

Phenotypic flexibility in respiratory traits is associated with improved aerial respiration in an
amphibious fish out of water

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Summary: The amphibious fish *Kryptolebias marmoratus* rapidly increases hematocrit and cutaneous angiogenesis (formation of new blood vessels) in response to aerial acclimation, which results in improved respiratory performance out of water.

Abstract

Amphibious fishes have evolved multiple adaptive strategies for respiring out of water, but there has been less focus on reversible plasticity. We tested the hypothesis that when amphibious fishes leave water, enhanced respiratory performance on land is the result of rapid functional phenotypic flexibility of respiratory traits. We acclimated four isogenic strains of *Kryptolebias marmoratus* to air for 0, 1, 3 or 7 days. We compared respiratory performance out of water with traits linked to the O₂ cascade. Aerial O₂ consumption rate was measured over a step-wise decrease in O₂ levels. There were significant differences between strains, but time out of water had the largest impact on measured parameters. *K. marmoratus* had improved respiratory performance (lower aerial critical oxygen tension (P_{crit}), higher regulation index (RI)) after only 1 day of air exposure and these changes were strongly associated with the change in hematocrit and dorsal cutaneous angiogenesis. Additionally, we found that 1-hour of air exposure induced the expression of four angiogenesis-associated genes, *vegfa*, *angpt2*, *pecam-1* and *efna1* in the skin. After 7 days in air, respiratory traits were not significantly linked to the variation in either aerial P_{crit} or RI. Overall, our data indicate that there are two phases involved in the enhancement of aerial respiration; an initial rapid response (1 day) and a delayed response (7 days). We found evidence for the hypothesis that respiratory performance on land in amphibious fishes is the result of rapid flexibility in both O₂ uptake and O₂ carrying capacity.

1. Introduction

The transition from an aquatic to terrestrial environment imposes many respiratory challenges for amphibious fishes (Brown et al., 1992; Sayer, 2005). As a result, amphibious fishes have evolved specific adaptations for life out of water (Graham, 1997). Many amphibious fishes switch their primary site of O₂ uptake from the gills to air-breathing organs (e.g. gas bladder, buccal-pharyngeal cavity, skin) to maintain O₂ demands (Graham, 1997). Epidermal capillaries have also been observed close to the skin surface (1-119µm) in amphibious fishes (Mittal and Munshi, 1971; Grizzle and Thiyagarajah, 1987; Park et al., 2006), whereas in most fishes capillaries are located deeper within the dermis (Feder and Burggren, 1985). The description of morphological adaptations for air breathing has a long history (e.g. Das, 1934; Hughes and Munshi, 1968), but less attention has been focused on reversible phenotypic flexibility in fishes out of water (Wright and Turko, 2016).

There is some evidence that amphibious fishes enhance aerial respiration out of water (emersion) by altering the efficiency of O₂ uptake. Aerial respiration may require some fish to undergo structural modifications (e.g. reduction in the diffusion distance or an increase in the number of cutaneous capillaries- angiogenesis) to maximize surface area for exchange (Marusic et al., 1981; Cooper et al., 2012; Glover et al., 2013; Turko et al., 2014). Angiogenesis is the development of new capillaries derived from pre-existing blood vessels (Djonov et al., 2000). This process can be controlled via different mechanisms (i.e. capillary intussusception and sprouting) and through multiple genes (i.e. vascular endothelial growth factor (*vegf*), angiopoietin-1 (*angpt1*), angiopoietin -2 (*angpt2*), ephrins (*efn*); Prior et al., 2004; Fagiani and Christofori, 2013). The gene coding for platelet endothelial cell adhesion molecule (*pecam-1*) expresses a protein (CD31) that helps form junctions between endothelial cells (Albelda et al., 1991). Angiogenesis during emersion would presumably increase blood flow near the respiratory epithelium maximizing gaseous exchange (Glover et al., 2013).

Plasticity in O₂ transport may also play a role in enhancing respiration in amphibious fishes out of water. For example, a faster rate of blood delivery (increased heart rate) would increase O₂ transport, as reported in mudskippers, *Periophthalmodon australis* (Kok et al, 1998; Garey, 1962). Reversible phenotypic plasticity of hemoglobin properties would also ameliorate the impact of

CO₂ accumulation and blood acidosis in amphibious fishes out of water. Increased hemoglobin-O₂ affinity during air exposure may be beneficial in offsetting the Bohr shift due to CO₂ retention in emersed amphibious fishes (Graham, 1997; Morris and Bridges, 1994). By altering oxygen carrying capacity (hemoglobin concentration and/or erythrocyte density; Delaney et al., 1976; Johansen et al., 1976; Marusic et al., 1981; Urbina and Glover, 2012; Turko et al., 2014), amphibious fishes may compensate for reduced O₂ carrying capacity (Root effect; Root, 1931). Some fishes do both. For example, *K. marmoratus*, *Protopterus amphibious* and *Protopterus aethiopicus* increase their hemoglobin-O₂ affinity (lower P₅₀) and increase hemoglobin concentration during emersion (Delaney, et al., 1976; Johansen et al., 1976; Turko et al., 2014). Thus, rapid responses to enhance O₂ transport would offset the negative effects of elevated blood CO₂ in air-exposed fishes (Graham, 1997).

We tested the hypothesis that amphibious fishes that leave water, have enhanced respiratory performance on land as a result of rapid functional phenotypic flexibility of respiratory traits. This hypothesis predicted that fish with increased cutaneous angiogenesis in response to air exposure will have a higher terrestrial respiratory performance (lower critical oxygen tension (P_{crit}), higher regulation index (RI)). As well, fish that have increased blood carrying capacity (increase hematocrit (Hct), increased number of red blood cells (nRBCs)) in response to air exposure should have a higher terrestrial respiratory performance (lower P_{crit}, higher RI). P_{crit} is defined as the point at which the O₂ consumption rate of an organism becomes dependent on environmental O₂ levels (Ultsch et al., 1978). In aquatic environments, P_{crit} has been found to be highly correlated to respiratory traits along the O₂ cascade (e.g. gill surface area and Hb-O₂ affinity; Mandic et al., 2009). Whereas, RI, an alternate performance measure, is the overall regulatory ability of the fish over the full range of atmospheric O₂ levels. This parameter provides insight as to whether an organism is a more conformer or a regulator (Mueller and Seymour, 2011).

K. marmoratus is an ideal species for studying the terrestrial respiratory performance of amphibious fish because they can tolerate weeks out of water (Taylor, 2012; Taylor et al., 2008; Wright, 2012), and prolonged air exposure results in angiogenesis of alternate respiratory surfaces (Cooper et al., 2012; Turko et al., 2014) and increased blood hemoglobin concentration (Turko et al., 2014). *K. marmoratus* are one of only two known self-fertilizing vertebrates,

creating isogenic offspring (Harrington, 1961), which allowed us to control genetic variation while manipulating the environment. Therefore, we compared respiratory traits (hematocrit, number of red blood cells, angiogenesis) and performance (aerial P_{crit} , RI) across multiple isogenic lineages of *K. marmoratus*.

2. Materials and Methods

a) Animals

Kryptolebias marmoratus hermaphroditic strains were obtained from the breeding colony housed at the Hagen Aqualab at the University of Guelph, Guelph, Ontario. The isogenic strains of FW2 (Freshwater) (Platek et al., 2017), 50.91 (Belize), HON11 (Honduras) and SLC (Florida) were used (Taterenkova et al., 2010). Fish were held individually in 120 ml semi-transparent plastic containers (FisherBrand Collection Containers; Fisher Scientific) and maintained under constant conditions (12:12h light-dark cycle; 25 °C, salinity (15 ppt) for Belize, Honduras and Florida (Frick and Wright, 2002) and salinity (0.3 ppt) for Freshwater fish (Platek et al., 2017)). Brackish water and freshwater were made with reverse osmosis water and marine salt (Instant Ocean™, Crystal Sea, Baltimore, MA, USA) to the appropriate salinity and changed weekly. The fish were fed *Artemia nauplii* three times a week until the beginning of experiments. This project was approved by the University of Guelph Animal Care Committee (AUP 2239).

b) Experimental Protocol

Fish (Honduras ($0.120 \text{ g} \pm 0.003$), Belize ($0.120 \text{ g} \pm 0.003$), Florida ($0.120 \text{ g} \pm 0.003$) and Freshwater ($0.140 \text{ g} \pm 0.004$)) were acclimated to water (control) or air (1, 3 or 7 days) at 25 °C. Air-acclimated fish were maintained on moist filter paper (15 ppt or 0.3 ppt) in plastic containers, as previously described (Ong et al., 2007). All treatment groups were subjected to a critical oxygen tension test (P_{crit}) and regulation index (RI) test in air in order to determine their respiratory performance ability. It was necessary to perform all experiments under the same conditions for comparisons. It is likely that some changes occurred very quickly when *K. marmoratus* left water (1-2 hours), but those potential changes are presumably minor relative to the more profound changes observed at 1, 3, and 7 days. Due to the small size of the fish all measurements could not be performed on the same individuals. New groups of fish were acclimated to air or water as described above and used for histological, blood or gene expression analyses (see below). At the end of the experiment, fish were euthanized with tricaine methanesulfonate (MS-222; 1.5mg/mL) and cut into transverse sections anterior to the dorsal fin.

Sections were covered with embedding medium (Shandon cryomatrix, ThermoFisher Scientific, Markham, ON, Canada), frozen using liquid nitrogen-chilled 2-methylbutane and stored at -80°C until sectioning (Brunt et al., 2016). An additional experiment on separate fish was conducted to measure hematocrit and the number of red blood cells. Fish from each strain were air exposed for 0, 1, or 7 days. Blood was collected by caudal severance using heparinized microhematocrit tubes (Kimble Chase, USA) (Turko et al., 2014). For gene expression analyses, fish (Honduras and Florida strains only) were sampled at 0 hrs (pre-emersion) and post-emersion at 1hr, 6hrs, 1 day, 3 days and 7 days. Skin samples were immediately transferred into RNAlater (ThermoFisher Scientific, Markham, ON, Canada) and stored at -20°C until analyses. It is important to note that we chose to only analyze the Honduras and Florida strains for gene expression analysis based on their differences in survival rate out of water. Honduras fish had a significantly higher survival rate after 7 days compared to Freshwater fish (Dong et al., in preparation). Additionally, the cost of RNA-seq limited the number of strains we could analyze.

c) Critical Oxygen Tension and Regulation Index

P_{crit} was measured in custom-made glass micro-respirometry chambers (~1 mL) in which an optode was used to measure oxygen saturation (Loligo Systems WITROX 4, Tjele, Denmark). Chambers were kept in an incubator (Innova 4230, New Brunswick Scientific, NJ, USA) to maintain a constant temperature of 25 °C. Before each experiment, wet filter paper was inserted into each respirometry chamber to maintain a humid environment during air exposure. All experiments were conducted between 12:00–18:00 to account for diurnal fluctuations in metabolic rate (Rodela and Wright, 2006). Preliminary experiments were conducted to determine the appropriate mass of fish: volume of chamber to achieve a significant change in atmospheric O₂ in a reasonable period of time. The volume of the chamber was adjusted by adding an inert material (wax). P_{crit} in air was measured using a modification of a step-wise hypoxia protocol previously described for an aquatic system (Borowiec et al., 2015; Crans et al., 2015), with a few exceptions to account for the differences in O₂ content between water and air. Fish were inserted into respirometry chambers and were acclimated to the chamber for 20 minutes at 100% air saturation. Preliminary experiments showed that 20 minutes was a sufficient acclimation period in air. This was determined by measuring MO₂ over a 2-hour period and we found that there was no statistically significant decrease or change across all MO₂ time points. Additionally, in preliminary experiments conducted on measuring maximum metabolic rate out of water, we

found that metabolic rate decreased back down to resting metabolic rate in only a few minutes (Blanchard, T.S., unpublished data). Which supports the idea that *K. marmoratus* recover quickly in response to handling stress.

O₂ consumption was initially measured at a PO₂ of 21.2kPa and then at a PO₂ of 14.8kPa to 10.6kPa in steps of 2.1kPa and from 10.6kPa to 1.1kPa in steps of 1.1kPa. At each step, the difference in PO₂ was recorded over 10 minutes in a sealed chamber. After each measurement, the chamber was flushed with the new PO₂ air and fish were left for 5 minutes before the next measurement began. Control of the PO₂ was achieved using a gas mixing system with air and N₂ (Wosthoff, Calibrated Instruments Inc., New York). Optodes were calibrated weekly using air (100% PO₂) and 2 M of sodium sulfite (0% DO) as described previously (Sutton et al., 2018). Routine O₂ consumption (RMR) was calculated as $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ by measuring the slope of the O₂ consumption curve over time at 21.2kPa. Background respiration was measured before and after each experiment, however, it was found to be negligible.

Aerial P_{crit} for each fish was calculated using nonlinear regressions, as described by Marshall et al. (2013) which better accommodates data sets where RMR more gradually declines with environmental O₂ levels, rather than a sharp transition at a specific O₂ level. To calculate RI, we first determined the curve that best fit the data and then we fitted a straight line at the start and end of MO₂. From there, we calculated RI as the area between the curve and straight line as described by Mueller and Seymour (2011). A RI of 1 represented complete regulation and a value of 0 represented total conformity to environmental O₂ levels (Mueller and Seymour, 2011).

d) Angiogenesis

Immunofluorescence was used to stain for the endothelial intercellular junction protein cluster of differentiation 31 (CD31), as previously described (Cooper et al., 2012), with a few modifications. The CD31 antibody has previously been used in the literature to quantify changes in endothelial cell proliferation in fishes (Cao et al., 2008; Cooper et al., 2012) and angiogenesis in mammals (DeLisser et al., 1997). Frozen transverse sections were cut (8 μm thick) using a cryostat at -22°C (Leica CM3050 S, Wetzlar, Germany) and slides were stored at -80°C until staining. Slides were defrosted for 2 hours prior to staining and then rinsed in phosphate buffer saline (PBS) with Triton-X (0.1% v/v) for two 5-minute washes to permeabilize the tissues. Samples were blocked for 1 hour at room temperature in blocking solution (PBS, 5% normal goat serum, 0.1% (v/v) Tween-20, 0.05% (v/v) sodium azide). All samples were incubated in a

humidified chamber overnight at 4°C in primary antibody (1:100 Rat anti-Mouse PECAM/CD31: PBS (Cat: 553370, BD Pharmingen), 0.1% (v/v) Tween-20, 0.05% (v/v) sodium azide). Samples were then rinsed in PBS with Tween-20 (0.1% v/v) three times for 5 minutes each. Samples were incubated in a humidified chamber for 2 hours at room temperature with Alexa-Fluor-488-labelled secondary antibody (1:400 goat anti-rat IgG: PBS; Invitrogen). Samples were washed five times for 5-minutes each with PBS and mounted with Fluoromount with DAPI (Sigma-Aldrich). A negative control, in which no primary antibody was applied, was used to ensure the specificity of the secondary antibody. Images were taken (20x) on the same day using a Nikon epifluorescent microscope (Nikon Eclipse 90i microscope, Nikon, Melville, NY, USA) using the same camera settings for all images. Using ImageJ, a line was traced around the epidermis (dorsal or ventral) and the integrated fluorescence density was calculated. To account for potential differences in tissue thickness across samples, values were normalized by dividing the integrated density of the epidermis by the integrated density of the skeletal muscle.

e) *Gene Expression/ RNA-Seq analysis*

Immediately following skin dissections, tissues were preserved in RNAlater and archived at -20°C. Skin tissues ($N=5$ per time point) were individually homogenized (Omni BeadRuptor) in RLT buffer with β -mercaptoethanol and RNA was purified from homogenates using Qiagen RNeasy Purification kits (Germantown, MD, USA). RNA-seq libraries were prepared using NEBNext RNA library preparations kits for Illumina (Ipswich, MA, USA). Libraries from 5 replicate individuals per strain, per treatment were prepared. Each sample was tagged with a unique barcode. All samples were multiplexed into a single pool (including samples not analyzed as part of this project), and this pool was sequenced across four lanes of Illumina HiSeq 4000 (PE-150). Sequencing yielded 1,236,473,082 raw reads across the 60 experimental samples. Sequencing failed for two samples, including one sample from strain Honduras from the 72-hr emersion sampling treatment ($N=4$), and one sample from the Honduras time 0 immersion control treatment ($N=4$). Short and low-quality reads were removed with Trimmomatic 0.36 (Bolger et al., 2014). Reads were mapped to the reference genome (RefSeq assembly accession: GCF_001649575.1) using STAR (Dobin et al., 2013). The average number of mapped reads per sample was 20,414,142. Read counts were generated using HTSeq (Anders et al., 2015). We removed genes from subsequent analyses when read counts were too low (criteria: read counts >

10 in at least 5 samples). Read counts were log₂ transformed and normalized for gene length and total library size in edgeR (Robinson et al., 2010).

f) Blood Analysis

Hematocrit was measured after centrifugation (International Clinical Centrifuge, Model CL, International Equipment, Needham, MA, USA) at 5,200 *g* for 2 minutes. Since blood volumes were minute (<1 µl/fish), images were taken of the microhematocrit tubes using a dissecting microscope (Wild of Canada Limited, Ottawa, Ontario) and the proportion of packed red blood cells was determined using ImageJ (Bianchini and Wright, 2013). To measure the number of red blood cells (nRBCs), whole blood was diluted in a 1:400 dilution (whole blood: Cortland's isotonic saline) (Wolf, 1963) and then further diluted 1:1 with 0.4% Trypan Blue solution to stain for non-viable red blood cells (Turko et al., 2014). Red blood cells were counted using a standard hemocytometer (American Optical, Buffalo, NY, USA) using a Nikon Eclipse 90i epifluorescent microscope (Nikon, Melville, NY, U.S.A). Red blood cells were manually counted from a single row in the center square of the hemocytometer. Rows were randomly selected by assigning each row a number and using a random number generator to determine which row to count. Unfortunately, we were unable to measure hemoglobin-O₂ affinity as in previous studies (Bianchini and Wright, 2013; Turko et al., 2014) due to equipment failure.

g) Statistical Analyses

Statistical analyses were performed using SigmaPlot v.11 (Systat Software, San Jose, CA, USA). The effects of time of air exposure and strain on aerial Pcrit, RMR, RI, hematocrit, nRBCs, and angiogenesis were individually tested using a two-way analysis of variance (ANOVA) with a post-hoc Holm-Sidak test. The relationship between the change in respiratory traits (hematocrit, nRBCs, angiogenesis, RMR) and respiratory performance (aerial Pcrit and RI) was tested by combining all data across strains by using separate simple linear regression tests. We calculated the change for each measurement using the following equation:

$$\frac{\text{Measurement}_{\text{time (x)}} - \text{Measurement}_{\text{time (0)}}}{\text{Measurement}_{\text{time (x)}}}$$

Data are presented as means ± SEM and tests were all evaluated at an alpha-level of 0.05. Differential gene expression analysis was performed in limma (Ritchie et al., 2015). The design

matrix specified two main effects, including time with six levels (time 0 immersion control, and emersion at 1 hr, 6 hrs, 1 day, 3 days, and 7 days post-transfer), and strain with two levels (Honduras and Freshwater), and a time-by-strain interaction term. We considered genes showing significant main effects or interaction if false discovery rate corrected p-values were <0.01 .

3. Results

a) Aerial Respiratory Performance

The aerial oxygen consumption rate of all four strains decreased with decreasing atmospheric PO_2 (**Figure 1**). Time and strain had significant effects on critical oxygen tension measured as aerial P_{crit} (time: $F_{3,156}=34.13$, $P<0.001$; strain: $F_{3,156}=3.14$, $P=0.02$; **Figure 2A**) and these did not interact ($F_{9,156}=1.53$, $P=0.14$). Aerial P_{crit} was significantly lower at 1, 3, and 7 days of air exposure relative to control (0 days), and 7 days was also significantly lower relative to 3 days of air exposure. The Belize strain had a significantly higher aerial P_{crit} than the Honduras strain ($P<0.01$), whereas the Florida and Freshwater strains were intermediate ($P>0.05$, **Figure 2A**).

Air exposure had a significant effect on RI. Fish acclimated to air for 1 day had a significantly higher RI than fish acclimated to air for 0 and 7 days, while 3 days was intermediate (time: $F_{3,156}=3.90$, $P=0.01$; **Figure 2B**). There was no effect of strain on RI (strain: $F_{3,156}=0.22$, $P=0.88$).

Routine metabolic rate (RMR) was also altered in fish out of water. RMR was significantly lower after 7 days in air relative to 0, 1 and 3 days of air exposure at 21.2 kPa, and this was not influenced by strain or their interaction (time: $F_{3,156}=7.62$, $P<0.001$; strain: $F_{3,156}=0.47$, $P=0.70$; interaction: $F_{3,156}=1.54$, $P=0.14$; **Figure 2C**).

b) Oxygen Uptake – Angiogenesis

Angiogenesis was enhanced by air exposure. CD31 expression was visible in both the dorsal and ventral region of the epidermis. Moreover, the expression of CD31 appeared more prominent at 3 and 7 days of air exposure in both regions (**Figure 3A-H**). In the dorsal region of the epidermis, fish acclimated to air for 1 day had a significantly higher CD31 fluorescence intensity than fish acclimated to air for 0 days. Moreover, fish acclimated to air for 3 and 7 days

had a significantly higher fluorescence intensity relative to fish acclimated to air for 0 and 1 day (time: $F_{3,105}=22.74$, $P<0.001$; strain: $F_{3,105}=0.15$, $P=0.93$; interaction: $F_{9,105}=0.73$, $P=0.68$; **Figure 3I**). In the ventral region of the epidermis, fish acclimated to air for 3 and 7 days had a significantly higher fluorescence intensity than fish acclimated to air for 0 and 1 day (time: $F_{3,107}=7.29$, $P<0.001$; strain: $F_{3,107}=0.70$, $P=0.55$; interaction: $F_{9,158}=0.33$, $P=0.96$; **Figure 3J**).

Angiogenesis was linked to respiratory performance across strains. The change in dorsal angiogenesis was positively related to the change in RI at 1 ($P=0.01$, **Table 1**) and 3 days of air exposure ($P=0.01$, **Table 1**), however, no significant relationship was detected at 7 days of air exposure (**Table 1**).

c) Gene Expression

We found a strong upregulation of three angiogenesis genes in the skin after 1 hour in air (*vegfa* 1.9-fold; *angpt2* 3.7-fold; *efna* 1.7-fold; $P<0.05$; **Figure 4A-C**) compared to control. Whereas, *pecan-1* was significantly up-regulated (1.6-fold; $P<0.05$; **Figure 4D**) by 6 hours following emersion. However, by 7 days of air exposure there is no significant difference in expression across all angiogenesis genes compared to control.

d) Oxygen Transport – O_2 Carrying Capacity

Hematocrit was altered by air exposure. Both strain and air exposure time had direct and interacting effects on hematocrit (time: $F_{2,82}=3.28$, $P=0.04$; strain: $F_{3,82}=3.73$, $P=0.01$; interaction: $F_{6,82}=4.27$, $P<0.001$; **Figure 5A**). At day 0, the Belize strain had a significantly higher hematocrit relative to both the Florida and Honduras strain, whereas the Freshwater strain was also higher than the Florida strain. Only the Florida strain showed a significant increase in hematocrit after 1 day of air exposure compared to 0 days, but both Honduras and Florida strains had higher hematocrits after 7 days. Neither air exposure nor strain influenced the number of red blood cells (all $P>0.05$, **Figure 5B**).

Initial changes in respiratory performance were related to oxygen-carrying capacity. The change in aerial P_{crit} was positively and significantly related to the change in hematocrit at 1 day of air exposure ($P=0.01$, **Table 1**). No other significant relations were detected between O_2 -carrying capacity (Hct, nRBC) and respiratory performance variables (aerial P_{crit} , RI; **Table 1**).

4. Discussion

In this study we have experimentally demonstrated that aerial acclimation improves respiratory performance in an amphibious fish. We compared respiratory performance out of water (aerial P_{crit} , RI) with traits linked to O_2 uptake (cutaneous angiogenesis) and O_2 transport (Hct, nRBCs) in 4 isogenic lineages of *K. marmoratus*. In general, we found that respiratory performance and traits varied across both emersion time and strain, but time was the stronger factor. Indeed, we found that time had the largest effect size in five of the seven traits measured, except for the two blood parameters (**Table 2**). *K. marmoratus* showed a consistently improved respiratory performance (lower aerial P_{crit} and higher RI) after only 1 day of aerial acclimation. The initial rapid improvement in aerial P_{crit} was most strongly linked to O_2 -carrying capacity (Hct) and the initial improvement in RI was significantly associated with dorsal angiogenesis. These results suggest that aerial P_{crit} and RI are regulated by different factors along the O_2 cascade. Overall, *K. marmoratus* showed modifications along both the O_2 uptake and transport system in response to air over time, however, only initial plastic changes in respiratory traits were related to improved respiratory performance out of water.

Respiratory Performance and Flexible Respiratory Traits

There appeared to be two phases of improved respiratory performance occurring over time in air. An initial rapid response (1 day; lower aerial P_{crit} and higher RI) was followed by a delayed response (7 days; lower aerial P_{crit}), suggesting that 2 different physiological responses may be involved. Both aerial P_{crit} (lower) and RI (higher) were significantly improved by 1 day in air. Interestingly, aerial P_{crit} stabilized between 1 and 3 days of emersion but was even lower by day 7. On the other hand, RI returned to control values by day 7. Moreover, the Honduras strain had an overall different aerial P_{crit} than the Belize strain across all time points; however, the largest variation in aerial P_{crit} appears to be at day 1. The Honduras strain appeared to have a more rapid respiratory response relative to the Belize strain. Interestingly, a previous study in our lab found that the *K. marmoratus* Honduras strain survived significantly longer in air compared to both the Florida and Belize strains (A. Turko, J. Doherty, P. Wright, unpublished data). Therefore, a higher emersion tolerance may be related, in part, to a greater initial respiratory ability during emersion.

1) *Initial Rapid Response*

The data indicate that the initial rapid plastic change in aerial P_{crit} was primarily driven by O_2 -carrying capacity. Our hypothesis predicted that fish with increased O_2 -carrying capacity would have a higher respiratory performance (lower aerial P_{crit} and higher RI). In support of this, we found that the change in Hct was strongly correlated ($R^2=0.97$) to the change in aerial P_{crit} at 1 day of air exposure, where strains with the largest increase in Hct also had the largest decrease in aerial P_{crit} . The relationship between Hct and P_{crit} has been shown in other species, for example hypoxia-tolerant aquatic fishes with a higher hematocrit tend to have a lower P_{crit} (Chapman et al., 2002). However, Mandic et al (2009) found no significant relationship between Hct and P_{crit} in various species of marine sculpins. Thus, the importance of O_2 -carrying capacity in fish respiration may be species-, time-, or environment-dependent.

The observed initial relationship between Hct and aerial P_{crit} in *K. marmoratus* may be influenced by inherent differences in Hct across strains or the ability to modify Hct in response to air exposure. Baseline O_2 -carrying capacity was significantly different across strains, both the Belize and Freshwater strains had higher Hct relative to the Florida strain. Elevated Hct is thought to be important for both O_2 uptake (gills or skin) and O_2 delivery to the tissues (Wells et al., 2003), as well as mitigating the effects of elevated tissue CO_2 on O_2 carrying capacity (Graham, 1997). Thus, having an inherently high O_2 -carrying capacity may be beneficial in sustaining O_2 demands during the initial transition onto land if the onset of respiratory plastic changes is delayed. Additionally, strains that had a higher baseline O_2 -carrying capacity exhibited no change in Hct in response to aerial acclimation. Increased Hct can be also a disadvantage as there is an exponential increase in blood viscosity with small changes in Hct, which can hinder blood flow through the epidermal capillaries and increase work output by the heart (Baldwin and Wells, 1990; Wells and Weber, 1991). Therefore, the costs associated with a higher Hct may exceed the advantages of having a higher O_2 -carrying capacity in strains with elevated Hct (Urbina and Glover, 2012).

The mechanisms involved in the rapid increase in Hct observed during emersion are unknown. Acute changes in Hct can arise due to a shift in fluid volume or through the release of red blood cells via the spleen (Jensen et al., 1993; Gallagher and Farrell, 1998). An acute

increase in Hct after 1 day of air exposure was only observed in the Florida strain, but there was no significant change in the nRBC. The increase in Hct could reflect a change in cell volume (Weber and Jensen, 1988). We estimated mean cell volume (MCV) from dividing mean Hct by mean nRBC (Turko et al., 2014). Indeed, an ~11% increase MCV was found in the Florida strain while in the other strains, MCV tended to decrease (Honduras, Belize) or the change in cell volume was negligible (~1% Freshwater; data not shown). Whether elevated catecholamines were involved in the Hct changes in the Florida strain is unknown, but β -adrenergic-stimulated erythrocyte swelling can result in response to air exposure (Nikinmaa, 1982; Perry et al., 1989). Further investigation will be necessary to tease apart the mechanism involved.

An increase in the number of blood vessels and/or epidermal blood perfusion may improve the ability to regulate O₂ consumption during emersion. We predicted that strains with higher cutaneous vascularization would exhibit a higher respiratory performance during emersion. Indeed, there was a strong relationship ($R^2=0.97$) between the change in dorsal angiogenesis and RI at 1 and 3 days of air exposure, where strains that showed the largest increase in CD31 expression also showed the largest increase in RI. In a separate study on *K. marmoratus*, RI was associated with gill surface area, implying that the ability to regulate O₂ consumption (RI) may be linked to modifications across the O₂-uptake system (Turko et al., 2012). This rapid adjustment in the dorsal region of the epidermis is also consistent with recent behavioural data showing *K. marmoratus* spent significantly more time exposing their dorsal surface to air relative to their ventral or lateral sides (Heffell et al., 2017). Mudskippers (*Boleophthalmus* and *Scartelaos*) also have a high degree of vascularization in the head and dorsal area, regions most often exposed to air (Zhang et al., 2000).

The rapid changes we observed in angiogenesis were consistent with the dramatic increase in the expression of genes involved with blood vessel development. Angiogenesis requires complex multi-step signaling that is mediated by molecules from three protein families – vascular endothelial growth factors (VEGFs), angiopoietins (ANGPTs), and ephrins (EFNs) – that act through receptor tyrosine kinases in endothelial cells (Gale and Yancopoulos, 1999). Transcripts for key members of each of these ligand families were significantly up-regulated within one hour of air exposure in *K. marmoratus* skin (Figure 4). Parallel up-regulation of both *angpt2* and *vegfa* is consistent with promotion of angiogenesis (Maisonpierre et al. 1997; Holash

et al. 1999). VEGF-induced angiogenesis requires ephrin-A2 (EPHA2) receptor activation, and VEGF induces expression of ephrinA1 (the ligand for EPHA2) in endothelial cells (Cheng et al. 2002). We found that 7-fold up-regulation of *epha2* occurs within one hour of emersion. The gene that codes for CD31, *pecam-1* was increased in expression by 1.6-fold within 6 hours of emersion. This delay relative to the other angiogenesis-associated genes may reflect the time required to grow new endothelial cells before cell-to-cell junctions are formed. It is important to note that CD31 (*pecam-1*) is also present in lymphocytes, platelets, leukocytes (neutrophils) and monocytes, thus other physiological roles of CD31 include involvement in the inflammatory response and vasculogenesis during embryonic development (DeLisser et al., 1994; Pinter et al., 1997). However, it is unlikely that our data are signalling these other physiological processes. Together, these data indicate that key initiators of angiogenesis signaling pathways are coordinately up-regulated almost immediately following exposure to air in *K. marmoratus* skin. Moreover, the gene expression and immunofluorescence data are strong evidence that *K. marmoratus* exhibit cutaneous angiogenesis during emersion, potentially as a mechanism to increase cutaneous gas exchange as well as the transfer of other molecules.

2) Delayed Response

Respiratory traits were not significantly linked to variation in either aerial P_{crit} or RI after 7 days in air. In fact, variation in cutaneous angiogenesis and P_{crit} were low between strains, although both were significantly enhanced relative to earlier time points across all strains. Cutaneous angiogenesis may be more important for other functional mechanisms (i.e. ion, water and nitrogen regulation) rather than respiration during more prolonged emersion. It is also possible that cutaneous respiration was augmented by angiogenesis in the buccal/opercular regions, as *K. marmoratus* are known to occasionally gulp air out of water (Turko et al., 2014). Finally, other physiological factors within the O_2 transport system may also play a role in the improved respiratory performance after 7 days in air (e.g. Hb- O_2 affinity; Johansen et al., 1976; Turko et al., 2014).

The O_2 -transport system (Hct) may be less important in long-term improvement of respiratory performance during emersion. An increase in O_2 -carrying capacity was found in both the Honduran and Florida, where both strains had a significantly higher Hct at 7 days of air exposure relative to the control values, but no change in nRBCs was observed. Thus, increased

Hct could be the result of a shift in plasma volume (Gallaugh and Farrell, 1998). In contrast to the findings at 1 day of air exposure, there was no significant relationship between the change in aerial P_{crit} and Hct at 7 days of air exposure nor was there a significant relationship between RI and the change in Hct or nRBC.

Routine metabolic rate (RMR) in response to air exposure is highly variable across air-breathing fish species. Some species increased (Gordon et al., 1970; Sacca and Burggren, 1982; Urbina et al., 2014), decreased (Delaney et al., 1974; Berg and Steen, 1965; Tamura et al., 1976) or did not change (Gordon et al., 1969; Pelster et al., 1988) aerial O_2 uptake compared to aquatic O_2 uptake. At 7 days of air exposure, we found a consistent and significant decrease in routine metabolic rate, possibly due to a programmed metabolic depression (Storey and Storey, 1990) or the inability to feed during emersion, which would reduce overall energy usage and decrease metabolic demands (O'Connor et al., 2000).

Small sample size in this study limited the statistical power. Due to the small size of the fish (~0.12g) and minute blood volumes, it was not possible to complete all measurements on the same individuals. Although the significant R^2 values were robust ($R^2 > 0.97$), future work should include a larger number of strains and/or other larger amphibious species.

Perspectives and Conclusions

Overall our findings support the hypothesis that reversible plasticity of the O_2 -cascade in amphibious fishes plays a functional role during emersion through the enhancement of respiratory performance. Moreover, we propose that *K. marmoratus* exhibit two different phases in the enhancement of aerial respiration; an initial rapid response (lower aerial P_{crit} , higher RI) and a delayed response (lower aerial P_{crit} and RMR). In turn, these findings may shed some light on the behaviour and ecology of *K. marmoratus* in the wild. *K. marmoratus* display two types of emersion behaviour in the field: (1) short-term emersion in which they move in and out of water throughout the day to escape either poor water conditions or to capture prey (Taylor, 2012) and (2) long-term emersion that occurs during the dry season in which they seek moist crevices, including excavated tunnels within decaying mangrove logs for weeks at a time (Taylor et al., 2008). Therefore, the observed initial rapid improvement in respiratory performance would allow *K. marmoratus* to satisfy metabolic demands during short term emersions, whereas the delayed reduction in RMR would be an energetic advantage during seasonal long-term fasts out of water.

Aerial P_{crit} varied across isogenic lineages, suggesting that strains may have different respiratory abilities in air that could affect their emersion tolerance. In turn, this could be the result of differences in their environment or genetics. The largest strain variation we observed was between Honduras and Belize. Thus, we could speculate that their geographic origin may be difference (e.g. less food availability, longer dry season, less predators) and thus might contribute to a more aerial phenotype as displayed in the Honduras strain. However, multiple genetic lineages (as a result of selfing) arise within each population at each geographic site and therefore, it is unlikely that habitat alone contributes to the physiological variation we observed. Self-fertilization is the prevalent form of reproduction in wild populations as indicated by a high proportion of homozygous individuals even though outcrossing is also possible (Mackiewicz et al., 2006a; Mackiewicz et al., 2006b). Evaluating whether the relative fitness of self-fertilizing lineages could be influenced by respiratory performance in air is a critical next step.

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6. Competing Interest

The authors declare no competing or financial interests.

7. Author Contributions

Experimental design: T.S.B and P.A.W.; Methodology: T.S.B, A.W., and Y.D.; Analysis: T.S.B, A.W., and Y.D.; Writing- original draft: T.S.B.; Writing – edits and revisions: T.S.B, P.A.W., A.W. and Y.D.

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9. Data availability

RNA-seq data have been deposited in the Sequence Read Archive at NCBI (SRA accession: SRP136920).

10. 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.
- Anders, S., Pyl, P. T. and Huber, W.** (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166-169.
- 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.
- Berg, T. and Steen, J. B.** (1965). Physiological mechanisms for aerial respiration in the eel. *Comp. Biochem. Physiol.* **15**, 469-484.
- 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.
- Bolger, A. M., Lohse, M. and Usadel, B.** (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120.
- Borowiec, B. G., Darcy, K. L., Gillette, D. M. and Scott, G. R.** (2015). Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **218**, 1198-1211.
- Brown, C. R., Gordon, M. S. and Martin, K. L. M.** (1992). Aerial and aquatic oxygen uptake in the amphibious red sea rockskipper fish, *Alticus kirki* (Family Blenniidae). *Copeia*. **4**, 1007-1013.
- Brunt, E., Turko, A., Scott, G.R. and Wright, P.A.** (2016). Amphibious fish jump better on land after acclimation to a terrestrial environment. *J. Exp. Biol.* **219**, 3204-3207.
- Cao, R., Jensen, L. D. E., Söll, I., Hauptmann, G. and Cao, Y.** (2008). Hypoxia-induced retinal angiogenesis in zebrafish as a model to study retinopathy. *PLoS ONE*, **3**, e2748.
- Chapman, L. J., Chapman, C. A., Nordlie, F. G. and Rosenberger, A. E.** (2002). Physiological refugia: swamps, hypoxia tolerance and maintenance of fish diversity in the Lake Victoria region. *Comp. Biochem. Physiol. A* **133**, 421-437.

- Cheng, N., Brantley, D. M., Liu, H., Lin, O., Enriquez, M., Gale, N., Yancopoulos, G., Cerretti, D. P., Daniel, T. O. and Chen, J.** (2002). Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis. *Mol. Cancer Res.* **1**, 2-11.
- 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. B* **161**, 268-274.
- Crans, K. D., Pranckevicius, N. A. and Scott, G. R.** (2015). Physiological tradeoffs may underlie the evolution of hypoxia tolerance and exercise performance in sunfish (Centrarchidae). *J. Exp. Biol.* **218**, 3264-3275.
- Das, B.K.** (1934). The habits and structure of *Pseudopocryptes lanceolatus*, a fish in the first stage of structural adaptations to aerial respiration. *Proc. Roy. Soc. Lond.* **115**, 422-430.
- DeLaney, R. G., Lahiri, S. and Fishman, A. P.** (1974). Aestivation of the African lungfish *Protopterus aethiopicus*: cardiovascular and respiratory functions. *J. Exp. Biol.* **61**, 111-128.
- DeLaney, R. G., Shub, C. and Fishman, A. P.** (1976). Hematologic observations on the aquatic and estivating African lungfish, *Protopterus aethiopicus*. *Copeia* 1976, 423-434.
- DeLisser, H. M., Newman, P. J. and Albelda, S. M.** (1994). Molecular and functional aspects of PECAM-1/CD31. *Immunol. Today.* **e15**, 490-495.
- DeLisser, H. M., Christofidoy-Solomidou, M., Strieter, R. M., Burdick, M. D., Robinson, C. S., Wexler, R. S., Kerr, J. S., Garlanda, C., Merwin, J. R., Madri, J. A. and Albelda, S. M.** (1997). Involvement of endothelial PECAM-1/CD31 in angiogenesis. *Am. J. Pathol.* **151**, 671-677.
- Djonov, V., Schmid, M., Tschanz, S. A. and Burri, P. H.** (2000). Intussusceptive angiogenesis: its role in embryonic vascular network formation. *Circ. Res.* **86**, 286-292.
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M. and Gingeras, T. R.** (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21.
- Fagiani, E. and Christofori, G.** (2013). Angiopoietins in angiogenesis. *Cancer Lett.* **328**, 18-26.
- Feder, M.E. and Burggren, W.W.** (1985) Cutaneous gas exchange in vertebrates: design,

- patterns, control and implications. *Biol. Rev.* **60**, 1–45.
- Frick, N. T. and Wright P. A.** (2002). Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus* II. Significant ammonia volatilization in a teleost during air exposure. *J. Exp. Biol.* **205**, 91-100.
- Gale, N. W., and Yancopoulos, G. D.** (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Gene Dev.* **13**, 1055-1066.
- Gallaugh, P. and Farrell, A. P.** (1998). Hematocrit and blood oxygen-carrying capacity. In: S.F. Perry and B. Tufts (eds) *Fish Respiration* (pp. 185-227). San Diego: Academic Press.
- Garey, W. F.** (1962). Cardiac responses of fishes in asphyxic environments. *Biol. Bull.* **122**, 362-353.
- Glover, C. N., Bucking, C. and Wood, C. M.** (2013). The skin of fish as a transport epithelium: a review. *J. Comp. Physiol. B* **183**, 877–891.
- Gordon, M. S., Boetius, I., Evans, D. H., McCarthy, R. and Oglesby, L. C.** (1969). Aspects of the terrestrial life in amphibious fish. I. The mudskipper, *Periophthalmus*. *J. Exp. Biol.* **50**, 141-149.
- Gordon, M. S., Fischer, S. and Tarifeno, E.** (1970). Aspects of the physiology of terrestrial life in amphibious fishes. II. The Chilean clingfish, *Sicyases sanguineus*. *J. Exp. Biol.* **53**, 559-572.
- Graham, J. B.** (1997). *Air-breathing fishes: Evolution, diversity, and adaptation*. San Diego: Academic Press.
- Grizzle, J. M. and Thiyagarajah, A.** (1987). Skin histology of *Rivulus ocellatus marmoratus*: apparent adaptation for aerial respiration. *Copeia* **1**, 237–240.
- Harrington, R. W. Jr.** (1961). Oviparous hermaphroditic fish with internal self-fertilization. *Science* **134**, 1749-1750.
- Heffell, Q., Turko, A. J. and Wright, P. A.** (2017). Plasticity of skin water permeability and skin thickness in the amphibious mangrove rivulus *Kryptolebias marmoratus*. *J. Comp. Physiol. B* 1-10.
- Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., Yancopoulos, G. D. and Wiegand, S. J.** (1999). Vessel cooption, regression, and

- growth in tumors mediated by angiopoietins and VEGF. *Science* **284**,1994-1998.
- Hughes, G.M. and Munshi, J.S.D.** (1968). Fine structure of the respiratory surface of an air-breathing fish, the climbing perch, *Analoas testudineus* (Bloch). *Nature Lond.* **219**, 1382-1384.
- Jensen, F.B., Nikinmaa, M. and Weber, R.E.** (1993). Environmental perturbations of oxygen transport in teleost fishes: causes, consequences and compensations. In: J.C. Rankin and F.B. Jensen (eds) *Fish Ecophysiology* (pp. 161–179). London: Chapman and Hall.
- Johansen, K., Lykkeboe, G., Weber, R. E. and Maloiy, G. M. O.** (1976). Respiratory properties of blood in awake and estivating lungfish, *Protopterus amphibius*. *Resp. Physiol.* **27**, 335-345.
- Kok, W. K., Lim, C. B., Lam, T. J. and Ip, Y. K.** (1998). The mudskipper *Periophthalmodon schlosseri* respire more efficiently on land than in water and vice versa for *Boleophthalmus boddarti*. *J. Exp. Zool.* **280**, 86-90.
- Mackiewicz, M., Tatarenkov, A., Taylor, D. S., Turner, B. J. and Avise, J. C.** (2006a). Extensive outcrossing and androdioecy in a vertebrate species that otherwise reproduces as a self-fertilizing hermaphrodite. *Proc. Natl. Acad. Sci. USA* **103**, 9924-9928.
- Mackiewicz, M., Tatarenkov, A., Turner, B. J. and Avise, J. C.** (2006b). A mixed-mating strategy in a hermaphroditic vertebrate. *Proc. R. Soc. B* **273**, 2449-2452.
- Maisonpierre, P. C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., Daly, T.J., Davis, S., Sato, T.N. and Yancopoulos, G. D.** (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277**, 55–60.
- Mandic, M., Todgham, A. E. and Richards, J. G.** (2009). Mechanisms and evolution of hypoxia tolerance in fish. *Proc. R. Soc. B* **276**, 735–744.
- Marshall, D. J., Bode, M. and White, C. R.** (2013). Estimating physiological tolerances - a comparison of traditional approaches to nonlinear regression techniques. *J. Exp. Biol.* **216**, 2176-2182.
- Marusic, E. T., Balbontin, F., Galli-Gallardo, S. M., Garreton, M., Pang, P. K. T. and Griffith, R.W.** (1981). Osmotic adaptations of the Chilean clingfish, *Sicyases sanguineus*, during emersion. *Comp. Biochem. Physiol.* **68A**, 123-126.

- Mittal, A. K. and Munshi, J. S.** (1971). A comparative study of the structure of the skin of certain air-breathing freshwater teleosts. *J. Zool.* **163**, 515-532.
- Morris, S. and Bridges, C. R.** (1994). Properties of respiratory pigments in bimodal breathing animals: air and water breathing by fish and crustaceans. *Am. Zool.* **34**, 216 – 228.
- 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.
- Nagy, J. A., Dvorak, A. M. and Dvorak, H. F.** (2007). VEGF-A and the induction of pathological angiogenesis. *Annu Rev Pathol-Mech.* **2**, 251-275.
- Nikinmaa, M.** (1982). Effects of adrenaline on red cell volume and concentration gradient of protons across the red cell membrane in the rainbow trout, *Salmo gairdneri*. *Mol. Physiol.* **2**, 287-297.
- O'Connor, K. I., Taylor, A. C. and Metcalfe, N. B.** (2000). The stability of standard metabolic rate during a period of food deprivation in juvenile Atlantic salmon. *J. Fish Biol.* **57**, 451.
- Ong, K., 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.
- Park, J. Y., Kim, I. S. and Lee, Y. J.** (2006). A study on the vascularization and structure of the epidermis of the air-breathing mudskipper, *Periophthalmus magnuspinnatus* (Gobiidae, Teleostei), along different parts of the body. *J. Appl. Ichthyol.* **22**, 62-67.
- Pelster, B., Bridges, C. R. and Grieshaber, M. K.** (1988). Physiological adaptations of the intertidal rockpool teleost *Blennius pholis* L. to aerial exposure. *Resp. Physiol.* **71**, 355-373.
- Perry, S. F., Kinkead, R., Gallagher, P. and Randall, D. J.** (1989). Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). *Resp. Physiol.* **77**, 351-364.
- Pinter, E., Barreuther, M., Lu, T., Imhof, B. A. and Madri, J. A.** (1997). Platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) tyrosine phosphorylation state changes during vasculogenesis in the murine conceptus. *Am. J. Pathol.* **150**, 1523-1530
- Platek, A., Turko, A., Doninni, A., Kelly, S. and Wright, P.A.** (2017). Environmental calcium

- regulates gill remodeling in a euryhaline teleost fish. *J. Exp. Zool. A* **2-3**, 139-142.
- Prior, B. M., Yang, H. T. and Terjung, R. L.** (2004). What makes vessels grow with exercise training? *J. Appl. Physiol.* **97**, 1119-1128.
- Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W. and Smyth, G. K.** (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47-e47.
- Robinson, M. D., McCarthy, D. J. and Smyth, G. K.** (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140.
- Root, R. W.** (1931). The respiratory function of the blood of marine fishes. *Biol. Bull.* **61**, 427-456.
- Regan, M.D. and Richards, J. G.** (2017). Rates of hypoxia induction alter mechanisms of O₂ uptake and the critical O₂ tension of goldfish. *J. Exp. Biol.* **220**, 2536- 2544.
- 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.
- Sacca, R. and Burggren, W.** (1982). Oxygen uptake in air and water in the air-breathing reedfish *Calamoichthys calabaricus*: role of skin, gills and lungs. *J. Exp. Biol.* **97**, 179–186.
- Sayer, M. D. J.** (2005). Adaptations of amphibious fish for surviving life out of water. *Fish Fish.* **6**, 186–211.
- Storey, K. B. and Storey, J. M.** (1990). Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q. Rev. Biol.* **65**, 145-174.
- Sutton, A. O., Turko, A. J., McLaughlin, R.L. and Wright, P.A.** (2018). Behavioural and physiological responses of an amphibious, euryhaline mangrove fish to acute salinity exposure. *Copeia* (in press).
- Tamura, S. O., Morii, H. and Yuzuriha, M.** (1976). Respiration of the amphibious fishes *Periophthalmus cantonensis* and *Boleophthalmus chinensis* in water and on land. *J. Exp. Biol.* **65**, 97–107.
- 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**, 1-9.

- 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.
- 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.
- Turko, A. J., Robertson, C. E., Bianchini, K., Freeman, M. and Wright, P. A.** (2014). The amphibious fish *Kryptolebias marmoratus* uses different strategies to maintain oxygen delivery during aquatic hypoxia and air exposure. *J. Exp. Biol.* **217**, 3988–3995.
- Ultsch, G.R., Boschung, H. and Ross, M.J.** (1978) Metabolism, critical oxygen tension, and habitat selection in darters (*Etheostoma*). *Ecology* **59**, 99–107.
- Urbina, M. A. and Glover, C. N.** (2012). Should I stay or should I go?: Physiological, metabolic and biochemical consequences of voluntary emersion upon aquatic hypoxia in the scaleless fish *Galaxias maculatus*. *J. Comp. Physiol. B* **182**, 1057–1067.
- Urbina, M. A., Walsh, P. J., Hill, J. V. and Glover, C. N.** (2014). Physiological and biochemical strategies for withstanding emersion in two galaxiid fishes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **176**, 49–58.
- Weber, R. E. and Jensen, F. B.** (1988). Functional adaptations in hemoglobins from ectothermic vertebrates. *Annu. Rev. Physiol.* **50**, 161–179.
- Wells, R. M. G. and Weber, R. E.** (1991). Is there an optimal haematocrit for rainbow trout, *Oncorhynchus mykiss* (Walbaum)? An interpretation of recent data based on blood viscosity measurements. *J. Fish Biol.* **38**, 53–65.
- Wells, R. M. G., Baldwin, J., Seymour, R. S., Baudinette, R. V., Christian, K. and Bennett, M. B.** (2003). Oxygen transport capacity in the air-breathing fish, *Megalops cyprinoides*: compensations for strenuous exercise. *Comp. Biochem. Phys. A* **134**, 45–53.
- 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.

- Wright, P. A. and Turko, A. J.** (2016). Amphibious fishes: evolution and phenotypic plasticity. *J. Exp. Biol.* **219**, 2245-2259.
- 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.

Tables

Table 1 Summary of univariate linear relationships between the change in respiratory performance (aerial P_{crit} and RI) and the change in each respiratory trait across four strains of *K. marmoratus* acclimated to air for 1, 3 and 7 days.

Respiratory Traits	Aerial P_{crit}			RI		
Day 1 ¹	β	95% CI	r^2	B	95% CI	r^2
Hct	0.74	0.35-1.12	0.97	0.16	-1.39-1.70	0.09
nRBC	0.96	-2.28-4.20	0.45	0.75	-1.31-2.82	0.55
Dorsal Angiogenesis	0.47	-3.15-4.09	0.13	0.90	0.70-1.10	0.99
Ventral Angiogenesis	-0.35	-1.20-0.50	0.61	0.10	-0.82-1.02	0.10
Day 3²						
Dorsal Angiogenesis	0.17	-2.09-2.43	0.05	0.70	0.32-1.08	0.97
Ventral Angiogenesis	0.10	-1.68-1.87	0.03	-0.12	-1.76-1.52	0.04
Day 7³						
Hct	-0.21	-1.09-0.68	0.33	0.39	-1.75-2.52	0.23
nRBC	-0.34	-1.46-0.77	0.47	0.47	-2.45-3.38	0.19
Dorsal Angiogenesis	0.48	-0.52-1.49	0.68	-0.76	-3.74-2.22	0.38
Ventral Angiogenesis	-0.06	-0.97-0.86	0.03	0.05	-1.91-2.02	0.01

Bolded values denote a significant relation between the two parameters.

¹ Change between 0 and 1 day of air exposure

² Change between 0 and 3 days of air exposure

³ Change between 0 and 7 days of air exposure

Table 2. Summary of *F*-statistic, *p*-value and effect size from two-way ANOVAs across respiratory and performance traits in *K. marmoratus*.

<i>Measurement</i>	<i>Variables</i>	<i>df</i>	<i>F</i>	<i>p-value</i>	<i>Effect Size</i>
Aerial P_{crit} ¹	Strain	3	3.144	0.027	0.057
	Time	3	34.126	<0.001	0.401
	Strain*Time	9	1.526	0.143	0.082
RI ¹	Strain	3	0.219	0.883	0.004
	Time	3	3.900	0.010	0.070
	Strain*Time	9	1.869	0.060	0.010
RMR ¹	Strain	3	0.440	0.724	0.008
	Time	3	7.976	<0.001	0.130
	Strain*Time	9	1.519	0.146	0.079
Hematocrit	Strain	3	3.731	0.014	0.119
	Time	2	3.277	0.043	0.073
	Strain*Time	6	4.273	<0.001	0.237
nRBC	Strain	3	2.582	0.059	0.090
	Time	2	0.685	0.507	0.017
	Strain*Time	6	0.271	0.949	0.020
Dorsal Angiogenesis	Strain	3	0.150	0.930	0.006
	Time	3	22.744	<0.001	0.392
	Strain*Time	9	0.679	0.679	0.059
Ventral Angiogenesis	Strain	3	0.701	0.553	0.019
	Time	3	7.285	<0.001	0.169
	Strain*Time	9	0.330	0.963	0.027

¹ Denotes that the same fish were used for these variables.

11. Figures

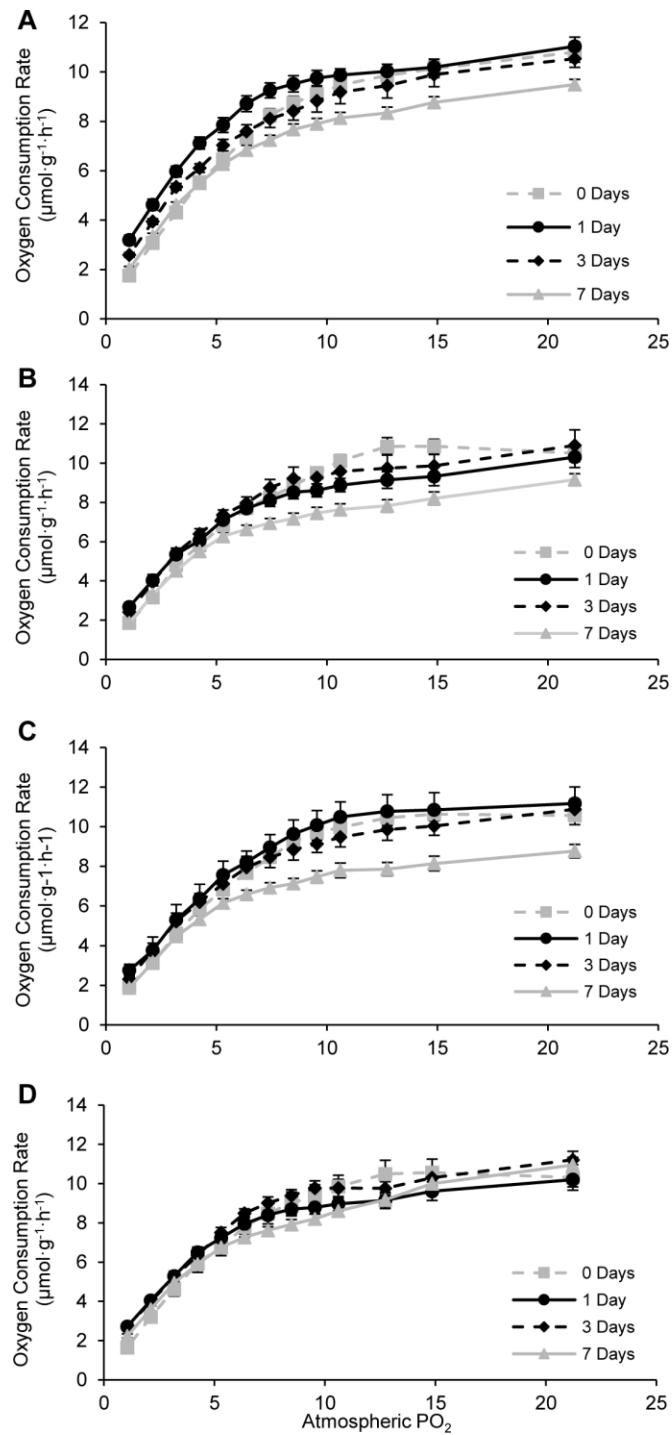


Figure 1. The effect of aerial hypoxia on O₂ consumption rate in four isogenic strains of *Kryptolebias marmoratus*. Oxygen consumption rates in response to varying atmospheric oxygen levels in (A) Florida, (B) Honduras, (C) Belize, and (D) Freshwater acclimated to air for 0, 1, 3 and 7 days. Data are presented as means \pm SEM ($N=8-16$).

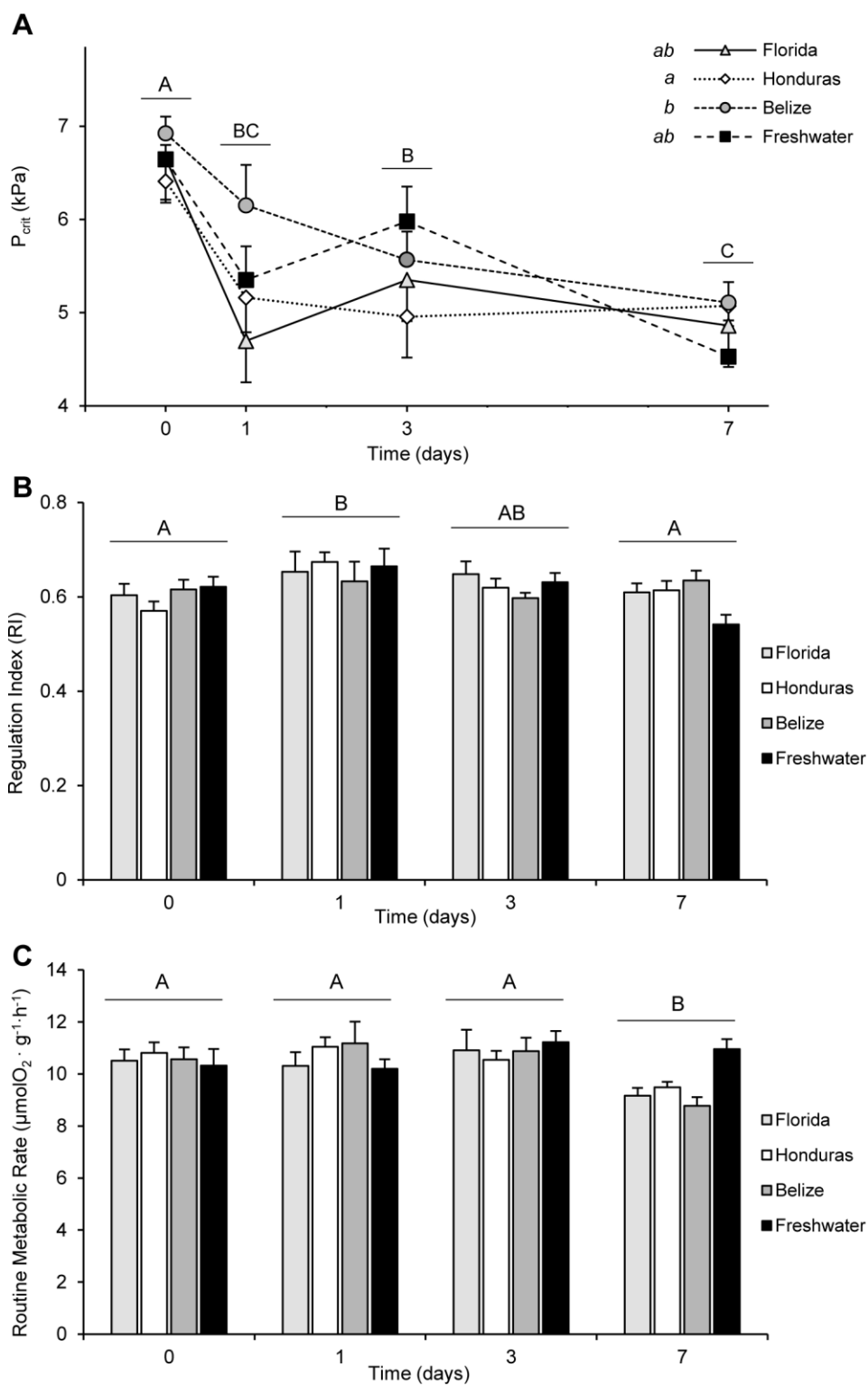


Figure 2. The effect of aerial hypoxia on (A) critical O₂ tension (P_{crit}), (B) regulation index (RI) and (C) routine metabolic rate (RMR) in four isogenic strains of *Kryptolebias marmoratus*. Fish were acclimated to air for 0, 1, 3 and 7 days. Data are presented as means \pm SEM ($N=8-16$). Different letters (upper-case) indicate statistically significant differences in P_{crit} across time and different letters (lower-case; shown in legend) indicate statistically significant differences in P_{crit} across strains, irrespective of time ($P<0.05$).

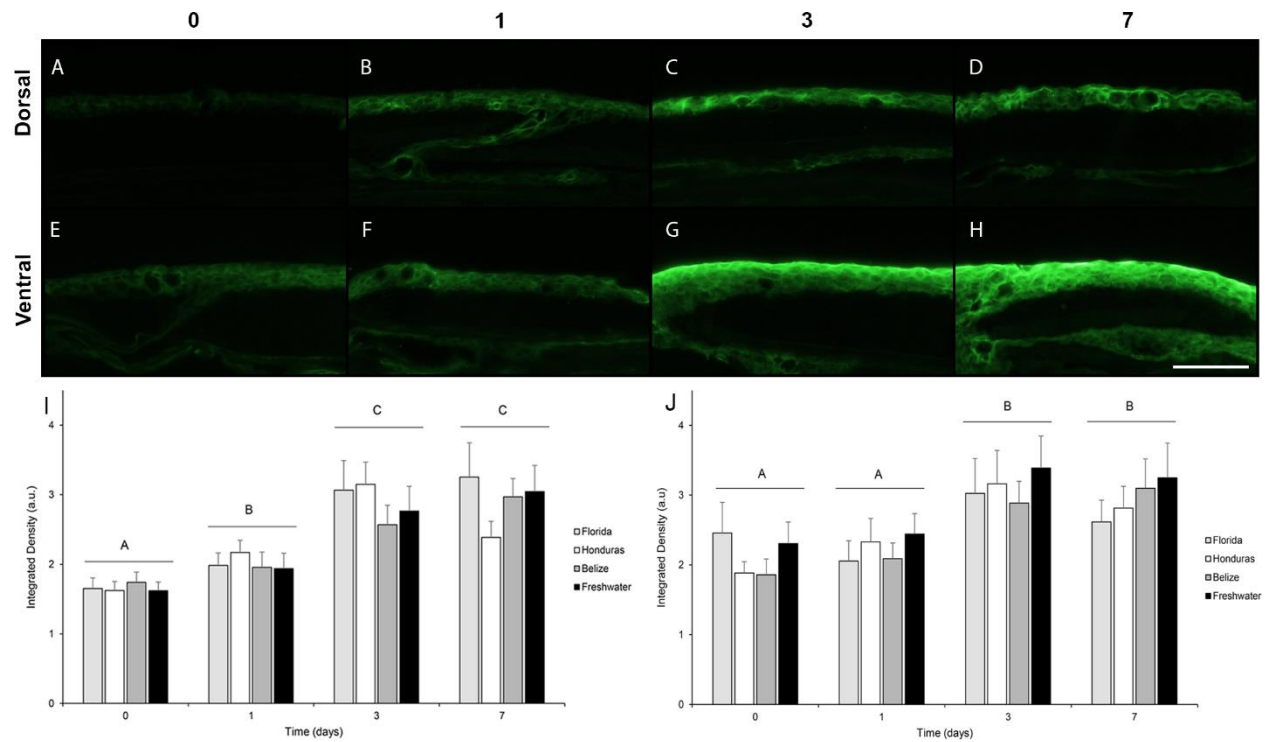


Figure 3. Air exposure induced dorsal and ventral cutaneous angiogenesis in *Kryptolebias marmoratus*. Representative images (FW strain; A-H) and expression (J-K) of the angiogenesis marker, CD31 stained, dorsal and ventral epidermis in fish acclimated to air to air for 0, 1, 3 and 7 days. A: Dorsal 0 days, B: Dorsal 1 day, C: Dorsal 3 days, D: Dorsal 7 days, E: Ventral 0 days, F: Ventral 1 day, G: Ventral 3 days, H: Ventral 7 days. Scale bar = 50 μ m. Data are presented as means \pm SEM ($N=6-8$). Groups not sharing the same letter are significantly different ($P < 0.05$).

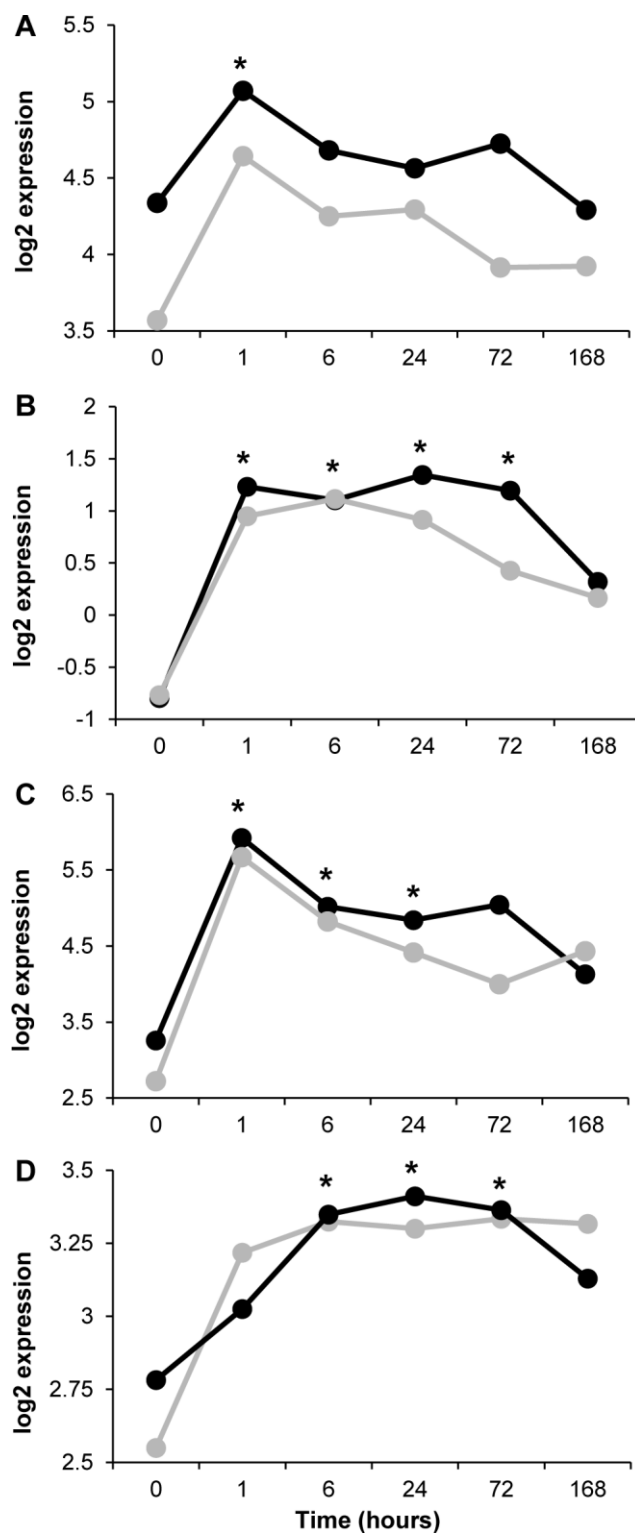


Figure 4. Air exposure induced an increase of angiogenesis gene expression in the skin of *Kryptolebias marmoratus*. RNAseq of pooled skin tissues ($n=5$) from Honduras (black) and

Freshwater (grey) strains showing levels of gene expression for (A) vascular endothelial growth factor – A (*vegfa*), (B) Angiopoietin-2 (*angpt2*), (C) ephrin-A2 (*ephna2*), (D) platelet endothelial cell adhesion molecule-1 (*pecam-1*). Data are presented as log2 expression and are normalized to t=0. *denotes significant differences in time from control (t=0; $P<0.05$).

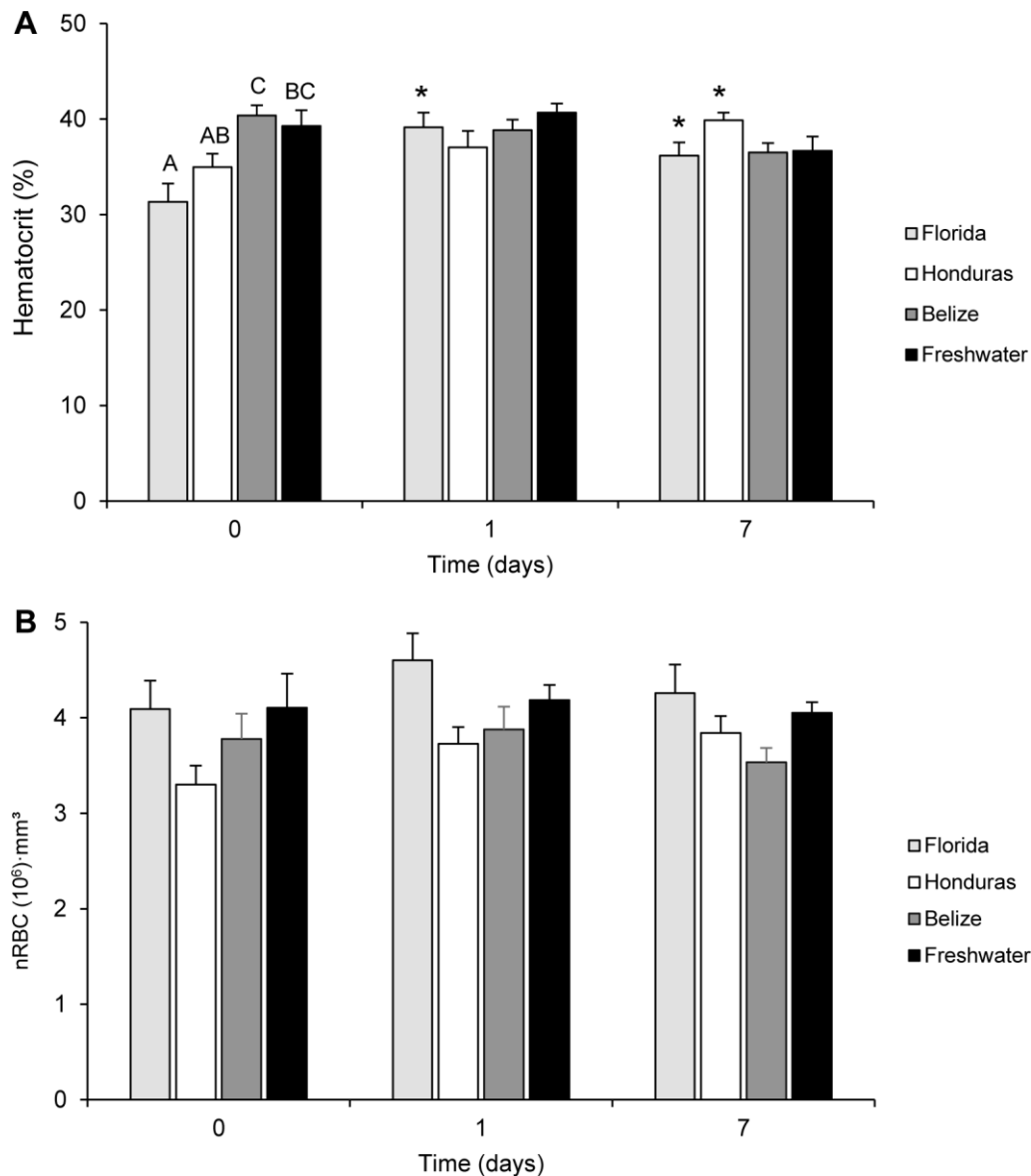


Figure 5. Air exposure changed blood parameters of four isogenic strains of *Kryptolebias marmoratus*. (A) Hematocrit and (B) number of red blood cells in fish acclimated to air for 0, 1 and 7 days. Data presented as means \pm SEM ($N=6-9$). Strains not sharing the same letter are significantly different ($P<0.05$). * denotes significant differences within a strain from the 0-day value (control).

Table S1. Summary of sample sizes used for each variable for each of the four strains of *K. marmoratus* acclimated to air for 0, 1, 3 and 7 days.

<i>Variables</i>	<i>Time</i>	<i>Strains</i>			
		<i>Florida</i>	<i>Honduras</i>	<i>Belize</i>	<i>Freshwater</i>
Aerial P_{crit} ¹	0 Days	12	12	12	12
	1 Day	8	8	8	8
	3 Days	8	8	8	8
	7 Days	16	16	16	12
RI ¹	0 Days	12	12	12	12
	1 Day	8	8	8	8
	3 Days	8	8	8	8
	7 Days	16	16	16	12
RMR ¹	0 Days	16	16	16	16
	1 Day	8	8	8	8
	3 Days	8	8	8	8
	7 Days	16	16	16	12
Hematocrit	0 Days	8	8	8	7
	1 Day	8	7	7	8
	7 Days	8	8	8	8
nRBC	0 Days	8	7	7	9
	1 Day	8	7	7	8
	7 Days	8	8	8	6
Dorsal Angiogenesis ²	0 Days	8	8	7	8
	1 Day	8	8	7	8
	3 Days	8	8	7	8
	7 Days	6	8	7	8
Ventral Angiogenesis ²	0 Days	8	8	7	8
	1 Day	8	8	7	8
	3 Days	8	8	7	8
	7 Days	6	8	7	8

^{1,2} Denotes that the same fish were used for these variables