

## SHORT COMMUNICATION

# Relationships between the peak hypoxic ventilatory response and critical O<sub>2</sub> 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<sub>2</sub> 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 ( $P_{W_{O_2}}$ ) at which this decline occurs is expected to be near the critical  $P_{W_{O_2}}$  ( $P_{crit}$ ), the  $P_{W_{O_2}}$  at which O<sub>2</sub> consumption begins to decline. Our results indicate that in zebrafish (*Danio rerio*), the relationship between peak HVR and  $P_{crit}$  is dependent on developmental stage. Peak ventilation occurred at  $P_{W_{O_2}}$  values higher than  $P_{crit}$  in larvae, but at a  $P_{W_{O_2}}$  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  $P_{W_{O_2}}$  below  $P_{crit}$ .

**KEY WORDS:** Hypoxia, Ventilation, Hypoxic ventilatory response, Critical O<sub>2</sub> tension

## INTRODUCTION

Environmental disturbances, particularly hypoxia, can compromise branchial gas transfer and thus rapid physiological adjustments are initiated to minimize the impact on O<sub>2</sub> 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  $P_{O_2}$  in the face of decreasing water  $P_{O_2}$  ( $P_{W_{O_2}}$ ; 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  $P_{W_{O_2}}$  termed the critical O<sub>2</sub> 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  $P_{W_{O_2}}$  (Rantin et al., 1992; Cerezo and Garcia Garcia, 2004; Scott et al., 2008; Monteiro et al., 2013). This decline in the 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  $\dot{M}_{O_2}$  (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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> uptake (Pan et al., 2019). Therefore, in zebrafish, both branchial and cutaneous respiration contribute to O<sub>2</sub> 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  $P_{W_{O_2}}$  range may be less important than in adult fish. Thus, we predict peak ventilation will occur at higher  $P_{W_{O_2}}$  than  $P_{crit}$ . For adult zebrafish, we predict that the  $P_{W_{O_2}}$  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  $P_{W_{O_2}}$  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<sub>2</sub> 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<sub>2</sub> uptake.

## MATERIALS AND 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

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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 sharpnose 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* (F. Hamilton 1822), 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). 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.

### $\dot{M}_{O_2}$ and $f_V$ in adult and larval zebrafish

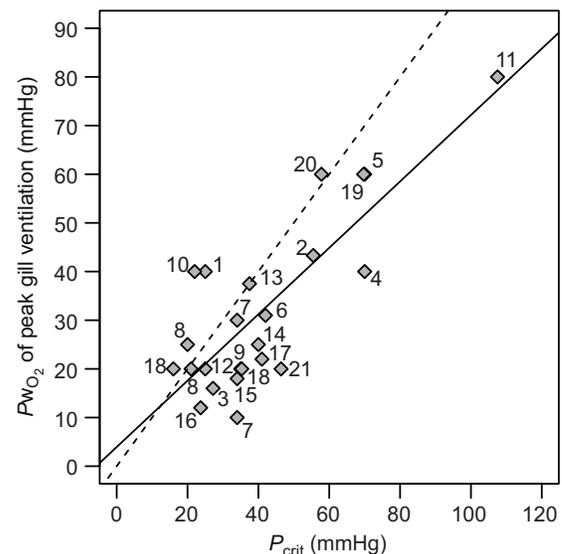
Simultaneous measurements of  $\dot{M}_{O_2}$  and  $f_V$  in response to declining  $P_{wO_2}$  were recorded in adult zebrafish and larvae at 4, 7, 10 and 15 dpf. Adult zebrafish were placed into 15.6 ml glass respirometers fitted with  $O_2$  sensor spots (horizontal mini chamber system; Loligo Systems, 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.  $P_{wO_2}$  was monitored continuously in the closed system respirometer using AutoResp (Loligo Systems).  $P_{wO_2}$  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 mass of each fish was determined using an analytical balance.

A larva was placed into an 80  $\mu$ l respirometry well, fitted with an  $O_2$  sensor spot (24-well glass microplate; Loligo Systems) 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, CA, 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, ON, Canada) at the beginning of the trial and  $P_{wO_2}$  levels were monitored using MicroResp (Loligo Systems) until the experiment was terminated upon the plateauing of  $P_{wO_2}$  levels. For the duration of the trial, the fish was video recorded using an iPhone SE camera mounted on the dissecting microscope. The mass of 4, 7, 10 and 15 dpf zebrafish larvae was 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 mass and respirometer volume. Water  $O_2$  concentration was 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/software/o2.exe](http://www.wfu.edu/~mudayja/software/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



**Fig. 1. Relationship between critical  $O_2$  tension ( $P_{crit}$ ) and the water  $P_{O_2}$  ( $P_{wO_2}$ ) at which fish reach peak ventilation frequency ( $f_V$ ) during exposure to acute hypoxia.** There was 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, traíra, *Hoplias malabaricus* (Sundin et al., 1999; Monteiro et al., 2013); 9, Trirão, *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), *Calophyus macropterus* (Scott et al., 2017); 14, sharpnose sea bream, *Diplodus puntazzo* (Cerezo and Garcia Garcia, 2004); 15, jeju, *Hoplerthrinus unitaeniatus* (Oliveira et al., 2004); 16, Mayan cichlid, *Mayaheros urophthalmus* (Burggren et al., 2019); 17, Atlantic killifish, *Fundulus heteroclitus* (Giacomin et al., 2019); 18, Nile tilapia, *Oreochromis 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).

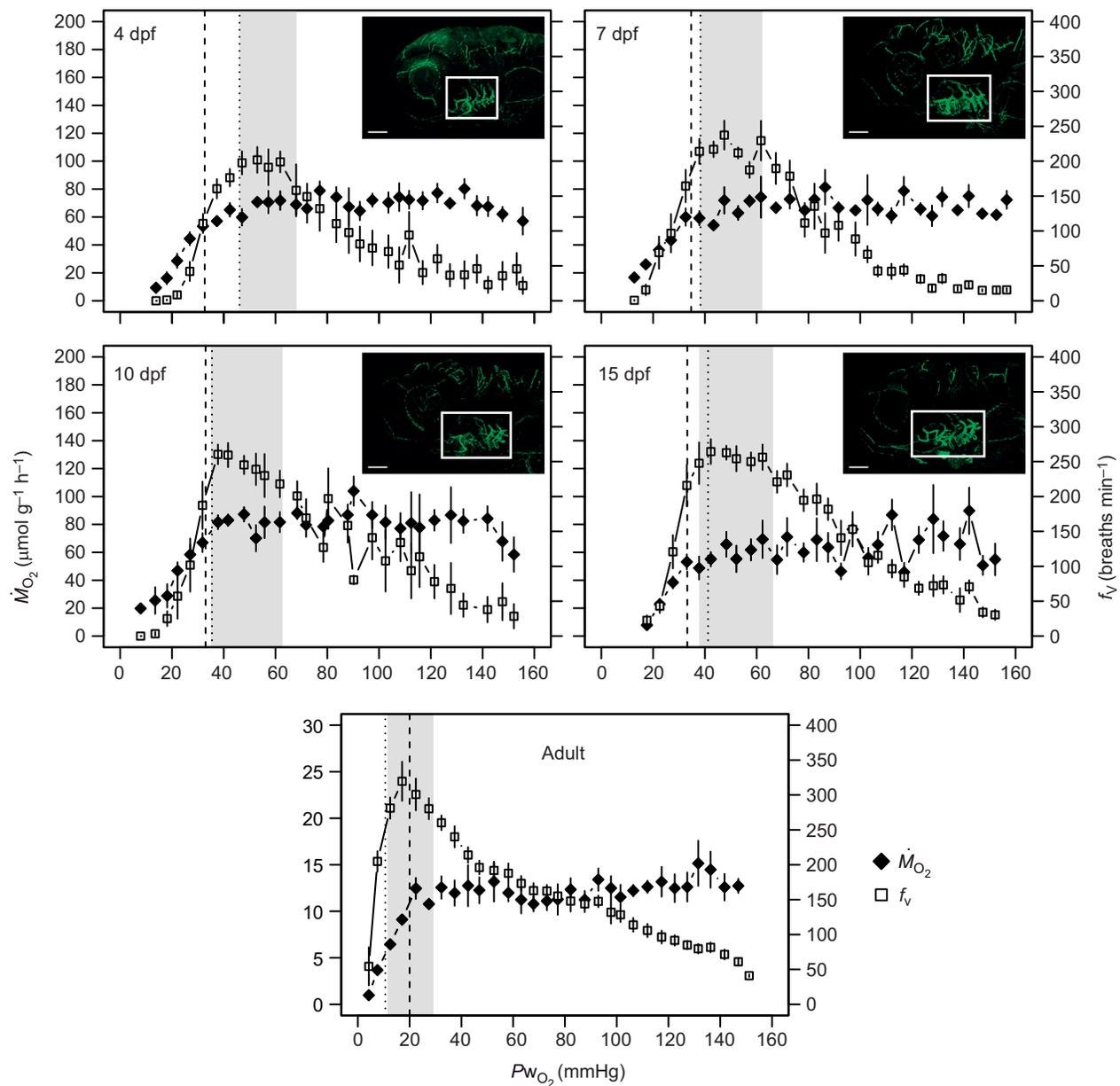
because adult zebrafish increase  $f_V$ , not breathing amplitude, during hypoxia (Vulesevic et al., 2006), and there is no established method to measure amplitude in larval zebrafish. Average  $f_V$  was determined for the first minute of each 3 min bin used to calculate  $\dot{M}_{O_2}$ . The  $f_V$  was plotted against  $Pw_{O_2}$  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  $Pw_{O_2}$  of peak ventilation, this value may not be fully representative of the response because often there is a range of  $Pw_{O_2}$  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 fluorescent 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  $\times 25/1.10$  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  $Pw_{O_2}$  at peak ventilation was determined using Pearson's product moment



**Fig. 2.**  $O_2$  consumption ( $\dot{M}_{O_2}$ ) and  $f_V$  in 4, 7, 10 and 15 days post-fertilization (dpf) larvae and adult zebrafish (*Danio rerio*) exposed to a progressive decrease in  $Pw_{O_2}$ .  $P_{crit}$  (dashed line),  $f_V$  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 ( $n=7$ ). Insets 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 means  $\pm$  s.e.m.

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 ( $\Delta$ ). 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  $P<0.05$ .

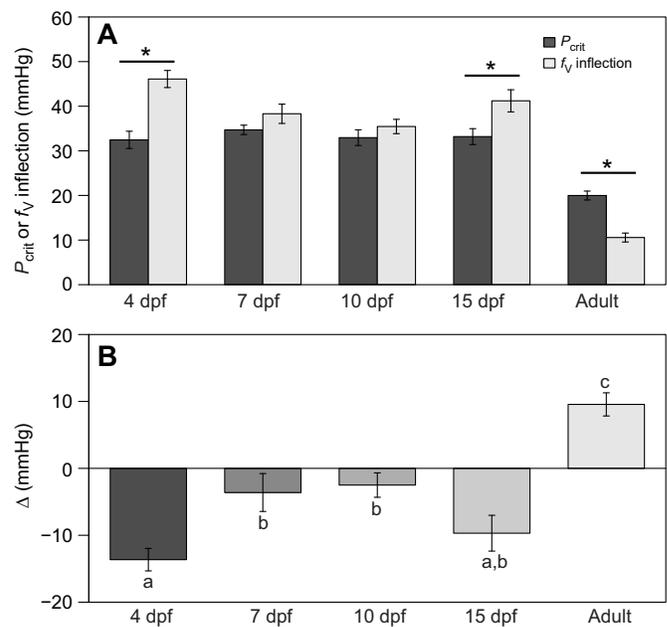
## RESULTS AND DISCUSSION

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  $P_{W_{O_2}}$  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  $P_{crit}$ , the  $P_{W_{O_2}}$  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  $P_{W_{O_2}}$  at peak ventilation ( $r=0.81$ ,  $P<0.01$ ; Fig. 1), indicating that in species with a lower  $P_{crit}$ , peak ventilation also occurred at a lower  $P_{W_{O_2}}$ . 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  $P_{W_{O_2}}$  lower than  $P_{crit}$ , and in some species, like the pacu (*Piaractus mesopotamicus*), peak ventilation occurred at a  $P_{W_{O_2}}$  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  $P_{W_{O_2}}$  falls below  $P_{crit}$ . Thus, the conclusion of Wood (2018) 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  $P_{W_{O_2}}$  that was significantly lower than  $P_{crit}$  (Fig. 2 and 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  $P_{crit}$  ( $19.9\pm 0.8$  mmHg) (Fig. 2 and Fig. 3A). Adult zebrafish are known to have high haemoglobin  $O_2$  affinity ( $P_{50}=4.4$  mmHg; Cadiz et al., 2019), indicating that at the  $P_{W_{O_2}}$  of maximal HVR, haemoglobin  $O_2$  saturation may have remained near 100%. It is possible that continued hyperventilation at  $P_{W_{O_2}}$  values below  $P_{crit}$  helps to maintain arterial  $P_{O_2}$ , bolstering  $\dot{M}_{O_2}$ .

In young (<7 dpf) larvae, peak ventilation occurred at a  $P_{W_{O_2}}$  higher than  $P_{crit}$  but as larvae aged, ventilation peaked at  $P_{W_{O_2}}$  closer to  $P_{crit}$  (Fig. 2 and Fig. 3). In 4 dpf larvae, the  $f_V$  inflection point and zone of maximal ventilation were significantly above  $P_{crit}$  (Figs 2 and 3A), indicating that the 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



**Fig. 3.**  $P_{crit}$  and  $f_V$  inflection, and  $P_{crit}$  minus  $f_V$  inflection ( $\Delta$ ) in 4, 7, 10 and 15 dpf larvae and adult zebrafish. (A) 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 adults ( $t=5.53$ ;  $P<0.01$ ). (B) There was a significant effect of life history stage on  $\Delta$  ( $F=12.80$ ,  $P<0.01$ ). Asterisks represent significant differences between  $P_{crit}$  and  $f_V$  inflection, and  $\Delta$  values with different letters are significantly different ( $P<0.05$ ). Data are means $\pm$ s.e.m.

region, as can be observed in the image collected using the Tg (*flil*: 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  $P_{W_{O_2}}$  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 and 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  $P_{W_{O_2}}$  above that of  $P_{crit}$  (Fig. 2 and Fig. 3A) and there was no statistical difference between 10 and 15 dpf larvae in  $P_{crit}-f_V$  inflection ( $\Delta$ ) (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  $P_{W_{O_2}}$  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 with 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  $P_{W_{O_2}}$  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  $P_{W_{O_2}}$  significantly higher than  $P_{crit}$  in 4 dpf larvae, but as larvae developed, the zone of peak ventilation shifted to lower  $P_{W_{O_2}}$  values, closer to  $P_{crit}$ . By adulthood, peak ventilation occurred well below  $P_{crit}$ . The mechanisms that determine the  $P_{W_{O_2}}$  of maximal HVR are unknown. However, the pattern of changes in the  $P_{W_{O_2}}$  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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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: M.M., Y.K.P., S.F.P.; Methodology: M.M., Y.K.P., S.F.P.; Validation: M.M., Y.K.P., K.M.G., S.F.P.; Formal analysis: M.M., Y.K.P., K.M.G., S.F.P.; Investigation: M.M., Y.K.P.; Resources: S.F.P.; Writing - original draft: M.M.; Writing - review & editing: M.M., Y.K.P., K.M.G., S.F.P.; Visualization: M.M., Y.K.P.; Supervision: S.F.P.; Funding acquisition: S.F.P.

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## Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.213942.supplemental>

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