlike that of Hif-1B$^{-/-}$ fish (79 breaths min^{-1}). The significantly lower Δf_V for Hif-1B$^{-/-}$ was the combined result of a higher resting f_V and a lower increase in f_V in hypoxia. In wild-type and Hif-1α knockout lines, average f_V during recovery was significantly lower than f_V at resting normoxia.

Progressive hypoxia

There was an increase in f_V at 60 mmHg hypoxia and a further increase in f_V at 30 mmHg hypoxia in adult wild-type and Hif-1α knockout lines (Fig. 6C). We also exposed fish to 15 mmHg hypoxia, but at this level there was mortality in all genotypes (wild-type 40%, Hif-1B$^{-/-}$ 83%, Hif-1A$^{-/-}$ 38% and Hif-1A$^{-/-}$B$^{-/-}$ 50% mortality). Because of the high mortality rate, we opted to exclude the 15 mmHg and the subsequent recovery data. There was no significant difference among genotypes for averaged and normalized f_V in response to hypoxia (Fig. 6D). However, if data from the Hif-1α paralogs were combined and tested against wild-type, there was a significant blunting of the

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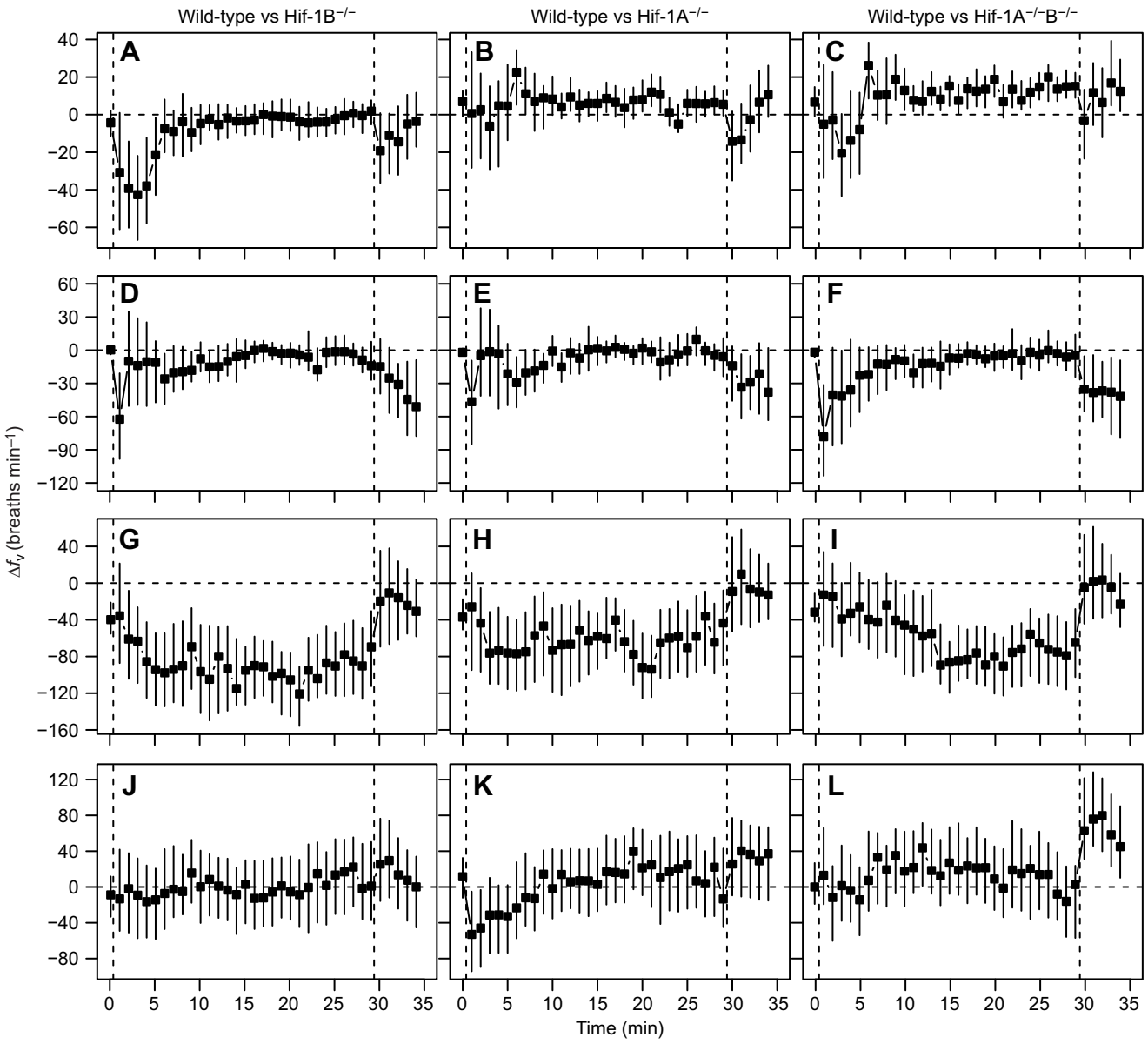

Fig. 2. The average difference in f_V (Δf_V) in Hif-1α mutant larvae relative to wild-types during 30 min of hypoxia (55 mmHg) and subsequent return to normoxia. Vertical dashed lines represent delineations among normoxia, hypoxia and recovery. Data are based on Fig. 1 and are of (A,D,G,J) Hif-1B$^{-/-}$ fish, (B,E,H,K) Hif-1A$^{-/-}$ fish and (C,F,I,L) Hif-1A$^{-/-}$B$^{-/-}$ fish relative to wild-type. Larvae were tested at 4 dpf (A–C), 7 dpf (D–F), 10 dpf (G–I) and 15 dpf (J–L). Estimates are presented with 95% credible interval (CI) based on Bayesian analysis. Each point represents the mode and the 95% CI of the posterior distribution associated with the difference in f_V of knockout relative to wild-type fish. Shown are mode (filled squares) and 95% CI (black lines) of the posterior distribution. Data are significant if the 95% CI lines do not intersect zero (dashed horizontal line).

hyperventilatory response in the Hif-1α knockout lines at 30 mmHg (two-way repeated measures ANOVA, genotype× oxygen: F=5.3, P=0.01, genotype: F=4.8, P=0.04, oxygen: F=54.4, P<0.01).

DISCUSSION

The goal of the study was to determine the involvement of Hif-1α paralogs in the hypoxic ventilatory response of larval and adult zebrafish. The role of each Hif-1α paralog was dependent on stage of development; in larvae, a knockout of either Hif-1α paralog generally caused a decrease in hypoxic f_V, while in adults, only Hif-1B knockout fish showed a significant decrease in hypoxic f_V. Our results suggest that the mechanism of action of Hif-1α may be through the NOS/NO pathway.

Hypoxic ventilation in wild-type larval zebrafish

The hypoxic hyperventilatory response is achieved through increases in either f_V or amplitude, or both, depending on the species (Perry et al., 2009). In this study, we focused on f_V because adult zebrafish solely increase f_V during hypoxia (Vulesevic et al., 2006) and, currently, there is no established method to measure breathing amplitude in zebrafish larvae.

During earlier larval stages (≤7 dpf), f_V significantly increased during hypoxia but the response was transient, with f_V returning to baseline levels within about 5–15 min. By 10 dpf, the increase in f_V was maintained throughout the hypoxia exposure similar to the response of adult fish. A similar breathing pattern during hypoxia was described previously in mammals as a biphasic response whereby neonates exposed to acute hypoxia exhibit an immediate

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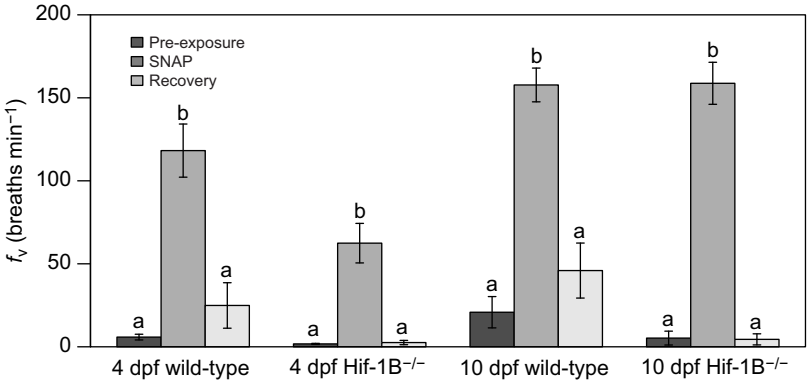

Fig. 3. f_V in wild-type and Hif-1B$^{-/-}$ larval zebrafish exposed to SNAP (nitric oxide donor) at 4 and 10 dpf. There was a significant effect of 400 µmol l^{-1} SNAP on f_V [one-way non-parametric ANOVA (Kruskal–Wallis): 4 dpf wild-type: χ^2=16.128, P<0.01, n=10; 4 dpf Hif-B$^{-/-}$: χ^2=17.007, P<0.01, n=11; 10 dpf wild-type: χ^2=20.117, P<0.01, n=8; 10 dpf Hif-1B$^{-/-}$: χ^2=15.398, P<0.01, n=8; Dunn's *post hoc*]. Data are presented as means±s.e.m. Values with different letters are significantly different (P<0.05) within each treatment group.

increase in ventilation followed by a decline to resting levels within a few minutes (<5 min) (Jansen and Chernick, 1983; Mortola et al., 1989; Powell et al., 1998). The decrease in ventilation during hypoxia was attributed primarily to a decrease in O_2 consumption, suggesting that metabolic rate is an important driver of the ventilatory response in neonatal mammals (Mortola et al., 1989). As neonates mature, elevated ventilation becomes sustained during hypoxia (Bonora et al., 1984), similar to the pattern observed in this study for developing zebrafish larvae. It is possible that, like mammals, early stage zebrafish larvae do not sustain the increased f_V during hypoxia because of a decrease in O_2 consumption. However, at an exposure of 55 mmHg it is unlikely that there was a decrease in O_2 consumption in zebrafish larvae given that O_2 consumption does not decrease until the critical O_2 tension (P_{crit}; the lowest Pw_{O_2} at which an animal can sustain routine O_2 consumption, below which O_2 consumption progressively decreases). For zebrafish larvae at 4 and 7 dpf, P_{crit} was measured at 33.7 and 30.1 mmHg, respectively (M.M., Y. K. Pan and S.F.P.,

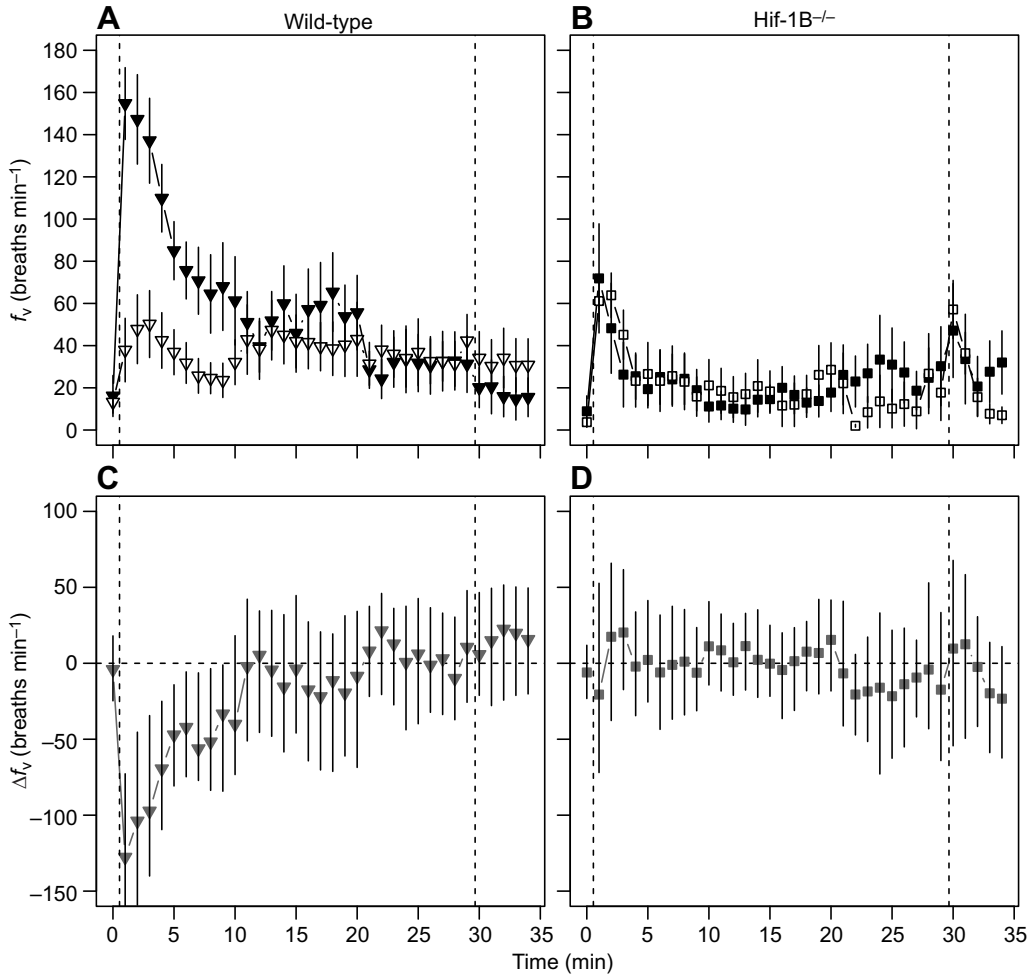

Fig. 4. f_V at 10 dpf in wild-type and Hif-1B$^{-/-}$ larval zebrafish exposed to L-NAME (nitric oxide inhibitor). (A,B) Wild-type and Hif-1B$^{-/-}$ zebrafish were exposed to hypoxia with (open symbols) or without (filled symbols) 1 mmol l^{-1} L-NAME. Data are presented as means±s.e. (C,D) Average difference in f_V (Δf_V) ±95% credible interval between untreated and L-NAME-treated fish during and after exposure to hypoxia, based on Bayesian analysis (n=8). Vertical dashed lines represent delineation among normoxia, hypoxia and recovery. Estimates are significant if the 95% CI do not intersect zero (dashed horizontal line).

Journal of Experimental Biology (2019) 222, jeb195198. doi:10.1242/jeb.195198

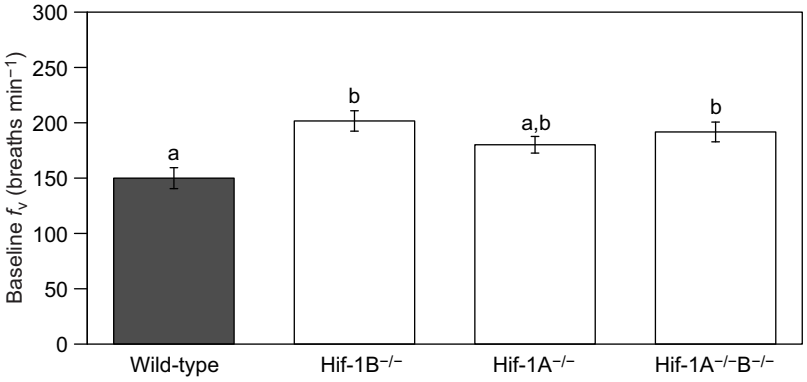

Fig. 5. Baseline f_V in wild-type, Hif-1B$^{-/-}$, Hif-1A$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$ adult zebrafish. Data were pooled from sustained and progressive hypoxia exposure experiments (Fig. 6). There was a significant effect of genotype on baseline f_V (ANOVA, F=6.83, P<0.01; n=16 for Hif-1A$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$, n=20 for wild-type and Hif-1B$^{-/-}$, Tukey *post hoc*). Data are presented as means±s.e.m. Values with different letters are significantly different (P<0.05).

unpublished data), Pw_{O_2} values that are much lower than the mild level of hypoxia used in our study. Alternatively, the hypoxic f_V patterns of the developing zebrafish larvae may be related to the transition between cutaneous and branchial respiration. During early development (<15 dpf), zebrafish larvae primarily rely on cutaneous respiration (Rombough, 2002), and in this study, the

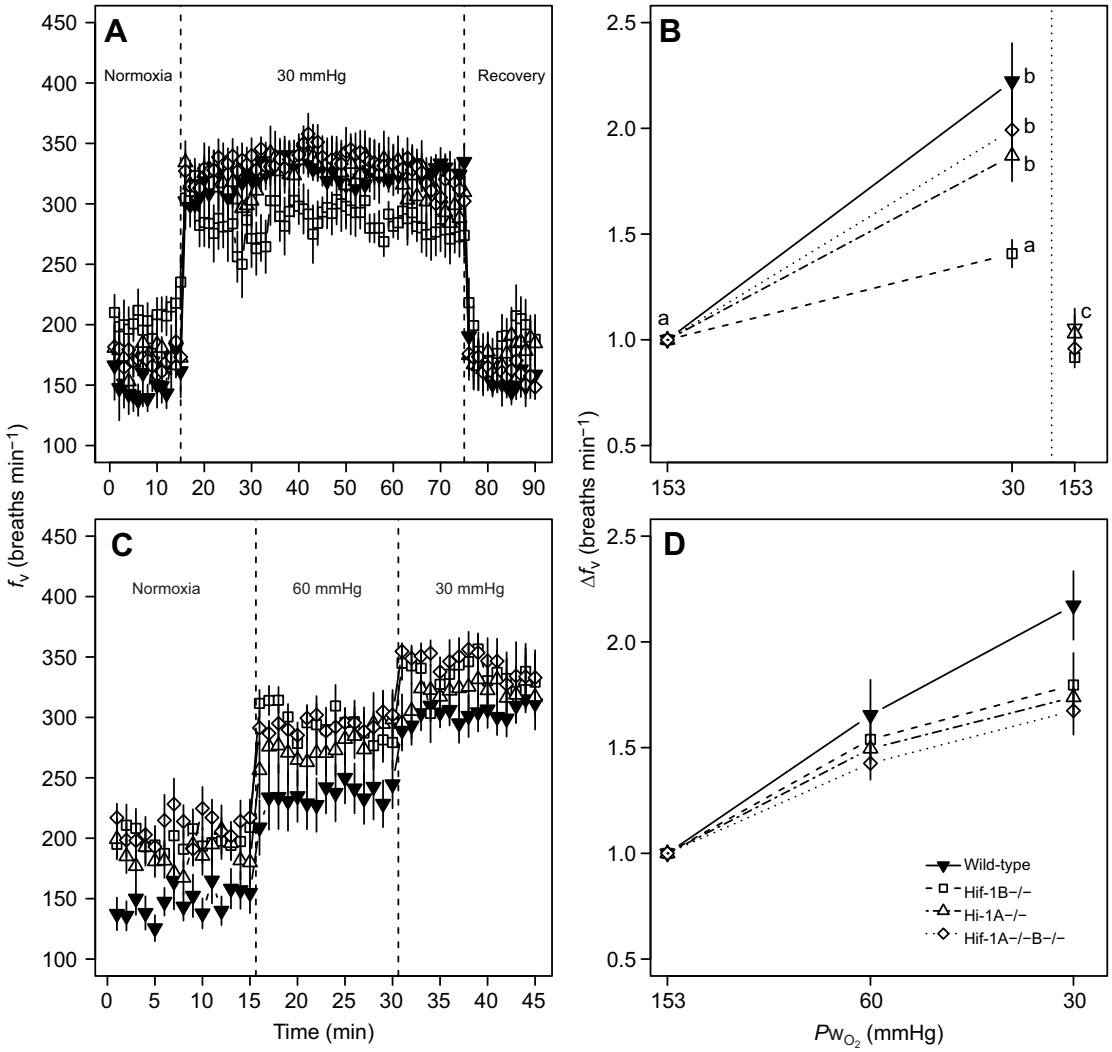

Fig. 6. f_V during sustained and progressive hypoxia in wild-type, Hif-1B$^{-/-}$, Hif-1A$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$ adult zebrafish. (A,B) Sustained hypoxia; (C,D) progressive hypoxia. A and C represent absolute ventilation frequency data while in B and D ventilation frequency was averaged across time for each O_2 level treatment and normalized to normoxic values. For sustained hypoxia (B), a significant interaction was detected (two-way repeated measures ANOVA, genotype×oxygen: F=5.25, P<0.01, genotype: F=4.51, P=0.011, oxygen: F=32.56, P<0.001, n=7–8, Tukey *post hoc*), while there was no significant interaction for progressive hypoxia (D; two-way repeated measures ANOVA, genotype×oxygen: F=1.78, P=0.11, genotype: F=1.66, P=0.19, oxygen: F=414.62, P<0.001, n=8 for all groups except for wild-type and Hif-1B$^{-/-}$ in progressive hypoxia where n=12). Values with different letters are significantly different (P<0.05) within each treatment group and across treatment groups. Data are presented as means±s.e.m.

larvae were exposed to an O_2 tension of 55 mmHg, a relatively mild level of hypoxia. It is possible that cutaneous respiration was sufficient to meet metabolic demand during the hypoxia exposure in fish at least until 7 dpf. Hyperventilation incurs a significant energetic cost (Hughes and Saunders, 1970; Jones and Schwarzfeld, 1974; Steffensen, 1985), and if cutaneous respiration is a sufficient means of obtaining O_2 from the environment during mild hypoxia, it may reduce the need for sustained hyperventilation, thus avoiding the costs associated with elevated f_V. As larvae develop and switch from cutaneous to branchial respiration (Rombough, 2002), elevated f_V is likely required to maintain arterial O_2 tension and may be the reason why older zebrafish larvae and adults sustain hyperventilation during hypoxia. Further investigation is required to pinpoint the factors that drive the change from transient to sustained hypoxic hyperventilation as zebrafish larvae mature.

Loss of Hif-1α function blunts the hypoxic ventilatory response

Hif-1α is thought to be one of the most critical transcription factors underlying the hypoxic phenotype in vertebrates because it is involved in numerous biological processes, including angiogenesis, erythropoiesis, apoptosis, metabolism, cell survival and cell proliferation (reviewed in Semenza, 2003). More recently, Hif-1α was implicated in the control of breathing (reviewed in Prabhakar and Semenza, 2012; Perry and Tzaneva, 2016). A knockout of Hif-1A or Hif-1B in larval zebrafish led to a significant blunting or abolishment of the hypoxic ventilatory response during the early stages of development. Specifically, there was no increase in f_V during hypoxia in Hif-1B$^{-/-}$ knockouts at 4 dpf and the duration of elevated f_V during hypoxia was shorter in both Hif-1α knockout lines at 7 dpf. Moreover, the magnitude of the f_V increase was lower in the two Hif-1α knockout lines at 10 dpf. However, by 15 dpf, the f_V changes during hypoxia were similar among the different genetic lines. A previous study (V.T., J. Ho, H. Li and S.F.P., unpublished data) showed that morpholino knockdown of Hif-1B significantly lowered f_V during acute hypoxia in comparison with f_V of wild-type larvae at 4 dpf. The blunted hypoxic ventilatory response associated with morpholino knockdown of Hif-1α is consistent with the results of the current study, in which Hif-1B knockout, and to a lesser extent Hif-1A knockout, diminished the hypoxic hyperventilatory response in larval zebrafish.

Adult Hif-1B$^{-/-}$ zebrafish exhibited a significantly lower f_V during exposure to sustained hypoxia (30 mmHg for 1 h) owing in part to a lower hypoxic f_V and in part to a higher resting f_V when compared with wild-type zebrafish. In contrast, there was no significant effect of Hif-1A knockout on the ventilatory response to this single level of hypoxia. When exposed to progressive and increasingly severe hypoxia (15 min at 60 and 30 mmHg), there was no significant difference between wild-type and the two Hif-1α knockout lines. However, the trend shows that there was blunting of the hyperventilatory response in both Hif-1α knockout lines at 30 mmHg which became significant when Hif-1α data were combined and tested against wild-type zebrafish. This suggests that there may be a small effect of Hif-1α knockout, but a lack of statistical power is preventing the detection of significant differences when individual Hif-1α paralogs are considered. It is possible that when Hif-1α knockout zebrafish are exposed to 30 mmHg with a prior exposure to 60 mmHg, the pre-exposure to a milder hypoxia may allow for compensatory mechanisms to alleviate at least partially the negative effects of knockout of Hif-1α. The importance of Hif-1α paralogs to the hypoxic ventilatory response in adult zebrafish remains equivocal, but the role the paralogs may play is likely dependent on the specific nature of the hypoxia exposure. Interestingly, in HIF-1α heterozygote adult mice, the ventilatory response was not impaired during exposure to acute hypoxia (Kline et al., 2002). However, when adult mice were subjected to chronic hypoxia followed by an acute hypoxic challenge, there was a blunted hypoxic ventilatory response in the HIF-1α heterozygous mice (Kline et al., 2002; Peng et al., 2006). It is possible that chronic exposure to hypoxia (sustained or intermittent) may reveal a more prominent and definitive involvement of Hif-1α in the hypoxic ventilatory response in adult zebrafish.

Consistent with other studies on larval zebrafish (Rahbar et al., 2016; Coe et al., 2017), older larvae (10–15 dpf) and adults rapidly returned f_V to normoxic levels after a bout of hypoxia. In contrast, f_V remained significantly elevated at 4 and 7 dpf during recovery in wild-type zebrafish, whereas in the Hif-1A$^{-/-}$ and Hif-1B$^{-/-}$ larvae at 4 and 7 dpf, f_V returned to normoxic levels rapidly following hypoxia. A study on the Siberian sturgeon (*Acipenser baeri*) demonstrated that ventilation remained significantly elevated for 30 min after a bout of hypoxia (Nonnotte et al., 1993). The post-hypoxic increase in ventilation was attributed to a persistent reduction of arterial P_{O_2} during recovery as tissue O_2 uptake increased to repay the O_2 debt accumulated during hypoxia (Nonnotte et al., 1993). While it is possible that a similar phenomenon may also explain the elevated post-hypoxic f_V in wild-type larvae at 4 and 7 dpf, the lack of ventilatory drive for much of the hypoxic exposure would suggest that arterial P_{O_2} and therefore tissue oxygenation were not sufficiently impacted by the decrease in environmental P_{O_2}. At 10 and 15 dpf, both Hif-1A$^{-/-}$ and Hif-1B$^{-/-}$ larvae exhibited elevated f_V during recovery, yet the hypoxic f_V among all genotypes was largely the same. The differences in the post-hypoxia f_V recovery pattern with increasing age and the interactive effects of Hif-1α knockout are intriguing and warrant further investigation.

Interactions between Hif-1α and NO in promoting the hypoxic ventilatory response

In mammals, a number of Hif-1α target genes known to influence breathing have been proposed as possible mechanisms by which Hif-1α modulates the hypoxic ventilatory response (reviewed in Powell and Fu, 2008; Prabhakar and Semenza, 2012). For example, vascular endothelial growth factor (VEGF; Jyung et al., 2000), erythropoietin (EPO; Soliz et al., 2005), endothelin-1 (ET-1; Kuwaki et al., 1996) and NO (Prabhakar et al., 1993) have been implicated in contributing to the mechanisms of action of Hif-1α. In particular, NO is an intriguing possibility because its production by NOS was shown to play a stimulatory role in the control of breathing in larval zebrafish (Porteus et al., 2015). We investigated the connection between Hif-1α and NO in Hif-1B$^{-/-}$ fish at 10 dpf because it was the first developmental time point of sustained elevated hypoxic f_V and the developmental stage at which knocking out functional Hif-1B had a significant impact on f_V. We focused on the Hif-1B paralog rather than Hif-1A because the Hif-1B$^{-/-}$ larvae showed a much greater blunting of the hyperventilatory response than the Hif-1A$^{-/-}$ larvae at 10 dpf. Wild-type larvae displayed a significantly lower hypoxic f_V when exposed to L-NAME in contrast to Hif-1B$^{-/-}$ larvae, which showed no effect of L-NAME. The lack of an effect of L-NAME on f_V in the Hif-1B knockout larvae indicates that NO is unable to contribute to the hypoxic control of breathing in larvae experiencing a loss of functional Hif-1B. These results suggest that the involvement of Hif-1B in hypoxic ventilation in zebrafish larvae is through NOS and, by extension,

NO and future work should focus on determining whether there is also a link between NOS and Hif-1A. Porteus et al. (2015) reported that neuronal NOS was localized to the O_2-sensing NECs of larval skin and adult zebrafish gill. Currently, there is no evidence to indicate that Hif-1α is co-localized with NOS in NECs although Hif-1α is found within the analogous O_2-sensing glomus cells of the carotid body (Roux et al., 2005). Although we have been repeatedly unsuccessful in using custom homologous or commercial heterologous HIF antibodies in immunohistochemistry experiments, future research should be aimed at co-localizing NOS and Hif-1α in zebrafish NECs. However, the involvement of Hif-1α in the hypoxic ventilatory response demonstrated in the current study for zebrafish and its possible link to NO signaling do not necessarily require their presence in NECs.

In adult zebrafish, unlike in larvae, NO exerts an inhibitory effect on ventilation during hypoxia (Porteus et al., 2015). Thus, if NO is the sole mechanism underlying the effects of Hif-1B on the control of breathing, we would expect that hypoxic f_V in Hif-1B adult fish would be greater than that in wild-type fish rather than less. However, similar to the effect in larvae, a loss of Hif-1B impaired the hypoxic ventilatory response in adult fish. These results suggest that in addition to NO, other mechanisms may be involved, such as VEGF, EPO or ET-1 (Prabhakar and Semenza, 2012), in the control of breathing via Hif-1α. These potential mechanisms may mask the inhibitory effect of NO on hypoxic ventilation in adult zebrafish.

Do the Hif-1α paralogs play equivalent roles in the hypoxic ventilatory response?

The possible outcomes in the evolution of gene duplicates after a genome-wide duplication event are (1) sub-functionalization, where the functions of the ancestral gene are divided between the paralogs, (2) neo-functionalization, where the paralogs acquire novel functions or (3) gene dosage, where the expression of the paralogs is higher than that of the ancestral gene (Zhang, 2003; Innan and Kondrashov, 2010; Glasauer and Neuhauss, 2014). As the ancestral Hif-1α role in the hypoxic ventilatory response is unknown, it is not possible to draw concrete conclusions regarding which mechanism governed the evolution of the Hif-1α paralogs. However, by examining the patterns of f_V in larval and adult zebrafish between Hif-1A$^{-/-}$ and Hif-1B$^{-/-}$, as well as the double-knockout line, we can begin to examine whether the two paralogs play similar or divergent roles in the control of breathing during hypoxia.

Both Hif-1α paralogs appear to be involved in the hypoxic ventilatory response of larval zebrafish. Loss of function of either paralog led to similar hypoxic breathing patterns, e.g. shorter duration of significantly elevated hypoxic f_V at 7 dpf, but also distinctly different breathing responses to hypoxia, e.g. abolishment of hypoxic f_V in Hif-1B$^{-/-}$ in contrast to no effect on hypoxic f_V in Hif-1A$^{-/-}$ larvae at 4 dpf. In adults, only the Hif-1B paralog appears to be involved in the hypoxic ventilatory response. Thus, it is conceivable that after the duplication of Hif-1α, relaxation of selection may have allowed the Hif-1α paralogs to evolve differences in the fine-tuning of the hypoxic control of breathing, possibly via different mechanistic pathways.

Evoking the loss of function of both paralog genes is a powerful method to determine how or whether the two paralogs interact with each other. If the effect of the double mutation is the sum of the effects of the single mutations, it is considered additive and thus the paralogs presumably are not interacting. If the effect is any other than additive, the paralogs are interacting with each other and this is termed epistasis. Interestingly, the hypoxic f_V of the double-knockout fish in relation to the single knockout of Hif-1α paralogs changes over developmental time. At 4 dpf, the effect of the double knockout was less than the sum of the individual Hif-1α knockout effects, possibly indicating positive epistasis, while at 7 dpf the effect was greater than the sum of the individual Hif-1α knockout effects, possibly indicating negative epistasis. In the adult zebrafish, the effects of the double knockout varied according to the type of hypoxia exposure. This would suggest that the way the two paralogs interact with each other and possibly with other mechanisms of control of breathing [e.g. HIF-2α, which was shown to inhibit the carotid body sensitivity; Prabhakar and Semenza, 2016] is dependent on age and type of hypoxia exposure. Elucidating the full effect of Hif-1α even with knockout models may be challenging if there is redundancy in the mechanisms of hypoxic control of breathing in zebrafish. Thus, the functional role of Hif-1α might be taken over by alternative mechanisms. Redundancy in the hypoxic response is a possibility given that zebrafish Hif-1α mutants are viable whereas a full knockout of HIF-1α in mice leads to embryonic lethality (Iyer et al., 1998), suggesting that in zebrafish some of the critical functions of Hif-1α may be performed even in the absence of Hif-1α. If this is the case, it would highlight greater hypoxic versatility and flexibility in the zebrafish as compared with the standard mammalian models (i.e. mouse).

Conclusions

In the current study, we demonstrate that Hif-1α plays a role in the hypoxic ventilatory response in zebrafish. Specifically, knockout of either of the Hif-1α paralogs decreases the hypoxic f_V response in larvae, but only Hif-1B knockout was found to have an effect in adult zebrafish. Control of hypoxic ventilation by Hif-1α may be, at least in part, through NO and its enzymatic catalyst NOS, a downstream target of Hif-1α. The effect of each Hif-1α paralog on the hypoxic f_V changes over developmental time and in response to different types of hypoxic exposure. These findings indicate that there is a complex set of interactions occurring between the paralogs and other mechanisms of control of breathing in determining the hypoxic f_V phenotype.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.M., S.F.P.; Methodology: M.M., V.T.; Validation: M.M.; Formal analysis: M.M., V.C.; Investigation: M.M.; Data curation: M.M.; Writing - original draft: M.M.; Writing - review & editing: M.M., V.T., V.C., S.F.P.; Visualization: M.M., S.F.P.; Supervision: S.F.P.; Project administration: M.M.; Funding acquisition: S.F.P.

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Supplementary information

Supplementary information available online at
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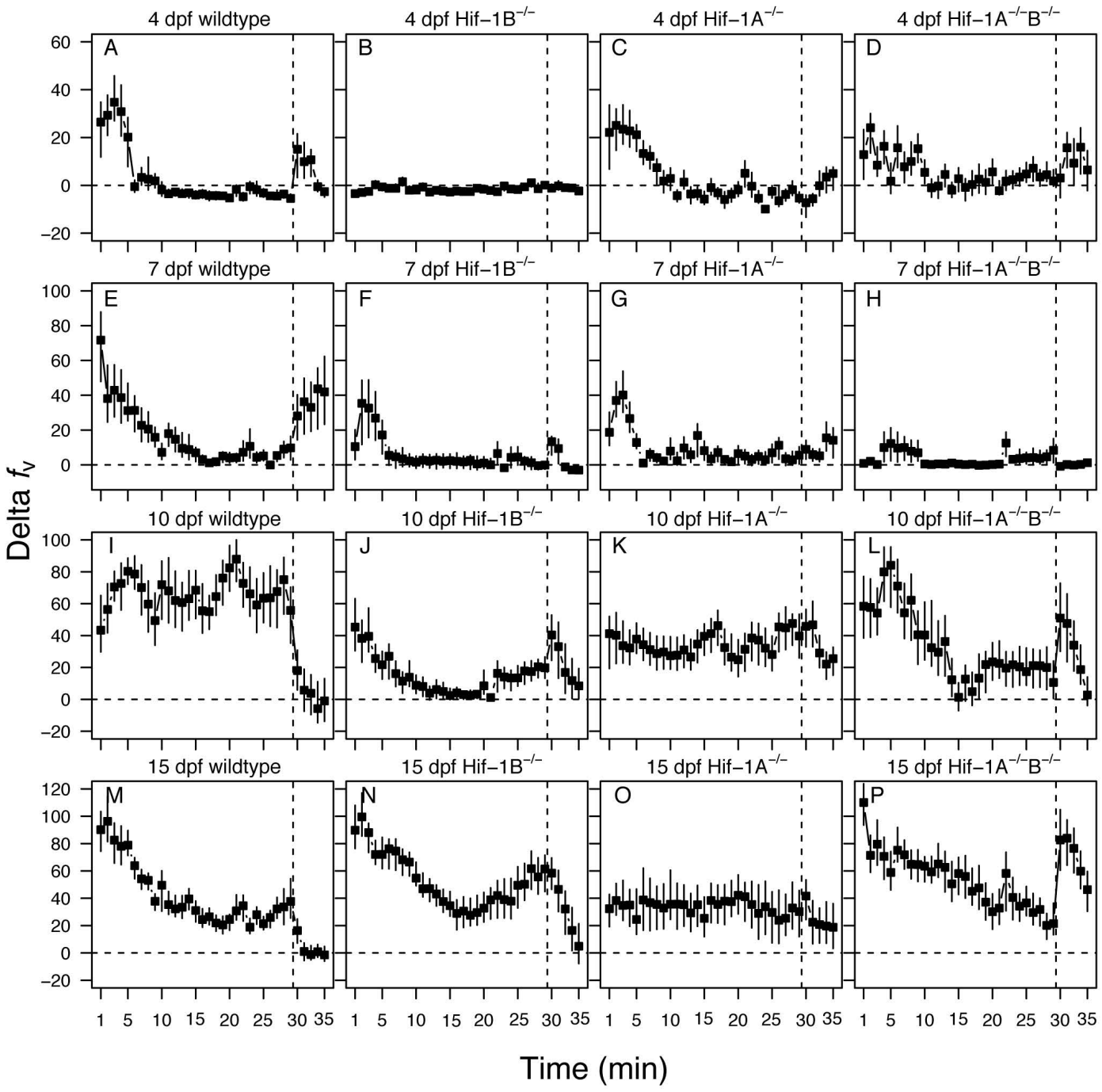

Figure S1. The average differences in f_V (delta f_V) between normoxia and hypoxia, and normoxia and recovery in wildtype, Hif-1B$^{-/-}$, Hif-1A$^{-/-}$ and Hif-1A$^{-/-}$B$^{-/-}$ larval zebrafish (data based on Figure 1). Panels A-D are larvae at 4 days post fertilization (dpf), panels E-H are larvae at 7 dpf, panels I-L are larvae at 10 dpf and panels M-P are larvae at 15 dpf. Panels A, E, I, M are wildtype larvae, panels B, F, J, N are Hif-1B$^{-/-}$ larvae, panels C, G, K, O are Hif-1A$^{-/-}$ larvae, and panels D, H, L, P are Hif-1A$^{-/-}$B$^{-/-}$ larvae. Estimates are presented with 95% credible interval based on Bayesian analysis. Data are significant if the 95% CI do not intersect zero (dotted horizontal line).