© 2014. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2014) 217, 3988-3995 doi:10.1242/jeb.110601

RESEARCH ARTICLE

The amphibious fish *Kryptolebias marmoratus* uses different strategies to maintain oxygen delivery during aquatic hypoxia and air exposure

Andy J. Turko*, Cayleih E. Robertson, Kristin Bianchini, Megan Freeman and Patricia A. Wright

ABSTRACT

Despite the abundance of oxygen in atmospheric air relative to water, the initial loss of respiratory surface area and accumulation of carbon dioxide in the blood of amphibious fishes during emersion may result in hypoxemia. Given that the ability to respond to low oxygen conditions predates the vertebrate invasion of land, we hypothesized that amphibious fishes maintain O_2 uptake and transport while emersed by mounting a co-opted hypoxia response. We acclimated the amphibious fish *Kryptolebias marmoratus*, which are able to remain active for weeks in both air and water, for 7 days to normoxic brackish water (15‰, ~21 kPa O_2; control), aquatic hypoxia (~3.6 kPa), normoxic air (~21 kPa) or aerial hypoxia (~13.6 kPa). Angiogenesis in the skin and bucco-opercular chamber was pronounced in air- versus water-acclimated fish, but not in response to hypoxia. Aquatic hypoxia increased the O_2-carrying capacity of blood via a large (40%) increase in red blood cell density and a small increase in the affinity of hemoglobin for O_2 (P_{50} decreased 11%). In contrast, air exposure increased the hemoglobin O_2 affinity (decreased P_{50}) by 25% without affecting the number of red blood cells. Acclimation to aerial hypoxia both increased the O_2-carrying capacity and decreased the hemoglobin O_2 affinity. These results suggest that O_2 transport is regulated both by O_2 availability and also, independently, by air exposure. The ability of the hematological system to respond to air exposure independent of O_2 availability may allow extant amphibious fishes, and may also have allowed primitive tetrapods to cope with the complex challenges of aerial respiration during the invasion of land.

KEY WORDS: Hemoglobin, Oxygen-carrying capacity, Hemoglobin–oxygen affinity, Air-breathing organ, Air-breathing fish, Mangrove rivulus

INTRODUCTION

The transition from aquatic to terrestrial life represents a major step in vertebrate evolution because the physical conditions between these environments are dramatically different. Oxygen solubility is relatively low in water and even well-oxygenated aquatic environments contain only about 3% of the O_2 available in atmospheric air (Dejours, 1988). Low concentrations of aquatic O_2, particularly in hypoxic habitats, have often been hypothesized to be one of the driving forces behind the evolution of amphibious or terrestrial life histories because invasion of land would allow animals to exploit the O_2-rich aerial environment (Graham, 1997).

Department of Integrative Biology, University of Guelph, Guelph, ON N1G 2W1, Canada.

*Author for correspondence (aturko@uoguelph.ca)

Received 4 July 2014; Accepted 15 September 2014

Taking advantage of aerial O_2 presents several challenges for fishes. Water-breathing fishes exchange respiratory gases across the gills but during emersion the gill lamellae typically collapse and coalesce, reducing the surface area available for respiration. Accumulation of CO_2 in the blood of emersed fishes, resulting from the low solubility of CO_2 in air versus water, can also impair the ability of hemoglobin (Hb) to bind and transport O_2 (Rahn, 1966; Ultsch, 1987). High blood partial pressure of CO_2 (P_{CO_2}) may reduce intraerythrocytic pH and reduce the affinity of Hb for O_2 by the Bohr effect, which prevents O_2 loading at the gas-exchange surface (Bohr et al., 1904). Impaired O_2 transport can also occur via the Root effect: a phenomenon found only in fishes, which prevents Hb from becoming fully O_2 saturated at high P_{CO_2} (Root and Irving, 1941; Gillen and Riggs, 1971). It has been hypothesized that well-adapted air-breathing and amphibious fishes should possess relatively pH-insensitive Hb to reduce the consequences of Bohr and Root effects on O_2 transport (Graham, 1997; Shartau and Brauner, 2014); however, not all air-breathing fishes follow this pattern (e.g. Farmer et al., 1979; Wells et al., 1997). Thus, despite the fact that amphibious fishes are surrounded by O_2-rich air during emersion, high blood P_{CO_2} coupled with a reduced respiratory surface area may cause amphibious fishes to become hypoxemic until physiological and morphological adjustments to the O_2 transport system can be made.

Fishes can increase their capacity for O_2 uptake, binding and transport in response to hypoxia or hypoxemia. For example, lamellar perfusion can be increased during exposure to aquatic hypoxia to increase the surface area available for O_2 uptake (Booth, 1979; Soivio and Tuurala, 1981). Fish adapted or acclimated to hypoxia often have blood with a high O_2-carrying capacity and possess Hb with a relatively high O_2-binding affinity (Weber and Fago, 2004; Brauner and Val, 2006; Wells, 2009). Air-breathing and amphibious fishes similarly tend to possess blood with a high O_2-carrying capacity, which has been hypothesized to compensate for the impaired O_2 uptake and transport caused by elevated blood P_{CO_2} during emersion (Farmer, 1979; Farmer et al., 1979; Graham, 1997).

During the evolution of amphibious lifestyles by early tetrapods or extant amphibious fishes, the hypoxia response could have been used to maintain O_2 transport in the face of high P_{CO_2} during emersion. The ability to sense and respond to hypoxia likely predates the invasion of land by animals, because even the basal *Caenorhabditis elegans* is known to accumulate the transcription factor hypoxia inducible factor-1 during hypoxic exposure (Rytkönen et al., 2011). After 24 h of emersion the amphibious fish *Gillichthys mirabilis* alters gene expression in a similar pattern to fish that experienced aquatic hypoxia, suggesting that similar strategies may be used to maintain metabolism (Gracey, 2008). However, it is unknown whether these transcriptional changes result in altered protein synthesis or have functional consequences. The

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110601

response of O_2-transport systems in amphibious fishes that spend longer periods (days to weeks) out of water is similarly not well understood.

Given the reduced capacity for transport of blood O_2 as a result of high P_{CO_2} and the loss of respiratory surface area from the gills during emersion, we hypothesized that amphibious fishes maintain O_2 uptake and transport while out of water by co-opting their hypoxia response. This hypothesis predicts that amphibious fishes acclimated to either aquatic hypoxia or a terrestrial environment would use a conserved suite of phenotypic changes to increase the O_2-carrying capacity, the affinity of Hb for O_2 and the flow of blood to extrabranchial sites of gas exchange. Alternatively, amphibious fishes may use different responses to tolerate aquatic hypoxia and air exposure. The mangrove rivulus *Kryptolebias marmoratus* (Poey 1880) is an ideal model for studying the adaptations used by amphibious fishes to maintain O_2 uptake and transport. In the wild, mangrove rivulus typically inhabit extremely hypoxic (P_{O_2}<2.5 kPa) puddles and crab burrows filled with water, but these fish also frequently emerse and find refuge in terrestrial habitats such as damp leaf litter or inside rotting logs that may also be hypoxic (Taylor et al., 2008, Ellison et al., 2012; Taylor, 2012). Emersions can last upwards of 2 months (Taylor, 1990), during which time mangrove rivulus remain active and are thought to obtain O_2 by cutaneous respiration (Grizzle and Thiyagarajah, 1987; Cooper et al., 2012; Wright, 2012). To test the hypothesis that air-exposed fish mount a hypoxia response, we exposed *K. marmoratus* to 7 days of aquatic normoxia, aquatic hypoxia (P_{O_2} ~3.6 kPa), aerial normoxia or aerial hypoxia (P_{O_2} ~13.6 kPa). We measured the O_2-carrying capacity of blood (number of red blood cells, hematocrit and [Hb]), Hb–O_2 affinity and extra-branchial angiogenesis, predicting that similar phenotypes should occur in fish exposed to aquatic hypoxia and air exposure.

RESULTS

Blood analysis

Both hypoxia and air exposure significantly increased blood [Hb] (two-way ANOVA: oxygen, P<0.001; medium, P<0.05; interaction, P=0.92; Fig. 1A). Hematocrit was significantly increased in all three treatment groups relative to normoxic control fish (two-way ANOVA: oxygen, P<0.01; medium, P=0.23; interaction, P<0.05; Fig. 1B). Hypoxia significantly increased the number of red blood cells (RBCs), but air exposure had no effect (two-way ANOVA: oxygen, P<0.001; medium, P=0.46; interaction, P=0.52; Fig. 1C). As a result of the small blood volumes used in this study, repeated sampling was impossible and therefore mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) could only be calculated from the mean values for each treatment group, precluding statistical analysis. None of the values varied dramatically between treatment groups (Table 1).

Air exposure significantly decreased P_{50}, the blood P_{O_2} at which Hb is 50% saturated with O_2 and P_{50} was increased by elevated *in vitro* CO_2, but there was no overall effect of oxygen availability (three-way ANOVA: medium, P<0.001; CO_2, P<0.001; O_2, P=0.06; Fig. 2). A significant interaction between respiratory medium and O_2 availability was found (P<0.001, all other interactions P>0.05) and *post hoc* tests revealed that air exposure significantly increased Hb–O_2 affinity (reduced P_{50}) to a greater extent in normoxic relative to aerial hypoxic conditions (Fig. 2). The Root effect was significantly larger between 0.3 kPa and 4.0 kPa P_{CO_2} than between 0.3 kPa and 0.6 kPa P_{CO_2}, but was not influenced by any treatment condition (three-way ANOVA: medium, P=0.21; O_2, P=0.06; CO_2, P<0.05; all interactions, P>0.05; Table 1). Hb–O_2 binding cooperativity (Hill coefficient, n_H) was significantly increased by hypoxia and high P_{CO_2}, but not air exposure (three-way ANOVA: medium, P=0.52; O_2, P<0.01; CO_2, P<0.01; all interactions, P>0.05; Table 1).

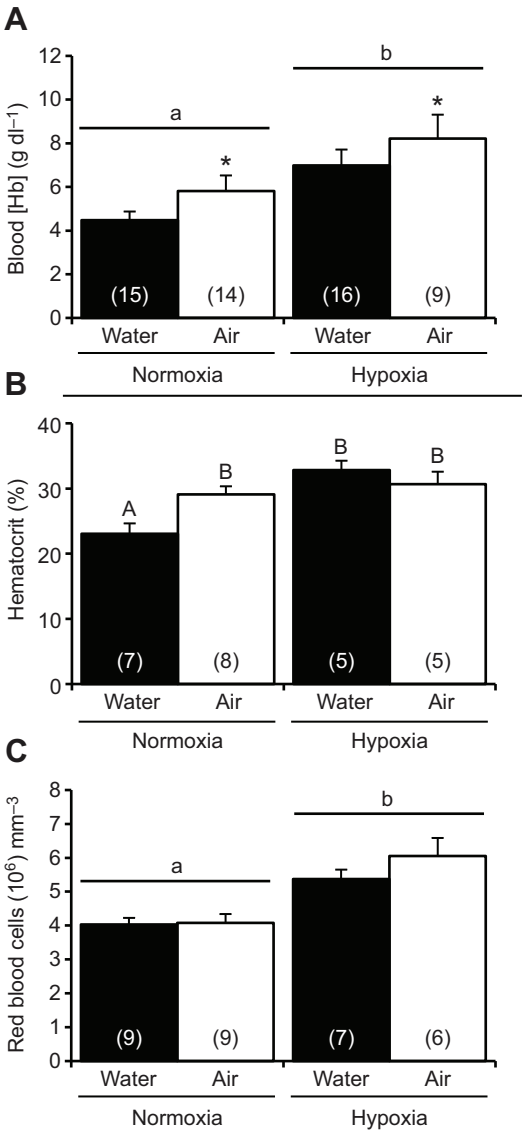

Fig. 1. Aquatic hypoxia and air exposure causes changes in the blood of *Kryptolebias marmoratus*. (A) Hb protein concentration, (B) hematocrit and (C) red blood cell counts. *Kryptolebias marmoratus* were acclimated for 7 days to one of four treatment groups: normoxic water, normoxic air, hypoxic water or hypoxic air. Hypoxia in water (P_{O_2}=3.59±0.06 kPa) and air (P_{O_2}=13.57±0.06 kPa) was achieved by mixing N_2 gas with atmospheric air. Different lower case letters indicate significant overall differences between normoxia and hypoxia (two-way ANOVA, P<0.05). Different upper case letters indicate significant differences between the four treatment groups if a significant interaction between oxygen availability and respiratory medium was found (two-way ANOVA, P<0.05). Asterisks denote a significant overall difference between fish in water versus air (two-way ANOVA, P<0.05). Sample sizes are given in parentheses. Error bars represent s.e.m.

Angiogenesis and aerial ventilation

Under conditions of aquatic normoxia (control), the marker for angiogenesis CD31 was visible in the dorsal and ventral cutaneous epithelium, as well as within the buccal and opercular chambers

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110601

Table 1. Hematological properties of *Kryptolebias marmoratus* acclimated for 7 days to different O_2 conditions

	Treatment			
	Aquatic normoxia	Aerial normoxia	Aquatic hypoxia	Aerial hypoxia
Root effect (%)				
0.3 kPa P_{CO_2} shift	–7.07±3.72 (9)	–11.41±3.66 (9)	–4.96±2.72 (10)	–6.55±1.69 (8)
3.7 kPa P_{CO_2} shift	–14.18±3.09 (9)*	–19.54±6.92 (9)*	–9.78±4.60 (10)*	–7.82±3.61 (8)*
Hill coefficient				
0.3 kPa CO_2	1.35±0.03 (9)^a	1.35±0.03 (9)^a	1.44±0.07 (10)^b	1.48±0.09 (8)^b
0.6 kPa CO_2	1.39±0.06 (9)^a	1.45±0.06 (9)^a	1.40±0.09 (10)^b	1.50±0.05 (8)^b
4.0 kPa CO_2	1.58±0.07 (9)^{a,*}	1.45±0.07 (9)^{a,*}	1.49±0.05 (10)^{b,*}	1.70±0.17 (8)^{b,*}
Mean corpuscular volume (fl)	59.07±5.22	73.68±5.84	63.10±4.24	50.68±5.47
Mean corpuscular hemoglobin (pg cell^{-1})	114.60±12.07	146.96±20.72	134.33±15.50	135.72±21.72
Mean corpuscular hemoglobin concentration (g dl^{-1})	19.40±2.21	19.95±2.62	21.29±2.39	26.78±3.95

Hypoxia in water (P_{O_2}=3.59±0.06 kPa) and air (P_{O_2}=13.57±0.06 kPa) was achieved by mixing N_2 gas with atmospheric air. Values are expressed as means ± s.e.m. Sample sizes are given in parentheses. Superscript letters within a row represent significant overall differences (P<0.05) between normoxia and hypoxia. Asterisks denote a significant overall difference (P<0.05) from all other CO_2 concentrations.

(Fig. 3A). After 7 days of air exposure, there was a striking increase in fluorescence of the buccal and opercular chambers (Fig. 3B). Across all four body regions, CD31 fluorescent intensity was significantly increased after air exposure, independent of O_2 availability (three-way ANOVA: medium, P<0.001; O_2, P=0.94; all interactions, P>0.05; Fig. 3C). Significantly reduced fluorescence was observed in the ventral epithelium compared with the other body regions measured (three-way ANOVA: region, P<0.001; Fig. 3C).

We observed air-exposed mangrove rivulus opening their mouths and 'gulping' air when out of water. Rates of aerial ventilation after 1 h of air-breathing were more than double the rates observed after 1 day or 7 days of air exposure (P<0.05; Fig. 4A). The activity of air-exposed fish (body movements h^{-1}) did not significantly change over the 7 day air-exposure period, although there was a trend towards decreased movement after 1 day (P=0.06; Fig. 4B). The majority of aerial ventilations occurred within 30 s of a body movement, although some ventilations were separated from body movements by more than 10 min (Fig. 4C).

DISCUSSION

Our results do not support the hypothesis that amphibious fishes maintain O_2 uptake and transport while out of water by co-opting the hypoxia response. Instead, we found that *K. marmoratus* have flexible O_2 uptake and delivery strategies that respond to changes in the respiratory environment. Behavioral and immunohistochemical data suggest that, during air exposure, the mouth and bucco-opercular chamber provide a supplementary gas exchange surface to the cutaneous epithelium. The hematological system also displays flexibility in response to changes in the respiratory environment. In contrast to our predictions, different responses were observed in fish exposed to air and hypoxia. Air exposure increased Hb–O_2 affinity and increased the concentration of Hb in the blood without increasing the number of circulating RBCs. In contrast, hypoxia in both aquatic and aerial environments substantially increased the O_2-carrying capacity of the blood via an increase in the density of RBCs, without any major effect on Hb–O_2 affinity. These results indicate that the changes in angiogenesis and binding affinity of Hb for O_2 observed during air exposure are not the result of altered O_2 availability, but instead may be controlled by a secondary pathway that can be activated concurrently with the hypoxia-activated pathway in the case of aerial hypoxia. Air-exposed mangrove rivulus might, for example, respond to changes in blood CO_2 or pH rather than O_2 (Putnam et al., 2004; Casey et al., 2010; Tresguerres et al., 2010).

If the binding affinity of Hb for O_2 reflects a balance between O_2 uptake and delivery, the abundance of O_2 in atmospheric air compared with water is predicted to result in relatively lower Hb–O_2

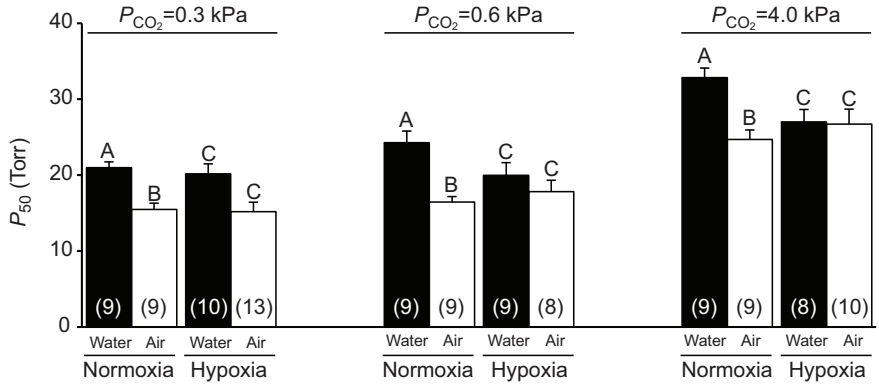

Fig. 2. Air exposure increases the affinity of hemoglobin for O_2 in *Kryptolebias marmoratus*. Fish were acclimated for 7 days to one of four treatment groups: normoxic water, normoxic air, hypoxic water or hypoxic air. Hypoxia in water (P_{O_2}=3.59±0.06 kPa) and air (P_{O_2}=13.57±0.06 kPa) was achieved by mixing N_2 gas with atmospheric air. Different upper case letters indicate significant overall differences in P_{50} (i.e. across all concentrations of CO_2, not within one P_{CO_2}) between the four treatment groups when a significant interaction between oxygen availability and respiratory medium was found (three-way ANOVA, P<0.05). P_{50} is presented in Torr; 1 Torr=133 Pa. There was a significant overall difference in P_{50} between each concentration of CO_2 used (three-way ANOVA, P<0.05), but there was no significant interaction between CO_2 and oxygen availability (P=0.71) or between CO_2 and respiratory medium (P=0.82). Sample sizes are given in parentheses. Error bars represent s.e.m.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.110601

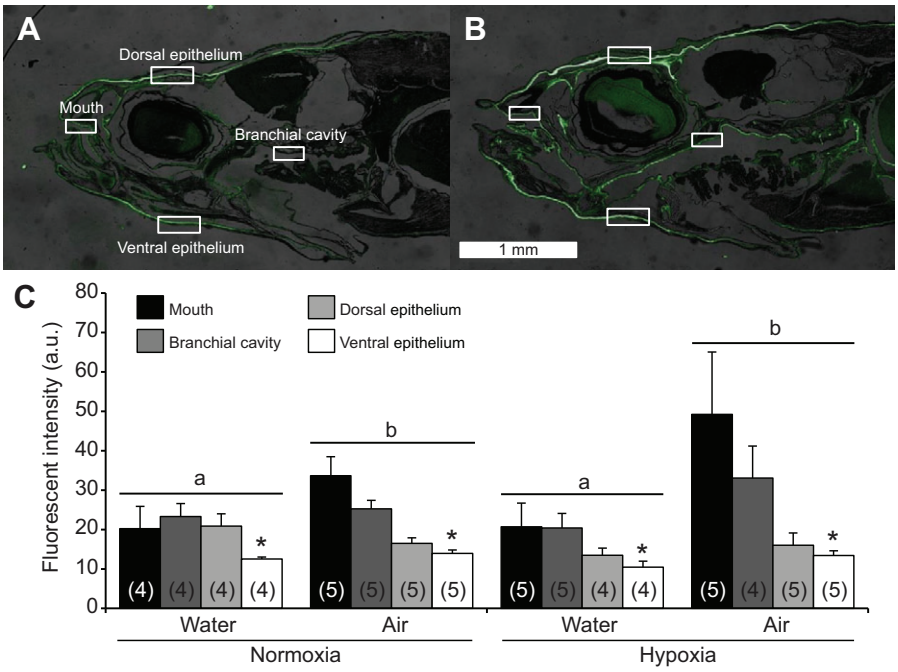

Fig. 3. Air exposure induces angiogenesis in the cutaneous epithelium and bucco-opercular chamber of *Kryptolebias marmoratus*. Representative composite images are presented of immunohistochemical staining for the angiogenesis marker CD31 (green) in a sagittal section through the head of *Kryptolebias marmoratus* acclimated to normoxic water (A) or air (B) for 7 days. Composite images were created from low-magnification (×4) fluorescent and differential interference contrast photomicrographs stitched together and overlaid using Adobe Photoshop. (C) Quantification of CD31 fluorescence (arbitrary units; a.u.) in fish from one of four treatment groups: normoxic water, hypoxic water, normoxic air or hypoxic air. Different letters indicate significant overall differences between fish in water versus air (three-way ANOVA, $P<0.05$). Asterisks denote a significant overall difference (three-way ANOVA, $P<0.05$) between the ventral epithelium and all other body regions. Error bars represent s.e.m.

affinity in air-breathing animals. Consistent with this hypothesis, the air-breathing fishes *Protopterus annectens*, *Polypterus senegalus* and *Clarias lazera* all reduce Hb–O_2 affinity during the ontogenetic shift from breathing in water to breathing in air (Graham, 1997). After 3 weeks out of water, the amphibious Canterbury mudfish *Neochanna burrowsius* also decreases Hb–O_2 affinity (Wells et al., 1984). Conversely, hypoxia-tolerant organisms are generally characterized by high Hb–O_2 affinities to maximize O_2 uptake (Graham, 1997; Wells, 2009). Why do mangrove rivulus contradict this pattern and increase affinity of Hb for O_2 after 7 days of air exposure? The lungfish *P. amphibius* increases Hb–O_2 affinity while air-breathing during aestivation, probably to enable reduced lung tidal volumes while inhabiting hypoxic subterranean environments (Johansen et al., 1976). In the non-aestivating mangrove rivulus, however, there was no significant effect of O_2 availability on Hb–O_2 affinity, suggesting that these fish are using a different strategy to aestivating lungfish.

We propose that the elevated affinity of Hb for O_2 observed in air-exposed mangrove rivulus serves to maintain a relatively stable effective P_{50} across respiratory media by counterbalancing the Bohr effect in emersed fish resulting from increased blood P_{CO_2} and/or decreased blood pH. The P_{50} of control (aquatic normoxia) mangrove rivulus at a P_{CO_2} of 0.6 kPa (P_{50}=24.7±1.3 Torr; 1 Torr=133 Pa) – a typical partial pressure in venous blood of aquatic tropical fishes – was approximately equal to that of blood from air-acclimated fish at a P_{CO_2} of 4.0 kPa (P_{50}=24.3±1.5 Torr), which is a typical level for emersed amphibious fishes. Thus, Hb–O_2 affinity may be maintained in *K. marmoratus* under a variety of environmental conditions, regardless of widely variable blood CO_2 levels. Like Hb in mangrove rivulus, protein function in other animals is often highly conserved during acclimation to changing environmental conditions (Somero, 1995). For example, expression of alternative troponin isoforms help to maintain cardiac function in *Oncorhynchus mykiss* and *Danio rerio* acclimated to varying

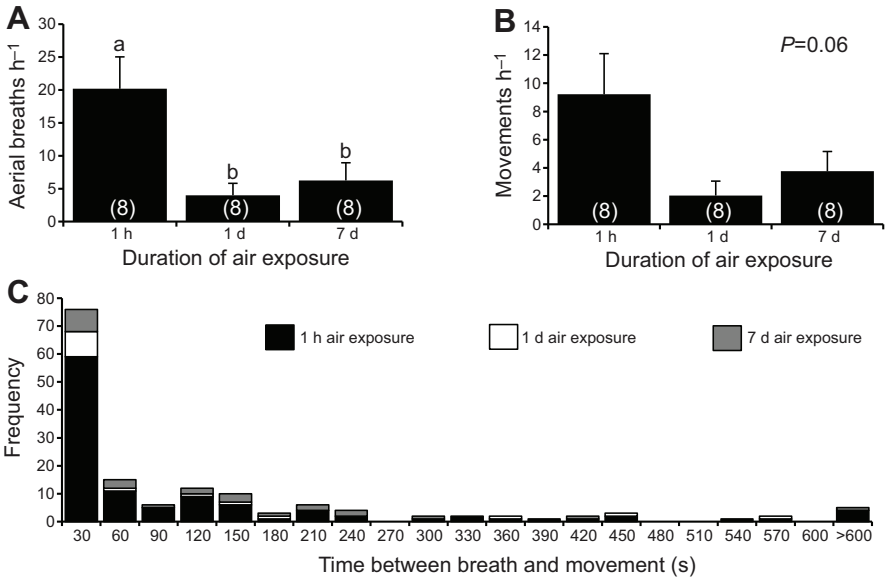

Fig. 4. Aerial ventilation and body movements in *Kryptolebias marmoratus* exposed to air. Frequency of aerial ventilation (A) and number of body movements (B) was measured over 7 days. Fish (N=8) were acclimated to air for 1 h, 1 day or 7 days. (C) Cumulative frequency of aerial ventilations in relation to whole body movements in 1 h recording periods after 1 h, 1 day and 7 days of air exposure. Significant differences (one-way ANOVA, $P<0.05$) between time points are indicated by different letters. Sample sizes are given in parentheses. Error bars represent s.e.m.

temperatures (Alderman et al., 2012; Genge et al., 2013). Conservation of Hb–O_2 affinity might allow mangrove rivulus to maintain consistent O_2 delivery in both air and water without requiring modifications to O_2-accepting tissues such as skeletal muscle (Hoppeler and Vogt, 2001; Fraser et al., 2006).

Fishes are able to modify Hb–O_2 affinity primarily by modifying the RBC intracellular environment or by changing the relative expression of Hb isoforms with different O_2-binding affinities (Grigg, 1969; Marinsky et al., 1990; Jensen, 1991; Val, 2000; Weber, 2000; Rutjes et al., 2007). Most commonly, air-breathing fishes increase organic phosphate concentrations relative to [Hb] during behavioral or ontogenetic shifts to air-breathing in order to facilitate O_2 unloading at the tissues (Babiker, 1984; Graham, 1997; Val, 2000). Conversely, hypoxia-tolerant fish species often maximize O_2 uptake using low ratios of organic phosphate to Hb compared with fishes that inhabit well-oxygenated water (Wells, 2009). If air-exposed mangrove rivulus are able to decrease organic phosphate concentrations to increase Hb–O_2 affinity, it is puzzling why fish acclimated to hypoxia did not increase affinity to the same extent. At a P_{CO_2} of 0.3 kPa, which is typical of arterial blood in a water-breathing fish, the P_{50} of hypoxia-acclimated fish was 20.2±1.3 Torr, compared with 21.0±0.7 Torr in control fish. When environmental O_2 is limited there is often a trade-off between optimizing O_2 uptake to RBCs and O_2 unloading at the tissues. Perhaps hypoxia-acclimated mangrove rivulus are constrained from increasing Hb–O_2 affinity by the need to facilitate O_2 delivery to the tissues and instead rely on other hypoxia-tolerance strategies such as increasing the density of red cells in the blood or enlarging gill surface area (Richards, 2011; Turko et al., 2012; Cook et al., 2013). At high partial pressures of CO_2 there was a trend towards increased Hb–O_2 affinity in hypoxia-acclimated fish; this may result from the significantly increased [Hb], which would provide extra pH-buffering capacity and thus lessen the magnitude of the Bohr effect at high P_{CO_2} (Weber, 2000; Wells, 2009).

Oxygen-carrying capacity was significantly increased, via increased RBC density, by hypoxic acclimation in either water or air but not during air exposure alone. Increasing the number of circulating RBCs in response to hypoxia to enhance O_2 transport, via erythropoiesis or splenic release, is a strategy used by many fishes (e.g. Tetens and Lykkeboe, 1981; Murad et al., 1990; Soldatov, 1996; Lai et al., 2006), but there are exceptions (e.g. McLeod et al., 1978; Routley et al., 2002). Increased O_2-carrying capacity allows fish to increase O_2 uptake from the environment without compromising unloading at the tissues and may therefore be a more efficient strategy for coping with hypoxia than adjusting Hb–O_2 affinity (Brauner and Wang, 1997). It is curious, then, why air exposure alone increased Hb–O_2 affinity but did not induce increased RBC density. Extra RBCs may increase blood viscosity and impede flow through the epidermal capillary beds used for aerial gas exchange (Baldwin and Wells, 1990).

Overall, we observed a relatively minor Root shift (12.2±2.1% mean reduction in maximum O_2 saturation in all treatment groups under normoxic conditions; P_{O_2}=22 kPa) across a large (3.7 kPa) CO_2 gradient. Neither air-breathing nor hypoxia exposure affected the magnitude of this shift, which was generally small compared with other teleosts (Pelster and Randall, 1998). This suggests that mangrove rivulus Hb is well adapted to maintaining near-maximal O_2 saturation at high P_{CO_2} during emersion, even immediately after leaving water.

Angiogenesis was observed in the skin and bucco-opercular region of air-exposed mangrove rivulus, but not in fish exposed to aquatic hypoxia. Instead of recruiting additional sites of gas exchange to tolerate hypoxia, mangrove rivulus appear to increase their capacity for branchial O_2 uptake by increasing gill surface area by reduction of the inter-lamellar cell mass (Turko et al., 2012). During emersion, amphibious fishes often rely on air-breathing organs such as lungs, buccal pouches or capillary-rich cutaneous surfaces (Johansen, 1970; Satchell, 1976; Olson, 1994; Graham, 1997). For example, bucco-pharyngeal blood vessels in *G. mirabilis* are filled with blood after 10–20 min of emersion (Todd and Ebeling, 1966). Phylogenetic analyses of mudskippers suggest that species with an increased reliance on air-breathing possess increased capillary density on the tongue and within the bucco-opercular chamber (Zhang et al., 2000; Gonzales et al., 2011). We also observed, for the first time, 'gulping' activity by emersed mangrove rivulus, strongly implicating this region as a site of gas exchange. These aerial ventilations were usually associated with body movements, suggesting that transient increases in O_2 demand might provide a stimulus to refresh the air in the bucco-opercular chamber. Aerial ventilation frequency significantly decreased between 1 h and 1 day of air exposure and was probably correlated with an increase in blood flow to extrabranchial respiratory surfaces. Augmented cutaneous blood flow increases O_2 uptake and probably allowed mangrove rivulus to reduce evaporative water loss by decreasing aerial ventilation frequency (Burggren and Mwalukoma, 1983; Burggren and Moalli, 1984). Air exposure also induces enlargement of the inter-lamellar cell mass, reducing the effective gill surface area, which may further limit water loss during bucco-opercular ventilation (Ong et al., 2007).

Observing bucco-opercular respiration was highly unexpected, considering gill surface area is reduced during air exposure and the branchial region was presumed to be non-functional (Ong et al., 2007; Cooper et al., 2012). Instead, aerial O_2 uptake in this species was thought to be achieved entirely across the cutaneous epithelium (Wright, 2012). The dorsal surface of mangrove rivulus has long been known to be covered with a dense network of epithelial capillaries (Grizzle and Thiyagarajah, 1987) and a recent paper from our laboratory demonstrated angiogenesis in the caudal fin of air-exposed fish (Cooper et al., 2012). However, given the small size (~100 mg) of mangrove rivulus and the infrequent nature of 'gulping' (1 breath every ~12 min), it is not surprising that this behaviour has been overlooked, especially considering that disturbances typically cause fish in both air and water to cease ventilatory activity (A.T., unpublished observation).

Overall, these results provide the first evidence that mangrove rivulus have a phenotypically flexible respiratory system that appears to be independently regulated by both O_2 availability and the respiratory medium. Although it initially appears that air-exposed fish increase the affinity of Hb for O_2, in contrast to most other amphibious fishes, we propose that this change represents an effort to maintain a stable P_{50} in the face of large changes in blood P_{CO_2} or pH when out of water. We also have shown for the first time that air exposure promotes angiogenesis in the bucco-opercular chamber, which mangrove rivulus ventilate by 'gulping' air. These results contradict the hypothesis that the hypoxia response was co-opted by fishes during the invasion of land and instead suggest that novel adaptations to the O_2 transport system have evolved.

MATERIALS AND METHODS

Experimental animals

Kryptolebias marmoratus hermaphrodites at least 6 months of age (*Slc8E* strain) (Tatarenkov et al., 2010) were obtained from the breeding colony maintained in the Hagen Aqualab, University of Guelph. Fish were reared individually in 120 ml semi-transparent plastic containers (FisherBrand

Collection Containers; Fisher Scientific) under constant conditions (25°C, 15–18‰, pH 8.1, 12 h:12 h light:dark cycle) (Frick and Wright, 2002). Water changes were performed weekly using artificial seawater (Crystal Sea Marinemix; Marine Enterprises International, Baltimore, MA, USA) made with reverse osmosis water. Fish were fed *Artemia nauplii* three to four times per week. The experiments in this study were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol 10G008).

Experimental fish were acclimated for 7 days (25°C) to one of four treatment groups: brackish water (control), air, aquatic hypoxia or aerial hypoxia. All fish were fasted for the duration of the acclimation period. Control fish were maintained under standard rearing conditions (~21 kPa O_2). Air-acclimated fish were maintained on moist filter paper (15‰ brackish water, ~21 kPa O_2) in plastic rearing containers (Ong et al., 2007). Hypoxia in both water and air was achieved by introducing a finely regulated flow of nitrogen into closed chambers containing experimental fish (Regan et al., 2011). Oxygen levels were measured daily (Hach LDO101 electrode connected to Hach HQ30d meter, Hach Company, Missisauga, ON, Canada) and averaged 3.59±0.06 kPa (mean ± s.e.m.; here and throughout) in water and 13.57±0.06 kPa in air over the course of the experiments. The P_{O_2} chosen for aquatic hypoxia has been previously found to be slightly above the critical O_2 tension of mangrove rivulus (Turko et al., 2012). The aerial O_2 concentration was selected after preliminary experiments indicated that more severe aerial hypoxia was lethal, whereas fish acclimated to less severe aerial hypoxia (~17 kPa) did not show any hematological response that differed relative to fish acclimated to normoxic air.

Blood analysis

To measure hematocrit, hemoglobin concentration and red blood cell counts, blood was collected by caudal severance using heparinised microhematocrit tubes (VWR, Missisauga, ON, Canada) (Bianchini and Wright, 2013). It should be noted that stress induced by this method of blood sampling may have caused the acute splenic release of red blood cells and thus our measurements may be consistent overestimates compared with the *in vivo* situation. The red blood cell counts at O_2-exchanging surfaces (e.g. the gills or epithelial capillaries) may also differ from the aortic blood we gathered (e.g. Klitzman and Duling, 1979; Hudetz et al., 1999). However, given the small size of *K. marmoratus*, this was the only practical method of blood collection. Hematocrit (Hct) was determined by centrifuging samples in sealed microhematocrit tubes at 7000 r.p.m. for 2 min in an International Clinical Centrifuge (Model CL, International Equipment, Needham, MA, USA). To accurately measure hematocrit of small blood volumes, images were taken under a dissecting microscope and analyzed using ImageJ (Bianchini and Wright, 2013). Hemoglobin concentration was quantified using the cyanmethemoglobin method (Blaxhall and Daisley, 1973). RBCs were counted in a standard hemocytometer using a 1:400 dilution of whole blood: Cortland's isotonic saline (Wolf, 1963) further diluted 1:1 with 0.4% Trypan Blue solution. The RBC indices, mean RBC volume (MCV), mean cellular Hb content (MCH) and mean cellular [Hb] (MCHC), were calculated from Hct/RBC count, ([Hb]/RBC count)×10 and [Hb]/Hct, respectively.

Hb–O_2 equilibrium curves were determined (25°C) at three different CO_2 partial pressures (0.3, 0.6, 4.0 kPa) using a spectrophotometer ($P_{wee}50$, La Trobe University, Melbourne, Australia) designed to utilize small blood volumes (Reeves, 1980; Henriksson et al., 2008; Bianchini and Wright, 2013). The lower CO_2 partial pressures approximated resting values for arterial (0.3 kPa CO_2) and venous (0.6 kPa CO_2) blood in tropical fishes (Heisler, 1982; Perry, 1986, Tufts and Perry, 1998), while the higher P_{CO_2} is typical of tropical amphibious fish during air exposure (Johansen, 1970; Graham, 1997). Blood samples were obtained via caudal severance and were immediately spread thinly between two gas-permeable membranes (Glad Go-Between Freezer Film, Hobart, Tasmania, Australia) held in place on a circular sample holder using an O-ring and inserted into the $P_{wee}50$. Oxygen saturation curves at each P_{CO_2} were created by measuring absorbance at 390 nm and 435 nm, respectively the isosbestic point and peak absorbance of deoxygenated Hb (Iuchi, 1973; Hoxter, 1979), at O_2 partial pressures between 0 and 22 kPa in 1 kPa increments. Each O_2 and CO_2 combination was mixed from compressed O_2, CO_2 and N_2 by the $P_{wee}50$, humidified and introduced to the sample within the temperature-controlled spectrophotometer. Hb-O_2 affinity (P_{50}), Root effect and cooperativity (Hill coefficient, n_H) were automatically calculated from the saturation curves by a manufacturer-supplied spreadsheet. The blood sampling method used may have overestimated Hb–O_2 affinity by causing the release of catecholamines, which activate NHE, increase red blood cell pH and ultimately reduce P_{50} (Nikinmaa and Boutilier, 1995; Reid et al., 1998). Blood was collected as quickly as possible to minimize the effects of a catecholaminergic response, but because of the small size of *K. marmoratus*, this was the only practical method of collecting blood. If stress impacted blood parameters, then the effect would be more or less consistent across all treatment groups. We considered measuring the pH of whole blood at each P_{CO_2} used for the Hb–O_2 affinity measurements to calculate the magnitude of the Bohr effect, but the small blood volumes and tendency of the blood to clot made this unfeasible.

Angiogenesis

Angiogenesis was determined by immunohistochemical staining for the endothelial integral membrane protein cluster of differentiation 31 (CD31), also known as platelet endothelial cell adhesion molecule (PECAM-1) (Albelda et al., 1991; Baluk and McDonald, 2008), which has previously been used to measure angiogenesis in *Kryptolebias marmoratus* (Cooper et al., 2012). Euthanized fish were fixed in 10% neutral buffered formalin at 4°C for 24 h, decalcified (Surgipath Decalcifier II, Winnipeg, Canada) for 1 h at 20°C and stored in 70% ethanol (4°C) until embedding. Gill arches were obtained from the left side. Dissected tissues were routinely embedded in paraffin, serially sectioned in 5 µm increments, deparaffinised in xylene and rehydrated with a graded ethanol series prior to staining. All samples were processed at the same time. Sections were incubated for 1 h at room temperature in primary antibody (1:400 Rat anti-Mouse PECAM/CD31:PBS; BD Pharmingen), rinsed briefly with PBS and incubated overnight with Alexa-Fluor-488-labelled secondary antibody (1:100 goat anti-rat IgG:PBS; Invitrogen) in PBS. Samples were then washed five times with PBS, mounted with Fluoromount (Sigma-Aldrich) and sealed with nail polish.

All slides were viewed on the same afternoon using a Nikon epifluorescent microscope (Nikon Eclipse 90i microscope, Nikon, Melville, NY, USA) and images were captured (×40) using identical camera settings (NIS Elements, Nikon). The background of each image was subtracted using the 'rolling ball' subtraction tool in ImageJ (US National Institutes of Health, Bethesda, MD, USA) and then the same intensity threshold was applied (Cooper et al., 2012). A box was then drawn around one of four body regions (the mouth, branchial cavity, dorsal epithelium or ventral epithelium) and the integrated density was calculated using ImageJ.

Aerial ventilation

Contrary to our predictions, the immunohistochemical data suggested that air exposure increased blood flow to the bucco-opercular cavity of mangrove rivulus. To confirm that mangrove rivulus are able to use these surfaces for gas exchange while emersed, we performed a study to measure rates of aerial ventilation. Mangrove rivulus were acclimated to air as described above. After 1 h, 1 day or 7 days of air exposure, fish were video recorded (Logitech Quickcam Pro, Fremont, CA, USA) under a dissecting microscope for 30 min and ventilatory activity (defined as an opening of the mouth and/or opercula) and body movements were recorded. Each fish was used only once.

Statistical analysis

One-way ANOVA and *post hoc* Holm–Sidak tests were used to test whether the duration of air exposure influenced ventilation rate. Two-way ANOVA with *post hoc* Holm–Sidak tests were used to determine the overall effects of respiratory medium and O_2 availability on [Hb], Hct and RBC counts. Three-way ANOVAs with *post hoc* Holm–Sidak tests were used to determine the overall effects of respiratory medium, O_2 availability and CO_2 on P_{50} and the magnitude of the Root effect. A three-way ANOVA was also used to determine the effect of respiratory medium, O_2 availability and body region on angiogenesis (CD31 fluorescence). Data were log transformed when necessary to meet the assumptions of normal distribution and equal variance.

SigmaPlot 11 (Systat Software, San Jose, CA, USA) was used for all analyses (critical α=0.05). Throughout the text values are given as means ± s.e.m.

Acknowledgements
The authors with to thank Dr D. Fudge for use of the microscope and hemocytometer and Dr G. Tattersall for help optimizing the $P_{wee}50$ equipment. We also thank Drs G. Scott, K. Gamperl and K. Gilmour for thoughtful discussions regarding the data. Thank you to B. Frank, M. Cornish, M. Davies and several volunteers for help with fish maintenance.

Competing interests
The authors declare no competing financial interests.

Author contributions
A.J.T., C.R., K.B., M.F. and P.A.W. conceived and designed the project. A.J.T., C.R., K.B., M.F. executed the experiments. A.J.T. analyzed the data and wrote the draft manuscript. A.J.T., C.R., K.B., M.F. and P.A.W. revised the manuscript.

Funding
Funding was provided by the Natural Sciences and Engineering Research Council of Canada Discovery grants program to P.A.W. (RGPIN-120513) and Natural Sciences and Engineering Research Council of Canada graduate scholarships to A.J.T., C.R. and K.B.

References

Albelda, S. M., Muller, W. A., Buck, C. A. and Newman, P. J. (1991). Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. *J. Cell Biol.* **114**, 1059-1068.
Alderman, S. L., Klaiman, J. M., Deck, C. A. and Gillis, T. E. (2012). Effect of cold acclimation on troponin I isoform expression in striated muscle of rainbow trout. *Am. J. Physiol.* **303**, R168-R176.
Babiker, M. M. (1984). Adaptive respiratory significance of organophosphates (ATP & GTP) in air-breathing fishes. *Hydrobiologia* **110**, 339-349.
Baldwin, J. and Wells, R. M. G. (1990). Oxygen transport potential in tropical elasmobranchs from the Great Barrier Reef: relationship between haematology and blood viscosity. *J. Exp. Mar. Biol. Ecol.* **144**, 145-155.
Baluk, P. and McDonald, D. M. (2008). Markers for microscopic imaging of lymphangiogenesis and angiogenesis. *Ann. N. Y. Acad. Sci.* **1131**, 1-12.
Bianchini, K. and Wright, P. A. (2013). Hypoxia delays hematopoiesis: retention of embryonic hemoglobin and erythrocytes in larval rainbow trout, Oncorhynchus mykiss, during chronic hypoxia exposure. *J. Exp. Biol.* **216**, 4415-4425.
Blaxhall, P. C. and Daisley, K. W. (1973). Routine haematological methods for use with fish blood. *J. Fish Biol.* **5**, 771-781.
Bohr, C., Hasselbalch, K. and Krogh, A. (1904). Concerning a biologically important relationship – the influence of the carbon dioxide content of blood on its oxygen binding. *Skand. Arch. Physiol.* **16**, 402-412.
Booth, J. H. (1979). The effects of oxygen supply, epinephrine, and acetylcholine on the distribution of blood flow in trout gills. *J. Exp. Biol.* **83**, 31-39.
Brauner, C. J. and Val, A. L. (2006). Oxygen transfer. In *Fish Physiology: The Physiology of Tropical Fish,* Vol. 22 (ed. A. L. Val, V. M. Almeida-Val and D. J. Randall), pp 277-306. New York, NY: Academic Press.
Brauner, C. J. and Wang, T. (1997). The optimal oxygen equilibrium curve: a comparison between environmental hypoxia and anemia. *Am. Zool.* **37**, 101-108.
Burggren, W. and Moalli, R. (1984). 'Active' regulation of cutaneous gas exchange by capillary recruitment in amphibians: experimental evidence and a revised model for skin respiration. *Respir. Physiol.* **55**, 379-392.
Burggren, W. and Mwalukoma, A. (1983). Respiration during chronic hypoxia and hyperoxia in larval and adult bullfrogs (*Rana catesbeiana*). I. Morphological responses of lungs, skin and gills. *J. Exp. Biol.* **105**, 191-203.
Casey, J. R., Grinstein, S. and Orlowski, J. (2010). Sensors and regulators of intracellular pH. *Nat. Rev. Mol. Cell Biol.* **11**, 50-61.
Cook, D. G., Iftikar, F. I., Baker, D. W., Hickey, A. J. R. and Herbert, N. A. (2013). Low-O_2 acclimation shifts the hypoxia avoidance behaviour of snapper (*Pagrus auratus*) with only subtle changes in aerobic and anaerobic function. *J. Exp. Biol.* **216**, 369-378.
Cooper, C. A., Litwiller, S. L., Murrant, C. L. and Wright, P. A. (2012). Cutaneous vasoregulation during short- and long-term aerial acclimation in the amphibious mangrove rivulus, *Kryptolebias marmoratus*. *Comp. Biochem. Physiol.* **161B**, 268-274.
Dejours, P. (1988). *Respiration in Water and Air. Adaptations-Regulation-Evolution*. Amsterdam: Elsevier.
Ellison, A., Wright, P., Taylor, D. S., Cooper, C., Regan, K., Currie, S. and Consuegra, S. (2012). Environmental diel variation, parasite loads, and local population structuring of a mixed-mating mangrove fish. *Ecol. Evol.* **2**, 1682-1695.
Farmer, M. (1979). The transition from water to air breathing: effects of CO_2 on hemoglobin function. *Comp. Biochem. Physiol.* **62A**, 109-114.
Farmer, M., Fyhn, H. J., Fyhn, U. E. H. and Noble, R. W. (1979). Occurrence of root effect hemoglobins in Amazonian fishes. *Comp. Biochem. Physiol.* **62A**, 115-124.
Fraser, J., de Mello, L. V., Ward, D., Rees, H. H., Williams, D. R., Fang, Y., Brass, A., Gracey, A. Y. and Cossins, A. R. (2006). Hypoxia-inducible myoglobin expression in nonmuscle tissues. *Proc. Natl. Acad. Sci. USA* **103**, 2977-2981.
Frick, N. T. and Wright, P. A. (2002). Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus* I. The influence of environmental salinity and external ammonia. *J. Exp. Biol.* **205**, 79-89.
Genge, C. E., Davidson, W. S. and Tibbits, G. F. (2013). Adult teleost heart expresses two distinct troponin C paralogs: cardiac TnC and a novel and teleost-specific ssTnC in a chamber- and temperature-dependent manner. *Physiol. Genomics* **45**, 866-875.
Gillen, R. G. and Riggs, A. (1971). The hemoglobins of a freshwater teleost *Cichlasoma cyanoguttatum*: the effects of phosphorylated organic compounds upon oxygen equilibria. *Comp. Biochem. Physiol.* **38B**, 585-595.
Gonzales, T. T., Katoh, M., Ghaffar, M. A. and Ishimatsu, A. (2011). Gross and fine anatomy of the respiratory vasculature of the mudskipper, *Periophthalmodon schlosseri* (Gobiidae: Oxudercinae). *J. Morphol.* **272**, 629-640.
Gracey, A. Y. (2008). The *Gillichthys mirabilis* Cooper array: a platform to investigate the molecular basis of phenotypic plasticity. *J. Fish Biol.* **72**, 2118-2132.
Graham, J. B. (1997). *Air-Breathing Fishes: Evolution, Diversity and Adaptation*. San Diego, CA: Academic Press.
Grigg, G. C. (1969). Temperature-induced changes in the oxygen equilibrium curve of the blood of the brown bullhead, *Ictalurus nebulosus*. *Comp. Biochem. Physiol.* **28**, 1203-1223.
Grizzle, J. M. and Thiyagarajah, A. (1987). Skin histology of *Rivulus ocellatus marmoratus*: apparent adaptation for aerial respiration. *Copeia* **1987**, 237-240.
Heisler, N. (1982). Intracellular and extracellular acid-base regulation in the tropical fresh-water teleost fish *Synbranchus marmoratus* in response to the transition from water breathing to air breathing. *J. Exp. Biol.* **99**, 9-28.
Henriksson, P., Mandic, M. and Richards, J. G. (2008). The osmorespiratory compromise in sculpins: impaired gas exchange is associated with freshwater tolerance. *Physiol. Biochem. Zool.* **81**, 310-319.
Hoppeler, H. and Vogt, M. (2001). Muscle tissue adaptations to hypoxia. *J. Exp. Biol.* **204**, 3133-3139.
Hoxter, G. (1979). Suggested isosbestic wavelength calibration in clinical analyses. *Clin. Chem.* **25**, 143-146.
Hudetz, A. G., Wood, J. D., Biswal, B. B., Krolo, I. and Kampine, J. P. (1999). Effect of hemodilution on RBC velocity, supply rate, and hematocrit in the cerebral capillary network. *J. Appl. Physiol.* **87**, 505-509.
Iuchi, I. (1973). Chemical and physiological properties of the larval and the adult hemoglobins in rainbow trout, *Salmo gairdnerii irideus*. *Comp. Biochem. Physiol.* **44B**, 1087-1101.
Jensen, F. B. (1991). Multiple strategies in oxygen and carbon dioxide transport by haemoglobin. In *Physiological Strategies for Gas Exchange and Metabolism* (ed. A. J. Woakes, M. K. Grieshaber and C. R. Bridges), pp. 55-78. Cambridge: Cambridge University Press.
Johansen, K. (1970). Air breathing in fishes. In *Fish Physiology: The Nervous System, Circulation and Respiration,* Vol. 4 (ed. W. S. Hoar and D. J. Randall), pp. 361-411. New York, NY: Academic Press.
Johansen, K., Lykkeboe, G., Weber, R. E. and Maloiy, G. M. O. (1976). Respiratory properties of blood in awake and estivating lungfish, Protopterus amphibius. *Respir. Physiol.* **27**, 335-345.
Klitzman, B. and Duling, B. R. (1979). Microvascular hematocrit and red cell flow in resting and contracting striated muscle. *Am. J. Physiol.* **237**, H481-H490.
Lai, J. C. C., Kakuta, I., Mok, H. O. L., Rummer, J. L. and Randall, D. (2006). Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. *J. Exp. Biol.* **209**, 2734-2738.
Marinsky, C. A., Houston, A. H. and Murad, A. (1990). Effects of hypoxia on hemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **68**, 884-888.
McLeod, T. F., Sigel, M. M. and Yunis, A. A. (1978). Regulation of erythropoiesis in the Florida gar, *Lepisosteus platyrhincus*. *Comp. Biochem. Physiol.* **60A**, 145-150.
Murad, A., Houston, A. H. and Samson, L. (1990). Haematological response to reduced oxygen-carrying capacity, increased temperature and hypoxia in goldfish, *Carassius auratus* L. *J. Fish Biol.* **36**, 289-305.
Nikinmaa, M. and Boutilier, R. G. (1995). Adrenergic control of red cell pH, organic phosphate concentrations and haemoglobin function in teleost fish. In *Advances in Comparative and Environmental Physiology: Mechanisms of Systemic Regulation,* Vol. 21 (ed. N. Heisler), pp. 107-133. Berlin: Springer.
Olson, K. R. (1994). Circulatory anatomy in bimodally breathing fish. *Am. Zool.* **34**, 280-288.
Ong, K. J., Stevens, E. D. and Wright, P. A. (2007). Gill morphology of the mangrove killifish (*Kryptolebias marmoratus*) is plastic and changes in response to terrestrial air exposure. *J. Exp. Biol.* **210**, 1109-1115.
Pelster, B. and Randall, D. (1998). The physiology of the root effect. In *Fish Physiology: Fish Respiration,* Vol. 17 (ed. S. F. Perry and B. L. Tufts), pp. 113-139. San Diego, CA: Academic Press.
Perry, S. F. (1986). Carbon dioxide excretion in fishes. *Can. J. Zool.* **64**, 565-572.
Putnam, R. W., Filosa, J. A. and Ritucci, N. A. (2004). Cellular mechanisms involved in CO_2 and acid signaling in chemosensitive neurons. *Am. J. Physiol.* **287**, C1493-C1526.
Rahn, H. (1966). Aquatic gas exchange: theory. *Respir. Physiol.* **1**, 1-12.
Reeves, R. B. (1980). A rapid micro method for obtaining oxygen equilibrium curves on whole blood. *Respir. Physiol.* **42**, 299-315.
Regan, K. S., Jonz, M. G. and Wright, P. A. (2011). Neuroepithelial cells and the hypoxia emersion response in the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* **214**, 2560-2568.

Reid, S. G., Bernier, N. J. and Perry, S. F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol.* **120C**, 1-27.
Richards, J. G. (2011). Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. *J. Exp. Biol.* **214**, 191-199.
Root, R. W. and Irving, L. (1941). The equilibrium between haemoglobin and oxygen in whole and hemolysed blood of the Tautog and a theory of the Haldane effect. *Biol. Bull.* **81**, 307-323.
Routley, M. H., Nilsson, G. E. and Renshaw, G. M. C. (2002). Exposure to hypoxia primes the respiratory and metabolic responses of the epaulette shark to progressive hypoxia. *Comp. Biochem. Physiol.* **131A**, 313-321.
Rutjes, H. A., Nieveen, M. C., Weber, R. E., Witte, F. and Van den Thillart, G. E. E. J. M. (2007). Multiple strategies of Lake Victoria cichlids to cope with lifelong hypoxia include hemoglobin switching. *Am. J. Physiol.* **293**, R1376-R1383.
Rytkönen, K. T., Williams, T. A., Renshaw, G. M., Primmer, C. R. and Nikinmaa, M. (2011). Molecular evolution of the metazoan PHD–HIF oxygen-sensing system. *Mol. Biol. Evol.* **28**, 1913-1926.
Satchell, G. H. (1976). The circulatory system of air breathing fish. In *Respiration of Amphibious Vertebrates* (ed. G. M. Hughes), pp. 105-124. New York, NY: Academic Press.
Shartau, R. B. and Brauner, C. J. (2014). Acid-base and ion balance in fishes with bimodal respiration. *J. Fish Biol.* **84**, 682-704.
Soivio, A. and Tuurala, H. (1981). Structural and circulatory responses to hypoxia in the secondary lamellae of *Salmo gairdneri* gills at two temperatures. *J. Comp. Physiol.* **145**, 37-43.
Soldatov, A. A. (1996). The effect of hypoxia on red blood cells of flounder: a morphologic and autoradiographic study. *J. Fish Biol.* **48**, 321-328.
Somero, G. N. (1995). Proteins and temperature. *Annu. Rev. Physiol.* **57**, 43-68.
Tatarenkov, A., Ring, B. C., Elder, J. F., Bechler, D. L. and Avise, J. C. (2010). Genetic composition of laboratory stocks of the self-fertilizing fish *Kryptolebias marmoratus*: a valuable resource for experimental research. *PLoS ONE* **5**, e12863.
Taylor, D. S. (1990). Adaptive specializations of the cyprinodont fish *Rivulus marmoratus*. *Florida Scientist* **53**, 239-248.
Taylor, D. S. (2012). Twenty-four years in the mud: what have we learned about the natural history and ecology of the mangrove rivulus, *Kryptolebias marmoratus*? *Integr. Comp. Biol.* **52**, 724-736.
Taylor, D. S., Turner, B. J., Davis, W. P. and Chapman, B. B. (2008). A novel terrestrial fish habitat inside emergent logs. *Am. Nat.* **171**, 263-266.
Tetens, V. and Lykkeboe, G. (1981). Blood respiratory properties of rainbow trout, *Salmo gairdneri*: responses to hypoxia acclimation and anoxic incubation of blood *in vitro*. *J. Comp. Physiol.* **145**, 117-125.
Todd, E. S. and Ebeling, A. W. (1966). Aerial respiration in the longjaw mudsucker *Gillichthys mirabilis* (Teleostei: Gobiidae). *Biol. Bull.* **130**, 265-288.
Tresguerres, M., Buck, J. and Levin, L. R. (2010). Physiological carbon dioxide, bicarbonate, and pH sensing. *Pflugers Arch.* **460**, 953-964.
Tufts, B. and Perry, S. F. (1998). Carbon dioxide transport and excretion. In *Fish Physiology: Fish Respiration,* Vol. 17 (ed. S. F. Perry and B. L. Tufts), pp. 229-282. San Diego, CA: Academic Press.
Turko, A. J., Cooper, C. A. and Wright, P. A. (2012). Gill remodelling during terrestrial acclimation reduces aquatic respiratory function of the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* **215**, 3973-3980.
Ultsch, G. R. (1987). The potential role of hypercarbia in the transition from water-breathing to air-breathing in vertebrates. *Evolution* **41**, 442-445.
Val, A. L. (2000). Organic phosphates in the red blood cells of fish. *Comp. Biochem. Physiol.* **125A**, 417-435.
Weber, R. E. (2000). Adaptations for oxygen transport: lessons from fish hemoglobins. In *Hemoglobin Function in Vertebrates* (ed. G. di Prisco, B. Giardina and R. E. Weber), pp. 23-37. Milano: Springer-Verlag Italia.
Weber, R. E. and Fago, A. (2004). Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Respir. Physiol. Neurobiol.* **144**, 141-159.
Wells, R. M. G. (2009). Blood-gas transport and hemoglobin function: adaptations for functional and environmental hypoxia. In *Hypoxia* (ed. J. G. Richards, A. P. Farrell and C. J. Brauner), pp. 255-299. San Diego, CA: Elsevier.
Wells, R. M. G., Forster, M. E. and Meredith, A. S. (1984). Blood oxygen affinity in the amphibious fish *Neochanna burrowsius* (Galaxiidae: Salmoniformes). *Physiol. Zool.* **57**, 261-265.
Wells, R. M. G., Baldwin, J., Seymour, R. S. and Weber, R. E. (1997). Blood oxygen transport and hemoglobin function in three tropical fish species from northern Australian freshwater billabongs. *Fish Physiol. Biochem.* **16**, 247-258.
Wolf, K. (1963). Physiological salines for fresh-water teleosts. *Prog. Fish-Cult.* **25**, 135-140.
Wright, P. A. (2012). Environmental physiology of the mangrove rivulus, *Kryptolebias marmoratus*, a cutaneously breathing fish that survives for weeks out of water. *Integr. Comp. Biol.* **52**, 792-800.
Zhang, J., Taniguchi, T., Takita, T. and Ali, A. B. (2000). On the epidermal structure of *Boleophthalmus* and *Scartelaos* mudskippers with reference to their adaptation to terrestrial life. *Ichthyol. Res.* **47**, 359-366.