

## RESEARCH ARTICLE

# Gill remodelling during terrestrial acclimation reduces aquatic respiratory function of the amphibious fish *Kryptolebias marmoratus*

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### SUMMARY

The skin-breathing amphibious fish *Kryptolebias marmoratus* experiences rapid environmental changes when moving between water- and air-breathing, but remodelling of respiratory morphology is slower (~1 week). We tested the hypotheses that (1) there is a trade-off in respiratory function of gills displaying aquatic *versus* terrestrial morphologies and (2) rapidly increased gill ventilation is a mechanism to compensate for reduced aquatic respiratory function. Gill surface area, which varied inversely to the height of the interlamellar cell mass, was increased by acclimating fish for 1 week to air or low ion water, or decreased by acclimating fish for 1 week to hypoxia (~20% dissolved oxygen saturation). Fish were subsequently challenged with acute hypoxia, and gill ventilation or oxygen uptake was measured. Fish with reduced gill surface area increased ventilation at higher dissolved oxygen levels, showed an increased critical partial pressure of oxygen and suffered impaired recovery compared with brackish water control fish. These results indicate that hyperventilation, a rapid compensatory mechanism, was only able to maintain oxygen uptake during moderate hypoxia in fish that had remodelled their gills for land. Thus, fish moving between aquatic and terrestrial habitats may benefit from cutaneously breathing oxygen-rich air, but upon return to water must compensate for a less efficient branchial morphology (mild hypoxia) or suffer impaired respiratory function (severe hypoxia).

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### INTRODUCTION

Adaptive phenotypic plasticity occurs when an organism can produce a range of relatively fit alternative phenotypes in response to the environment. Alternative physiological phenotypes can be expressed relatively quickly in response to an environmental change, while morphological changes often take longer (Piersma and van Gils, 2011). Therefore, the advantages of plasticity may be limited by lag-times between experiencing environmental change and remodelling the phenotype accordingly (DeWitt et al., 1998; Pigliucci, 2001; Callahan et al., 2008; Auld et al., 2010). Plasticity of functionally related traits, however, may be temporally integrated in order to cope with the environmental change immediately and over time (Schlichting and Pigliucci, 1998; Pigliucci, 2001; Relyea, 2003). Amphibious fishes experience abrupt environmental changes when moving between aquatic and terrestrial habitats. Can the temporal integration of physiological and morphological respiratory phenotypes ease the transition between respiratory media?

Amphibious fishes require unique gills that can tolerate exposure to both water and air. During air exposure (emersion), gills are vulnerable to desiccation and, when the lamellae are unsupported by water, these collapse and coalesce, which can cause permanent damage. Therefore, many amphibious fishes have gills with relatively short and thick lamellae that are able to function during frequent transitions between air and water with few morphological adjustments (Graham, 1997; Sayer, 2005). Conversely, the Amazonian fish *Arapaima gigas* dramatically and permanently

remodel their gills, reducing surface area, during ontogeny as the fish transition from obligate water breathers to obligate air breathers (Brauner et al., 2004). The amphibious mangrove rivulus (*Kryptolebias marmoratus* Poey 1880; formerly mangrove killifish *Rivulus marmoratus*) is able to reversibly remodel its gill morphology in response to air exposure by developing a mass of cells between the lamellae, the interlamellar cell mass (ILCM) (Ong et al., 2007). The ILCM develops after 7 days in air and reduces functional gill surface area, likely to protect the delicate lamellae or reduce water loss (Ong et al., 2007). Frequent voluntary emersions can also cause ILCM enlargement (Turko et al., 2011). In the wild, mangrove rivulus typically inhabit extremely hypoxic (<1 mg l<sup>-1</sup>) crab burrows and puddles, but will emerge if hypoxia becomes too severe or in response to elevated hydrogen sulfide, hunger or aggression (Abel et al., 1987; Davis et al., 1990; Taylor, 1990; Dunson and Dunson, 1999). Emersions can last upwards of 2 months (Taylor, 1990), during which time oxygen is obtained through cutaneous respiration (Grizzle and Thiyagarajah, 1987). In addition, mangrove rivulus are synchronous self-fertilizing hermaphrodites capable of producing large numbers of isogenic offspring, making it possible to isolate environmental effects on phenotype (Harrington, 1961; Vrijenhoek, 1985; Tateronkov et al., 2010). The ability to reversibly remodel their gills combined with their unique reproductive strategy makes mangrove rivulus an excellent model for studying respiratory trade-offs and physiological compensatory mechanisms during the transition between aquatic and terrestrial habitats.

To assess the physiological compensation for reduced gill surface area, we studied the hypoxic ventilatory response and oxygen uptake ability of mangrove rivulus expressing different gill morphologies. In water, fishes typically respond to environmental hypoxia initially by increasing activity and using avoidance behaviour (Chapman and McKenzie, 2009). If this fails, an acute hypoxic ventilatory response occurs within seconds to minutes: hyperventilation of the gills increases oxygen uptake by increasing the frequency and/or amplitude of opercular movements (Perry et al., 2009; Porteus et al., 2011). In the few other fishes capable of reversible ILCM development, the water-breathing goldfish (*Carassius auratus*) and crucian carp (*C. carassius*), large ILCMs increased the critical partial pressure of oxygen ( $P_{crit}$ ), the level of dissolved oxygen (DO) at which oxygen uptake can no longer be maintained (Sollid et al., 2003; Fu et al., 2011). Despite this, the sensitivity of the hypoxic ventilatory response in goldfish was not affected (Tzaneva and Perry, 2010). However, these fish do not need to cope with sudden transitions between respiratory media. Mangrove rivulus rapidly switch between air- and water-breathing, and the ability to adjust gill ventilation rates may be a strategy to moderate any negative consequences of reduced gill surface area (large ILCMs) acquired during acclimation to terrestrial conditions. This would be especially important during the lag period after fish return to water and prior to the gills being remodelled for water breathing.

We hypothesised that there is a trade-off in the aquatic respiratory function of mangrove rivulus acclimated to terrestrial habitats as a result of changes in functional gill surface area. Furthermore, we hypothesised that rapidly modifiable physiological mechanisms are mobilized to compensate for the costs of gill remodelling during the transition between respiratory media. Therefore, we tested the predictions that (1) fish with low gill surface area will have a more sensitive hypoxic ventilatory response, (2) physiological (ventilation) and morphological (gill surface area) respiratory phenotypes are integrated to maintain oxygen uptake (unchanged  $P_{crit}$  and regulation index) and (3) recovery from hypoxia will be unaffected by reduced gill surface area. Alternatively, if  $P_{crit}$  is increased or recovery from hypoxia is impaired in fish with reduced gill surface area, it would suggest that increased ventilation cannot compensate for the lag-time effects of gill remodelling.

## MATERIALS AND METHODS

### Experimental animals

*Kryptolebias marmoratus* hermaphrodites (DAN06 strain, at least 6 months of age, 0.04–0.11 g, originating from Belize) (Tatarenkov et al., 2010) were obtained from the breeding colony maintained in the Hagen Aqualab, University of Guelph. Fish were held individually in 120 ml semi-transparent plastic containers (FisherBrand Collection Containers, Fisher Scientific, Ottawa, ON, Canada) under constant conditions (25°C, 15–18‰, pH 8.1, DO at 100% saturation ≈ 160 Torr, 12h:12h light:dark cycle) (Frick and Wright, 2002). Water changes were performed weekly using artificial seawater (Instant Ocean Synthetic Sea Salt, Blacksburg, VA, 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 Utilisation Protocol 10G008).

### Experimental protocol

#### Treatment conditions

In preliminary experiments, we established that gill morphology could be altered not just by air exposure (Ong et al., 2007), but also by aquatic hypoxia and ion composition. Therefore, mangrove

rivulus were acclimated (25°C) for 7 days prior to experimentation to brackish water (control: 15‰ Instant Ocean Synthetic Sea Salt,  $\text{Na}^+=7.50 \text{ mmol l}^{-1}$ ,  $\text{Ca}^{2+}=4.35 \text{ mmol l}^{-1}$ ,  $\text{Mg}^{2+}=7.05 \text{ mmol l}^{-1}$ ,  $\text{Cl}^-=4.8 \text{ mmol l}^{-1}$ , pH 8.1) or one of three treatment conditions: air exposure, chronic hypoxia (15‰, DO  $18.1 \pm 0.3\%$  sat ≈ 30 Torr) or low-ion water (1‰ Instant Ocean Synthetic Sea Salt,  $\text{Na}^+=0.50 \text{ mmol l}^{-1}$ ,  $\text{Ca}^{2+}=0.29 \text{ mmol l}^{-1}$ ,  $\text{Mg}^{2+}=0.47 \text{ mmol l}^{-1}$ ,  $\text{Cl}^-=0.32 \text{ mmol l}^{-1}$ , pH 8.1). Air-acclimated fish were maintained on moist filter paper (15‰ brackish water) in plastic rearing containers (Ong et al., 2007). For other treatments, fish were held in standard rearing containers within an aerated recirculating apparatus (50l), which prevented fish from voluntarily emerging, a behaviour known to cause gill remodelling (Turko et al., 2011). Hypoxia was achieved by introducing a finely regulated flow of compressed nitrogen rather than air into the system to reduce the level of DO (Regan et al., 2011). DO was measured daily (Hach LDO101 electrode connected to Hach HQ30d metre, Hach Company, Mississauga, ON, Canada) and averaged  $18.1 \pm 0.3\%$  DO saturation (mean  $\pm$  s.e.m) over the course of the experiment. All fish were fasted for the duration of the acclimation period, as mangrove rivulus are unable to feed while emersed. The acute response to hypoxia and metabolic rate experiments (detailed below) were conducted using the same water in which fish were acclimated; air-acclimated fish were tested in control water (15‰).

### Acute ventilatory response to hypoxia

Ventilatory movements were quantified non-invasively by detecting impedance changes caused by opercular displacement in a custom-built ventilation recording chamber (modified from Altimiras and Larsen, 2000; Vulesevic et al., 2006). Chambers consisted of standard 1 ml syringes (BD Syringe, Franklin Lakes, NJ, USA) cut to 40 mm in length, with two solder-coated copper wires inserted through small holes 15 mm from the tapered end of the syringe and sealed with epoxy. These electrodes were connected, via an amplifier (Vernier Instrumentation Amplifier, Vernier Software and Technology, Beaverton, OR, USA) set at 0–200 mV, to an impedance converter (Model 2991, Biocom, Culver City, CA, USA). The amplified impedance signals were digitized (Vernier LabPro, Vernier Software and Technology) and recorded (20 Hz) on a computer (LoggerPro 3.8, Vernier Software and Technology). To validate these impedance data, fish were video recorded (Logitech Quickcam Pro, Fremont, CA, USA) at a wide range of ventilation frequencies (10–147 ventilations  $\text{min}^{-1}$ ;  $N=5$ ) while inside the ventilation chamber. The resulting video recordings and impedance data files were randomised to blind the observer (A.T.), and ventilation frequencies were measured. Linear regression of impedance data plotted against video-recorded values showed that the two measurement techniques were highly similar ( $R^2=0.99$ ,  $P<0.001$ ).

Water (25°C) was circulated through the ventilation recording chamber at  $\sim 5 \text{ ml min}^{-1}$ , a rate sufficient to maintain water DO in the chamber. Fish were left to acclimate for at least 1 h in the ventilation chamber before exposure to stepwise hypoxia. Oxygen concentrations in water supplying the recording chamber were maintained using a DIGAMIX gas mixing pump (Wösthoff, Dortmund, Germany) and monitored with an oxygen electrode (Vernier DO-BTA, Vernier Software and Technology). Observations were initiated when ventilatory frequency had reduced to  $<10 \text{ ventilations min}^{-1}$ , as preliminary experiments showed that this level of ventilation was typical of control fish. Ventilation measurements were recorded for 10 min at each of the following DO levels: 100, 50, 40, 30, 20 and 10% DO saturation, followed

by recovery measurements at 40 and 100% DO saturation. The gas mixture was adjusted to the next step in the series immediately after each recording period and DO was allowed to stabilise for 10 min before the next ventilation measurement. Preliminary experiments using an oxygen microelectrode (Unisense OX-25, Aarhus, Denmark) inserted into the ventilation chamber showed no detectable difference between oxygen levels in the reservoir and the chamber within 7–8 min of changing the gas mixture; therefore, fish were exposed to the new oxygen level for ~2–3 min before recording of ventilation began. Measurements were gathered between 11:00 and 17:00 h each day to minimize the impact of the diel metabolic cycle (Rodela and Wright, 2006). After each experiment, fish were immediately euthanized (2 ml<sup>-1</sup> 2-phenoxyethanol) and fixed in 10% neutral buffered formalin for histological processing.

Preliminary results indicated that most fish acclimated to both air and low ion water initiated a ventilatory response at water DO levels greater than 50%. In order to more accurately assess the oxygen level at which a respiratory response occurred in this group of fish, a second experiment was conducted. Fish were acclimated and ventilation rates were measured as described previously, but instead of immediately reducing DO saturation to 50%, steps of 100, 90, 80, 70, 60, 50, 40, 30 and 10% DO saturation were used. Fish were fixed for histology immediately after each experiment.

Ventilatory frequency ( $f_V$ ), relative amplitude ( $A_V$ ), gill ventilation ( $V_G$ ) and apnoea were quantified at each level of hypoxia. Frequency was quantified by counting the number of voltage peaks recorded over the 10 min recording period. Amplitude recordings varied based on the size of each fish and its precise location within the recording chamber, and therefore only a relative measure of amplitude could be assessed. The amplitude of ventilatory movements at 10% DO was taken to be maximal, and the ventilatory amplitude at all other oxygen concentrations was calculated as the proportion of this value. Visual observations of fish exposed to 10% DO suggested that these fish were ventilating at maximum capacity, and prolonged exposure to this degree of hypoxia was potentially lethal (Davis et al., 1990). Relative gill ventilation was calculated as the product of  $f_V$  and  $A_V$ .

#### Oxygen regulation and $P_{crit}$

Oxygen consumption rates,  $P_{crit}$  and the regulation index (RI) were measured using closed glass respirometry chambers (~12 ml, 25°C) in which an electrode (Vernier DO-BTA) continuously measured DO (modified from Rodela and Wright, 2006). Chambers were sealed immediately after fish were inserted, and oxygen consumption was recorded over a 2–4 h period until DO dropped below 5% of saturation. Background oxygen consumption by the electrodes was measured in chambers without fish for 1 h immediately prior to each experiment and this value was subtracted from the oxygen consumption values of the fish.

Oxygen consumption was calculated as  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$  by measuring the slope of the oxygen consumption curve at 100, 75, 50, 40, 30, 20, 10 and 5% DO saturation.  $P_{crit}$  was calculated using Jeffrey Muday's Physiological Regulation and Conformation program (available at [www.wfu.edu/~mudayja/software/o2.exe](http://www.wfu.edu/~mudayja/software/o2.exe)), which uses Yeager and Ultsch's (Yeager and Ultsch, 1989) algorithm. The RI, a measure of the degree to which an organism regulates its oxygen uptake independently of environmental oxygen (RI=1) or conforms to environmental oxygen levels (RI=0), was calculated according to the protocol of Mueller and Seymour (Mueller and Seymour, 2011).

#### Gill histology

Euthanized fish were fixed in 10% buffered formalin for 24 h, embedded in paraffin and routinely processed for haematoxylin and eosin staining as described previously (Turko et al., 2011). Slides were viewed using light microscopy (Nikon Eclipse 90i epifluorescent microscope, Nikon, Melville, NY, USA) and measurements were taken digitally (NIS Elements software, Nikon). The height of the ILCM was measured parallel to the lamellae, from the edge of the filament to the most distal extension of the ILCM along the lamellae (Ong et al., 2007). Functional gill surface area is inversely related to the height of the ILCM, as the ILCM occupies the space between adjacent lamellae and determines the proportion of each lamella exposed to the environment. The ILCMs adjacent to five randomly selected lamellae from each gill arch were measured, and the 20 measurements were averaged to provide a mean value for each individual (LeBlanc et al., 2010).

#### Statistical analysis

A one-factor ANOVA with *post hoc* Holm–Sidak tests was used to test whether treatments had a significant effect on ILCM height,  $P_{crit}$  or RI. A two-factor repeated-measures ANOVA with *post hoc* Holm–Sidak tests was used to compare ventilatory parameters and oxygen consumption relative to both normoxia (100% DO saturation) and brackish water controls. Data were transformed where necessary to better meet homoscedasticity and distribution assumptions. A square-root transformation ( $\sqrt{+3}$ ) was used for  $f_V$ , while natural logarithm transformations ( $\ln+1$ ) were used for quantitative variables ( $A_V$ ,  $V_G$  and oxygen consumption) (Zar, 1998). Apnoea was measured as a proportion; therefore, these data were arcsin transformed (Zar, 1998). SigmaStat 3.5 or SigmaPlot 11 (Systat Software, San Jose, CA, USA) were used for all analyses (critical  $\alpha=0.05$ ). Throughout the text, values are given as means  $\pm$  s.e.m.

## RESULTS

### Gill histology

Treatment conditions had significant effects on gill morphology (Fig. 1). A substantial cell mass between lamellae with a distinct cleft at approximately the midpoint of the ILCM was observed in fish acclimated for 7 days to either air or low ion water (Fig. 1B,C), relative to control fish (Fig. 1A). The height of the ILCM of air- and low-ion-water-acclimated fish was significantly increased relative to brackish water controls by 34% ( $P<0.0001$ ) and 24% ( $P<0.01$ ), respectively (Fig. 1E). Conversely, hypoxia acclimation significantly reduced ILCM height by ~45% ( $P<0.0005$ ; Fig. 1D,E).

### Acute hypoxic ventilatory response

In normoxia, mangrove rivulus exhibited episodic breathing, ventilating infrequently in short bursts (Fig. 2A). As the severity of hypoxia increased, ventilatory bursts increased in duration and the apnoeic periods between them shortened (data not shown), until fish were ventilating continuously (Fig. 2B). In response to acute hypoxia, control fish significantly increased  $f_V$  at 40% DO saturation ( $P<0.05$ ; Fig. 3A), while  $A_V$  was not significantly increased until 10% DO saturation ( $P<0.05$ ; Fig. 3B). A significant increase in  $V_G$  occurred at 40% DO saturation ( $P<0.05$ ; Fig. 3C).

Fish acclimated to air for 7 days showed an increased sensitivity to hypoxia compared with controls. Air-acclimated fish significantly increased  $f_V$ ,  $A_V$  and  $V_G$  over normoxic rates at 50, 20 and 50% DO saturation, respectively (compared with 40, 10 and 40% in the control group;  $P<0.05$ ; Fig. 3). Air-acclimated fish also had significantly higher  $f_V$  relative to controls at 50, 40 and 30% DO



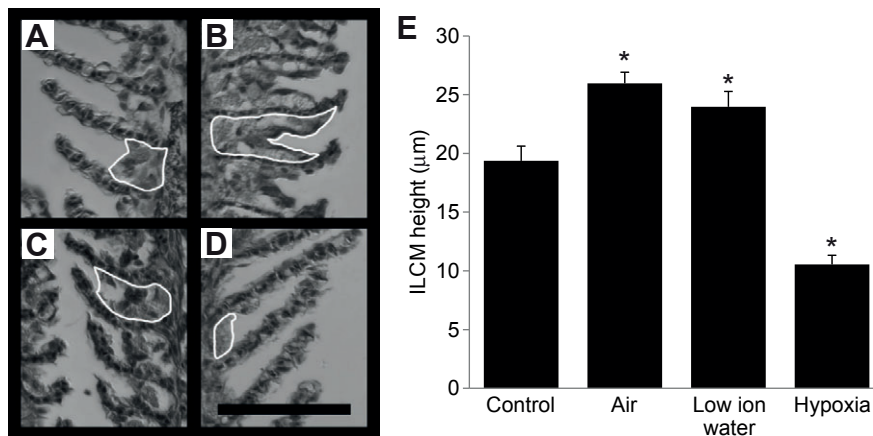


Fig. 1. Representative light micrographs of *Kryptolebias marmoratus* gill morphology in fish acclimated for 7 days to (A) 15‰ brackish water (control), (B) air, (C) low ion water and (D) hypoxia. One interlamellar cell mass (ILCM) in each acclimation condition is outlined in white. Scale bar, 50 µm. The histogram (E) depicts the height of the ILCM in gills of *K. marmoratus* acclimated for 7 days to control ( $N=8$ ), air ( $N=16$ ), low ion water ( $N=16$ ) and hypoxia ( $N=8$ ). Asterisks denote a significant difference from the brackish water control group ( $P<0.05$ ). Values are means  $\pm$  s.e.m.

saturation ( $P<0.05$ ; Fig. 3A), while  $V_G$  was significantly elevated compared with control fish at 50% DO saturation ( $P<0.05$ ; Fig. 3C). After recovery, the  $f_V$  and  $V_G$  of air-acclimated fish remained significantly increased compared with pre-hypoxic exposure normoxic values ( $P<0.05$ ; Fig. 3A,C) and ventilation remained significantly elevated for at least 1 h after the return to normoxia (data not shown), compared with control fish that returned to pre-hypoxic exposure ventilation rates within 10 min (Fig. 3). Similar to air-acclimated fish, mangrove rivulus acclimated to low ion water showed an increased sensitivity to hypoxia relative to controls. Low-ion-water-acclimated fish significantly increased  $f_V$ ,  $A_V$  and  $V_G$  from normoxic values at 50% DO saturation ( $P<0.05$ ; Fig. 3).  $f_V$  and  $V_G$  were also significantly higher in low-ion-water-acclimated fish than control fish at 50 and 40% DO saturation ( $P<0.05$ ; Fig. 3A,C). After recovery, the  $f_V$  and  $V_G$  of low-ion-water-acclimated fish remained significantly increased compared with pre-hypoxic exposure normoxic values ( $P<0.05$ ; Fig. 3A,C). These values remained significantly elevated for at least 1 h after the return to normoxia (data not shown), whereas control fish returned to pre-hypoxic ventilatory rates within 10 min (Fig. 3). Hypoxia-acclimated fish showed significantly decreased  $V_G$  at 30% DO saturation relative to brackish water controls ( $P<0.05$ ; Fig. 3C).

Follow-up studies to determine the ventilatory response of air and low-ion-water-acclimated fish on a finer scale (i.e. 100 through 50% DO saturation) showed that air-acclimated fish significantly

increased  $f_V$  and  $V_G$  relative to normoxia at 70% DO saturation ( $P<0.05$ ; supplementary material Fig. S1), and low-ion-water-acclimated fish increased  $f_V$  and  $V_G$  at 80% DO saturation ( $P<0.05$ ; supplementary material Fig. S1), compared with 40% DO saturation in control fish.

Variation in apnoea followed the inverse pattern to ventilation. At 50% DO saturation, both air- and low-ion-water-acclimated fish exhibited apnoea for significantly less time than control fish ( $P<0.05$ ; Fig. 4). Similarly, the duration of apnoea after recovery to normoxia in these groups was significantly lower than controls and relative to pre-exposure normoxic values ( $P<0.05$ ; Fig. 4). Hypoxia-acclimated fish exhibited significantly more apnoea at 50% DO saturation relative to brackish water controls ( $P<0.05$ ; Fig. 4).

#### Oxygen regulation and $P_{crit}$

Normoxic oxygen consumption rates were approximately equal across treatments (Fig. 5A). Fish acclimated to air or low ion water for 7 days had significantly lower rates of oxygen consumption relative to control fish at 10% DO saturation ( $P<0.05$ ; Fig. 5A). The  $P_{crit}$  of air- and low-ion-water-acclimated fish was 2.7- and 2-fold higher than control fish, respectively ( $P<0.05$ ; Fig. 5B), while there was no change in the  $P_{crit}$  of hypoxia acclimated fish ( $P>0.05$ ; Fig. 5B). There was no significant difference in the RI between any treatment and brackish water control fish ( $P>0.05$ ; Fig. 5C).

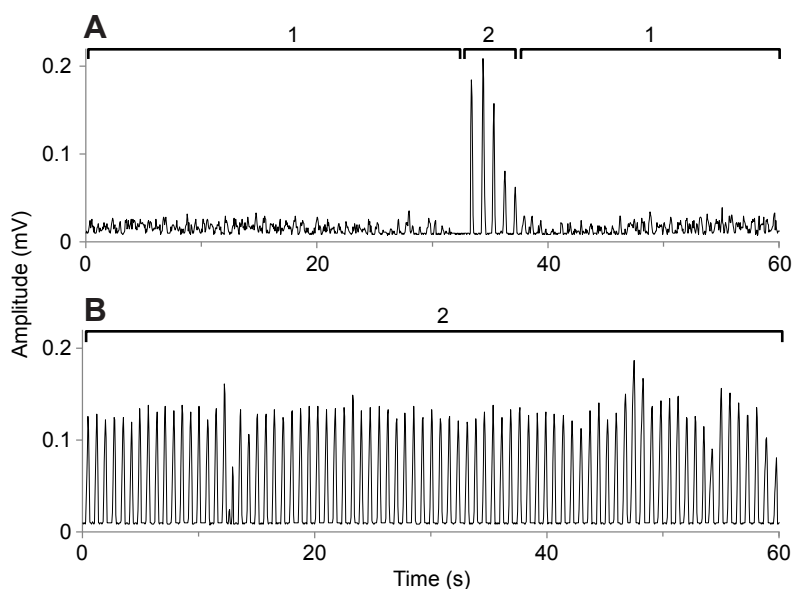


Fig. 2. Representative raw ventilatory impedance recordings illustrating *Kryptolebias marmoratus* in (A) normoxic water and (B) hypoxic water. The label '1' represents apnoea and '2' represents opercular movements (ventilation).

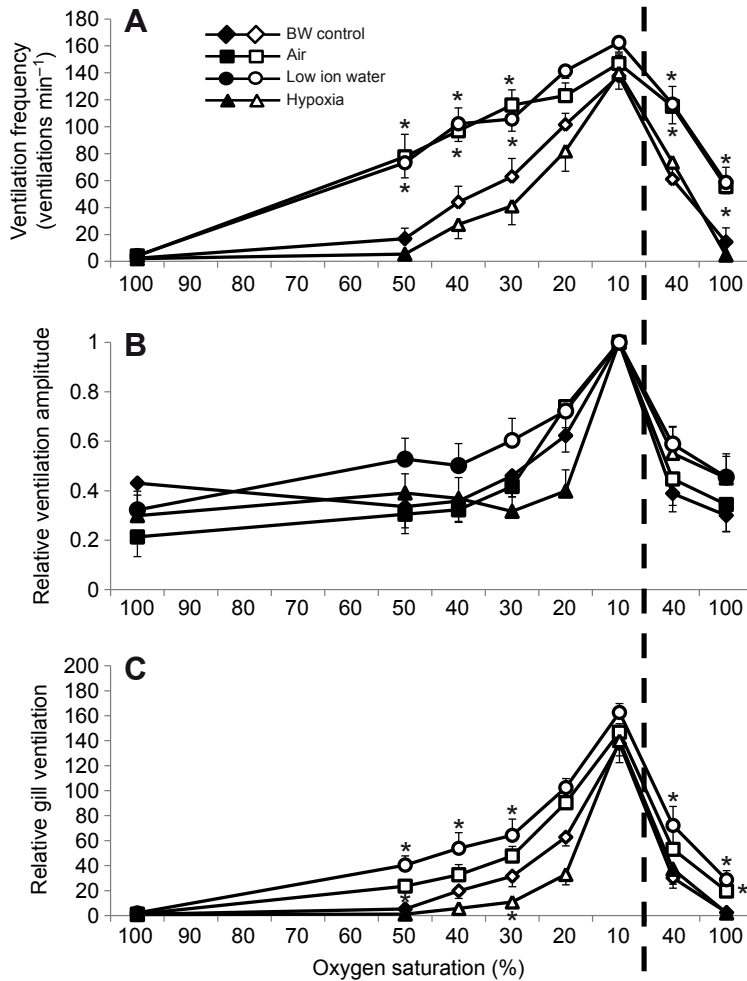


Fig. 3. (A) Ventilatory frequency, (B) relative ventilatory amplitude and (C) relative gill ventilation of *Kryptolebias marmoratus* acclimated for 7 days to air (squares,  $N=8$ ), low ion water (circles,  $N=8$ ) or hypoxia ( $\sim 20\%$  dissolved oxygen saturation; triangles,  $N=8$ ) compared with the responses of control fish acclimated to 15‰ brackish water (BW; diamonds,  $N=8$ ). Open symbols denote a significant difference from initial normoxic (100% oxygen saturation) values ( $P < 0.05$ ). Asterisks denote a significant difference from the control group at a given oxygen saturation ( $P < 0.05$ ). The vertical dashed line indicates the transition between hypoxia exposure and recovery to normoxia. Values are means  $\pm$  s.e.m.

## DISCUSSION

Our findings demonstrate that gill remodelling in mangrove rivulus had significant negative consequences for aquatic respiratory function and hypoxia tolerance. Fish acclimated for 7 days to air or low ion water increased ventilation at a higher level of DO relative to control fish, and maintained significantly elevated ventilation rates as oxygen levels were progressively lowered. This enhanced ventilatory activity maintained oxygen uptake initially (above  $\sim 50\%$  DO saturation), but the significantly higher  $P_{crit}$  we observed in these fish suggests that increased ventilation was insufficient to compensate for reduced gill surface area at more extreme levels of hypoxia. Additionally, ventilatory activity did not immediately recover to pre-exposure levels in air- and low-ion-water-acclimated fish as it did in controls, suggesting that an oxygen debt had accumulated (Heath, 1973; Rantin et al., 1998). This provides further evidence that increased ventilation could not fully compensate for the negative effects of enlarged ILCMs at levels of DO between 50 and 10% of saturation. On the one hand, these results support our first prediction that gill remodelling negatively impacts hypoxia tolerance in mangrove rivulus. On the other hand, the data do not support the prediction that integration of ventilatory and morphological respiratory phenotypes maintains oxygen consumption. Instead, these results support the alternative hypothesis that there is a lag-time limit to gill remodelling that can only be partially moderated with a more sensitive hypoxic ventilatory response. Branchial respiratory function in mangrove rivulus acclimated to terrestrial conditions

appears to be limited in hypoxic aquatic environments until gill morphological changes can occur.

Theory predicts that if the costs of gill remodelling are large, then adaptive phenotypic plasticity should only evolve if transitions between environments occur relatively infrequently (León, 1993; Padilla and Adolph, 1996). In the wild, however, mangrove rivulus emerge relatively frequently on daily and seasonal time scales (Taylor, 1990; Taylor et al., 2008). Is the theory wrong or do rivulus compromise under field conditions? A blunted degree of gill plasticity would maintain aquatic respiratory function, but at a cost of potentially increasing the risks of desiccation or lamellar damage during emersion (Ong et al., 2007). We have previously shown that under laboratory conditions where mangrove rivulus were raised in oxygen-saturated water, frequent and voluntary transitions between terrestrial and aquatic habitats caused ILCM enlargement (Turko et al., 2011). Thus, profound gill remodelling occurs even when emersions are of short duration. Further studies assessing the variability of environmental parameters in the field and the costs and benefits of gill remodelling under these conditions are required.

Our results imply that gill surface area significantly affects the oxygen uptake of water-breathing mangrove rivulus. Similarly, Sakuragui et al. (Sakuragui et al., 2003) found that increased ventilation could not maintain arterial oxygen partial pressure in obligately water-breathing wolf fish (*Hoplias malabaricus*) possessing gills with increased blood–water diffusion distances caused by ionocyte proliferation, although  $P_{crit}$  was unaffected in this species. Together with our data, these results suggest that

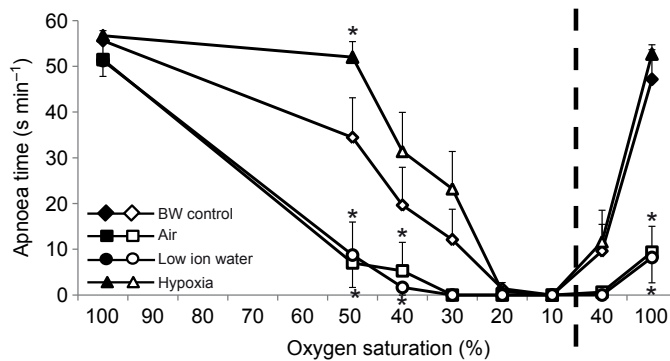


Fig. 4. Proportion of time (s min<sup>-1</sup>) *Kryptolebias marmoratus* exhibited apnoea following acclimation for 7 days to air (squares, N=8), low ion water (circles, N=8) or hypoxia (~20% dissolved oxygen saturation; triangles, N=8) compared with control fish acclimated to 15‰ brackish water (BW; diamonds, N=8). Open symbols denote a significant difference from initial normoxic (100% oxygen saturation) values (P<0.05). Asterisks denote a significant difference from the control group at a given oxygen saturation (P<0.05). The vertical dashed line indicates the transition between hypoxia exposure and recovery to normoxia. Values are means ± s.e.m.

increased gill ventilation may only compensate for morphological impairment during moderate hypoxia, because under extreme hypoxia the cost of hyperventilation could exceed any gains made in oxygen uptake (Perry and Gilmour, 2002). It is interesting, however, that *H. malabaricus* were able to maintain  $P_{crit}$  while the facultatively air-breathing mangrove rivulus did not. This suggests that perhaps the trade-off between gill morphology and aquatic respiratory function is influenced by the availability of alternate respiratory strategies available to different taxa (Graham, 1997).

There are other quickly altered phenotypes besides hyperventilation that fishes may use to respond to hypoxia (Chapman

and McKenzie, 2009). For example, mangrove rivulus emerge in response to extreme hypoxia ( $EC_{50}$ =3% DO saturation) (Regan et al., 2011). The threshold for emersion is well below the  $P_{crit}$  (~18% DO saturation), however, and may be preceded by the use of aquatic surface respiration (ASR) (A.J.T., personal observation). ASR allows fishes to exploit the relatively high DO that occurs at the air–water interface, but also may increase the risk of predation, and therefore ASR often does not begin until oxygen levels drop to near  $P_{crit}$  (Kramer and McClure, 1982; Sloman et al., 2006; Perry et al., 2009). Like the mangrove rivulus, inanga (*Galaxias maculatus*) respond to progressive hypoxia first by hyperventilating and utilising ASR, but if environmental oxygen drops below  $P_{crit}$  these fish emerge and switch to air breathing (Urbina et al., 2011). Thus it appears that emersion is used as a last resort. Functional gill surface area may impact the emersion threshold of mangrove rivulus, an interesting question for future investigations.

We observed significant increases in the ILCMs of fish exposed to air or low ion water, as has been previously observed (Ong et al., 2007; LeBlanc et al., 2010). We also found that 7 days of hypoxic acclimation significantly decreased the height of the ILCM in mangrove rivulus. While this is the first report of hypoxia-induced gill plasticity in *K. marmoratus*, this response was not surprising based on previous studies in hypoxia-acclimated cyprinid fishes, where an increase in gill surface area would likely facilitate oxygen uptake (Sollid et al., 2003; Matey et al., 2008; Mitrovic et al., 2009). Enlarged ILCMs during acclimation to low ion water is thought to reduce ion loss at the expense of reduced respiratory function according to the osmorepiratory compromise hypothesis (Gonzalez and McDonald, 1992; LeBlanc et al., 2010).

The resting ventilatory frequencies observed in this study were remarkably low compared with those of other tropical teleosts (Perry et al., 2009), but share the characteristic pattern of episodic breathing with fishes having low respiratory demands (Milsom, 1991). Episodic breathing is thought to minimize the energetic cost of respiration while maintaining oxygen uptake (Milsom, 1991).

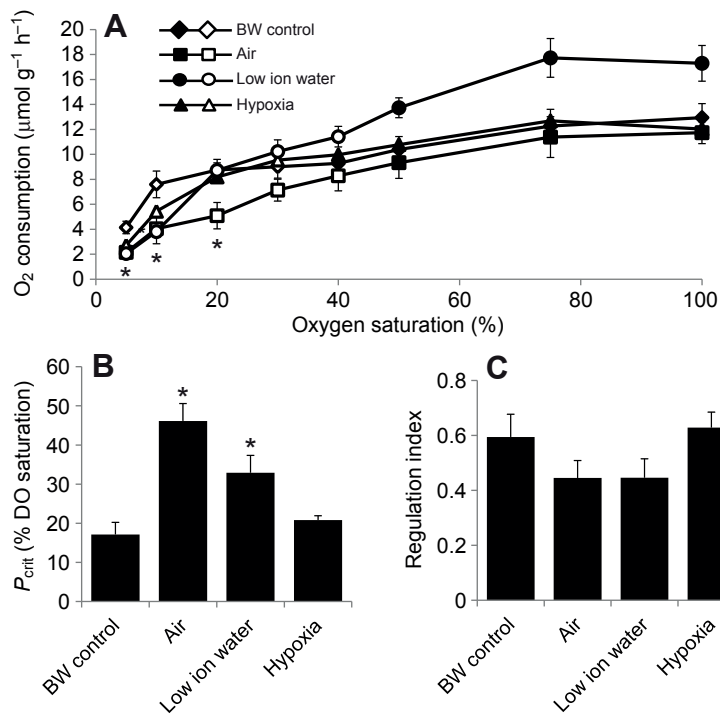


Fig. 5. (A) Oxygen uptake ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ ) of *Kryptolebias marmoratus* acclimated for 7 days to air (squares, N=8), low ion water (circles, N=8) or hypoxia (~20% dissolved oxygen saturation; triangles, N=8) compared with control fish acclimated to 15‰ brackish water (BW; diamonds, N=8). Open symbols denote a significant difference from initial normoxic (100% oxygen saturation) values (P<0.05). Asterisks denote a significant difference from the control group at a given oxygen saturation (P<0.05). (B) Critical oxygen tension ( $P_{crit}$ ) of *K. marmoratus* acclimated for 7 days to 15‰ BW control conditions (N=8), air (N=8), low ion water (N=8) and hypoxia (N=8). Asterisks denote a significant difference from the control group (P<0.05). (C) Regulation index of *K. marmoratus* acclimated for 7 days to 15‰ BW control conditions (N=8), air (N=8), low ion water (N=8) and hypoxia (N=8). A value of 1 represents perfect regulation of oxygen consumption independent of environmental levels, while a value of 0 represents perfect conformity. Values are means ± s.e.m.



Perhaps mangrove rivulus are able to accomplish such low ventilatory frequencies under normoxia because of the presence of dense epidermal capillary beds, which allow them to respire cutaneously (Grizzle and Thiyagarajah, 1987). The tropical amphibious swamp eel *Synbranchus marmoratus* (~80 g) was able to achieve an average aquatic  $f_V$  of 1–4 ventilations  $\text{min}^{-1}$  in normoxia by obtaining 33–53% of required oxygen *via* the skin (Graham et al., 1987). Cutaneous oxygen uptake scaled with mass in *S. marmoratus* and could theoretically account for 100% of oxygen requirements in a 10 g fish. Mangrove rivulus used in the present study weighed ~100-fold less than this theoretical size limit, and should therefore be able to predominantly use cutaneous respiration; indeed, this appears to be the case during emersion (Ong et al., 2007; Cooper et al., 2012). Therefore, while the scope of ventilatory increase in these fish appears remarkable (~50-fold  $f_V$  increase and ~100-fold  $V_G$  increase!), we propose that it is the result of low resting ventilation enabled by cutaneous respiration. Moreover, the maximum ventilation frequency observed in mangrove rivulus was low compared with that of other similarly sized tropical species (e.g. ~150 ventilations  $\text{min}^{-1}$  in mangrove rivulus *versus* 350 ventilations  $\text{min}^{-1}$  in zebrafish, *Danio rerio*) (Vulesevic and Perry, 2006).

Our results indicate that the sensitivity of the mangrove rivulus hypoxic ventilatory response is dependent on the size of the ILCM. This is in contrast to the findings of Tzaneva and Perry (Tzaneva and Perry, 2010), who found that ILCM height did not affect the hypoxia sensitivity of *C. auratus*. However, goldfish with large ILCMs were tested at a colder temperature than those with small ILCMs, raising the possibility that differences in oxygen demand may have been a contributing factor (Tzaneva and Perry, 2010). In contrast, the oxygen demand of mangrove rivulus with different gill morphologies was equal, suggesting that the height of the ILCM rather than oxygen demand influenced the sensitivity of the hypoxic ventilatory response.

Fish with reduced gill surface area had significantly increased  $P_{\text{crit}}$  values compared with control fish, suggesting that gill remodelling imposes a dramatic respiratory cost on mangrove rivulus. Enlarged ILCMs in *C. carassius* and *C. auratus* also have been correlated with an increased  $P_{\text{crit}}$  compared with fish with smaller ILCMs (Sollid et al., 2003; Fu et al., 2011). As well, many fish populations inhabiting relatively oxygen-poor habitats have both enlarged gills and reduced  $P_{\text{crit}}$  compared with conspecifics from well-oxygenated habitats (e.g. Olowo and Chapman, 1996; Chapman et al., 2002; Timmerman and Chapman, 2004; Mandic et al., 2009). Given the impact of enlarged ILCMs, it was surprising that the  $P_{\text{crit}}$  of hypoxia-acclimated mangrove rivulus did not change with decreased ILCM height. However, hypoxia-acclimated fish exhibited significantly more apnoea at 50% DO saturation and decreased  $V_G$  at 30% DO saturation, suggesting that the increased gill surface area may reduce the energy expenditure required to ventilate under moderate hypoxia. Gill surface area had no significant impact on the RI of mangrove rivulus, although the trend ( $P=0.11$ ) suggests that fish with enlarged ILCMs were possibly less able to regulate oxygen compared with control or hypoxia-acclimated fish (Mueller and Seymour, 2011).

Overall, these results indicate that gill remodelling in mangrove rivulus is a relatively complex phenomenon, dependent on respiratory medium, environmental ionic composition and oxygen availability. However, any benefits of gill remodelling are limited by the cost of displaying a large ILCM during water breathing, and by the relatively long lag-times required for ILCMs to be modified. Increased gill ventilation was only able to compensate for large

ILCMs during moderate, but not severe, hypoxia. These results highlight some of the difficulties in switching between respiratory media, and demonstrate that extreme phenotypes can have consequences for tolerating rapid environmental change.

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## REFERENCES

- Abel, D. C., Koenig, C. C. and Davis, W. P. (1987). Emersion in the mangrove forest fish *Rivulus marmoratus*: a unique response to hydrogen sulfide. *Environ. Biol. Fishes* **18**, 67–72.
- Altimiras, J. and Larsen, E. (2000). Non-invasive recording of heart rate and ventilation rate in rainbow trout during rest and swimming. Fish go wireless! *J. Fish Biol.* **57**, 197–209.
- Auld, J. R., Agrawal, A. A. and Relyea, R. A. (2010). Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proc. Biol. Sci.* **277**, 503–511.
- Brauner, C. J., Matey, V., Wilson, J. M., Bernier, N. J. and Val, A. L. (2004). Transition in organ function during the evolution of air-breathing: insights from *Arapaima gigas*, an obligate air-breathing teleost from the Amazon. *J. Exp. Biol.* **207**, 1433–1438.
- Callahan, H. S., Maughan, H. and Steiner, U. K. (2008). Phenotypic plasticity, costs of phenotypes, and costs of plasticity: toward an integrative view. *Ann. N. Y. Acad. Sci.* **1133**, 44–66.
- Chapman, L. J. and McKenzie, D. J. (2009). Behavioural responses and ecological consequences. In *Hypoxia* (ed. J. G. Richards, C. J. Brauner and A. P. Farrell), pp. 26–79. San Diego, CA: Academic Press.
- Chapman, L. J., Chapman, C. A., Nordlie, F. G. and Rosenberger, A. E. (2002). Physiological refugia: swamps, hypoxia tolerance, and maintenance of fish biodiversity in the Lake Victoria region. *Comp. Biochem. Physiol.* **133A**, 421–437.
- 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.
- Davis, W. P., Taylor, D. S. and Turner, B. J. (1990). Field observations of the ecology and habits of mangrove rivulus (*Rivulus marmoratus*) in Belize and Florida. *Ichthyol. Explor. Freshw.* **1**, 123–134.
- DeWitt, T. J., Sih, A. and Wilson, D. S. (1998). Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* **13**, 77–81.
- Dunson, W. A. and Dunson, D. B. (1999). Factors influencing growth and survival of the killifish, *Rivulus marmoratus*, held inside enclosures in mangrove swamps. *Copeia* **1999**, 661–668.
- 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.
- Fu, S.-J., Brauner, C. J., Cao, Z.-D., Richards, J. G., Peng, J.-L., Dhillon, R. and Wang, Y.-X. (2011). The effect of acclimation to hypoxia and sustained exercise on subsequent hypoxia tolerance and swimming performance in goldfish (*Carassius auratus*). *J. Exp. Biol.* **214**, 2080–2088.
- Gonzalez, R. J. and McDonald, D. G. (1992). The relationship between oxygen consumption and ion loss in a freshwater fish. *J. Exp. Biol.* **163**, 317–332.
- Graham, J. B. (1997). *Air-Breathing Fishes: Evolution, Diversity, and Adaptation*. San Diego, CA: Academic Press.
- Graham, J. B., Baird, T. A. and Stockmann, W. (1987). The transition to air breathing in fishes IV. Impact of branchial specialisations for air breathing on the aquatic respiratory mechanisms and ventilatory costs of the swamp eel *Synbranchus marmoratus*. *J. Exp. Biol.* **129**, 83–106.
- Grizzle, J. M. and Thiyagarajah, A. (1987). Skin histology of *Rivulus ocellatus marmoratus*: apparent adaptation for aerial respiration. *Copeia* **1987**, 237–240.
- Harrington, R. W., Jr (1961). Oviparous hermaphroditic fish with internal self-fertilization. *Science* **134**, 1749–1750.
- Heath, A. G. (1973). Ventilatory responses of teleost fish to exercise and thermal stress. *Am. Zool.* **13**, 491–503.
- Kramer, D. L. and McClure, M. (1982). Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. *Environ. Biol. Fishes* **7**, 47–55.
- LeBlanc, D. M., Wood, C. M., Fudge, D. S. and Wright, P. A. (2010). A fish out of water: gill and skin remodelling promotes osmo- and ionoregulation in the mangrove killifish *Kryptolebias marmoratus*. *Physiol. Biochem. Zool.* **83**, 932–949.
- Leon, J. A. (1993). Plasticity in fluctuating environments. In *Adaptation in Stochastic Environments* (ed. J. Yoshimura and C. W. Clark), pp. 105–121. Berlin: Springer Verlag.
- Mandic, M., Todgham, A. E. and Richards, J. G. (2009). Mechanisms and evolution of hypoxia tolerance in fish. *Proc. Biol. Sci.* **276**, 735–744.
- Matey, V., Richards, J. G., Wang, Y., Wood, C. M., Rogers, J., Davies, R., Murray, B. W., Chen, X. Q., Du, J. and Brauner, C. J. (2008). The effect of hypoxia on gill morphology and ionoregulatory status in the Lake Qinghai scaleless carp, *Gymnocypris przewalskii*. *J. Exp. Biol.* **211**, 1063–1074.

- Milsom, W. K. (1991). Intermittent breathing in vertebrates. *Annu. Rev. Physiol.* **53**, 87-105.
- Mitrovic, D., Dymowska, A., Nilsson, G. E. and Perry, S. F. (2009). Physiological consequences of gill re-modelling in goldfish (*Carassius auratus*) during exposure to long-term hypoxia. *Am. J. Physiol.* **297**, R224-R334.
- Mueller, C. A. and Seymour, R. S. (2011). The regulation index: a new method for assessing the relationship between oxygen consumption and environmental oxygen. *Physiol. Biochem. Zool.* **84**, 522-532.
- Olowo, J. P. and Chapman, L. J. (1996). Papyrus swamps and variation in the respiratory behaviour of the African fish *Barbus neumayeri*. *Afr. J. Ecol.* **34**, 211-222.
- 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.
- Padilla, D. K. and Adolph, S. C. (1996). Plastic inducible morphologies are not always adaptive: the importance of time delays in a stochastic environment. *Evol. Ecol.* **10**, 105-117.
- Perry, S. F. and Gilmour, K. M. (2002). Sensing and transfer of respiratory gases at the fish gill. *J. Exp. Zool.* **293**, 249-263.
- Perry, S. F., Jonz, M. G. and Gilmour, K. M. (2009). Oxygen sensing and the hypoxic ventilatory response. In *Hypoxia* (ed. J. G. Richards, C. J. Brauner and A. P. Farrell), pp. 194-255. San Diego, CA: Academic Press.
- Piersma, T. and van Gils, J. A. (2011). *The Flexible Phenotype*. Oxford: Oxford University Press.
- Pigliucci, M. (2001). *Phenotypic Plasticity*. Baltimore, MD: The Johns Hopkins University Press.
- Porteus, C., Hedrick, M. S., Hicks, J. W., Wang, T. and Milsom, W. K. (2011). Time domains of the hypoxic ventilatory response in ectothermic vertebrates. *J. Comp. Physiol. B* **181**, 311-333.
- Rantin, F. T., Guerra, C. D. R., Kalinin, A. L. and Glass, M. L. (1998). The influence of aquatic surface respiration (ASR) on cardio-respiratory function of the serrasalmid fish *Piaractus mesopotamicus*. *Comp. Biochem. Physiol.* **119A**, 991-997.
- 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.
- Relyea, R. A. (2003). Predators come and predators go: the reversibility of predator-induced traits. *Ecology* **84**, 1840-1848.
- Rodela, T. M. and Wright, P. A. (2006). Metabolic and neuroendocrine effects on diurnal urea excretion in the mangrove killifish *Rivulus marmoratus*. *J. Exp. Biol.* **209**, 2704-2712.
- Sakuragui, M. M., Sanches, J. R. and Fernandes, M. N. (2003). Gill chloride cell proliferation and respiratory responses to hypoxia of the neotropical erythrinid fish *Hoplias malabaricus*. *J. Comp. Physiol. B* **173**, 309-317.
- Sayer, M. D. J. (2005). Adaptations of amphibious fish for surviving life out of water. *Fish Fish.* **6**, 186-211.
- Schlichting, C. D. and Pigliucci, M. (1998). *Phenotypic Evolution: A Reaction Norm Perspective*. Sunderland, MA: Sinauer Associates.
- Sloman, K. A., Wood, C. M., Scott, G. R., Wood, S., Kajimura, M., Johannsson, O. E., Almeida-Val, V. M. F. and Val, A. L. (2006). Tribute to R. G. Boulter: the effect of size on the physiological and behavioural responses of oscar, *Astronotus ocellatus*, to hypoxia. *J. Exp. Biol.* **209**, 1197-1205.
- Sollid, J., De Angelis, P., Gundersen, K. and Nilsson, G. E. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol.* **206**, 3667-3673.
- 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 specialisations of the cyprinodont fish *Rivulus marmoratus*. *Fla. Sci.* **53**, 239-248.
- 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.
- Timmerman, C. M. and Chapman, L. J. (2004). Hypoxia and interdemec variation in the sailfin molly (*Poecilia latipinna*). *J. Fish Biol.* **65**, 635-650.
- Turko, A. J., Earley, R. L. and Wright, P. A. (2011). Behaviour drives morphology: voluntary emersion patterns shape gill structure in genetically identical mangrove rivulus. *Anim. Behav.* **82**, 39-47.
- Tzaneva, V. and Perry, S. F. (2010). The control of breathing in goldfish (*Carassius auratus*) experiencing thermally induced gill remodelling. *J. Exp. Biol.* **213**, 3666-3675.
- Urbina, M. A., Forster, M. E. and Glover, C. N. (2011). Leap of faith: voluntary emersion behaviour and physiological adaptations to aerial exposure in a non-aestivating freshwater fish in response to aquatic hypoxia. *Physiol. Behav.* **103**, 240-247.
- Vrijenhoek, R. C. (1985). Homozygosity and interstrain variation in the self-fertilizing hermaphroditic fish, *Rivulus marmoratus*. *J. Hered.* **76**, 82-84.
- Vulesevic, B. and Perry, S. F. (2006). Developmental plasticity of ventilatory control in zebrafish, *Danio rerio*. *Respir. Physiol. Neurobiol.* **154**, 396-405.
- Vulesevic, B., McNeill, B. and Perry, S. F. (2006). Chemoreceptor plasticity and respiratory acclimation in the zebrafish *Danio rerio*. *J. Exp. Biol.* **209**, 1261-1273.
- Yeager, D. P. and Uitsch, G. R. (1989). Physiological regulation and conformation: a BASIC program for the determination of critical points. *Physiol. Zool.* **62**, 888-907.
- Zar, J. H. (1998). *Biostatistical Analysis*, 4th edn. Upper Saddle River, NJ: Prentice Hall.