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RESEARCH ARTICLE

Fluctuating environments during early development can limit adult phenotypic flexibility: insights from an amphibious fish

Giulia S. Rossi*,‡, Paige V. Cochrane* and Patricia A. Wright

ABSTRACT

The interaction between developmental plasticity and the capacity for reversible acclimation (phenotypic flexibility) is poorly understood, particularly in organisms exposed to fluctuating environments. We used an amphibious killifish (*Kryptolebias marmoratus*) to test the hypotheses that organisms reared in fluctuating environments (i) will make no developmental changes to suit any one environment because fixing traits to suit one environment could be maladaptive for another, and (ii) will be highly phenotypically flexible as adults because their early life experiences predict high environmental variability in the future. We reared fish under constant (water) or fluctuating (water–air) environments until adulthood and assessed a suite of traits along the oxygen cascade (e.g. neuroepithelial cell density and size, cutaneous capillarity, gill morphology, ventricle size, red muscle morphometrics, terrestrial locomotor performance). To evaluate the capacity for phenotypic flexibility, a subset of adult fish from each rearing condition was then air-exposed for 14 days before the same traits were measured. In support of the developmental plasticity hypothesis, traits involved with O_2 sensing and uptake were largely unaffected by water–air fluctuations during early life, but we found marked developmental changes in traits related to O_2 transport, utilization and locomotor performance. In contrast, we found no evidence supporting the phenotypic flexibility hypothesis. Adult fish from both rearing conditions exhibited the same degree of phenotypic flexibility in various O_2 sensing- and uptake-related traits. In other cases, water–air fluctuations attenuated adult phenotypic flexibility despite the fact that phenotypic flexibility is hypothesized to be favoured when environments fluctuate. Overall, we conclude that exposure to environmental fluctuations during development in *K. marmoratus* can dramatically alter the constitutive adult phenotype, as well as diminish the scope for phenotypic flexibility in later life.

KEY WORDS: Acclimation, Gill lamellae, Heart size, Locomotor performance, Neuroepithelial cells, Oxygen cascade, Phenotypic plasticity, Skeletal muscle

INTRODUCTION

Phenotypic plasticity is the ability of an organism with a given genotype to express environmentally mediated alternative phenotypes (Travis, 1994; West-Eberhard, 2003). Alternative phenotypes can occur in response to different developmental conditions, with potential lasting effects on the adult phenotype (developmental plasticity), and/or can occur reversibly throughout an organism's adult life (phenotypic flexibility) (Piersma and Drent, 2003). Phenotypic flexibility is hypothesized to be advantageous under fluctuating environmental conditions because it can enable phenotype–environment matches across a wider range of adult environmental conditions than could be achieved if traits were fixed during early development (West-Eberhard, 2003). In contrast, developmental plasticity is hypothesized to be advantageous when environments are relatively stable as, under these circumstances, phenotypic changes made during early life could prepare the organism for future environmental conditions (Levins, 1963; Gabriel and Lynch, 1992).

Developmental plasticity and adult phenotypic flexibility have historically been viewed as separate processes, but recent studies have demonstrated that these two forms of plasticity can be linked (for review, see Beaman et al., 2016). In other words, the environmental conditions an organism experiences during development can alter both the constitutive adult phenotype and the capacity for phenotypic flexibility during adulthood. For example, zebrafish (*Danio rerio*) raised at different embryonic temperatures differed in their adult swimming performance, and in their capacity to alter swimming performance with thermal acclimation (Scott and Johnston, 2012). Alterations in the scope for phenotypic flexibility may be adaptive to fine-tune developmental changes (Gabriel and Lynch, 1992).

The majority of studies that have investigated the interaction between developmental plasticity and phenotypic flexibility have reared organisms under constant environmental conditions (e.g. Scott and Johnston, 2012; Schnurr et al., 2014; Seebacher and Grigaltchik, 2014), in which developmental plasticity is hypothesized to be favoured. In recent years, however, the importance of evaluating the phenotypic responses of organisms to ecologically relevant fluctuations in environmental conditions has become increasingly apparent (Morash et al., 2018; Targett et al., 2019; Williams et al., 2019), as phenotypic responses to fluctuating conditions can be considerably different from those invoked by exposure to a new stable environment. For example, adult mummichogs (*Fundulus heteroclitus*) acclimated to intermittent versus constant hypoxia exhibited different plastic responses in a suite of physiological traits along the O_2 cascade (Borowiec et al., 2015) – a complex and integrated pathway that involves the sensing, uptake, transport and utilization of O_2 (Dzal et al., 2015). Nevertheless, very few studies have considered fluctuations during early development, despite the fact that many fishes encounter marked fluctuations in environmental O_2 availability during early life. For instance, several fish species deposit their embryos in intertidal environments where they are periodically exposed to air (Middaugh, 1981; Taylor, 1999). Air holds ∼30 times more O_2 than water and O_2 diffuses ∼8000 times faster in air than in water, resulting in higher O_2 availability for fishes on land (Dejours, 1988). How does ecologically relevant exposure to fluctuating environmental O_2 levels during development (e.g. repeated air exposure in fishes) affect the adult phenotype and the subsequent capacity for adult phenotypic flexibility in traits along the O_2 cascade?

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Fluctuating water–air transitions occur in amphibious fishes (~200 species; Wright and Turko, 2016). Amphibious fishes can be powerful models for understanding the impact of fluctuating extreme environmental conditions on phenotypic plasticity because the properties of air and water are so dramatically different. Although air provides higher O_2 availability compared with water, air has a lower density and viscosity relative to water and thus air exposure often results in a loss of gill function due to the collapse of gill lamellae (Lam et al., 2006). The loss of gill function may result in hypoxemia until modifications are made to enhance the uptake of atmospheric O_2. Moreover, the loss of gill respiration on land, and the low solubility of carbon dioxide in air relative to water, results in an elevation of internal CO_2 levels (reviewed by Wright and Turko, 2016; Bayley et al., 2019). Thus, although air exposure enhances O_2 availability, it is accompanied by several challenges (e.g. hypercapnia, nitrogen waste accumulation, desiccation) that may differentially affect fish at different life stages depending on a number of factors, including properties of respiratory exchange surfaces. Although many amphibious fishes repeatedly traverse the water–air interface during development (Ishimatsu et al., 2018), the consequences for the adult phenotype and the subsequent capacity for adult phenotypic flexibility remain unknown.

We tested the complementary hypotheses that fish exposed to fluctuating water–air environments during development (i) will exhibit no developmental changes along the O_2 cascade compared with fish reared under constant O_2 conditions because fixing mean trait values to suit one environment (e.g. land) could result in a phenotype–environment mismatch during exposure to another environment (e.g. water) and (ii) will therefore be highly phenotypically flexible as adults because their early life experiences (i.e. environmental fluctuations) predict high environmental variability in the future. We used the amphibious mangrove rivulus (*Kryptolebias marmoratus*) to test these hypotheses because it is an ideal vertebrate model for the study of phenotypic plasticity. *Kryptolebias marmoratus* is self-fertilizing and therefore allows for the production of isogenic lineages that minimize the confounding factor of genotypic variation, and reveal only the environmentally mediated phenotypic responses (Tatarenkov et al., 2010; Earley et al., 2012). Furthermore, *K. marmoratus* tolerates terrestrial exposure during all life stages (Taylor, 2012), and demonstrates considerable phenotypic flexibility along the O_2 cascade in response to air exposure. Previous studies from our laboratory have demonstrated that air-exposed *K. marmoratus* increase cutaneous expression of genes involved with O_2 sensing (Dong et al., 2019, preprint), proliferate epidermal capillaries (Cooper et al., 2012; Blanchard et al., 2019) and remodel their skeletal muscle towards a more aerobic phenotype (i.e. hypertrophy of red muscle fibers and increased red muscle capillarity; Brunt et al., 2016; Rossi et al., 2018), which probably enhances O_2 sensing, uptake, transport and utilization on land. In addition, air-exposed *K. marmoratus* also remodel their gills (i.e. increase the height of the interlamellar cell mass) – a change that may provide structural support to delicate lamellae and/or reduce water loss on land (Ong et al., 2007; Turko et al., 2011; 2018). To test the developmental plasticity hypothesis, we reared *K. marmoratus* under constant (water) or fluctuating (water–air) environmental conditions until adulthood (94 days post-hatch) and examined anatomical, behavioural and biochemical traits associated with O_2 sensing, uptake, transport and utilization by the skeletal muscle, as well as terrestrial locomotor performance as a collective measure of aerobic performance. The developmental plasticity hypothesis predicts that fish reared under constant and fluctuating conditions will exhibit the same phenotype in all measured traits at adulthood. Thus, phenotypic differences between rearing groups at adulthood (94 days post-hatch) would not support the developmental plasticity hypothesis. To evaluate the capacity for adult phenotypic flexibility, a subset of adult fish from each rearing condition was then air-exposed for 14 days before the same traits were measured. The phenotypic flexibility hypothesis makes trait-specific predictions. Adult air exposure was predicted to increase neuroepithelial cell (NEC) density (Dong et al., 2019, preprint) as well as decrease NEC size, as exposure to aquatic hypoxia causes an increase in NEC size in this species (Regan et al., 2011). Adult air exposure was also predicted to increase cutaneous capillarity (Blanchard et al., 2019), red muscle capillarity, red muscle fiber size (Brunt et al., 2016; Rossi et al., 2018), mitochondrial density of the skeletal muscle, and improve terrestrial locomotor performance (Brunt et al., 2016). Additionally, we expected a decrease in gill surface area (Ong et al., 2007) and ventricle size with air exposure, as O_2-limiting conditions increase ventricle size in other species (McClelland et al., 2005; Simonot and Farrell, 2007). We also examined hypoxia-induced emersion behaviour (i.e. when fish leave water in response to aquatic hypoxia) as a measure of whole-body hypoxia sensitivity because hypoxia-induced emersion behaviour has previously been demonstrated to be controlled, in part, by NEC stimulation in *K. marmoratus* (Regan et al., 2011). We hypothesized that changes in NEC density and/or size in the gills and/or skin would alter whole-body hypoxia sensitivity. We predicted that fish possessing a greater density of NECs and/or larger NECs would exhibit higher whole-body hypoxia sensitivity. In all traits, however, we predicted that fish raised under fluctuating conditions would exhibit a greater acclimation response to adult air exposure, regardless of the direction of phenotypic change.

MATERIALS AND METHODS

Experimental animals

We collected embryos within 24 h of release from adult *Kryptolebias marmoratus* Poey 1880 hermaphrodites (50.91 strain, originating from Twin Cayes, Belize; Tatarenkov et al., 2010), which were held under constant laboratory conditions (15‰ salinity, 25°C, 12 h:12 h light:dark cycle; Frick and Wright, 2002). As *K. marmoratus* embryos can be released from adults at varying developmental stages (Harrington, 1961), we discarded any embryos that exceeded developmental stage 21 (onset of pigmentation; Mourabit et al., 2011) to ensure that the remaining embryos were approximately the same age (Wells et al., 2015). We maintained the remaining embryos individually in water under standard conditions (~25 ml, pH 8.0, 15‰, 25°C, 12 h:12 h light:dark cycle) for 30 days, after which we induced hatching by manual removal of the chorion (Wells et al., 2015), as natural hatching can be delayed (≤108 days) after reaching hatching competency (Furness et al., 2018). Water changes were not performed during the 30-day embryonic period. Larvae were then exposed to either fluctuating or constant conditions until adulthood (94 days post-hatch). Experimental procedures were approved by the University of Guelph Animal Care Committee (AUP 3891).

Experimental protocol

Fish reared under constant conditions (constant fish; N=58) were maintained in 350 ml clear plastic containers that were filled with ~250 ml water (pH 8.0, 15‰, 25°C), but were handled on the same protocol as the fluctuating group to account for any stress related to handling. Fish reared under fluctuating water–air conditions (fluctuating fish; N=67) were manually transitioned between water and air, spending 50% of the experimental period in water (47 days) and 50% in air (47 days). Preliminary experiments revealed that 1-day post-hatch larvae survived <3 days of air exposure. Therefore, the air exposure period was progressively lengthened accordingly (1 day air

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for the first 3 weeks post-hatch, and then 3-, 5- and ultimately 7-day periods as fish aged; Fig. 1). Between air exposure periods, fish were maintained in water for an equal period (e.g. 3 days air, then 3 days water). Air exposure was accomplished as previously described (Ong et al., 2007). Briefly, fish were individually placed on moistened filter paper over cotton balls soaked in 50 ml of water (pH 8.0, 15‰, 25°C; >99% relative humidity; Ong et al., 2007) in experimental containers that were identical to those used to maintain fish in water. Fish reared under fluctuating conditions were fed *Artemia* sp. nauplii each day they were in water, and the constant group were fed on the same days. Water changes were performed every second week during the 94-day experimental period. Previous studies from our laboratory have demonstrated that phenotypic changes induced by air exposure in adult *K. marmoratus* are generally reversed within the same time frame upon return to water (e.g. Ong et al., 2007). Consequently, all fish spent the final week of the experimental period (days 88–94) in water to allow for the reversal of any phenotypically flexible responses induced by the previous air exposure period, and therefore reveal only permanent developmental changes.

At 94 days post-hatch, subsets of fish from both rearing treatments were used to examine emersion behaviour, terrestrial locomotor performance, and tissue differences. We euthanized the fish in MS-222 (500 mg l^{-1}) prior to tissue collection. We air-exposed the remaining fish from each rearing treatment for 14 days and repeated all measurements. It should be noted that *K. marmoratus* reach adulthood at 90 days post-hatch (Gresham et al., 2020) and do not exhibit significant age-related changes in the adult phenotype until >2 years of age (Rossi et al., 2019). Therefore, trait differences following air exposure are unlikely to be the result of age-related phenotypic changes during the 14-day air exposure period. Fish from all treatments were approximately the same size (Fig. S1).

Neuroepithelial cell density and size, and whole-body hypoxia sensitivity

We examined the density and size of serotonergic neuroepithelial cells (NECs) in the gills and skin by collecting whole gill baskets and skin samples directly posterior to the opercula (N=7–12 per treatment). We chose to examine serotonergic NECs because serotonin is an important mediator of ventilatory and behavioural responses to acute hypoxia in fishes (Regan et al., 2011; Shakarchi et al., 2013). We performed indirect double immunofluorescence labelling with antibodies raised in rabbits against serotonin (5-HT, 1:250; MilliporeSigma, Burlington, MA, USA) and a zebrafish neuron specific antibody raised in mouse (zn-12, 1:100; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), as previously described (Regan et al., 2011). Secondary antibodies included goat anti-rabbit fluorescein isothiocynante (FITC, 1:50; MilliporeSigma) and Alexa Fluor 594 goat anti-mouse IgG (1:100; Invitrogen, Carlsbad, CA, USA), which indirectly labelled 5-HT and zn-12 immunoreactivity of NECs and nerve fibers, respectively (Regan et al., 2011).

We determined the density of NECs in the gills as previously described by Robertson et al. (2015). To determine the density of NECs in the skin, we took images of the ventral skin (2.0 mm×1.6 mm), overlaid a 0.26 mm×0.26 mm grid on each image, and then counted the number of NECs in 50% of the grids to obtain an average number of NECs per grid. We estimated NEC size by manually measuring the area of all clearly visible NECs using ImageJ (http://image j.nih.gov/ij/) (Cochrane et al., 2019).

To determine if changes in NEC density and/or size in the gills and skin influenced whole-body hypoxia sensitivity, we examined hypoxia-induced emersion behaviour in a separate group of fish, as previously described (N=7–9 per treatment; Regan et al., 2011). Immediately following emersion, fish were euthanized as above. The thoracic cavity was then immediately opened so that the heart could be rinsed in physiological saline (mmol l^{-1}: 94 NaCl, 24 $NaCO_3$, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 0.7 $CaCl_2$; pH 7.6), and then bathed in 1 mol l^{-1} potassium chloride *in situ* to cause maximal contraction prior to fixation of the entire thorax (Johnson et al., 2014). Fish were then fixed in 10% buffered formalin for 72 h, decalcified (1 h Cal-EX; Fisher Scientific, Waltham, MA, USA), and embedded in paraffin wax for gill and heart histology.

Cutaneous capillarity and gill morphology

To assess cutaneous capillarity, a ∼3 mm transverse muscle steak immediately anterior to the dorsal fin was excised and processed for cryosectioning as previously described (N=9–10 per treatment; Rossi et al., 2018, 2019). Using 8 µm cryosections, we visualized capillarity in the ventral skin by staining for an endothelial cell–cell adhesion molecule, CD31 (PECAM-1; Blanchard et al., 2019). The primary and secondary antibodies included rat anti-mouse PECAM/CD31 (1:100; BD Pharmingen, San Jose, CA, USA) and

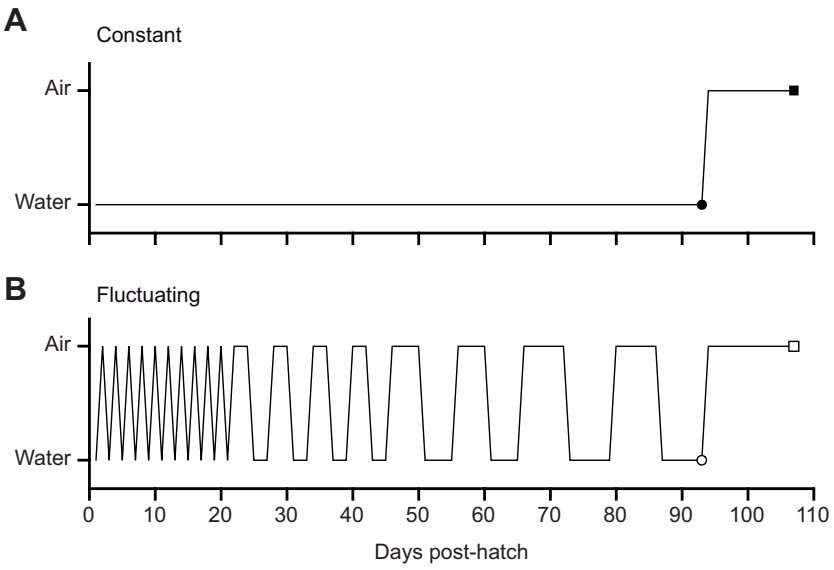

Fig. 1. Schematic representation of the experimental timeline. Circles (filled, constant; open, fluctuating) indicate when *Kryptolebias marmoratus* were sampled to evaluate the adult phenotype after rearing in (A) constant water or (B) a fluctuating water–air environment (developmental plasticity). Squares (filled, constant; open, fluctuating) represent when fish were sampled to evaluate the capacity for adult phenotypic flexibility in response to 14 days of air exposure.

Alexa Fluor 488 goat anti-rat IgG (1:400; Invitrogen), respectively. We cut the heads of paraffin-embedded fish into 5 µm sagittal sections to examine gill morphology (N=8–9 per treatment). We then stained and analysed the sections containing gill tissue for interlamellar cell mass (ILCM) coverage, as described previously (Turko et al., 2018).

Ventricle size and red muscle capillarity

We stained all 5 µm paraffin sections that contained heart tissue using hematoxylin and eosin to determine the maximal cross-sectional area of the ventricle. We estimated the cross-sectional area of the ventricle every 15 µm (i.e. one in every three sections; 17±3 sections per fish) by tracing around the perimeter of the ventricle in ImageJ. We plotted the distribution of ventricle area in each fish (N=5–7 per treatment; Fig. S2) and determined the maximal cross-sectional area of the ventricle (standardized to individual body mass) from the resulting distribution. If paraffin sections were lost from near the peak of the distribution, fish were excluded from the analysis so as not to under-estimate ventricle size. We visualized the capillarity of the red muscle located at the lateral line using 8 µm transverse cryosections of muscle obtained immediately anterior to the dorsal fin using CD31, as above (N=9–10 per treatment). All capillaries in contact with the red skeletal muscle fibers were counted, and then standardized to the total number of red muscle fibers as a proxy for O_2 transport to the muscle (Rossi et al., 2019).

Red skeletal muscle phenotype

We quantified the size and number of red skeletal muscle fibers using 8 µm transverse cryosections (described above) that we stained for slow myosin using a mouse IgA primary antibody (S58, 1:10; Developmental Studies Hybridoma Bank) and an Alexa Fluor 488 goat anti-mouse IgG secondary antibody (1:400; Invitogen), as described previously (N=9–10 per treatment; Rossi et al., 2018, 2019). We also measured the aerobic capacity of the red muscle using a succinate dehydrogenase (SDH) stain (N=9–10 per treatment; Borowiec et al., 2015; McFarlane et al., 2019). We measured citrate synthase (CS) activity in a separate subset of fish to assess the mitochondrial density of the red and white muscle combined (N=8–10 per treatment; McClelland et al., 2005). Briefly, a ∼25 mg muscle steak was excised from the posterior end of each fish, frozen in liquid nitrogen, and homogenized on ice in 20 volumes of homogenization buffer (20 mmol l^{-1} Hepes, 1 mmol l^{-1} sodium EDTA and 0.1% Triton X-100; pH 7.4). We determined CS activity at 25°C, by measuring the rate of change in absorbance at 412 nm for 10 min. The assay buffer contained 0.1 mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid), 0.3 mmol l^{-1} acetyl CoA and 0.5 mmol l^{-1} oxaloacetate, in 50 mmol l^{-1} Tris-HCl (pH 8.0). A control well lacking oxaloacetate was used to correct for background thiolase activity. We standardized CS activity to protein content (Bradford, 1976).

Terrestrial locomotor performance

To assess terrestrial locomotor performance, we jumped fish to exhaustion, as before (N=9–10 per treatment; McFarlane et al., 2019). Briefly, we placed fish in a terrarium (30 cm×60 cm) lined with moist filter paper. Following a 2 min adjustment period, we encouraged fish to jump by gently prodding them with a consistent touch using a clicker ballpoint pen until exhaustion (i.e. when fish were unresponsive to prodding). We video recorded jumping trials and quantified the number of jumps, as well as the total and average jump distance (standardized to individual body length) from video recordings (Brunt et al., 2016).

Statistical analyses

Statistical analyses were performed using RStudio (version 1.1.447). Data and residuals were originally assessed for homogeneity of variance using Levene's tests and normality using Shapiro–Wilk tests, respectively. Data were appropriately transformed when necessary. We used two-way ANOVAs to analyse the effect of rearing condition and adult air exposure on all examined traits. When a significant interaction was present in the data, the data were divided by rearing condition to determine the effect of air exposure on the adult phenotype using two-tailed t-tests. We performed a linear regression to determine the relationship between the size of red muscle fibers and the total jump distance. Results were considered significant at α<0.05. All raw data are available in the electronic supplementary material file 'Dataset 1'.

RESULTS

Neuroepithelial cell density and size, and whole-body hypoxia sensitivity

5-HT-immunopositive NECs were located on the efferent aspect of the gill filaments as well as on the skin, and were in close proximity to zn-12-immunopositive nerve fibers (Fig. 2). The O_2 sensing system exhibited little developmental plasticity (Fig. 3), with rearing condition only influencing cutaneous NEC size (two-way ANOVA: P<0.001; Fig. 3D). The cutaneous NECs of fluctuating fish were 18% smaller than those of constant fish. In contrast, we found evidence for phenotypic flexibility in the density and size of NECs in both the gills and skin (Fig. 3). Interestingly, the rearing environment affected the capacity for phenotypic flexibility in gill NEC density, as evident by an interaction between rearing treatment and adult air exposure (two-way ANOVA: interaction P=0.03; Fig. 3A). Adult air exposure increased (23%) the NEC density of constant fish (t-test: P=0.04), but did not affect that of fluctuating fish (t-test: P=0.39). A similar trend was evident in cutaneous NEC density (Fig. 3B). Adult air exposure caused a significant decline in NEC size in both tissues (gills two-way ANOVA: P<0.001; skin two-way ANOVA: P<0.01; Fig. 3C,D), regardless of rearing condition.

Rearing environment had an impact on the scope for phenotypic flexibility in the emersion response to acute aquatic hypoxia (two-way ANOVA: interaction: P=0.02; Fig. 3E). Constant (t-test: P=0.02), but not fluctuating (t-test: P=0.52) fish emersed at significantly higher [O_2] following adult air exposure.

Cutaneous capillarity and gill morphology

Cutaneous capillarity was not developmentally plastic (two-way ANOVA: P=0.69), but was phenotypically flexible (two-way ANOVA: P<0.01) (Fig. 4A). Fish exhibited a 1.5-fold increase in CD31 intensity in the ventral skin following adult air exposure. We found no significant changes in gill lamellae coverage by ILCM with treatment (two-way ANOVA: P=0.58, P=0.25; Fig. 4B).

Ventricle size and red muscle capillarity

The maximum cross-sectional area of the ventricle demonstrated significant developmental plasticity (two-way ANOVA: P=0.03) (Fig. 5A). The maximum cross-sectional area of the ventricle of constant fish was 14% larger than that of fluctuating fish. The number of capillaries in the red muscle was not altered by any treatment (two-way ANOVA: P=0.55, P=0.16; Fig. 5B).

Red skeletal muscle phenotype

Muscle phenotype was modulated by both rearing environment and adult air exposure. Fish raised under fluctuating conditions had 10%

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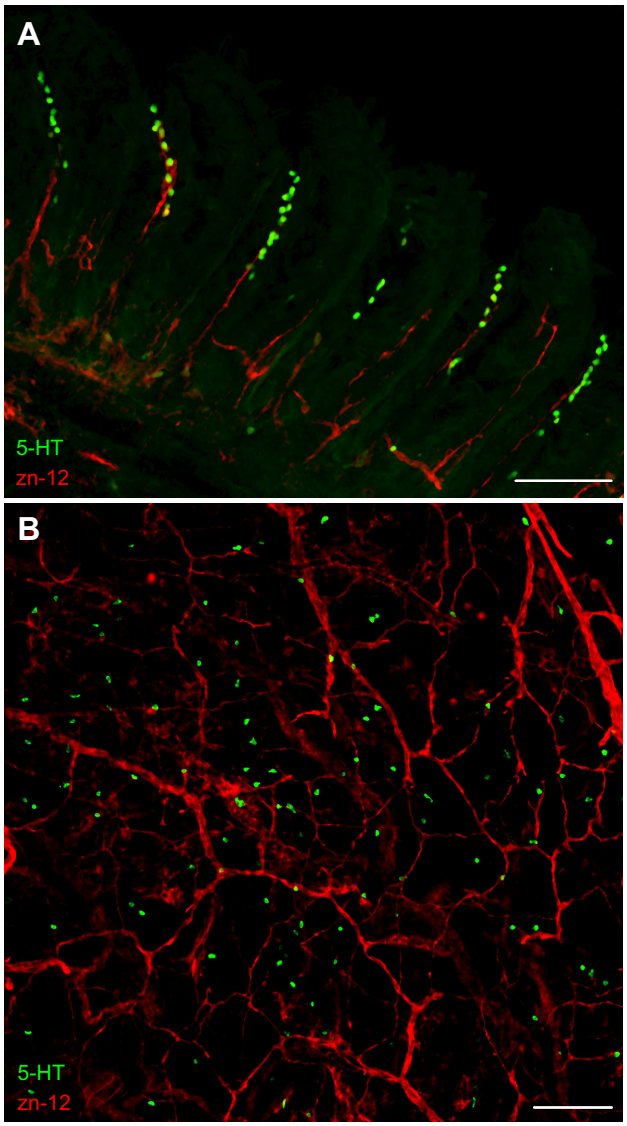

Fig. 2. Representative confocal images of 5-HT-immunopositive NECs (green) and zn-12-immunopositive nerve fibers (red). NECs were present on (A) the efferent aspect of the gill filaments and (B) the skin of *K. marmoratus*. Nerve fibers were found in close association with NECs in both tissues. Scale bars: 100 µm.

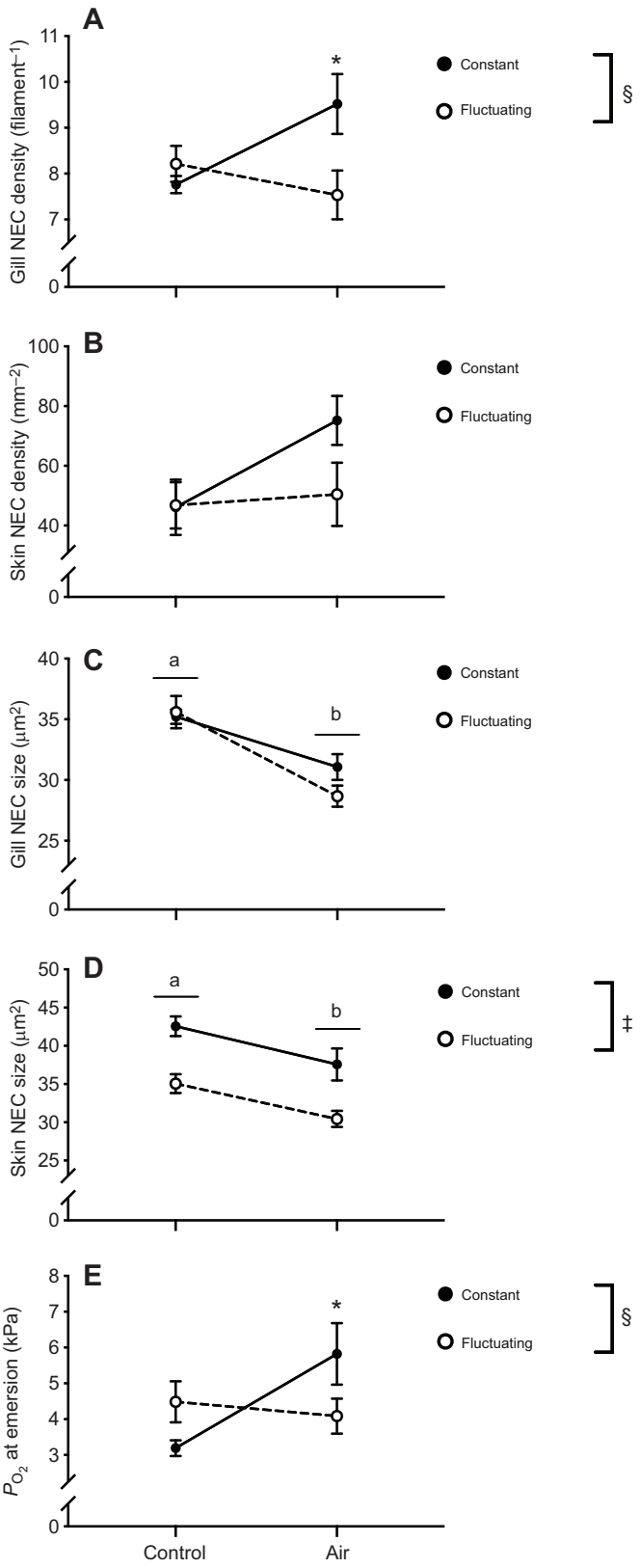

Fig. 3. See next page for legend.

more red muscle fibers than fish reared under constant conditions (two-way ANOVA: P=0.03) (Fig. 6A). Similarly, fluctuating fish had 29% larger red muscle fibers than constant fish (Fig. 6B). The rearing environment also affected the capacity for phenotypic flexibility in the size of red muscle fibers (two-way ANOVA: P=0.02). Adult air exposure resulted in significant hypertrophy of red muscle fibers in constant fish (t-test: P<0.01), with no change in fluctuating fish (t-test: P=0.18) (Fig. 6B). We found no evidence of phenotypic flexibility in the number of red muscle fibers (two-way ANOVA: P=0.59) (Fig. 6A). We found no change in SDH staining intensity (two-way ANOVA: P=0.39, P=0.16) or CS activity in the skeletal muscle (two-way ANOVA: P=0.11, P=0.28) with treatment (Fig. 6C,D).

Terrestrial jumping performance

Terrestrial jumping performance exhibited both developmental plasticity and phenotypic flexibility. The number of jumps that fish performed before exhaustion was developmentally plastic (two-way ANOVA: P<0.01); fluctuating fish could perform >15 more jumps than constant fish (Fig. 7A). We found a similar trend in the average and total jump distance (Fig. 7B,C). Rearing condition also affected

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Fig. 3. Phenotypic plasticity in the O_2 sensing system of *K. marmoratus*. The adult phenotype after rearing (Control) and after 14 days of air exposure (Air) in (A,B) the density of neuroepithelial cells (NECs) in the gills and skin, (C, D) the size of NECs in the gills and skin, and (E) the P_{O_2} at emersion. §Significant interaction between the rearing condition and air exposure. When a significant interaction was detected, we compared control and air-exposed fish within each rearing group. *Significant difference between control and air-exposed fish within the constant rearing group (phenotypic flexibility). ‡Significant main effect of rearing condition (developmental plasticity). Different lowercase letters (a,b) denote a significant main effect of air exposure (phenotypic flexibility). *N*=7–12 per treatment.

the scope for phenotypic flexibility in both the average (two-way ANOVA: interaction $P<0.001$) and total jump distance (two-way ANOVA: interaction $P=0.02$). Constant fish jumped significantly further (>100 body lengths) following adult air exposure (*t*-test: $P<0.01$), while the distance travelled by fluctuating fish did not change (*t*-test: $P=0.62$) (Fig. 7C). The average jump distance followed a very similar trend (Fig. 7B). Finally, we found a significant positive correlation between the size of red muscle fibers and the total distance fish travelled before reaching exhaustion (linear regression: $P=0.01$, $R^2=0.18$) (Fig. S3).

Summary

Overall, we found that exposure to fluctuating water–air environments induced developmental plasticity in several traits along the O_2 cascade (i.e. skin NEC size, maximum cross-sectional area of the ventricle, number of red muscle fibers, number of jumps before exhaustion). We also found that the rearing environment had an impact on the capacity for phenotypic flexibility in response to adult air exposure; constant fish exhibited phenotypic flexibility in eight traits (i.e. gill NEC density, gill NEC size, skin NEC size, P_{O_2} at emersion, cutaneous capillarity, red muscle fiber size, average jump distance, total jump distance), whereas fluctuating fish exhibited phenotypic flexibility in only three traits (i.e. gill NEC size, skin NEC size, cutaneous capillarity).

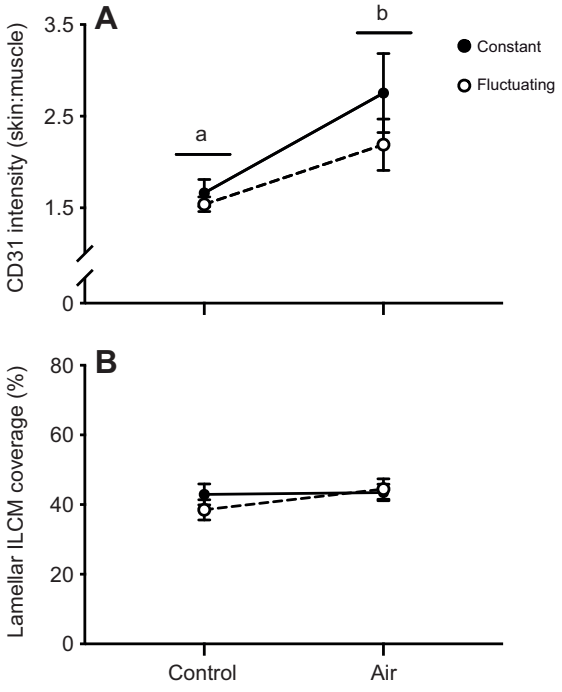

Fig. 4. Phenotypic plasticity in traits involved with O_2 uptake in *K. marmoratus*. The adult phenotype after rearing (Control) and after 14 days of air exposure (Air) in (A) the cutaneous capillarity (CD31) and (B) the percentage of the lamellae covered by an interlamellar cell mass (ILCM). Different lowercase letters (a,b) denote a significant main effect of air exposure (phenotypic flexibility). *N*=8–10 per treatment.

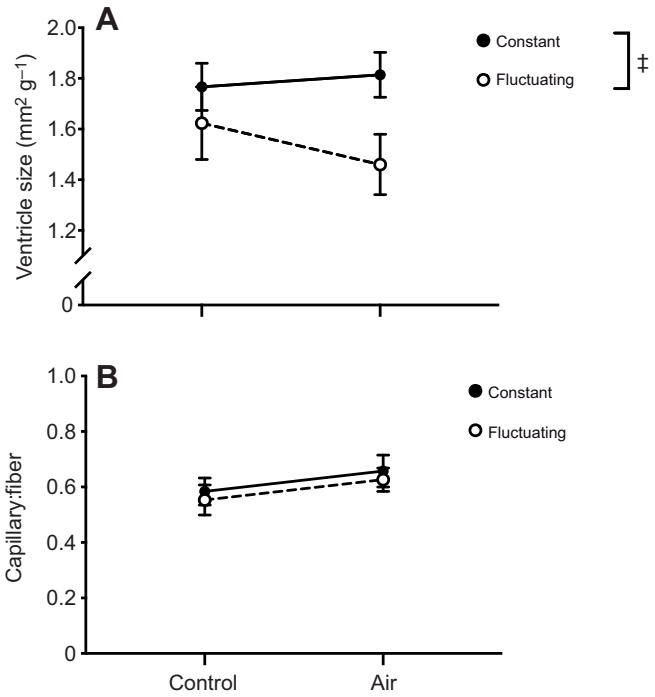

Fig. 5. Phenotypic plasticity in traits involved with O_2 transport in *K. marmoratus*. The adult phenotype after rearing (Control) and after 14 days of air exposure (Air) in (A) the maximum ventricle size and (B) the red muscle capillarity. See Fig. 3 for explanation of symbols. *N*=5–10 per treatment.

DISCUSSION

We used the amphibious *K. marmoratus* to test the hypothesis that fish exposed to fluctuating water–air environments during development exhibit no permanent developmental changes along the O_2 cascade compared with fish reared under constant conditions because fixing mean trait values to suit one environment (e.g. land) could be maladaptive when exposed to another environment (e.g. water). In support of our hypothesis, the development of the O_2 sensing system in *K. marmoratus* was largely unaffected by water–air fluctuations during early life, as were traits related to O_2 uptake. The lack of developmental plasticity, also termed developmental canalization (Debat and David, 2001), implies that irreversibly altering these traits for repeated air exposure may result in a costly phenotype–environment mismatch when fish spend time in water. Unexpectedly, we found evidence for marked developmental changes in traits related to O_2 transport and utilization. We suggest that the water–air phenotype induced during development (e.g. larger red muscle fibers) is not maladaptive in an aquatic environment and/or that the energetic cost of repeated remodelling in response to adult air exposure is significant. We also tested the hypothesis that fish reared under fluctuating conditions would be highly phenotypically flexible as adults. However, fish from both rearing environments exhibited the same degree of phenotypic flexibility in traits involved with O_2 sensing (NEC size) and O_2 uptake (cutaneous capillarity). Surprisingly, in other cases, exposure to fluctuating water–air conditions during development attenuated phenotypic flexibility in later life, despite the fact that phenotypic flexibility is hypothesized to be favoured when early environments are unstable. It is important to note, however, that our experiment was

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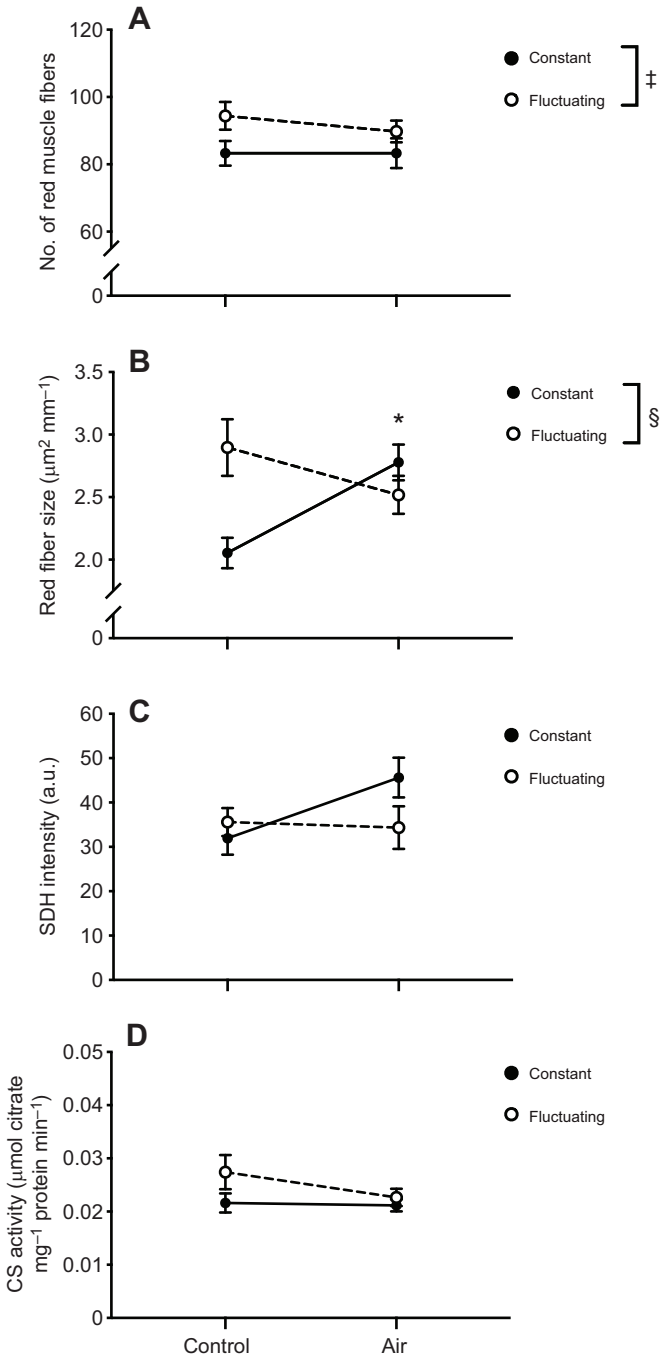

Fig. 6. Phenotypic plasticity in traits involved with O_2 utilization in the skeletal muscle of *K. marmoratus*. The adult phenotype after rearing (Control) and after 14 days of air exposure (Air) in (A) the number of red muscle fibers, (B) the size of red muscle fibers, (C) the succinate dehydrogenase (SDH) staining intensity in the red muscle (a.u., arbitrary units) and (D) the citrate synthase (CS) activity in the red and white muscle. See Fig. 3 for explanation of symbols. *N*=8–10 per treatment.

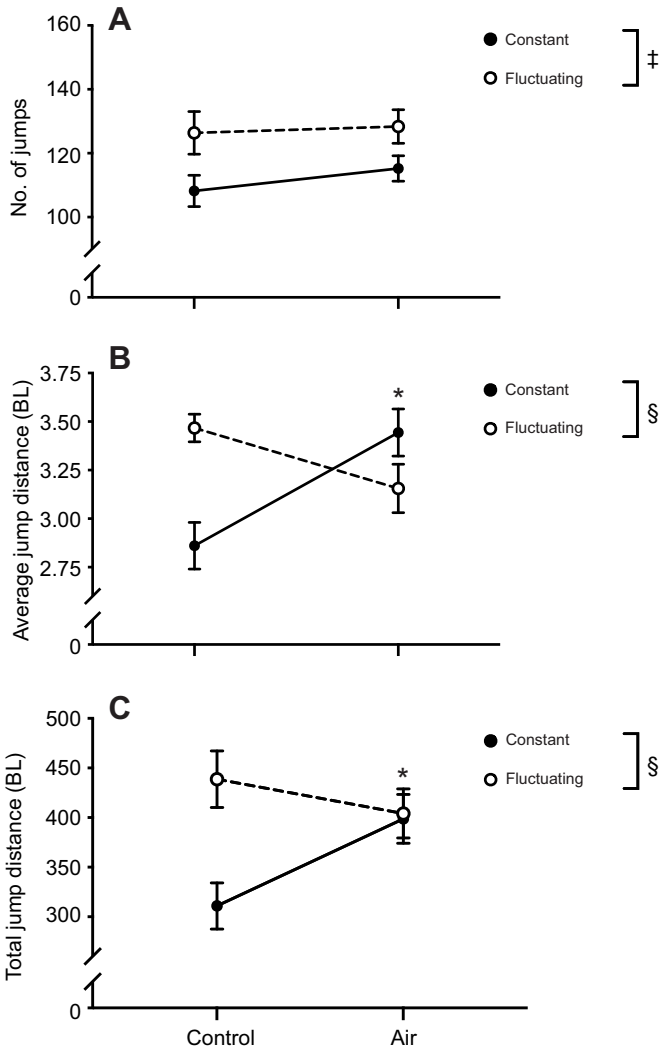

Fig. 7. Phenotypic plasticity in the terrestrial locomotor performance of *K. marmoratus*. The adult phenotype after rearing (Control) and after 14 days of air exposure (Air) in (A) the number of jumps, (B) the average jump distance (BL, body lengths) and (C) the total jump distance (BL). See Fig. 3 for explanation of symbols. *N*=8–10 per treatment.

'one-sided' in the sense that fish reared in a fluctuating environment had exposure to air while those in constant conditions did not. It is unknown whether the scope for phenotypic flexibility would similarly be blunted if fish were reared solely in air (although this rearing condition is an impossibility). Nevertheless, our findings suggest that energetic and/or performance trade-offs may mediate the phenotypic responses of *K. marmoratus* at different levels of the O_2 cascade. Moreover, exposure to fluctuating water–air environments during early life can diminish the scope for plasticity in response to air exposure in later life stages.

O_2 sensing

We found little developmental plasticity in the O_2 sensing system, as only cutaneous NEC size was affected by the rearing environment. Fish reared under fluctuating conditions possessed significantly smaller cutaneous NECs relative to fish reared under constant conditions. Similar findings have been reported in mammalian studies, in which early life exposure to hyperoxia significantly reduces the volume of the carotid body (i.e. the analogue of gill NECs in mammals; Hockman et al., 2017) – a change that persists into adulthood (Bavis et al., 2011). Interestingly, NECs in the gills did not change in size with repeated air exposure during early development, suggesting that the mechanisms underpinning NEC development may differ between the gills and skin and/or that the sensitivity of NECs to environmental O_2 levels during early life differs between these tissues.

The O_2 sensing system exhibited a high degree of phenotypic flexibility in response to adult air exposure. We found that NECs in

both the gills and skin decreased in size following adult air exposure regardless of rearing treatment. Our findings are consistent with previous work from our laboratory showing that hypoxia acclimation increased NEC size in the gills and skin of adult *K. marmoratus* (Regan et al., 2011), thus suggesting that O_2 availability in adulthood modulates NEC size in this species. Interestingly, we also found that fish reared under constant, but not fluctuating conditions, exhibited an increase in NEC density in the gills and skin following adult air exposure, as well as increased whole-body hypoxia sensitivity. The opposite trend has been reported in zebrafish (*Danio rerio*), where gill NEC density and whole-body hypoxia sensitivity was decreased after hyperoxia acclimation (Vulesevic et al., 2006). Therefore, we suggest that whole-body hypoxia sensitivity in fish may be controlled, in part, by NEC density. Alternatively, increased whole-body hypoxia sensitivity in constant fish following adult air-exposure may have been caused by a reduction in hypoxia tolerance (i.e. increased critical partial pressure of oxygen; Turko et al., 2012). Moreover, we suggest that other factors (besides O_2 levels) associated with air exposure may drive NEC proliferation in the gills and skin of *K. marmoratus*. For example, air exposure elicits a significant rise in internal CO_2 levels in *K. marmoratus* (L. Tunnah, C. Robertson, A. Turko and P.A.W., unpublished data). Elevated internal CO_2 levels during air exposure may cause proliferation of gill and skin NECs in *K. marmoratus*, as exogeneous exposure to high CO_2 has previously been demonstrated to cause proliferation of gill NECs in this species (Robertson et al., 2015). Our findings also indicate that the exposure to fluctuating environments blunts flexibility in NEC density in response to adult air exposure, but the underlying mechanism remains unknown.

O_2 uptake and transport

The traits related to O_2 uptake that we measured (i.e. gill morphology, cutaneous capillarity) were not developmentally plastic. While some studies have similarly demonstrated that environmental O_2 availability during development has no effect on the respiratory structures of fishes (e.g. *Trichopodus trichopterus*; Mendez-Sanchez and Burggren, 2019), others have shown the opposite effect (e.g. *Pseudocrenilabrus multicolor victoriae*, Chapman et al., 2008; *Polypterus senegalus*, Turko et al., 2019). In terms of O_2 transport, we found that water–air fluctuations during development significantly reduced ventricle size. Surprisingly, very few studies have examined how perturbations in environmental O_2 availability can alter the cardiac morphology of fishes (Gamperl and Farrell, 2004; Motyka et al., 2017), particularly during early life. There is some evidence to suggest that chronic hypoxia exposure has no effect on the heart size of adult fishes (e.g. *Sciaenops ocellatus*; Pan et al., 2017), but can alter other cardiac traits (e.g. mitochondrial size in *Platichthys flesus*; Lennard and Huddard, 1992). Even less is known about cardiac remodelling in response to hyperoxia/air exposure among fishes, but mammalian studies have shown that relatively high O_2 availability can result in a reduction of heart mass (Fan et al., 2005). The persistent effects of water–air fluctuations on the cardiac morphology of *K. marmoratus* may indicate that any performance costs associated with a reduced ventricle size (e.g. reduced cardiac output) during aquatic exposure are marginal.

Cutaneous capillarity was highly phenotypically flexible in *K. marmoratus*. Adult air exposure proliferated cutaneous capillaries, as previously reported in this species (Cooper et al., 2012; Turko et al., 2014; Blanchard et al., 2019). Increased cutaneous capillarity is presumed to enhance the O_2 uptake capacity of amphibious species on land (Graham, 1997; Blanchard et al., 2019), but may be detrimental in aquatic environments if less blood is available for branchial gas exchange. Thus, the use of phenotypic flexibility over developmental plasticity may buffer against a potential phenotype–environment mismatch in water. Although the O_2 transport capacity (ventricle size, red muscle capillarity) was unaffected by adult air exposure in this study, in previous studies we reported increased blood O_2 transport (i.e. increased [hemoglobin] and hematocrit) in air-exposed *K. marmoratus* (Turko et al., 2014; Blanchard et al., 2019). Similarly, rainbow trout (*Oncorhynchus mykiss*) improved blood O_2-carrying capacity by increasing the concentration of hemoglobin in their blood following exposure to intermittent aquatic hyperoxia (Ritola et al., 2002). Therefore, we suggest that flexibly altering hematological parameters, rather than heart size, in response to elevated O_2 availability may be preferable for modulating O_2 transport during terrestrial sojourns.

O_2 utilization and aerobic performance

The early life environment had a pronounced impact on skeletal muscle phenotype. Fluctuating fish developed more red muscle than constant fish, which correlated with better terrestrial performance, as previously demonstrated in this species (Rossi et al., 2019; McFarlane et al., 2019). These skeletal muscle changes are unlikely to be the result of terrestrial activity because air-exposed *K. marmoratus* remain quiescent (Turko et al., 2014), and we found no change in the aerobic capacity (SDH intensity) of the red muscle, which increases considerably after terrestrial exercise training (McFarlane et al., 2019), but not air exposure alone (Rossi et al., 2018). Rather, the structure of skeletal muscle in fishes can be highly sensitive to environmental O_2 availability, particularly during early life (Matschak et al., 1997). For instance, rainbow trout (*O. mykiss*) reared in hyperoxia developed more red muscle relative to controls in normoxia, whereas trout reared in hypoxia developed less (Lefèvre et al., 2007). Despite having more red muscle, we found no change in total muscle mitochondrial density in fish reared under fluctuating conditions. However, the vast majority of the skeletal musculature in fishes is white muscle (>90%; Bone, 1978), which may have masked red muscle-specific changes in mitochondrial content. The fact that the locomotor performance correlated positively with red muscle fiber size is suggestive of enhanced O_2 utilization, but further studies are required to understand the mechanism(s).

Finally, we found that the early life environment influenced the scope for phenotypic flexibility in the red muscle. Constant fish exhibited hypertrophy of their red muscle fibers after adult air exposure, matching that of fluctuating fish. However, phenotypic flexibility was not observed in the red muscle of fluctuating fish, possibly because developmental modifiers had maximally increased their red muscle fiber size. Interestingly, wild-caught *K. marmoratus* also have more red muscle (i.e. more and larger red muscle fibers) than laboratory-reared fish and are less phenotypically flexible in response to adult air exposure (G.S.R. and A. J. Turko, unpublished data). The phenotypic similarities between wild and laboratory-reared (fluctuating) *K. marmoratus* implies that water–air fluctuations frequently occur during the early life stages of fish in the wild. Indeed, *K. marmoratus* embryos (Taylor, 2012) and larvae (D. S. Taylor and P.A.W., personal observation) have been found out of water in the wild. The fact that *K. marmoratus* has evolved under fluctuating conditions may explain the fundamentally different patterns of phenotypic flexibility observed in the muscle of constant versus fluctuating laboratory-reared fish, as well as in other traits (e.g. gill NEC density, P_{O_2} at emersion, average and total jump distance) throughout this study. It is important to note, however, that there are

numerous traits that we could have examined along the O_2 cascade, and that each may have exhibited a slightly different response to the rearing environment and to adult air exposure. On the one hand, the O_2 cascade is an integrated system and thus phenotypic changes at one level of the cascade are often accompanied by parallel changes at other levels (Di Prampero, 1985; Hoppeler and Weibel, 1998). On the other hand, only a broader survey of traits under the same experimental conditions would elucidate whether this is the case in *K. marmoratus*, and whether the patterns of plasticity observed in our study apply to the O_2 cascade more broadly.

Conclusions and perspectives

The environment during early life can provide important information about the mean environmental conditions as well as the variability in those conditions in the future (Beaman et al., 2016; Stamps and Frankenhuis, 2016), particularly when animals occupy the same environment throughout life. The variability we subjected fish to was not consistently predictable, as the length of air exposure increased over time. Therefore, we anticipated that fish exposed to fluctuating conditions during development would be highly phenotypically flexible as adults because the cues from their early life environment would predict high environmental variability in the future. Paradoxically, our study demonstrates that various phenotypically flexible responses were blunted in *K. marmoratus* when reared under fluctuating water–air conditions. Fish reared under constant conditions exhibited phenotypic flexibility in response to adult air exposure in eight traits measured along the O_2 cascade, whereas fluctuating fish only exhibited flexibility in three traits. Why?

One possible explanation is that when environments fluctuate on rapid and/or unpredictable time scales, organisms may express a fixed phenotype that reduces temporal variation in fitness rather than relying on phenotypic flexibility (i.e. bet-hedging; Childs et al., 2010). For example, some birds alter the size and number of eggs produced in each clutch depending on the prevailing environmental conditions (i.e. phenotypic flexibility), while others consistently produce eggs of an intermediate size that may not be optimal for any environment, but reduces variation in fitness over time (Marshall et al., 2008; Olofsson et al., 2009). Thus, *K. marmoratus* may permanently alter some traits during development (e.g. red fiber size) in order to reduce the variation in fitness experienced when transitioning between water and land. This unexpected pattern of plasticity could presumably occur in any trait, and in response to any stressor, if the time required for reversible remodelling exceeds the duration of environmental change. Alternatively, phenotypic flexibility may have been blunted in *K. marmoratus* due to the energetic costs of phenotype remodelling. Organisms may express a fixed phenotype if the fitness advantage gained from repeated remodelling to two (or more) differing environments is outweighed by the energetic cost (DeWitt et al., 1998). Although phenotypic flexibility is generally presumed advantageous under fluctuating conditions, we suggest that the frequency and/or predictability of environmental fluctuations as well as the fitness and/or energetic costs associated with repeated reversible remodelling are important factors that mediate phenotypic responses to environmental change.

In some traits, there was no evidence of developmental plasticity in response to environmental fluctuations, but surprisingly the adult response was significantly curtailed (e.g. gill NEC density). Such a scenario suggests that water–air fluctuations may have altered the underlying molecular mechanisms of these traits during development, resulting in the apparent attenuation of adult phenotypic flexibility. Alternatively, the expression of phenotypic flexibility in constant fish may represent a maladaptive response to adult air exposure, as phenotypic flexibility is not universally adaptive (Velotta and Cheviron, 2018). Taken together, our data provide compelling evidence that early life exposure to fluctuating water–air environments alters mean adult traits in the O_2 cascade, as well as fine-tunes the reversible adult response to the water–air transition.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.S.R., P.V.C., P.A.W.; Methodology: G.S.R., P.V.C., P.A.W.; Validation: G.S.R., P.V.C., P.A.W.; Formal analysis: G.S.R., P.V.C.; Investigation: G.S.R., P.V.C.; Writing - original draft: G.S.R., P.V.C.; Writing - review & editing: G.S.R., P.V.C., P.A.W.; Visualization: G.S.R., P.V.C.; Supervision: P.A.W.; Funding acquisition: G.S.R., P.V.C., P.A.W.

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Supplementary information

Supplementary information available online at https://jeb.biologists.org/lookup/doi/10.1242/jeb.228304.supplemental

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