Relationships between the peak hypoxic ventilatory response and critical O_2 tension in larval and adult zebrafish (*Danio rerio*)

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Abstract

Fish increase ventilation during hypoxia, a reflex termed the hypoxic ventilatory response (HVR). The HVR is an effective mechanism to increase O_2 uptake, but at a high metabolic cost. Therefore, when hypoxia becomes severe enough, ventilation declines, as its benefit is diminished. The water oxygen partial pressure (P_wO_2) at which this decline occurs is expected to be near the critical P_wO_2 (P_{crit}), the P_wO_2 at which O_2 consumption begins to decline. Our results indicate that in zebrafish (*Danio rerio*), the relationship between peak HVR and P_{crit} was dependent on developmental stage. Peak ventilation occurred at P_wO_2's higher than P_{crit} in larvae, but at a P_wO_2 significantly lower than P_{crit} in adults. Larval zebrafish use cutaneous respiration to a greater extent than branchial respiration and the cost of sustaining the HVR may outweigh the benefit, whereas adult zebrafish, which rely on branchial respiration, may benefit from using HVR at P_wO_2 below P_{crit}.

Keywords: Hypoxia, Ventilation, Hypoxic ventilatory response, Critical O_2 tension

Introduction

Environmental disturbances, particularly hypoxia, can compromise branchial gas transfer and thus rapid physiological adjustments are initiated to minimize the impact on O_2 uptake ($\dot{M}O_2$; Perry and Wood, 1989). Most teleost species increase ventilation volume through a change in ventilation frequency (f_V) and/or amplitude (reviewed by Perry et al. 2009), referred to as the hypoxic ventilatory response (HVR). The HVR helps to maintain arterial PO_2 in the face of decreasing water PO_2 (P_WO_2; Perry et al., 2009) and typically, the magnitude of the HVR is dependent on the severity of hypoxia (e.g. Sundin et al., 1999; Vulesevic et al, 2006; Pan et al. 2019). The HVR is an important factor delaying an inevitable decrease in $\dot{M}O_2$ as the severity of hypoxia increases, but despite the benefits of the HVR, $\dot{M}O_2$ eventually declines in severe hypoxia at a P_wO_2 termed the critical O_2 tension (P_{crit}). Similarly, in many fish species, ventilation volume increases with the severity of hypoxia to a peak, after which ventilatory effort declines with further decreases in P_wO_2 (Rantin et al., 1992; Cerezo and Garcia Garcia, 2004; Scott et al., 2008; Monteiro et al., 2013). This decline in HVR in severe hypoxia may be a result of diminishing benefits of the HVR (Perry et al. 2009). The metabolic cost of ventilation is high, and even at rest may account for 10% of routine MO_2 (Cameron and Cech, 1970; Jones and Schwarzfeld, 1974; Randall and Daxboeck, 1984) owing to the high density and viscosity of water combined with high ventilation convection requirements (Perry and Wood, 1989; Gilmour, 1997). In severe hypoxia, the increase in ventilation volume incurs a metabolic cost at a time when O_2 is limited, leading to a possible mismatch between a reduced capacity for ATP production and increased metabolic demand of respiratory tissues. Therefore, any benefit of increased O_2 uptake

from the HVR may not be sufficient to sustain the cost of ventilation, resulting in a decline in HVR during hypoxia. However, to date no study has explicitly determined the cause of the decline in HVR and it is possible that other limitations may play a role. Based on a correlation of data gleaned from the literature, it was suggested that peak ventilation occurs near the P_{crit} (Perry et al. 2009), and this observation led Wood (2018) to suggest that fish "abandon" hyperventilation at or near the P_{crit}. However, the relationship has not been tested experimentally by collecting ventilation and P_{crit} data from the same individual.

Moreover, we predict that this relationship will change over development. In zebrafish (*Danio rerio*), cutaneous diffusion is the dominant mechanism of O_2 uptake in larvae until the gills become the primary site of gas transfer at around 15 days post fertilization (dpf; Rombough, 2002; Rombough, 2004). Despite the apparently limited respiratory role of the gills during early developmental stages, larval zebrafish begin to hyperventilate in response to hypoxia as early as 3 dpf (Jonz and Nurse, 2005) and by 7 dpf, preventing hyperventilation impairs O_2 uptake (Pan et al., 2019). Therefore, in zebrafish, both branchial and cutaneous respiration contribute to O_2 uptake during larval stages, with the proportional contribution of each shifting over developmental time. During stages when cutaneous respiration is dominant, maintaining hyperventilation over a wide P_wO_2 range may be less important than in adult fish. Thus, we predict peak ventilation will occur at higher P_wO_2's than P_{crit}. For adult zebrafish, we predict that the P_wO_2 corresponding to peak ventilation during progressive hypoxia will be near P_{crit} but unlike the assertion of Wood (2018), we expect that hyperventilation will continue as P_wO_2 falls below P_{crit}. In addition to characterizing the relationship between P_{crit} and peak

ventilatory effort in adult and larval zebrafish across developmental time, we updated the survey of the literature to include data on peak HVR and P_{crit} of 11 more species not included in the analysis of Perry et al. (2009). Maintaining peak ventilatory effort is metabolically costly, particularly when O_2 is limited, and discerning peak ventilation patterns in relation to $\dot{M}O_2$ during progressive hypoxia may provide an important indicator as to when the metabolic cost of maintaining HVR outweighs the benefit of increased O_2 uptake.

Methods

Data mining for peak ventilation and P_{crit} in fishes exposed to hypoxia

Species for which peak ventilation (typically reported as ventilation volume with the exception of a few studies that measured water flow) during hypoxia and P_{crit} were known were used in a correlation analysis of the P_wO_2 of peak ventilation and P_{crit}. With a few exceptions, peak ventilation and P_{crit} were obtained from different batches of fish within a single study. In four studies, one on sharpsnout sea bream (*Diplodus puntazzo*) (Cerezo and Garcia Garcia, 2004), two on Nile tilapia (*Oreochromis niloticus*), and one on Amazonian Oscar (*Astronotus ocellatus*), peak ventilation and P_{crit} were measured simultaneously in response to progressive hypoxia. For rainbow trout (*Oncorhynchus mykiss*), peak ventilation and P_{crit} were obtained from two separate sources.

Experimental animals

Adult zebrafish, *Danio rerio*, were held in 10 L polycarbonate tanks in a recirculating aquatic system (Aquatic Habitats, Apopka, FL, USA) at the University of Ottawa aquatic care facility. Fish were kept under a 14 h:10 h light:dark cycle in 28°C dechloraminated city of Ottawa tap water and were fed to satiation with GEMMA 300

fish feed (Skretting USA, Westbrook, ME, USA) twice daily. Standard breeding protocols (Westerfield, 2000) were followed to obtain embryos during controlled breeding events. The night before breeding, a male zebrafish was separated by a divider from two female zebrafish in a 2 L breeding tank. The following morning, the water was changed and the divider was removed, allowing the fish to breed. Embryos were collected and reared in 50 mL Petri dishes (40 embryos per dish) containing dechloraminated city of Ottawa tap water and 0.05% methylene blue maintained at 28°C. Water in the Petri dishes was replaced daily. At 5 dpf, the larvae were transferred to static 2 L tanks and water was changed in the tanks every second day. At this stage, the larvae begin to feed exogenously and fish were fed daily to satiation with GEMMA Micro 75 fish feed (Skretting, USA, Westbrook, ME, USA). The larvae were raised to 7, 10 and 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.

O_2 consumption ($\dot{M}O_2$) and breathing frequency (f_V) in adult and larval zebrafish

Simultaneous measurements of $\dot{M}O_2$ and f_V in response to declining P_wO_2 were recorded in adult zebrafish and larvae at 4, 7, 10 and 15 dpf. Adult zebrafish were placed into 15.6 mL glass respirometers fitted with O_2 sensor spots (horizontal mini chamber system; Loligo Systems, Viborg, Denmark) and allowed to recover overnight. The respirometers were flushed continuously with 28°C water from a 20 L recirculating tank gassed with air. At the beginning of the trial, the flush pump to the glass respirometer was turned off while the recirculating pump remained on to provide mixing to ensure stable

P_wO_2 readings. Water PO_2 was monitored continuously in the closed system respirometer using AutoResp (Loligo Systems, Viborg, Denmark). Water PO_2 fell as fish consumed the O_2 in the respirometer and the experiment was terminated when the P_wO_2 levels plateaued. Each individual fish was video recorded for the duration of the $\dot{M}O_2$ trial using an iPhone SE camera and f_V data were extracted from the videos by manual counting as described below. The weight of the fish was determined using an analytical balance.

A larva was placed into an 80 µL respirometry well fitted with an O_2 sensor spot (24-well glass microplate; Loligo Systems, Viborg) and situated on an O_2 sensor reader (SDR SensorDish Reader, PreSens, Regensburg, Germany). Both the microplate and the fluorescence sensor were placed under a dissecting microscope (stereo trinocular microscope, AmScope, Irvine, USA) focused on the well containing the larva. The experiment was conducted in a temperature-controlled room maintained at 28°C. The well was sealed with adhesive tape (AB0580, ThermoFisher Scientific, Mississauga, Canada) at the beginning of the trial and P_wO_2 levels were monitored using MicroResp (Loligo Systems, Viborg, Denmark) until the experiment was terminated upon the plateauing of PO_2 levels. For the duration of the trial, the fish was video recorded using an iPhone SE camera mounted on the dissecting microscope. The weights of 4, 7, 10 and 15 dpf zebrafish larvae were determined on a separate batch of fish using the protocol of Pan et al. (2019).

The $\dot{M}O_2$ was calculated over sequential 3 min intervals using the slope of the relationship of P_wO_2 *versus* time, standardized for fish weight and respirometer volume. Water O_2 concentrations were calculated using the solubility coefficient of O_2 in freshwater at 28°C (Boutilier et al., 1984). The $\dot{M}O_2$ was plotted as a function of P_wO_2

and an inflection point representing P_{crit} was calculated for each fish using the broken-stick (or segmented) regression approach (Yeager and Ultsch, 1989) and REGRESS software (www.wfu.edu/~mudayja/sofware/o2.exe).

In both adults and larvae, f_V was quantified by counting either buccal or opercular movements depending on the orientation of the fish in the chamber or well and the visibility of the mouth and/or operculum. We focused on f_V as an index of ventilation volume because adult zebrafish increase f_V and not breathing amplitude during hypoxia (Vulesevic et al., 2006), and there is no established method to measure amplitude in larval zebrafish. Average f_V was determined for the first minute of each 3 min bin used to calculate $\dot{M}O_2$. The f_V was plotted against water PO_2 and an inflection point, termed the 'f_V inflection point', was determined using the broken-stick (or segmented) regression approach, the same technique used to calculate P_{crit}. Although it is straightforward to determine the P_wO_2 of peak ventilation, this value may not be fully representative of the response because often there is a range of P_wO_2 values over which ventilation plateaus near maximum values. We found that the variance around the mean P_{crit} for larvae and adults was on average approximately 17%, and we chose this value to represent the range of ventilation near peak value, which we termed the 'zone of maximal ventilation'.

Confocal imaging of gills in larval zebrafish

Tg(fli1:eGFP) larvae that express enhanced green fluorescence protein (GFP) in the vasculature under the control of the fli1 promoter were raised to 4, 7, 10 and 15 dpf and fixed overnight by immersion in 4% paraformaldehyde in phosphate-buffered saline at 4°C. Larvae were mounted in 1% low melt agarose (BioShop, Burlington, ON, Canada) on a depression slide (VWR, Mississauga, ON, Canada) and images were

acquired using a Nikon A1R MP confocal microscope with Apo x25/1.10 NA water objective and NIS-elements software (Nikon Instruments Inc., Melville, NY, USA).

Statistical analysis

Statistical analyses were performed in R (https://www.R-project. org/). The linear association between P_{crit} and P_wO_2 at peak ventilation was determined using Pearson's product moment correlation coefficient. Whether P_{crit} was significantly different from the f_V inflection at each larval stage and in adult zebrafish was tested using a two-tailed Student's *t*-test. An ANOVA in the car package (Fox and Weisberg, 2011) was used to determine whether the difference between P_{crit} and f_V inflection varied with developmental stage, and Tukey's post hoc test was performed on (P_{crit}-f_V inflection). All data were tested for normality using the Shapiro-Wilk test and equal variance using Bartlett's test. Data that failed normality or equal variance were log transformed. Significance was set at $P<0.05$.

Results and Discussion

The goal of this study was to characterize the relationship between P_{crit} and peak HVR in larval and adult zebrafish. During hypoxia, ventilation increases as the severity of hypoxia increases and subsequently falls when P_wO_2 drops to a severely low tension that is species-specific (Rantin et al., 1992; Cerezo and Garcia Garcia, 2004; Scott et al., 2008; Monteiro et al., 2013). Perry et al. (2009) proposed that the decline in ventilation occurs around the P_{crit}, the P_wO_2 at which aerobic metabolism is compromised and $\dot{M}O_2$ begins to decrease.

Focusing on 21 fish species for which P_{crit} and ventilation volume during hypoxia are known (for adult fish), a significant positive correlation was found between P_{crit} and the P_wO_2 at peak ventilation ($r = 0.82$, $p < 0.01$; Fig. 1), indicating that in species with a lower P_{crit}, peak ventilation also occurred at a lower P_wO_2. A similar survey of the literature on fewer species also obtained a significant correlation between peak HVR and P_{crit} (Perry et al. 2009). A correlation between peak HVR and P_{crit} is not surprising given that ventilatory effort is metabolically costly and the effective contribution of ventilation during hypoxia is diminished at P_{crit} as evidenced by a fall in $\dot{M}O_2$ (Perry et al. 2009). However, when a line of identity was plotted, most species fell below the line (Fig. 1), indicating that peak ventilation was achieved at a P_wO_2 lower than P_{crit}, and in some, like the pacu, (*Piaractus mesopotamicus*), the peak ventilation occurred at a P_wO_2 far below P_{crit} (approximately 20 mmHg lower; Rantin et al., 1998). Thus, despite the apparent significant metabolic cost, in some species, the HVR appears to be maintained even when P_wO_2 falls below P_{crit}. Thus, the conclusion of Wood (2019) that fish often "abandon hyperventilation" at P_{crit} does not appear to be supported by existing data presented in Fig. 1.

In adult zebrafish, maximal ventilatory effort occurred at a P_wO_2 that was significantly lower than P_{crit} (Fig. 2; Fig. 3A), similar to patterns observed in species such as the spangled perch (*Leiopotherapon unicolor*; Gehrke and Fielder, 1988), the pacu (Rantin et al., 1998) and the jeju *Hoplerythrinus unitaeniatus*; Oliveira et al., 2004) (see Fig. 1). Peak ventilatory effort, quantified either as f_V inflection point or zone of maximal ventilation, occurred around 10 mmHg, well below P_{crit} (19.9 ± 0.8 mmHg) (Fig. 2; Fig. 3A). Adult zebrafish are known to have high hemoglobin O_2 affinity (P_{50} = 4.4 mmHg;

Cadiz et al., 2019), indicating that at the P_wO_2 of maximal HVR, hemoglobin O_2 saturation may have remained near 100%. It is possible that continued hyperventilation at P_wO_2 values below P_{crit} helps to maintain arterial PO_2, bolstering $\dot{M}O_2$.

In young (<7 dpf) larvae, peak ventilation occurred at a P_wO_2 higher than P_{crit} but as larvae aged, ventilation peaked at P_wO_2's closer to P_{crit} (Fig. 2; Fig. 3). In 4 dpf larvae, the f_V inflection point and zone of maximal ventilation were significantly above P_{crit} (Fig. 2; Fig. 3A), indicating that HVR was decreasing even as $\dot{M}O_2$ remained constant. At 4 dpf, zebrafish primarily rely on cutaneous respiration (Rombough, 2002; Rombough 2004) and blood vessels are just beginning to form in the pharyngeal arches region as can be observed in the image collected using the Tg(fli1:eGFP) line (Fig. 2; Fig. S1). Thus the HVR is not necessary to maintain O_2 uptake at this stage (Jonz and Nurse, 2005; Pan et al., 2019), and a decrease in maximal ventilation at P_wO_2 well above P_{crit} may be effective in conserving limited metabolic energy. There was a left shift in both the f_V inflection point and zone of maximal ventilation in 7 and 10 dpf larvae, moving them closer to the P_{crit} (Fig. 2; Fig. 3A). By 7 dpf, respiratory lamellae begin to form (Jonz and Nurse, 2005), which is apparent in the images collected in the current study as increased vascularization in the buccal cavity (Fig. 2; Fig. S1). Moreover, at 7 dpf (unlike at 4 dpf), preventing hypoxic hyperventilation in zebrafish impedes O_2 uptake (Pan et al., 2019). In older larvae, the HVR, coupled with cutaneous respiration, becomes an important mechanism to maintain $\dot{M}O_2$, and a shift of maximal ventilatory effort closer to that of P_{crit} would be beneficial to O_2 uptake.

The f_V inflection point and zone of maximal ventilation for 15 dpf occurred at a P_wO_2 above that of P_{crit} (Fig. 2; 3A) and there was no statistical difference between 10 and 15 dpf larvae in Δ between P_{crit} and f_V inflection (Fig. 3B). Branchial respiration is thought to dominate in developing zebrafish beginning around 15 dpf (Rombough, 2002). Accordingly, we had expected that the relationship between peak HVR and P_{crit} at 15 dpf would be similar to that of adult fish, but in contrast, it was more similar to that of younger larvae. In steelhead trout (*Oncorhynchus mykiss*), P_{crit} decreases as larvae develop, suggesting an increase in the capacity for O_2 uptake at lower P_wO_2 as development progresses (Rombough, 1988a). In zebrafish larvae, however, P_{crit} was constant across development to 15 dpf at 32 - 34 mmHg, whereas in adult fish, P_{crit} was markedly lower (20 mmHg). Despite the greater reliance on branchial respiration, the full capacity of the adult gill has not yet developed in 15 dpf larvae, likely limiting the capacity to improve O_2 uptake in hypoxia. Regulation of functional gill surface area, ventilation and perfusion is thought to be critical in promoting gas transfer and hypoxia tolerance (Rombough 1988b). It is possible that these factors cannot be maximized during hypoxia to the same degree in a larval gill as in the adult gill. Aside from changes in convection (e.g. as result of HVR), larval gills show little plasticity compared to adult gills (Sackville and Brauner, 2018), supporting the idea that there may be greater constraints on branchial gas transfer during hypoxia in larvae than in adults. Therefore, it is possible that at 15 dpf, the cost of HVR far exceeds the benefit and the HVR begins to decline at a higher P_wO_2 than P_{crit}.

Conclusions

By simultaneously measuring $\dot{M}O_2$ and f_V during progressive hypoxia, we evaluated the relationship between peak ventilation frequency and P_{crit} in developing larvae and adult zebrafish. Peak ventilation occurred at a P_wO_2 significantly higher than P_{crit} in 4 dpf larvae, but as larvae developed, the zone of peak ventilation shifted to lower P_wO_2's, closer to P_{crit}. By adulthood, peak ventilation occurred well below P_{crit}. The mechanisms that determine the P_wO_2 of maximal HVR are unknown. However, the pattern of changes in the P_wO_2 of peak HVR and P_{crit} across life history allows us to speculate that a driving factor may be the relationship between the metabolic cost of the HVR versus its benefit. It is likely that in early stage larvae, the metabolic cost of the HVR significantly outweighs its benefit, while the opposite is true in adult fish. However, it is important to consider that the decrease in HVR may not be a result of a shift in balance between metabolic benefit and cost, but rather a result of a different limitation. It is possible that the constraining effects of viscosity on larval fish owing to their small size may produce high demands on ventilatory effort during hypoxia, leading to fatigue of the respiratory muscles. Further research is warranted to determine the underlying cause of the decline in HVR in larval and adult fish.

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Author contributions

M.M., Y.K.P. and S.F.P. designed the experiment; M.M. and Y.K.P. carried out the experiment; M.M. and Y.K.P. carried out the statistical analyses, M.M., Y.K.P., K.M.G. and S.F.P. provided input in data interpretation, M.M. wrote the original draft of the manuscript and all authors provided input and approved the manuscript.

Competing interests

We have no competing interests.

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Data availability

Images of Tg(fli1:eGFP) zebrafish larvae at 4, 7, 10, and 15 dpf can be found in Supplemantary Materials, Figure S1.

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Figures

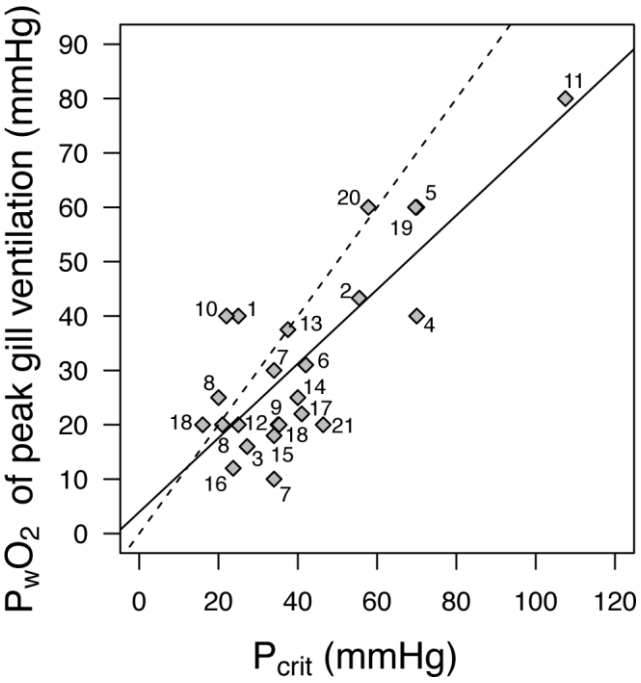

Figure 1. The relationship between critical O_2 tension (P_{crit}) and the P_wO_2 at which fish reach peak ventilation frequency (f_V) during exposure to acute hypoxia. There is a significant correlation ($r = 0.81$, $p < 0.01$) between P_{crit} and P_wO_2 at peak ventilation. The dashed line is the line of identity and the solid line is the line of best fit. 1, turbot *Scophthalmus maximus* (Maxime et al., 2000); 2, Atlantic cod *Gadus morhua* (Mckenzie et al., 2009); 3, spangled perch *Leiopotherapon unicolor* (Gehrke and Fielder, 1988); 4, European eel *Anguilla Anguilla* (Le Moigne et al., 1986); 5, flounder *Platichthys flesus* (Steffensen et al., 1982); 6, flounder *Paralichthys dentatus* (Capossela et al., 2012); 7, pacu, *Piaractus mesopotamicus*; (Rantin et al., 1998) (Leite et al., 2007); 8, traira *Hoplias malabaricus* (Sundin et al., 1999; Monteiro et al., 2013); 9, Triarão *Hoplias lacerdae* (Rantin et al., 1992); 10, rainbow trout *Oncorhynchus mykiss* (Ott et al., 1980; Perry and Gilmour, 1996); 11, Japanese eel *Anguilla japonica* (Chan, 1986); 12, matrinxã *Brycon*

amazonicus (Monteiro et al., 2013); 13, piracatinga (catfish) *Calophysus macropterus* (Scott et al., 2017); 14, sharpsnout sea bream *Diplodus puntazzo* (Cerezo and Garcia Garcia, 2004); 15, jeju *Hoplerythrinus unitaeniatus* (Oliveira et al., 2004); 16, Mayan cichlid *Mayaheros uropthalmus* (Burggren et al., Accepted); 17, Atlantic killifish *Fundulus heteroclitus* (Giacomin et al., 2019); 18, Nile tilapia *Oreochromic niloticus* (Thomaz et al. 2009) (Martins et al., 2011); 19, bowfin *Amia calva* (Porteus et al., 2014); 20, striped catfish *Pangasianodon hypophthalmus* (Lefevre et al., 2011); 21, Amazonian Oscar *Astronotus ocellatus* (Scott et al., 2008).

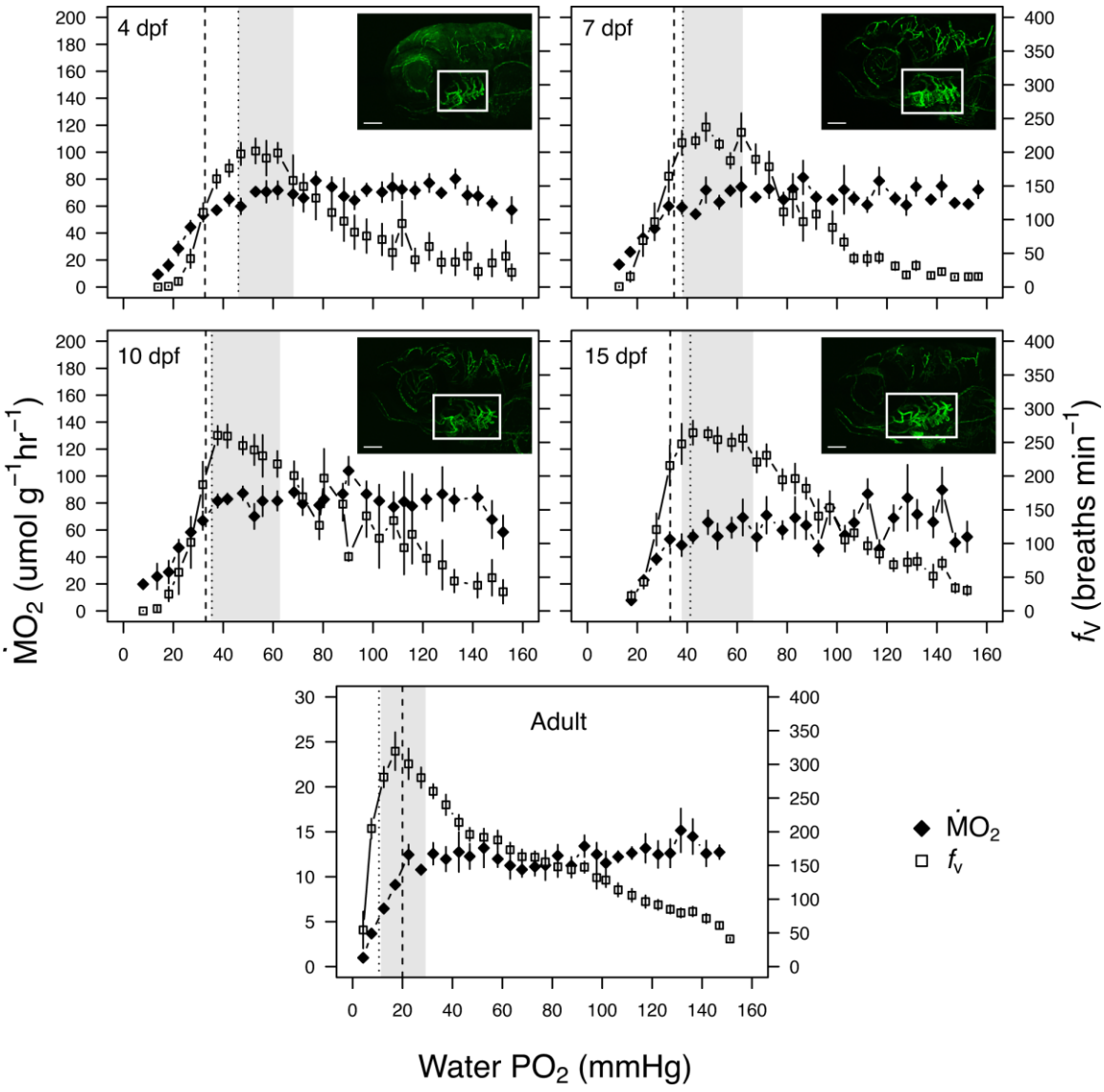

Figure 2. O_2 consumption ($\dot{M}O_2$) and breathing frequency (f_V) in 4, 7, 10 and 15 day post fertilization (dpf) larvae and adult zebrafish (*Danio rerio*) exposed to a progressive decrease in P_wO_2. Critical O_2 tension (P_{crit}; dashed line), breathing frequency inflection (dotted line) and zone of maximal ventilation (grey band) were calculated at each larval stage (n=9 for 4, 7 and 10 dpf and n=11 for 15 dpf) and in adult zebrafish (n=7). Inserts are images of Tg(fli1:eGFP) larvae at 4, 7, 10 and 15 dpf focusing on the head region to show the vasculature of the pharyngeal arches/gill regions (see Fig. S1 for greater detail). Data are presented as means ± SEM.

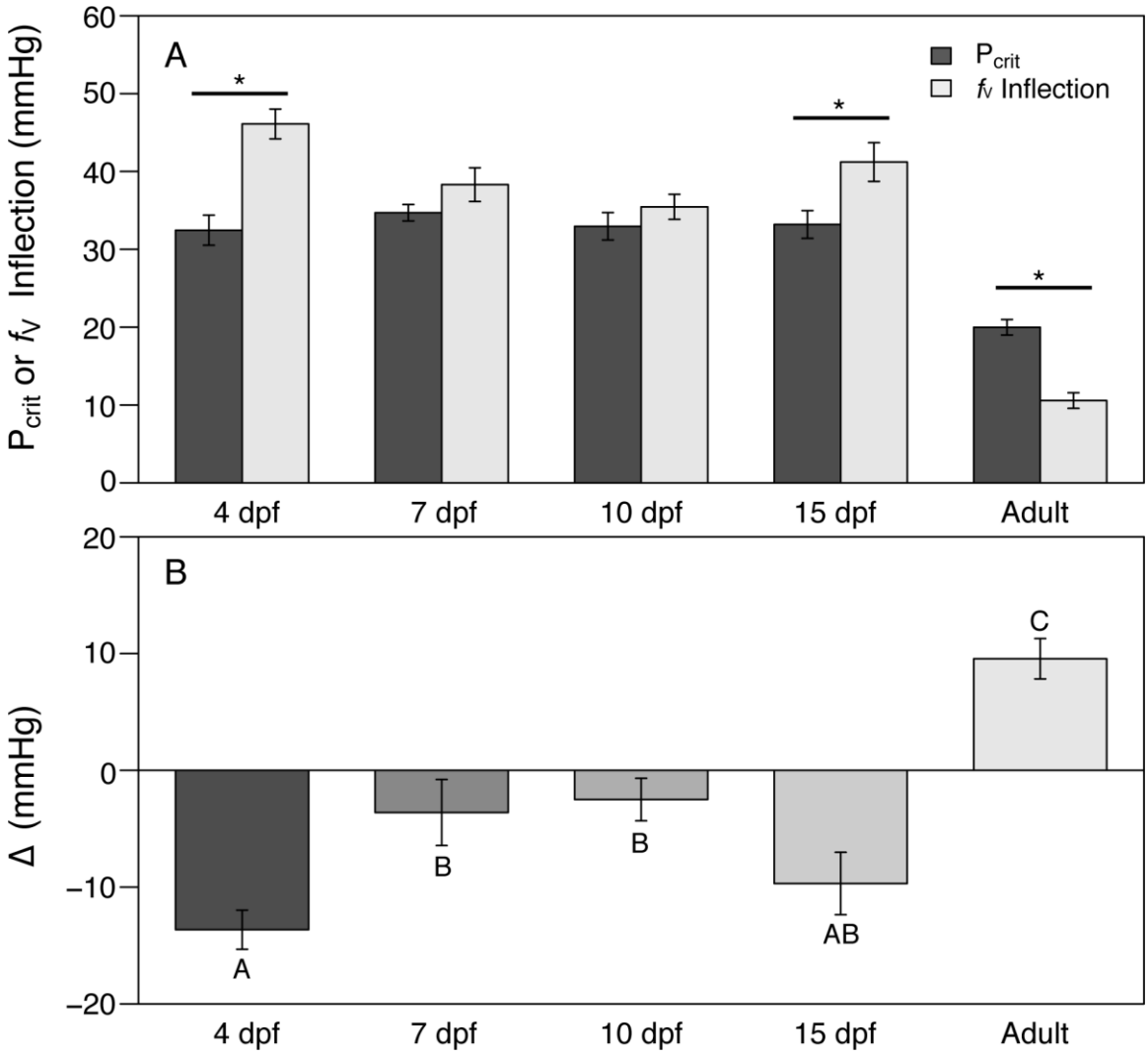

Figure 3. Critical O_2 tension (P_{crit}) and breathing frequency inflection (f_V inflection) (A) and Δ (P_{crit} minus f_V inflection) (B) in 4, 7, 10 and 15 day post fertilization (dpf) larvae and adult zebrafish. There was a significant difference between P_{crit} and f_V inflection in 4 dpf larvae ($t = -8.14$; $P < 0.01$), 15 dpf larvae ($t = -3.752$, $P < 0.01$) and in adults ($t = 5.53$; $P < 0.01$). There was a significant effect of life history stage on Δ ($F=12.80$, $P < 0.01$). Asterisks represent significant differences between P_{crit} and f_V inflection, and Δ values with different letters are significantly different ($P < 0.05$). Data are presented as means ± SEM.

Figure S1. Images of Tg(fli1:eGFP) zebrafish larvae (A) focusing on the head region. Vasculature of the pharyngeal arches/gill regions (arrowheads) can be observed in 4, 7, 10, and 15 dpf larvae (B-E), with vasculature being more complex in 7, 10, and 15 dpf larvae compared to that of the 4 dpf larva. Scale bar represents 100 µm.