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RESEARCH ARTICLE

Evaluating the physiological significance of hypoxic hyperventilation in larval zebrafish (*Danio rerio*)

Yihang K. Pan*,‡, Milica Mandic*, Alex M. Zimmer and Steve F. Perry

ABSTRACT

In water-breathing fishes, the hypoxic ventilatory response (HVR) represents an increase in water flow over the gills during exposure to lowered ambient O_2 levels. The HVR is a critical defense mechanism that serves to delay the negative consequences of hypoxia on aerobic respiration. However, the physiological significance of the HVR in larval fishes is unclear as they do not have a fully developed gill and rely primarily on cutaneous gas transfer. Using larval zebrafish (4, 7, 10 and 15 days post-fertilization; dpf), we examined HVR under three levels of hypoxia (25, 45 and 60 mmHg). The larvae exhibited widely different HVRs as a function of developmental age and level of the hypoxia. Yet, critical O_2 tensions (P_{crit}) remained constant (30–34 mmHg) over the same period of development. Micro-optrode O_2 sensors were used to measure a significant decrease in buccal cavity water O_2 tensions in 4 and 7 dpf larvae compared with the water they inspired, demonstrating significant extraction of O_2 from the buccal cavity. To assess the physiological significance of the HVR, ventilatory water flow was prevented in larvae at 4 and 7 dpf by embedding their heads in agar. An increase in P_{crit} was observed in larvae at 7 dpf but not 4 dpf, suggesting that buccal ventilation is important for O_2 extraction by 7 dpf. Combined, these data indicate that branchial/buccal gas transfer plays a significant role in O_2 uptake during hypoxia, and supports a physiological benefit of the HVR in early life stages of zebrafish.

KEY WORDS: Oxygen consumption, Critical P_{O_2}, Control of breathing, Cutaneous gas transfer, Gill

INTRODUCTION

The hypoxic ventilatory response (HVR) is a widely conserved physiological phenomenon in fish exposed to lowered ambient water O_2 tension (P_{O_2}). By increasing ventilation frequency (f_V) and/or ventilation amplitude, the vast majority of water-breathing fishes studied to date exhibit an increase in the volume of water flowing over the gills when exposed to hypoxia (Perry et al., 2009). By decreasing the residence time of water flowing over the gill lamellae, an increase in ventilation volume serves to decrease the difference in P_{O_2} between inspired and expired water, hence raising the average P_{O_2} of the ventilatory water and increasing the water-to-blood P_{O_2} gradient (ΔP_{O_2}). In addition to raising O_2 diffusive conductance (O_2 uptake/ΔP_{O_2}), the increased average ventilatory water P_{O_2} minimizes the decline in arterial P_{O_2} during hypoxia (Perry et al., 2009; Perry and Gilmour, 2002). Thus, hyperventilation during hypoxia ultimately delays the transition from aerobic to anaerobic metabolism. A drawback of the HVR is an increase in energy expenditure for ventilation, which is believed to be relatively high, even under normoxic conditions (Jones and Schwarzfeld, 1974; Steffensen, 1985). As water P_{O_2} first begins to drop, ventilation rises to a maximal rate, and then decreases during the most severe levels of hypoxia (Cerezo and García García, 2004; Scott et al., 2008), presumably at the point where the cost of increasing ventilation exceeds the benefits to O_2 transfer (Perry et al., 2009).

Similarly to adults, some larval fish species also hyperventilate in response to hypoxia (Burggren et al., 2016; Holeton, 1971; Jonz and Nurse, 2005; McDonald and McMahon, 1977; Peterson, 1975). The hyperventilatory response of larvae is intriguing because at this stage of development these fish possess undeveloped gills that contribute little to gas and ion exchange in comparison to skin (Fu et al., 2010; Rombough, 2002; Rombough and Ure, 1991; Wells and Pinder, 1996; Zimmer et al., 2014).

At the time of hatch, the gill of the rainbow trout (*Oncorhynchus mykiss*) larva possesses filaments, with rudimentary lamellae developing soon after (González et al., 1996; Morgan, 1974; Rombough, 1999). However, the gill of post-hatch trout larvae is not the primary site for gas or ion transfer. Indeed, at hatch, the gill does not contribute to ammonia excretion (Zimmer et al., 2014) and accounts for merely 10% of Na^+ uptake and approximately 20% of total O_2 uptake (Fu et al., 2010; Zimmer et al., 2014). The gill does not become the primary site of O_2 uptake until 27 days post-hatch (dph) (Fu et al., 2010; Zimmer et al., 2014). Nonetheless, it is clear that larval trout exhibit a HVR as early as 1 dph (Holeton, 1971). Similarly, in zebrafish (*Danio rerio*), gill development does not occur until just after hatching [at 3 days post-fertilization (dpf) at 28°C], beginning with the appearance of gill filament primordia on the pharyngeal arches, followed by the appearance of lamellae at 7 dpf (Jonz and Nurse, 2005). Like trout, early larval zebrafish do not utilize gills as a primary site for O_2 uptake (Rombough, 2002). Based on the empirical relationship between skin surface area per unit O_2 uptake and body mass (Rombough, 2004) as well as the results of experiments that prevented buccal water flow (Rombough, 2002), it was determined that gills are not required for O_2 uptake until 10–14 dpf. Yet, despite the gills not being the primary O_2 uptake site in early development, larval zebrafish initiate the HVR as early as 3 dpf (Jonz and Nurse, 2005).

Given the limited role of the larval gill in O_2 uptake, the physiological significance of a hyperventilatory response to hypoxia in larval fishes is unclear. It has been suggested, however, that the development of the HVR before complete formation of the gill may act to ensure that O_2-sensing pathways are functional by the time the larvae become dependent on branchial respiration (Jonz and Nurse, 2005). Because small larvae reside in a viscous environment (with a low Reynolds number), fluid accelerated by ventilation rapidly decelerates between cycles, potentially incurring a higher ventilation energy expenditure than in adult fish (Rombough, 1988). Thus, it is

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario K1N 6N5, Canada.
*These authors contributed equally to this work

‡Author for correspondence (ypan034@uottawa.ca)

Y.K.P., 0000-0003-1455-1439

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conceivable that increasing ventilation might decrease survival time in hypoxia if the hyperventilatory response is of no physiological benefit. We therefore hypothesize that zebrafish larvae can regulate the HVR depending on P_{O_2} levels, and that the HVR plays a physiological role in maintaining O_2 uptake in larval zebrafish. The first objective was to examine the HVR at several development stages (4, 7, 10 and 15 dpf) and to determine whether the dynamic characteristics of the HVR are related to critical O_2 tension (P_{crit}), the P_{O_2} below which the fish can no longer maintain a stable rate of O_2 consumption ($\dot{M}_{O_2}$) (Ultsch et al., 1978). The second objective was to measure P_{O_2} within the buccal cavity of 4 and 7 dpf larvae to determine whether O_2 is extracted from water in the buccal cavity. Lastly, we prevented larvae from ventilating and observed whether the fish exhibited a reduced capacity for O_2 uptake.

MATERIALS AND METHODS

Experimental animals

Zebrafish [*Danio rerio* (F. Hamilton 1822)] were obtained from in-house stock at the University of Ottawa aquatic care facility. 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 daily. Adults were bred using breeding traps as per standard methods (Westerfield, 2000) to obtain embryos. Briefly, one male and two females were separated the night before breeding using a divider in 2 liter breeding traps. Once lights turned on the next day, water in the tank was changed and the dividers were removed allowing the zebrafish to breed. Embryos were collected and reared initially in an incubator (28.5°C) in 50 ml Petri dishes containing dechloraminated tap water and 0.05% Methylene Blue. At 5 dpf, larvae were transferred to static 1 liter tanks and fed once daily except on the day of the experiment when food was withheld. Water was changed daily in the Petri dishes and every other day in the static tanks. The larvae were raised in static tanks until 7, 10 or 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.

Ventilation in developing larvae

The hypoxic ventilatory response, specifically ventilation frequency (f_V), was measured at 4, 7, 10 and 15 dpf during acute exposure to hypoxia (60, 45 or 25 mmHg). These developmental stages were chosen based on the timing of the physical development of the gills, as well as the development of O_2-sensing pathways within the gills of zebrafish larvae. At 4 dpf, gill filament primordia are present on the pharyngeal arches yet the putative O_2 chemoreceptors – neuroepithelial cells (NECs) of the gill filaments – do not appear until 5 dpf (Jonz and Nurse, 2005). By 7 dpf, lamellae begin to form, and all NECs receive innervation (Jonz and Nurse, 2005), yet regular buccal movements are not observed until 8 dpf (Jonz and Nurse, 2005). Lastly, the 15 dpf point was chosen as it is believed that gills become the dominant site for gas transfer at 14 dpf (Rombough, 2002). An individual larva was placed in a glass micro-capillary tube with an inner diameter of 1 mm. Water containing 0.05 mg ml^{-1} Tris-buffered (pH 7.6) MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Syndel Laboratories, Nanaimo, Canada) maintained at 28.5°C was gravity-fed through the microcapillary tube at a rate of 1.6–1.8 ml min^{-1}. Tris-buffered MS-222 was used to minimize the movement in larval zebrafish to facilitate f_V analysis (Jonz and Nurse, 2005). Each larva was allowed to recover from handling in the chamber for 10 min in normoxic water (P_{O_2}=153 mmHg) prior to the start of the trial. During each trial, f_V was recorded by video for 5 min under normoxic conditions (baseline), followed by 15 min under hypoxia and an additional 5 min after returning to normoxia. Videos were recorded using an iPhone SE mounted onto a dissecting microscope (stereo trinocular microscope, AmScope, Irvine, USA). Average f_V was determined for each minute by counting either buccal or opercular movements depending on the fish orientation in the chamber and the visibility of the mouth and/or operculae. Each larval zebrafish was exposed to only a single level of hypoxia; different animals were used for different levels of hypoxia.

Respirometry in developing larvae

Routine metabolic rate (RMR) and P_{crit} were measured using closed system respirometry. Larvae of the same developmental age were placed into individual respirometry wells (80 µl for 4 and 7 dpf; 500 µl for 10 and 15 dpf) using a 24-well glass microplate (Loligo Systems, Viborg, Denmark) fitted with O_2 sensor spots. The microplate was sealed with adhesive plate seals (AB0580, ThermoFisher Scientific, Mississauga, Canada) and maintained at 29°C using a water bath. The sealed microplate and water bath were attached to an O_2 fluorescence sensor (SDR SensorDish Reader, PreSens, Regensburg, Germany) and P_{O_2} levels were measured until they plateaued, at which point the experiment was ended. Larval fish mass was obtained by raising separate batches of fish to the same developmental age. Fish (n=11–18) were pooled into a single mini 5 µm cell strainer (pluriSelect, San Diego, USA) and excess water was removed by centrifugation (100 ***g***). Mass was measured using an analytical balance, and at least six pooled values were obtained for each developmental age and used in the following calculation of $\dot{M}_{O_2}$ (µmol g^{-1} h^{-1}):

$$\dot{M}_{O_2} = \frac{\Delta P_{O_2} \times \alpha_{O_2} \times V}{M_b}.$$

Where ΔP_{O_2} is the slope of P_{O_2} over time (mmHg h^{-1}), α_{O_2} is the solubility coefficient of O_2 (µmol l^{-1} mmHg^{-1}) in freshwater at 28°C (Boutilier et al., 1984), V is the respirometer volume (l) and M_b is the body mass of fish (g). The volume of the fish accounted for less than 0.4% of the respirometer volume and thus was not considered to displace a significant volume in the respirometer. Data were binned in 5 mmHg intervals. Metabolic rate was calculated as the average $\dot{M}_{O_2}$ before P_{O_2} levels dropped below 90 mmHg. This cut-off was chosen because both 7 dpf larvae and adults do not hyperventilate when P_{O_2} is above 90 mmHg (unpublished observations; Vulesevic and Perry, 2006). P_{crit} was calculated by the 'broken-stick' regression approach, adopted from Yeager and Ultsch (1989). This approach estimates P_{crit} as the intersection of the two linear regression lines that best fit the P_{O_2} versus $\dot{M}_{O_2}$ plot and was carried out using the REGRESS software (www.wfu.edu/~mudayja/software/o2.exe). P_{O_2} versus $\dot{M}_{O_2}$ plots and P_{crit} regression lines were inspected 'blindly' by two researchers independently for quality control, and only those that both researchers deemed to be valid traces were retained. Traces that were excluded either did not have a stable oxy-regulatory phase in the P_{O_2} versus $\dot{M}_{O_2}$ curve to fit a tightly fitted line or used less than 4 data points to fit the oxy-conforming phase of the curve, indicating that the fit was likely driven by an outlier.

Respirometry was also conducted on 4 and 7 dpf larvae whose heads were embedded in agar (applying an 'agar helmet') to prevent gill ventilation (Rombough, 2002). To apply the agar, larvae were lightly anesthetized in 0.05 mg ml^{-1} Tris-buffered MS-222. A larva was transferred onto a glass slide and excess water around the larva was removed using a pipette. 1–2 µl of 2.5% low melting point

agarose (ThermoFisher Scientific, Mississauga, Canada) was applied to the head and was allowed to set before excess agar was trimmed off using a micro scalpel. Control fish were subjected to the same treatment excluding the application of agar. The treated larvae were allowed to recover from anaesthesia and handling for 1 h before RMR and P_{crit} were measured, as described above. To determine whether the agar might also be impeding O_2 diffusion (in addition to preventing ventilatory water flow), a 'proof of principle' experiment was conducted in which RMR and P_{crit} were compared in larvae with agar applied to the head only and in larvae experiencing encasement of the whole body in agar. The results (data not shown) demonstrated that RMR (P=0.75, Mann–Whitney rank sum test) and P_{crit} (P=0.13, Student's t-test) did not differ in the two treatment groups, suggesting that applying agar to the head is a viable method of preventing ventilation without affecting cutaneous O_2 uptake.

Visualization of buccal cavity water flow

Water flow through the buccal cavity of a ventilating 4 dpf larval zebrafish was visualized by injecting 0.5% Phenol Red (Sigma-Aldrich, Oakville, Canada) solution in front of the mouth. Video was recorded using an iPhone SE mounted onto a Nikon SMZ 1500 stereo dissecting microscope (Nikon Instruments, Melville, USA).

Local O_2 consumption within the buccal cavity

To test the hypothesis that O_2 is extracted from water upon passage through the buccal cavity of 4 and 7 dpf larval zebrafish, an O_2 micro-optrode technique was used. This system (Hughes et al., 2019) consists of an O_2-sensing fibre-optic micro-optrode (tip diameter=20 µm) attached to a detector system (Applicable Electronics, New Haven, CT, USA), which was operated by custom software (ASET-LV4; Science Wares, Falmouth, USA). Optrodes were calibrated with a 'zero-solution' containing 40 mg ml^{-1} anyhydrous sodium sulfite dissolved in water in which measurements were made and a well-aerated sample of water.

Larvae were anaesthetized with 0.3 mg ml^{-1} of Tris-buffered MS-222 to the point at which ventilation ceased but heartbeat continued, and placed in a Petri dish containing water with the same anaesthetic level. Using a dissecting microscope (SMZ 1500; Nikon Instruments, Melville, NY, USA) the micro-optrodes were placed either directly in front of the mouth to obtain P_{O_2} of the inspired water or into the mouth to obtain buccal cavity P_{O_2}. In both cases, optrodes were left in place until a stable signal was achieved (usually within 3–5 min), after which P_{O_2} was measured every 10 s over 5 replicate measurements.

A separate experiment was designed to measure the P_{O_2} of the water leaving the buccal cavity in a ventilating 7 dpf zebrafish. Larvae were exposed to a light anaesthetic dose (0.05 mg ml^{-1} Tris-buffered MS-222) and to 10 µmol l^{-1} L-adrenaline (+)-bitartrate salt (Sigma-Aldrich, Oakville, Canada) in the surrounding water (to stimulate ventilation) because the fish needed to be ventilating in order to assess the ability of oxygen extraction through the buccal cavity. In the absence of adrenaline, ventilation rates were too low (1–2 breaths min^{-1}) under normoxic conditions. Upon exposure to adrenaline, ventilation rates initially were high (~60 breaths min^{-1}), but these rates eventually slowed to 5–10 breaths min^{-1} at which point measurements were made. Given that the cutaneous surfaces surrounding the opercula of larval zebrafish likely contribute to O_2 uptake (Hughes et al., 2019), absolute expired P_{O_2} could not be assessed. Rather, the change in local P_{O_2} behind the operculum that occurred following a breath was assessed as a measure of O_2 extraction capacity in ventilating 7 dpf larvae.

Statistical analysis

Breathing frequencies of zebrafish exposed to hypoxia were analyzed using Markov chain Monte Carlo (MCMC) sampler for multivariate generalized linear models using MCMCglmm package (Hadfield, 2010) in R (https://www.R-project.org/). In two of the models, all f_V measurements were fitted as dependent variables and either developmental age or P_{O_2} was fitted as a fixed effect (fitted separately for each trait). In a separate model, f_V measurements in hypoxia were fitted as dependent variables and the last minute of the 5 min normoxia treatment was fitted as a fixed effect. 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. 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 the fixed effect we determined whether the 95% HPDs included or excluded zero. For similar analysis, see Mandic et al. (2019).

All other statistical analyses were carried out in Sigmaplot (Systat Software, USA). Respirometry data were analyzed using one-way analysis of variance (ANOVA). Routine metabolic rate data did not pass a normality test (Shapiro–Wilk test), thus the Kruskal–Wallis ANOVA on ranks was used followed by Dunn's *post hoc* test. Critical P_{O_2} data passed both normality and equal variance tests (Levene's test), thus ANOVA was performed on these data. The difference between inspired and buccal cavity P_{O_2} in fish of the same developmental age was compared using a paired t-test. For 4 dpf larvae, the data did not pass the normality test, and thus a Wilcoxon signed rank test was used. Difference in ΔP_{O_2} between 4 and 7 dpf larvae was compared using a Student's t-test. Respirometry data for fish with and without agar were analyzed by comparing only the difference in RMR or P_{crit} between these two groups at the same developmental age. RMR data for 4 dpf larvae was analyzed using a Student's t-test. RMR data for 7 dpf larvae, and P_{crit} data for 4 and 7 dpf larvae either failed the normality or equal variance test, and thus Mann–Whitney rank sum test was performed. Slopes of $\dot{M}_{O_2}$ versus P_{O_2} before P_{crit} for each group in the agar experiment were tested against 0 for significance using a linear regression. For all tests, significance was set at P<0.05.

RESULTS

Ventilation frequency, resting metabolic rate and critical O_2 tension across developmental age groups

For larvae at all developmental ages, exposure to hypoxia resulted in an increase in f_V, which returned to baseline levels within minutes when larvae were switched back to normoxic conditions (Fig. 1, Fig. S1). However, the duration and intensity of hyperventilation were determined by both developmental age and severity of the hypoxia. In 4 dpf larvae (Fig. 1A), f_V increased and remained significantly elevated during exposure to 25 mmHg hypoxia (Fig. S1A), whereas the significant increase in f_V was transient (5–10 min) when larvae were exposed to either 45 or 60 mmHg hypoxia (Fig. S1B,C). By 7 dpf (Fig. 1B), f_V increased and remained elevated in larvae exposed to both 25 and 45 mmHg hypoxia (Fig. S1D,E), with hyperventilation being transient for the

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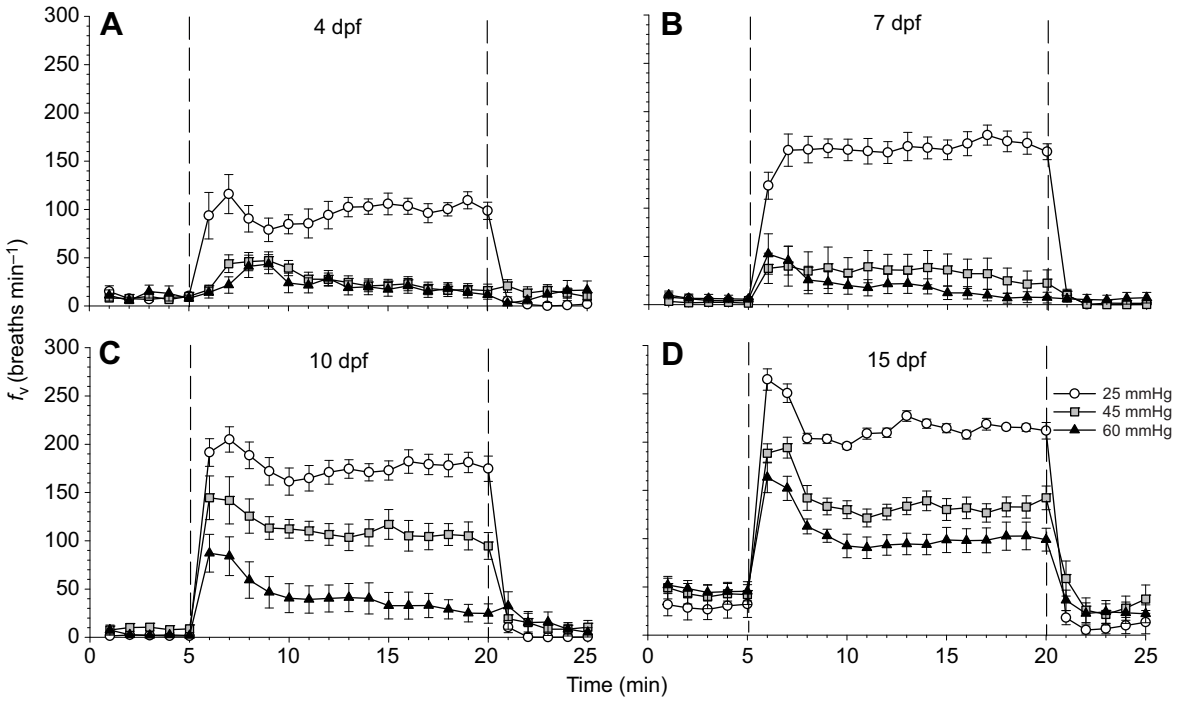

Fig. 1. The interactive effects of hypoxia and developmental age on ventilation frequency (f_V) in larval zebrafish. Larvae at (A) 4 days post fertilization (dpf), (B) 7 dpf, (C) 10 dpf and (D) 15 dpf. Larvae were exposed to normoxia for 5 min (153 mmHg) followed by 15 min of hypoxia at 60 mmHg, 45 mmHg or 25 mmHg and subsequently returned to normoxia for 5 min. Vertical dashed lines represent delineations between normoxia, hypoxia and normoxic recovery. Data are presented as means±s.e.m. Traces were analyzed using Markov chain Monte Carlo sampler for multivariate generalized linear models, and the statistical analysis can be found in Figs S1–S3. *N*=9 for 7 dpf larvae exposed to 60 mmHg; *N*=17 for 4 dpf exposed to 45 mmHg; *N*=10 for all other groups.

first 10 min when exposed to 60 mmHg hypoxia only (Fig. S1F). In both 10 dpf (Fig. 1C) and 15 dpf larvae (Fig. 1D), f_V increased and remained elevated at all three hypoxia levels tested (Fig. S1G–L). Interestingly, f_V decreased to below baseline levels in 15 dpf larvae when returned back to normoxia (Fig. S1J–L). Comparing larvae of the same developmental age, exposure to 25 mmHg resulted in a significantly higher f_V than in the 45 or 60 mmHg exposures at all developmental ages (Fig. S2). At 4 dpf and 7 dpf, 45 and 60 mmHg exposure resulted in the same level of increase in f_V (Fig. S2B,E), but by 10 dpf and 15 dpf, 45 mmHg exposure resulted in a significantly greater increase of f_V compared with 60 mmHg exposure (Fig. S2H,K). For larvae at different development ages exposed to the same level of hypoxia, there was a general trend for the maximum f_V to increase as the larvae developed (Fig. S3).

Oxygen consumption was measured at developmental ages matching those of larvae in the ventilation experiment (Fig. 2A). Routine metabolic rate was significantly lower in 7 dpf larvae compared with the other developmental ages (Fig. 2B), but P_{crit} was the same across all developmental ages (30–34 mmHg, Fig. 2C).

Buccal cavity P_{O_2}

At 4 dpf, larvae were able to inspire water through the mouth and expire water from their operculae (Fig. 3, Movie 1). Larvae at 4 and 7 dpf were able to extract O_2 in the buccal cavity (Fig. 4). Buccal cavity P_{O_2} was significantly lower than inspired P_{O_2} in both 4 and 7 dpf larvae; however, the younger larvae showed a significantly greater difference between inspired and buccal cavity P_{O_2} (Fig. 4B). True expired water P_{O_2} could not be assessed, thus the local changes in P_{O_2} behind the operculum that occurred following a breath were assessed in ventilating 7 dpf larvae, showing a decrease after each breath when f_V was between 5 and 10 breaths min^{-1} (see representative trace, Fig. 4C).

The effects of blocking ventilation on resting metabolic rate and critical O_2 tension

$\dot{M}_{O_2}$ was measured in 4 and 7 dpf larvae that were prevented from ventilating by applying an agar helmet to the head (Fig. 5). At 4 dpf, larvae showed a significantly lower RMR (Fig. 5C) when ventilation was blocked compared with larvae that were able to ventilate, yet P_{crit} (Fig. 5D) was not different between the two treatment groups. On the other hand, RMR (Fig. 5C) was not different in 7 dpf larvae between the ventilating and non-ventilating larvae, but P_{crit} (Fig. 5D) was significantly higher for larvae that were unable to hyperventilate. $\dot{M}_{O_2}$ increased as water P_{O_2} levels gradually decreased to P_{crit} for ventilating larvae of both developmental ages; the slopes (4 dpf, −0.12; 7 dpf, −0.13) were significantly lower than zero (P<0.0001 for both 4 dpf and 7 dpf). However, $\dot{M}_{O_2}$ remained constant until P_{crit} for larvae that were unable to ventilate; the slopes (4 dpf: −0.03, 7 dpf: 0.01) were not significantly different from zero (4 dpf, P=0.15; 7 dpf, P=0.58, Fig. 5A,B).

DISCUSSION

The goals of this study were to determine whether larval zebrafish can regulate their HVR and whether this physiological response is beneficial. Based on previous studies, it is known that the gills are not the primary site of O_2 uptake in early larval stages of fishes (Fu et al., 2010; Rombough, 2002; Rombough and Ure, 1991; Wells and Pinder, 1996; Zimmer et al., 2014), leading to the question of why larval fishes hyperventilate in response to hypoxia. The results of this study clearly show that larval zebrafish are not only able to regulate their HVR, but also rely on it to maintain O_2 uptake under hypoxic conditions as early as 7 dpf.

Depending on species, the HVR consists of an increase in f_V or ventilation amplitude, or both, to increase the volume of water flowing over the gills (Perry et al., 2009). In this study, we focused

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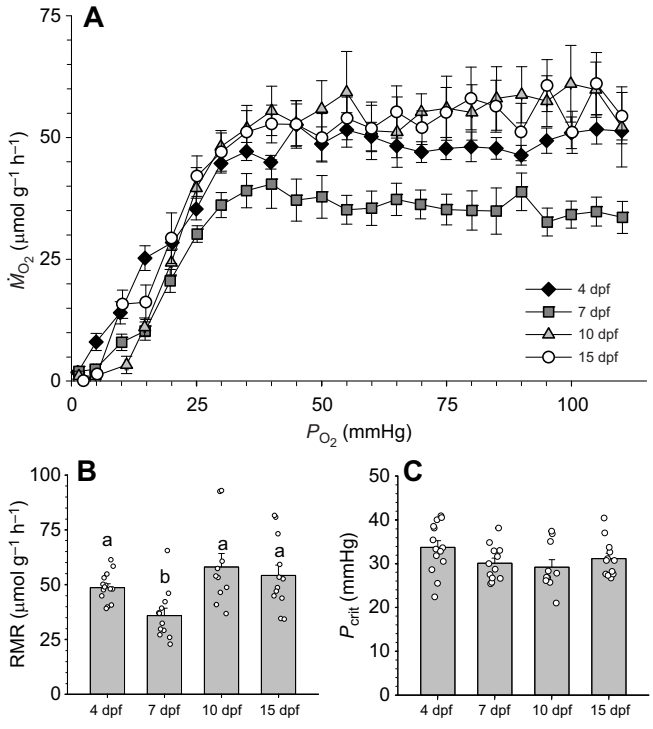

Fig. 2. Respirometry data for larvae at different stages of development. (A) Water oxygen tension (P_{O_2}) versus oxygen consumption rate ($\dot{M}_{O_2}$) for 4 dpf (N=14), 7 dpf (N=12), 10 dpf (N=10) and 15 dpf (N=12) larvae. (B) Routine metabolic rate (RMR) and (C) critical oxygen tension (P_{crit}) data calculated from the P_{O_2} versus $\dot{M}_{O_2}$ plot. Data are presented as means±s.e.m. Values with different letters are significantly different (P<0.05).

on f_V because adult zebrafish increase f_V, but not ventilation amplitude, during hypoxia (Vulesevic et al., 2006) and there is currently no reliable method for measuring ventilation amplitude in larval zebrafish. The results of the present study demonstrate that larval zebrafish are not only able to hyperventilate during hypoxia as early as 4 dpf, as has been previously reported (Jonz and Nurse, 2005; Mandic et al., 2019), but that they are also able to modify the magnitude and the duration of the HVR based on the level of hypoxia imposed and the developmental age. In 4 dpf and 7 dpf larvae exposed to mild hypoxia (60 mmHg), the increase in f_V was transient, reducing to baseline levels after 5–10 min. It is possible that up to 7 dpf, cutaneous respiration is sufficient to meet metabolic demands, even in mild hypoxic conditions, and thus sustained hyperventilation is avoided as ventilation itself is an energetically costly response (Jones and Schwarzfeld, 1974; Steffensen, 1985). Alternatively, the return of f_V to baseline levels could reflect a gradual desensitization of the oxygen chemoreceptor NECs. In zebrafish larvae, NECs are located on the skin (Coccimiglio and Jonz, 2012) and gill filament primordia (Jonz and Nurse, 2005), but NECs on the gill filament primordia are not innervated until 7 dpf (Jonz and Nurse, 2005). It is possible that gill NECs are able to sense internal and external P_{O_2} levels owing to their location within a permeable epithelium between the respiratory water flow and the arterial blood supply, whereas NECs on the skin are more likely to sense external P_{O_2} levels only (Jonz et al., 2004). Thus, it is possible that the increase in f_V arises from the skin NECs sensing a change in ambient P_{O_2} and under mild hypoxia the fall in blood P_{O_2} may not be severe enough to provide additional sensory input required to sustain the HVR. Therefore, it is proposed that NECs gradually desensitize and f_V returns to baseline levels.

Although f_V was significantly elevated throughout the duration of hypoxia in 10 and 15 dpf larvae, the greatest increase in f_V occurred in the first 5 min of exposure. Upon their return to normoxia, f_V of 15 dpf larvae dropped to below baseline levels. This post-hypoxic hypoventilation is characteristic of short-term depression within the hypoxic ventilatory time domain, whereby a transient overshoot in f_V at the onset of hypoxic stimulation or a transient undershoot in f_V at the termination of hypoxic stimulation occurs. These transients in f_V

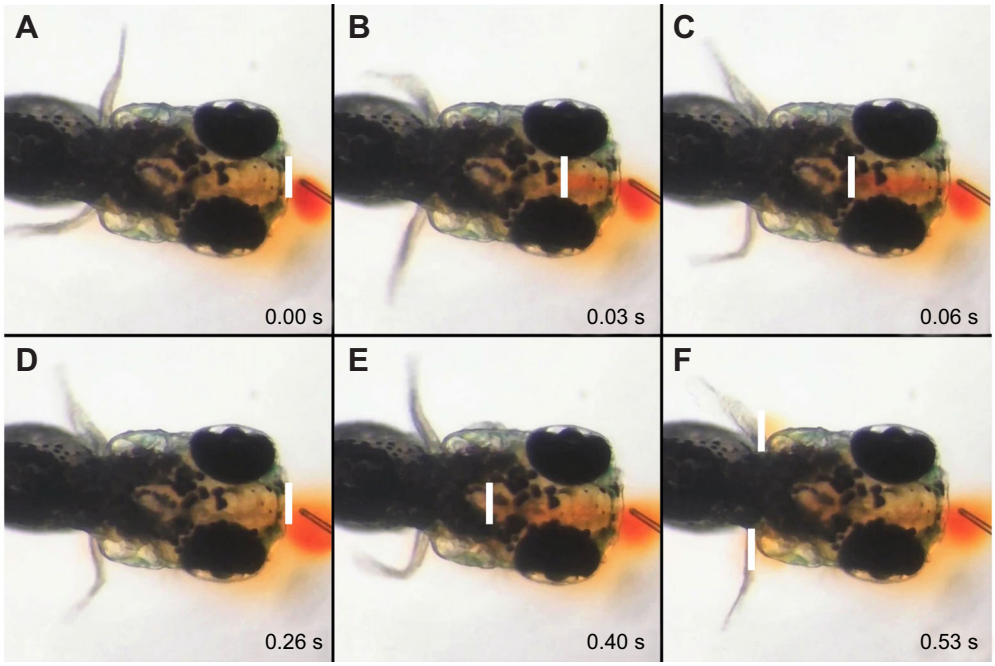

Fig. 3. Image sequence of 4 dpf zebrafish larva inspiring water from the mouth and expiring water out of the operculae. Concentrated Phenol Red solution was injected at the mouth (A), and was inspired by the ventilating larva (B,C). The Phenol Red solution was ejected during 'coughing' (D) and reabsorbed into the buccal cavity (E), prior to exiting the operculae (F). The white line represents the front of the Phenol Red solution. The image sequence was extracted from Movie 1.

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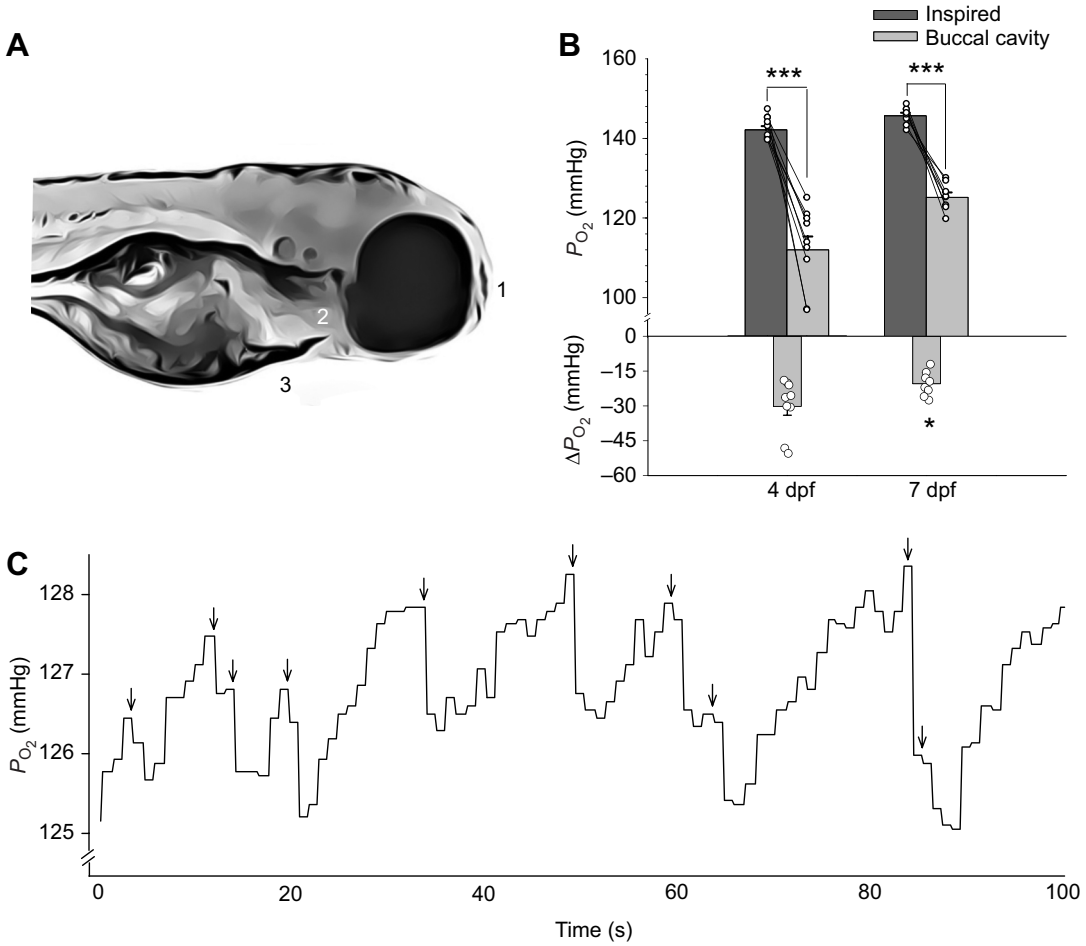

Fig. 4. P_{O_2} levels measured at the head region of larval zebrafish using micro-optrodes. (A) Inspired P_{O_2} was measured at position 1; buccal cavity P_{O_2} was measured just above the gill basket shown in position 2; and expired P_{O_2} was measured at position 3. (B) Inspired and buccal cavity P_{O_2} levels measured in 4 dpf (N=9) and 7 dpf (N=8) larvae, as well as the ΔP_{O_2} between the two groups. Data are presented as means±s.e.m. Asterisks represent the level of significance. (*P<0.05, ***P<0.001). (C) Representative trace of expired P_{O_2} levels measured at position 3 in a 7 dpf larva. Arrows represent distinct breaths.

last seconds to minutes (Powell et al., 1998) and are also observed in adult fish species (Maxime et al., 1995) although the specific mechanism is unclear. In mouse, however, the short-term depression is likely associated with an inhibition of f_V rather than a decrease in responsiveness to the hypoxic stimulus (Hayashi et al., 1993). Thus, we suggest that larval zebrafish also exhibit a high level of ventilatory control, as they are able to initiate a hyperventilatory response of variable magnitude and duration with the possibility of inhibitory and excitatory inputs simultaneously regulating the overall ventilatory response.

Although larvae at different development ages initiate slightly different hyperventilatory responses, their RMR and P_{crit} are the same, with the exception of a lower RMR in 7 dpf larvae. Rombough and Drader (2009) reported a similar phenomenon in zebrafish in which RMR was lower in 7 dpf larvae compared with 5 dpf and 14 dpf larvae. Similarly, it was observed that RMR decreased between 5 dpf and 7 dpf in zebrafish (Grillitsch et al., 2005). Complete yolk sac absorption occurs at 5 dpf in zebrafish (Kimmel et al., 1995), whereas respiratory lamellae do not appear until 7 dpf (Jonz and Nurse, 2005). The yolk sac epithelium can serve as a respiratory tissue, contributing up to 33% of O_2 uptake (Rombough, 1998; Wells and Pinder, 1996). Thus, a drop in RMR at 7 dpf could reflect a decreased ability for cutaneous O_2 uptake, at a time when branchial O_2 transfer is not yet fully functional.

Critical P_{O_2} was constant across the four developmental ages, indicating that differences in the temporal dynamics of the HVR were not correlated with P_{crit}. In larvae at 4 dpf, the HVR was sustained for the duration of exposure only at the most severe level of hypoxia (25 mm Hg) or below P_{crit}. With increasing age, the larval HVR was sustained for the duration of exposure at higher and higher P_{O_2} values compared with P_{crit}. This finding suggests that at 4 dpf, the hyperventilatory response is not necessary and that cutaneous respiration alone is likely sufficient to meet O_2 demand. It is interesting that the HVR is not sustained in 4 dpf and 7 dpf larvae until external P_{O_2} levels fall near to P_{crit}, because at this point $\dot{M}_{O_2}$ is already under decline, which challenges the physiological benefit of hyperventilation, as it no longer delays the transition from aerobic to anaerobic metabolism. However, from 10 to 15 dpf, the hyperventilatory response begins to play a greater role at external P_{O_2} levels well above P_{crit}, arguably contributing more to total O_2 uptake. The fact that the HVR was sustained at higher P_{O_2} values with increasing developmental age (10–15 dpf) presumably reflects the inability of cutaneous O_2 uptake to meet metabolic demands in the older larvae.

We present direct evidence that larvae as young as 4 dpf were able to inspire water into the buccal cavity and extract O_2 prior to the expired water exiting the operculae. The decrease in buccal cavity

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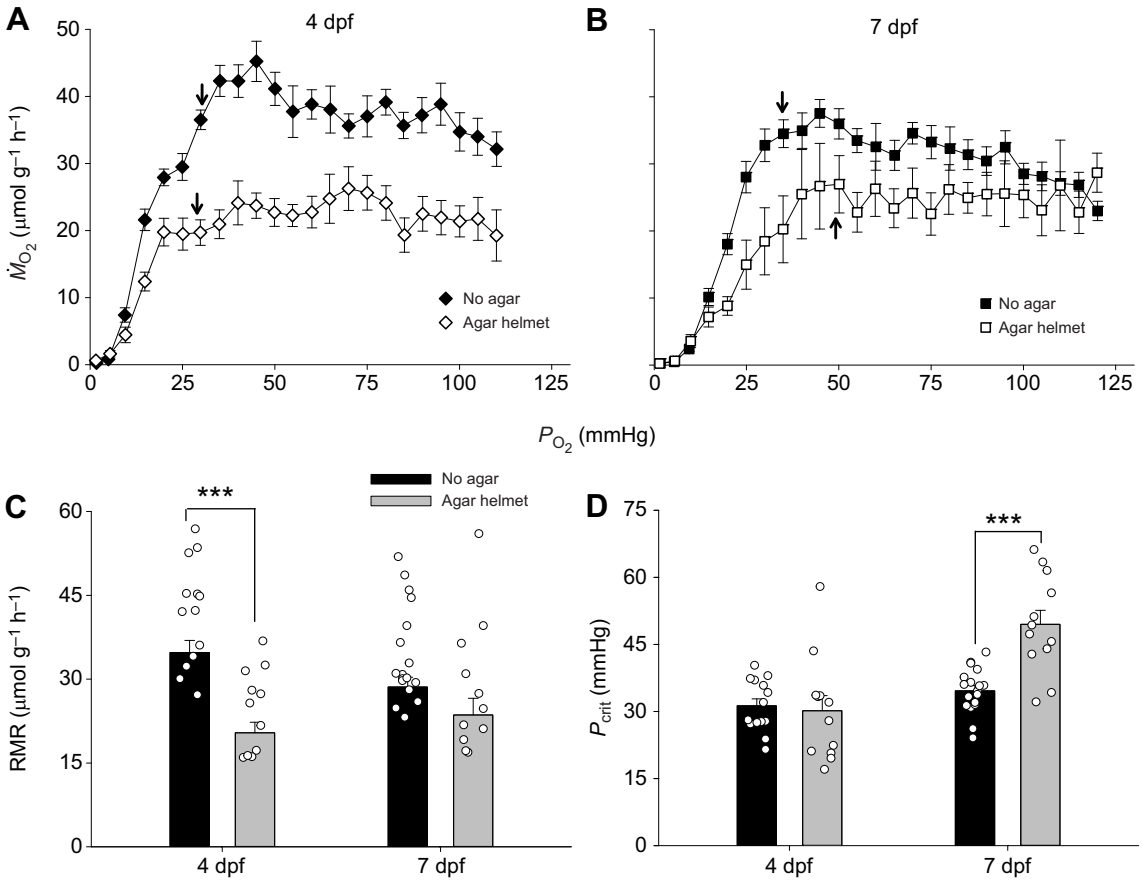

Fig. 5. Respirometry data for agar helmet- versus non-agar helmet-treated larvae. P_{O_2} versus $\dot{M}_{O_2}$ for agar helmet-treated larvae (unfilled symbols) and non-agar helmet-treated larvae (filled symbols) in (A) 4 dpf (N=13 for no agar larvae, N=11 for agar helmet larvae) and (B) 7 dpf (N=17 for no agar larvae, N=11 for agar helmet larvae) zebrafish. Arrows indicate the P_{crit} for each treatment. (C) RMR and (D) P_{crit} data were calculated from the P_{O_2} versus $\dot{M}_{O_2}$ plots. Data are presented as means±s.e.m. Asterisks represent the level of significance. (**P<0.01).

P_{O_2} compared with inspired water, as well as a fall in P_{O_2} at the operculae where water is expired following each breath clearly demonstrated that O_2 was being removed from the ventilatory water. The effect of water residence time within the buccal cavity was assessed by examining the relationship between inter-breath duration and the relative change in local P_{O_2} at the operculum following a breath. The decrease in P_{O_2} was positively correlated with the water residence time within the buccal cavity, with water residence time contributing to 13% of the observed variation (data not shown). It should be noted that with our experimental design, it was not possible to determine whether the decline in buccal cavity P_{O_2} was caused by localized O_2 consumption or by the movement of O_2 into the blood to be transported throughout the body.

In addition to showing that O_2 can be extracted in the buccal cavity of 4 and 7 dpf larvae, we also examined the physiological importance of buccal cavity O_2 extraction by preventing ventilation. In 4 dpf larvae, blocking ventilation by applying an agar helmet resulted in a decrease in RMR, yet P_{crit} remained the same. This decrease in RMR was unlikely to be caused by the agar impeding cutaneous gas exchange, as agar itself is highly porous to water (Movie 2) and there was no difference in RMR between fish fully encased in agar and fish with agar covering the head only (data not shown). As larvae with agar helmets were still able to move their fins (Movie 3), it is also unlikely that this decrease in RMR can be attributed to less water being ventilated across their cutaneous respiratory surfaces. This decrease in RMR may have resulted from reduced movement, as fish with agar helmets swam less. However, given that the P_{crit} is the same between 4 dpf larvae that can and cannot ventilate, it is likely that cutaneous respiration alone is able to satisfy their metabolic demands. These data suggest that the HVR does not have a physiological benefit in zebrafish larvae at 4 dpf. Thus, as previously hypothesized, the HVR may be an innate response in 4 dpf larvae, acting to ensure that O_2-sensing pathways are functional by the time the larvae become dependent on branchial respiration (Jonz and Nurse, 2005). However, this is clearly not the case in 7 dpf larvae, because P_{crit} was significantly higher in fish that were unable to hyperventilate, suggesting that hyperventilation is aiding O_2 uptake during hypoxia. This result is surprising considering that 4 dpf larvae have a lower P_{O_2} within their buccal cavity compared with 7 dpf larvae. However, it should be noted that for the micro-optrode experimental design, it was not possible to determine whether the decline in buccal cavity P_{O_2} was caused by localized O_2 consumption or by the movement of O_2 into the blood to be transported throughout the body. Thus, a possible explanation is that protein turnover (related to gill development) is higher in the 4 dpf larvae, resulting in a greater proportion of regional O_2 consumption, as gills have been shown to be a sink for O_2 in larval trout immediately after hatch (Rombough, 1992). Alternatively, the gills or buccal cavity of 7 dpf larvae may be more highly vascularized than in 4 dpf larvae, resulting in more blood containing O_2 that had been extracted from cutaneous sites of gas exchange supplying the buccal/branchial vasculature of 7 dpf larvae. This would potentially reduce O_2 extraction from the water in 7 dpf

compared with 4 dpf larvae. This lower P_{O_2} within the buccal cavity of 4 dpf larvae also does not contradict the idea that hyperventilation is beneficial at 7 dpf but not necessarily at 4 dpf, because all O_2 measurements were conducted under normoxic conditions owing to limitations of the experimental set up. In normoxia, cutaneous O_2 uptake alone may be able to satisfy O_2 demands in 7 dpf larvae as well, but under hypoxic conditions, O_2 uptake through the buccal cavity plays a more important role.

In conclusion, the current study demonstrated that larval zebrafish are able to regulate their HVR and that by 7 dpf, this response significantly contributes to overall O_2 uptake under hypoxic conditions. This finding suggests that hyperventilation and branchial respiration are of physiological benefit in larvae as early as 7 dpf and thus contrasts with previous studies suggesting that cutaneous respiration alone provides sufficient O_2 to meet respiratory demands of early stage larvae (Rombough, 2002, 2007). These previous studies, however, examined the partitioning of cutaneous versus branchial O_2 uptake under normoxia. Rombough and Drader (2009) found that hemoglobin enhances O_2 uptake in larval zebrafish only in hypoxia; similarly, it is possible that branchial respiration and hyperventilation play a significant role in zebrafish larvae, but only in hypoxia.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: Y.K.P., M.M., S.F.P.; Methodology: Y.K.P., M.M.; Validation: Y.K.P., M.M., A.M.Z., S.F.P.; Formal analysis: Y.K.P., M.M., A.M.Z.; Investigation: Y.K.P., M.M., A.M.Z.; Writing - original draft: Y.K.P.; Writing - review & editing: Y.K.P., M.M., A.M.Z., S.F.P.; Visualization: Y.K.P., M.M.; Supervision: S.F.P.; Project administration: Y.K.P., M.M.; Funding acquisition: S.F.P.

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