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SHORT COMMUNICATION

Oxygen drives skeletal muscle remodeling in an amphibious fish out of water

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ABSTRACT

Skeletal muscle remodeling in response to terrestrial acclimation improves the locomotor performance of some amphibious fishes on land, but the cue for this remodeling is unknown. We tested the hypothesis that muscle remodeling in the amphibious *Kryptolebias marmoratus* on land is driven by higher O_2 availability in atmospheric air, and the alternative hypothesis that remodeling is induced by a different environmental or physiological condition that fish experience on land. Fish were acclimated to 28 days of air, or to aquatic hyperoxia, hypercapnia, hypoxia, elevated temperature or fasting conditions. Air, fasting and hyperoxic conditions increased (>25%) the size of oxidative fibers in *K. marmoratus* while hypoxia had the reverse effect (23% decrease). Surprisingly, hyperoxia acclimation also resulted in a transformation of the musculature to include large bands of oxidative-like muscle. Our results show that *K. marmoratus* is highly responsive to environmental O_2 levels and capitalizes on O_2-rich opportunities to enhance O_2 utilization by skeletal muscle.

KEY WORDS: Oxidative muscle, Hyperoxia, Slow myosin, Alkaline phosphatase, Succinate dehydrogenase, *Kryptolebias marmoratus*

INTRODUCTION

The physical disparities between air and water make locomotory movement on land far more difficult than movement in water (Schmidt-Nielsen, 1972). Nevertheless, there are >200 extant species of amphibious fish that leave water (i.e. emerse) and spend time on land as part of their natural history (Gordon et al., 1969; Ord and Cooke, 2016; Wright and Turko, 2016). When on land, amphibious fishes must be mobile to exploit terrestrial resources, find refuge from predation and desiccation, and eventually return to water (Sayer, 2005). Thus, skeletal muscle plays an important role in locomotion on land (Cediel et al., 2008; Brunt et al., 2016; Du and Standen, 2017).

Skeletal muscle is one of the most phenotypically plastic tissues in the body of fishes (Sänger, 1993). It is generally composed of two anatomically and functionally distinct fiber types: slow-oxidative (red) and fast-glycolytic (white) fibers that use aerobic and anaerobic pathways, respectively, for ATP production. In some species, fast-oxidative (pink) fibers – intermediate between red and white fibers – are also present (Johnston, 1981). Fishes can modify structural and metabolic properties of skeletal muscle in response to changes in demand and the environment to bring their phenotype closer to a functional optimum (Johnston, 2006; McClelland and Scott, 2014). For instance, the amphibious *Polypterus senegalus* use their pectoral fins to swim slowly in water, but when emersed, the pectoral fins are often used to generate rapid bursts of power (Standen et al., 2014, 2016). When reared in a terrestrial environment, *P. senegalus* possess a greater proportion of glycolytic fibers in the pectoral muscles than aquatically reared fish, which is thought to improve muscle function for locomotion on land (Du and Standen, 2017). A recent study from our laboratory showed that the amphibious *Kryptolebias marmoratus* reversibly remodeled skeletal muscle towards a more aerobic phenotype (i.e. increased the total cross-sectional area of oxidative muscle via hypertrophy) after 14 days of emersion, despite being fasted and inactive (Brunt et al., 2016). This change in muscle phenotype was positively correlated with terrestrial locomotor performance. Air-acclimated *K. marmoratus* also generated less lactate during locomotion than their water-acclimated cohorts. What drives changes in muscle phenotype in *K. marmoratus* out of water? One potential cue for muscle remodeling is the higher O_2 availability in atmospheric air that allows for enhanced O_2 utilization during terrestrial locomotion.

In the present study, we tested the hypothesis that the hypertrophic growth of oxidative skeletal muscle fibers in *K. marmoratus* acclimated to air is driven by higher environmental O_2 availability. Even with the same partial pressure of O_2 (P_{O_2}), O_2 availability is higher in air than in water, both because the diffusivity of O_2 in air is ∼8000 times greater and because the concentration of O_2 in air is ∼30 times higher than that of water. Both factors result in much thinner boundary layers and steeper P_{O_2} gradients next to the respiratory surface of animals in air (Dejours, 1988). The hypothesis predicts that the muscle phenotype of fish exposed to aquatic hyperoxia would closely resemble that of air-acclimated fish. There are also several physiological challenges that *K. marmoratus* experiences on land that could serve as cues for muscle remodeling, given the highly plastic nature of skeletal muscle. For example, the loss of gill function on land, and the low solubility of CO_2 in air relative to water, results in the accumulation of CO_2 in the body (i.e. hypercapnia), which leads to respiratory acidosis (Wright and Raymond, 1978; Daxboeck and Heming, 1982; Heisler, 1982). Hypoxemia may also occur initially when gills become non-functional until modifications are made to enhance the uptake of atmospheric O_2. Moreover, fish may be challenged by higher ambient air temperature relative to water (Tytler and Vaughan, 1983; Gibson et al., 2015), particularly with direct solar radiation (Graham et al., 1985). Some amphibious fishes have been shown to lower their body temperature below that of ambient air through evaporative water loss from moist skin, but high-humidity environments (e.g. tropics) can significantly reduce the capacity for evaporative cooling (Tytler and Vaughan, 1983; Gibson et al., 2015). Fasting could also be a cue, as many amphibious fishes rely solely on intrinsic energy stores to maintain routine metabolic functions during emersion, because, as suction

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feeders, they require water to swallow food (Alexander, 1970; Ferry-Graham and Lauder, 2001). Thus, we tested the alternative hypotheses that the cue for muscle remodeling during emersion is hypercapnia, hypoxemia, elevated temperature and/or fasting. We acclimated *K. marmoratus* to 28 days of air, or one of the following aquatic exposures: hyperoxia (P_{O_2}=41.8 kPa), hypercapnia (P_{CO_2}=5.1 kPa), hypoxia (stepwise decrease to P_{O_2}=4.1 kPa), a 5°C increase in temperature and fasting conditions. The cross-sectional area (CSA), the number of fibers, the number of capillaries and the staining intensity of succinate dehydrogenase (SDH) of oxidative skeletal muscle at the lateral line was measured.

MATERIALS AND METHODS

Animals

All experimental fish were adult hermaphrodites of the self-fertilizing (50.91 strain; Tatarenkov et al., 2010) *K. marmoratus* (Poey 1880) (0.13±0.03 g, mean±s.d.). Prior to experiments, fish were individually maintained in 120 ml plastic holding cups (~60 ml water, 15‰ salinity, 25°C) in the Hagen Aqualab at the University of Guelph on a 12 h:12 h light:dark cycle (Frick and Wright, 2002). Fish were fed live *Artemia* sp. three times weekly. All experimental procedures were approved by the University of Guelph Animal Care Committee (AUP 3891).

Experimental protocol

Fish were exposed for 28 days to one of seven treatments: control, hyperoxia, hypoxia, elevated CO_2, elevated temperature, air or fasting. In all experiments, fish were individually maintained in plastic holding cups (120 ml) with ~60 ml water. All experiments were conducted in brackish water (15‰), except for elevated temperature and hypoxia acclimations, which were conducted in well water (0.2‰; Platek et al., 2017) to accommodate a separate study (A.J.T., B. Cisternino and P.A.W., in preparation). Control fish were compared at both salinities (0.2‰ n=15, 15‰ n=14) and muscle parameters were not statistically different (ANOVA; P>0.27). Thus, control fish from each experiment were combined into a single group. In all experiments, except the air-exposed and fasted groups, fish were fed *Artemia* sp. three times weekly. At the end of each experiment, fish were killed in tricaine methanesulfonate (MS222; 500 mg l^{-1}), weighed and the standard length measured. Body condition (Fulton's K) was determined using mass and standard length measurements as described by Froese (2006). A ~3 mm transverse steak immediately anterior to the dorsal fin was removed, coated in embedding medium (Shandon Cryomatrix™, Fisher Scientific, Hampton, NH, USA), frozen in liquid nitrogen-cooled isopentane and stored at −80°C.

Hyperoxia

Hyperoxia (P_{O_2}=41.8 kPa) acclimation was achieved by placing fish (0.14±0.04 g) in hyperoxic water for 28 days maintained at 25°C (15‰). Fish were individually maintained in perforated plastic holding cups (120 ml) that filled to ~60 ml when resting in a 20 l tank. Hyperoxia was maintained by continuously bubbling compressed O_2 into the 20 l tank. Normoxic (control) fish were held in an identical apparatus and aerated with air. In a follow-up experiment to examine an unusual skeletal muscle response to hyperoxia in *K. marmoratus*, we compared *K. marmoratus* (0.12±0.01 g) and a closely related non-amphibious freshwater guppy *Poecilia wingei* Poeser, Kempkes and Isbrücker 2005 (0.16±0.18 g) held in hyperoxic or normoxic (control) water (0.2‰) for 21 days. Oxygen levels were measured periodically in all experiments and were always between 36.0 and 46.2 kPa for hyperoxia acclimation, and between 19.5 and 20.5 kPa for control (Hach LDO101 electrode connected to Hach HQ30d meter, Hach Company, Mississauga, ON, Canada).

Hypercapnia

Hypercapnia (P_{CO_2}=5.1 kPa) acclimation was achieved by placing fish in individual holding cups with ~60 ml of water in a CO_2 incubator (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) for 28 days (25°C, 15‰; Robertson et al., 2015). In an earlier experiment, we determined that a water P_{CO_2} of 5.1 kPa results in a whole-body P_{CO_2} of ~4 kPa in *K. marmoratus* – the same P_{CO_2} as air-acclimated fish (C. Robertson, A.J.T. and P.A.W., unpublished). Control fish were also maintained in plastic individual holding cups with ~60 ml of water (25°C, 15‰).

Hypoxia

Kryptolebias marmoratus (0.17±0.01 g) were held for 28 days in hypoxic or normoxic (control) water maintained at 25°C (0.2‰). Fish were individually maintained in perforated plastic holding cups that rested in a 20 l tank where the O_2 level was reduced from 50% (P_{O_2}=10.3 kPa) to 20% (P_{O_2}=4.1 kPa) air saturation by 10% decrements weekly with N_2. The appropriate O_2 level was maintained using an automated oxygen control system (OXY-REG, Loligo Systems, Viborg, Denmark). Normoxia (control) was maintained as above. Oxygen levels were verified periodically as for the hyperoxia experiments (see above).

Temperature

Fish (0.12±0.02 g) were individually placed in perforated plastic holding cups resting in a 20 l tank. Water temperature in the 20 l tank was maintained with an aquarium heater at either 30 or 25°C (control) for 28 days (0.2‰) and periodically verified with a thermometer.

Air and fasting

Air acclimation was achieved by placing fish (0.13±0.02 g) on moist filter paper (15‰) in individual holding cups as previously described (Ong et al., 2007). *Kryptolebias marmoratus* are unable to eat out of water (Pronko et al., 2013) and therefore an additional group of fish (0.13±0.01 g) were fasted in water for 28 days (25°C, 15‰).

Analysis

Frozen muscle steaks were cut into 9 µm transverse sections in a cryostat (Leitz Cryostat Microtome, Labequip Ltd, Markham, ON, Canada) at −20°C, mounted on Superfrost Plus slides (Fisher Scientific), and stored at −80°C until staining. Oxidative muscle fibers were identified by staining for slow myosin using a mouse IgA primary antibody (S58; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) as previously described (Johnston et al., 2004). Thus, if fast-oxidative (pink) muscle is present in this species, it was not included in the analysis. An alkaline phosphatase (AP) stain was used to visualize capillaries in the oxidative muscle, and a succinate dehydrogenase (SDH) stain was used as a proxy for the aerobic capacity of oxidative muscle (Borowiec et al., 2015; Brunt et al., 2016).

All stained slides were viewed using an epifluorescence microscope (Nikon Eclipse 90i microscope, Nikon, Tokyo, Japan) and photographed using NIS Elements software (Nikon). Slides were randomized to reduce observational bias and analyzed using ImageJ (http://imagej.nih.gov/ij/). Fluorescent slow myosin-stained sections were used to quantify the total number of oxidative fibers on one lateral half of each fish. Thirty of these fibers were randomly selected for cross-sectional area (CSA) measurements. The total

CSA of oxidative muscle (in one lateral half) was determined by multiplying the number of oxidative fibers by the average size of the 30 random fibers. The size of individual oxidative fibers and the total CSA of oxidative muscle were standardized to standard length to account for variation in body size as there were no differences in standard length across treatment groups (P=0.12). Using the AP-stained slides, capillaries in contact with oxidative fibers were counted and reported as a capillary:oxidative fiber ratio. The SDH staining intensity was determined by circumscribing a square (500 μm^2) over the oxidative muscle (centered over the horizontal septum) and using ImageJ to calculate the integrated density within the square. The nearby glycolytic muscle showed non-specific background staining, and therefore the integrated density of glycolytic muscle was subtracted from that of the oxidative muscle.

Statistics

All data were assessed for normality and homogeneity of variance using Shapiro–Wilk and Bartlett's tests, respectively. One-way analyses of variance (ANOVA), followed by Tukey *post hoc* tests, were used to determine the effect of each experimental acclimation on the number of oxidative fibers, the size of individual oxidative fibers, the total CSA of oxidative muscle and the capillary:oxidative fiber ratio. Non-parametric Kruskal–Wallis tests, followed by Dunn's *post hoc* tests, were used to determine the effect of each experimental acclimation on SDH staining intensity and on Fulton's K, as normality and homogeneity of variance assumptions were violated. Unpaired t-tests were used to determine the effects of hyperoxia acclimation on the percentage CSA occupied by oxidative and oxidative-like muscle in *K. marmoratus* and *P. wingei* relative to their respective controls. Significance was designated at α=0.05.

RESULTS AND DISCUSSION

Oxidative muscle at the lateral line showed the typical triangular shape with slow myosin, AP and SDH markers (Fig. 1A–C). The total CSA of oxidative muscle at the lateral line was significantly larger in fasted, hyperoxia- and air-acclimated fish relative to controls (Tukey; P<0.01) (Fig. 2A). In contrast, the CSA of oxidative muscle in hypoxia-acclimated fish was smaller than that of control fish (Tukey; P<0.01; Fig. 2A). The size of individual oxidative fibers showed the same significant changes (ANOVA; P<0.01; Fig. 2B). There was no significant difference in the number of oxidative fibers across treatment groups (ANOVA; P=0.29; Fig. 2C). The capillary:oxidative fiber ratio was not significantly different across treatment groups (ANOVA; P=0.42; Fig. S1A), nor was SDH staining intensity different in fasted, or air-, hypercapnia-, warm temperature- or hypoxia-acclimated fish relative to control (Dunn's test; P>0.05). However, fish acclimated to hyperoxia showed significantly higher SDH staining intensity than control fish (Dunn's test; P=0.03) (Fig. S1B). There was no significant difference in Fulton's K between treatment groups relative to control (Dunn's test; P>0.05), but hypoxia-acclimated fish had a higher Fulton's K than warm temperature-acclimated fish (Dunn's test; P<0.05) (Fig. S2).

Unusual bands of oxidative-like muscle were noted in *K. marmoratus* dorsal and ventral to the lateral line with hyperoxia acclimation (Fig. 3A). Oxidative-like fibers were not statistically different in size (3.2±0.3 μm^2 mm^{-1}) from oxidative fibers at the lateral line (2.6±0.4 μm^2 mm^{-1}; Tukey; P=0.11), but were significantly smaller than glycolytic fibers (80.3±8.0 μm^2 mm^{-1}; Tukey; P<0.01). Several attempts to stain with SDH and slow myosin markers were only partially successful, and therefore we use the term 'oxidative-like' muscle. There was no evidence of oxidative-like muscle bands in *P. wingei* following hyperoxia acclimation (Fig. 3B). There was a 7-fold increase in the percentage CSA occupied by oxidative and oxidative-like muscle in *K. marmoratus* relative to control (t-test; P=0.01; Fig. 3C).

We hypothesized that the hypertrophic growth of oxidative skeletal muscle fibers in *K. marmoratus* acclimated to air is driven by higher environmental O_2 availability. Indeed, hypertrophy in oxidative muscle at the lateral line in response to aquatic hyperoxia closely resembled the response to air acclimation. Hyperoxia acclimation also increased the SDH staining intensity in *K. marmoratus*, suggesting that high environmental O_2 increases the aerobic capacity of skeletal muscle in these fish. Moreover, aquatic hyperoxia caused a transformation of the musculature to include discrete bands of oxidative-like muscle in *K. marmoratus*, but not in the closely related aquatic species *P. wingei*. To the best of our knowledge, these additional bands of oxidative muscle have not been reported in any other species. Thus, the amphibious *K. marmoratus* is highly responsive to environmental O_2 levels and may capitalize on O_2-rich opportunities (e.g. air, aquatic hyperoxia) to enhance O_2 utilization. The fact that hypoxia induced the reverse

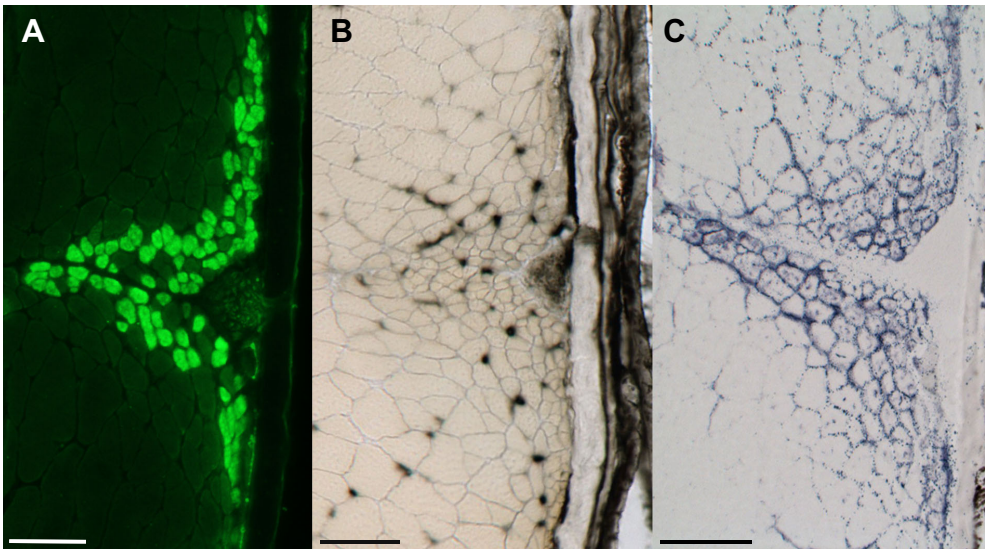

Fig. 1. Representative images of *Kryptolebias marmoratus* oxidative skeletal muscle at the lateral line. (A) Fluorescent slow myosin, (B) alkaline phosphatase and (C) succinate dehydrogenase markers. Scale bars: 100 μm.

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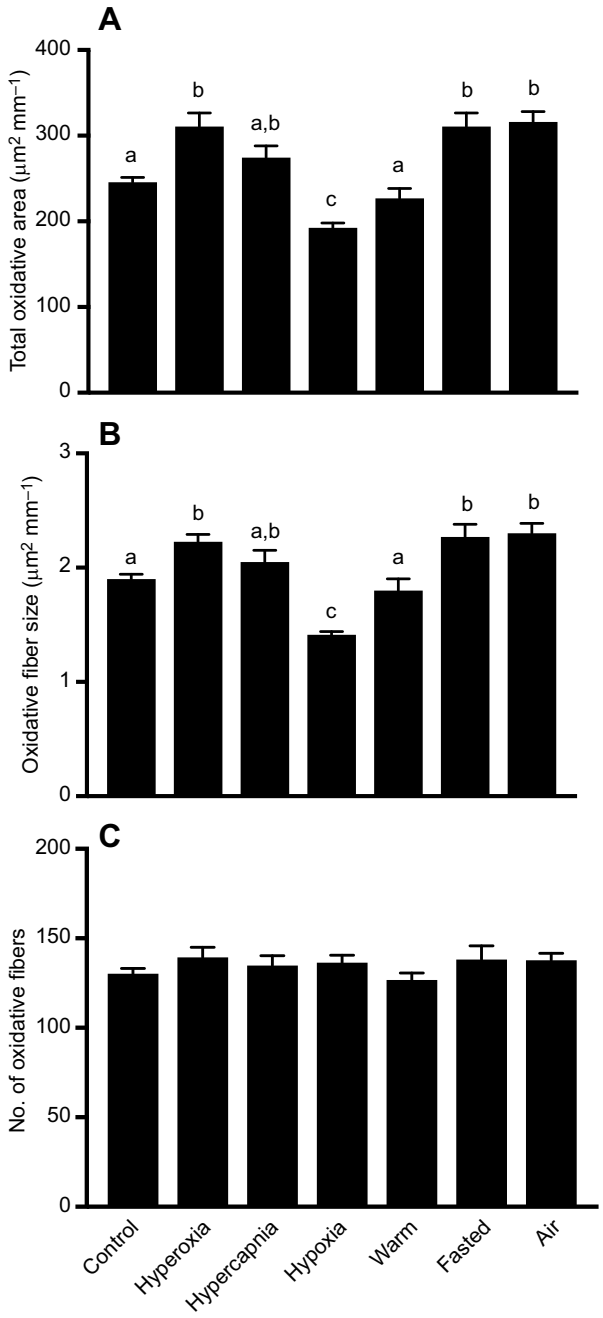

Fig. 2. Characteristics of *K. marmoratus* oxidative skeletal muscle at the lateral line. Fish were acclimated to control (n=30), air (n=9), fasted (n=8), hypercapnic (n=8), warm (n=8), hypoxic (n=7) or hyperoxic (n=10) conditions for 28 days. (A) The total cross-sectional area of oxidative muscle relative to body length. (B) The average size of individual oxidative muscle fibers relative to body length. (C) The total number of oxidative muscle fibers. Data are means±s.e.m. Letters denote significant differences between treatments (P<0.05).

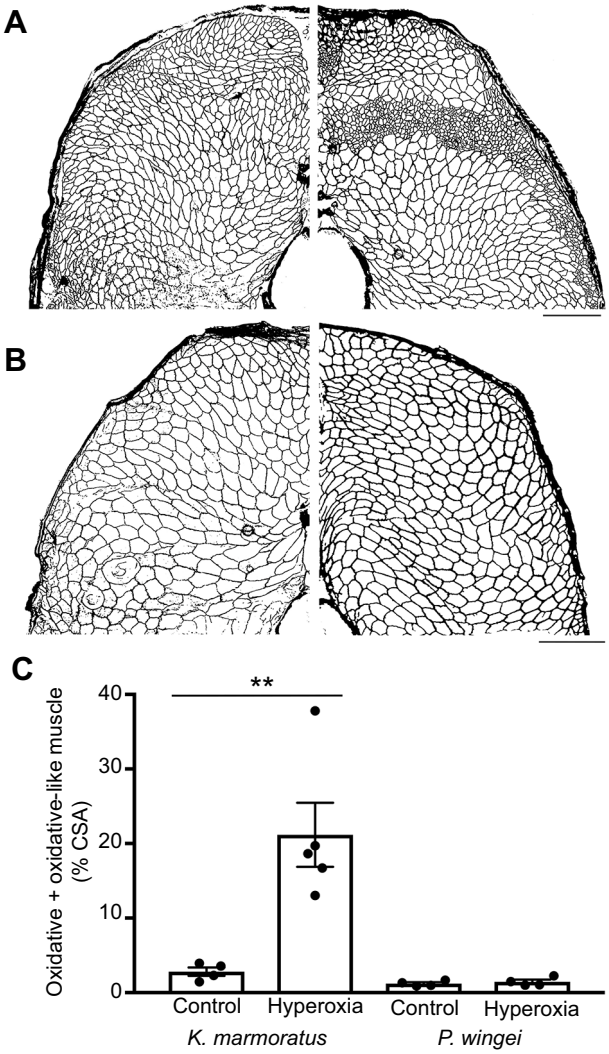

Fig. 3. Comparison of skeletal muscle of amphibious *K. marmoratus* and non-amphibious *Poecilia wingei*. Digitized cross-sections of (A) control (left) and hyperoxia-acclimated (right) *K. marmoratus* showing a thick band of oxidative-like muscle, and (B) control (left) and hyperoxia-acclimated (right) *P. wingei*. (C) The percentage cross-sectional area (CSA) in *K. marmoratus* and *P. wingei* occupied by oxidative and oxidative-like muscle in control fish (n=4, 4) and following hyperoxia acclimation (n=5, 4). Data are means±s.e.m. Asterisks denote significance (**P<0.01). Scale bars: 500 μm.

response in *K. marmoratus*, i.e. reduced oxidative muscle fiber size, also underlines the O_2 sensitivity of skeletal muscle in this species.

Environmental O_2 availability is known to induce phenotypic changes in the skeletal muscle of many fishes. Chronic hypoxia typically causes a decrease in oxidative fiber size and the aerobic capacity of skeletal muscle, reducing the rate of O_2 utilization (for reviews, see Sänger, 1993; McClelland and Scott, 2014). In contrast, when the amphibious Japanese mudskipper *Periophthalmus modestus* was acutely exposed to aerial hyperoxia during terrestrial exercise, it showed improved endurance, suggesting enhanced O_2 utilization by the skeletal muscle at higher environmental O_2 levels (Jew et al., 2013). But whether *P. modestus* or other mudskippers remodel skeletal muscle during terrestrial sojourns remains unknown and worthy of study.

We also found evidence for the alternative hypothesis that fasting conditions caused the skeletal muscle phenotype observed in air-acclimated *K. marmoratus*. However, the reason why fasting elicits this change in muscle phenotype is unclear. Prolonged fasting in animals often leads to a loss in muscle protein but a concomitant increase in water content (McCue, 2010). In fact, some animals are so effective at replacing lost tissue with water that there is no net change in body mass with prolonged starvation (Marsden et al., 1973; Comoglio et al., 2004). It is unlikely, however, that the increase in oxidative fiber size in response to fasting in our study resulted from an increase in muscle water content because we observed no change in glycolytic fiber size. Alternatively, functional prioritization of

oxidative muscle may have occurred as several fishes maintain the integrity of oxidative fibers during prolonged fasting by preferentially atrophying tissues with a lower functional priority for energy (Greer-Walker, 1971; Beardall and Johnston, 1983; Simpkins et al., 2003). The increase in oxidative muscle in *K. marmoratus* in response to fasting may, therefore, reflect a reallocation of energy from lower priority tissues (e.g. gut) to ensure effective locomotor performance is maintained. Additionally, if blood flow is reallocated towards skeletal muscle, then muscle O_2 levels may be elevated, leading to O_2-induced muscle remodeling – an idea that requires further exploration.

Perspectives

Enhanced O_2 utilization on land and improved terrestrial locomotion are hypothesized to have fueled the diversification and ecological radiation of early tetrapods (Graham et al., 1995). The vertebrate transition to land was accompanied by radical changes in skeletal muscle structure, from anatomical separation of muscle fiber types in fishes to muscles with mixed and more diverse fiber types in tetrapods (Schilling, 2011). The reason why tetrapod muscles differ from the ancestral state remains unresolved but several hypotheses have been proposed (Schilling, 2011; Forgan and Forster, 2012). One hypothesis suggests that the evolution of mixed muscle fibers in tetrapods is related to the higher O_2 availability on land (O_2 availability hypothesis), which allowed for increased tissue complexity and metabolic rate (Forgan and Forster, 2012). Sometimes, phenotypically flexible responses in amphibious fishes recapitulate the evolution of constitutively expressed traits in tetrapods (e.g. Standen et al., 2014). Here, we show that aquatic hyperoxia – typical of eutrophic ponds or tide pools – and air provide a stimulus for enhanced skeletal oxidative capacity and tissue complexity, providing evidence for the O_2 availability hypothesis.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.S.R., A.J.T., P.A.W.; Methodology: G.S.R., A.J.T., P.A.W.; Validation: G.S.R., A.J.T., P.A.W.; Formal analysis: G.S.R.; Investigation: G.S.R., A.J.T.; Resources: G.S.R., A.J.T., P.A.W.; Data curation: G.S.R.; Writing - original draft: G.S.R.; Writing - review & editing: G.S.R., A.J.T., P.A.W.; Visualization: G.S.R., A.J.T., P.A.W.; Supervision: P.A.W.; Funding acquisition: A.J.T., P.A.W.

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Supplementary information

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