

SHORT COMMUNICATION

Amphibious fish jump better on land after acclimation to a terrestrial environment

Emily M. Brunt¹, Andy J. Turko¹, Graham R. Scott² and Patricia A. Wright^{1,*}

ABSTRACT

Air and water differ dramatically in density and viscosity, posing different biomechanical challenges for animal locomotion. We asked how terrestrial acclimation influences locomotion in amphibious fish, specifically testing the hypothesis that terrestrial tail flip performance is improved by plastic changes in the skeletal muscle. Mangrove rivulus *Kryptolebias marmoratus*, which remain largely inactive out of water, were exposed to water or air for 14 days and a subgroup of air-exposed fish was also recovered in water. Tail flip jumping performance on land improved dramatically in air-acclimated fish, they had lower lactate levels compared with control fish, and these effects were mostly reversible. Muscle plasticity significantly increased oxidative muscle cross-sectional area and fibre size, as well as the number of capillaries per fibre. Our results show that reversible changes to the oxidative skeletal muscle of *K. marmoratus* out of water enhance terrestrial locomotory performance, even in the absence of exercise training.

KEY WORDS: Exercise performance, Muscle remodelling, Phenotypic flexibility, Invasion of land

INTRODUCTION

Effective locomotion both in water and on land requires many physiological and behavioural changes in order to cope with the decreased viscosity and density of air versus water. Amphibious fishes have evolved many different biomechanical strategies for movement on land, from axial undulations in ropefish *Erpetoichthys calabaricus* to appendicular-based movement in Oxudercine mudskippers (Pace and Gibb, 2014). In addition to fixed evolutionary changes, amphibious fishes may also use plastic responses that enhance performance out of water. For example, when amphibious bichir *Polypterus senegalus* were raised permanently on land and encouraged to move daily, they developed stronger pectoral girdles and a more efficient gait relative to fish in water (Standen et al., 2014). Can short-term acclimation to terrestrial environments also cause reversible plasticity in skeletal muscle and locomotory performance in amphibious fishes? The amphibious mangrove rivulus *Kryptolebias marmoratus* reversibly remodels respiratory surfaces and blood O₂ transport after a week out of water remaining relatively quiescent (Ong et al., 2007; Turko et al., 2014); however, it is unknown whether locomotory performance is also altered.

We tested the hypothesis that plastic changes in the skeletal muscle of adult amphibious fishes during terrestrial acclimation improve locomotory performance. This hypothesis predicts that fish acclimated to a terrestrial environment – and that experience increased effective gravity – should have improved movement capacity and associated plastic changes to the skeletal muscle compared with individuals maintained solely in water, where they are buoyant and effectively weightless. We studied *K. marmoratus*, an amphibious fish that moves on land by tail flip jumping (Taylor, 2012; Pronko et al., 2013), tolerates emersion for up to 66 days in the laboratory and has been found packed into rotting logs in the dry season (Taylor, 2012).

MATERIALS AND METHODS

Experimental animals

Hermaphrodites of the self-fertilizing *Kryptolebias marmoratus* (Poey 1880), originating from Twin Cayes, Belize, were used for this experiment (strain 50.91, standard length 15.84±0.04 mm, mass 53.24±2.01 mg) (Tatarenkov et al., 2010). Fish were maintained at the Hagen Aqualab, University of Guelph, in individual 100 ml plastic containers (~60 ml water, 15‰, 25°C) and exposed to a 12 h:12 h photoperiod (Ong et al., 2007). Fish were fed live *Artemia nauplii* three times weekly.

Experimental protocol

Fish were subjected to one of three treatments: control in water (14 days; *N*=15–26), air (14 days; *N*=13–26), or air followed by fasted recovery in water (28 days; *N*=13–26). *Kryptolebias marmoratus* are suction feeders and do not feed out of water, and therefore fish were fasted for the duration of the experiment (Pronko et al., 2013). Fish were tested for jumping performance and then processed for histological analysis. For air exposure, fish were held on moistened filter paper over a reservoir of water (15‰) in the same 100 ml plastic containers as above (Ong et al., 2007).

Immediately following treatment, fish were subjected to a jumping test in air. Fish were placed on moist filter paper in a terrarium (60 cm×30 cm). After a 2 min adjustment period, the fish were encouraged to jump with repeated gentle prods of a clicker ballpoint pen directed at the tail. When the fish reached exhaustion, i.e. when the clicking no longer stimulated jumping motion, they were given 2 min to rest. The jump test was then repeated until the fish reached exhaustion once again. These two bursts of jumping were summed for all variables measured. Each trial was video recorded for later analysis. Videos were analysed to quantify the duration of each jumping bout, the number of jumps, the total distance covered, the longest jump and the position of the longest jump in the sequence. Duration was taken as the time from the first jump until the fish stopped jumping in response to the pen. The distance travelled was calculated by adding up the length of each individual jump. Jump length was measured from where the centre of the body landed on the filter paper. A ‘jump’ was considered to

¹Department of Integrative Biology, University of Guelph, Guelph, ON, Canada N1G2W1. ²Department of Biology, McMaster University, Hamilton, ON, Canada L8S4K1.

*Author for correspondence (patwright@uoguelph.ca)

 P.A.W., 0000-0003-4041-8885

be when the entire body of the fish left the ground (Pronko et al., 2013).

To compare lactate accumulation between groups, a separate group of fish were subject to a submaximal jumping test, where fish were only made to perform 10 jumps. This standardized number of jumps was chosen based on the initial experiments to ensure that all fish could perform the same number of jumps without reaching exhaustion. Immediately after the 10th jump, the fish were killed using MS-222 and frozen in liquid nitrogen for later measurement of lactate.

To analyse skeletal muscle, fish were killed using MS-222, coated in embedding medium (Shandon cryomatrix, ThermoFisher Scientific, Markham, ON, Canada), and immediately frozen in liquid nitrogen-chilled isopentane. Transverse cryosections (7 μm) of the frozen muscle, taken immediately behind the dorsal fin, were cut in a cryostat (Leitz Cryostat Microtome, LABEQUIP Ltd, Markham, ON, Canada) at -20°C and stored at -80°C until staining. Succinate dehydrogenase staining was used to discern oxidative from glycolytic muscle fibres and alkaline phosphatase staining was used to visualize capillaries (Borowiec et al., 2015). Stained slides were photographed using NIS Elements software (Nikon, Melville, NY, USA) and analysed using ImageJ (<http://imagej.nih.gov/ij/>). Slides were randomized to reduce observational bias. The cross-sectional area of oxidative muscle was standardized to standard length to account for variation in body size. The number of oxidative fibres was determined by counting all oxidative fibres on one lateral half of the fish and multiplying by two. The average transverse area of the oxidative fibres was estimated by dividing the total area of the oxidative region by the number of counted fibres. We also measured the transverse area of 10 randomly chosen oxidative and glycolytic fibres per fish as an independent measurement of muscle fibre size. All capillaries were counted within the aerobic muscle region and reported as capillary density (capillaries/area) and capillary:fibre ratio. Because of the much larger area of glycolytic muscle, capillaries were counted in a

randomized subset of regions (representing $\sim 25\%$ of the total area). Whole-body lactate was measured enzymatically (Robertson et al., 2014).

One-way analyses of variance (ANOVA) and *post hoc* Holm–Sidak tests were used to determine the effect of treatment on jumping performance muscle phenotype. Data were square root or \ln transformed when necessary to meet assumptions of normality and equal variance. Multiple linear regression was used to determine whether skeletal muscle parameters (oxidative and glycolytic fibre size, number, area) were significant predictors of terrestrial performance. For all tests, critical $\alpha=0.05$.

RESULTS

Air-exposed fish performed 1.6-fold more jumps ($P<0.001$; Fig. 1A), travelled 2-fold further ($P<0.001$; Fig. 1B) and continued jumping 1.7-fold longer ($P<0.001$; Fig. 1C) than control fish before exhaustion, and this effect was reversed after fish were returned to water for 14 days. The longest jump was significantly greater in air-exposed fish compared with control fish ($P=0.005$; Fig. 1D). Moreover, significantly less lactate was produced by air-exposed fish relative to control fish (1.87 ± 0.31 versus 3.11 ± 0.56 mmol l^{-1} ; $P=0.041$) after 10 jumps, even though air-exposed fish jumped further than control fish (61.4 ± 6.5 versus 41.6 ± 2.9 cm) over this period ($P<0.001$).

The total cross-sectional area of oxidative muscle was significantly greater in air-exposed fish compared with control fish ($P=0.018$; Fig. 2A), but returned to control values after recovery in water ($P>0.05$; Fig. 2A). A trend was also seen towards larger individual oxidative muscle fibres in air-exposed fish, suggestive of hypertrophy, both when fibre size was calculated by dividing the total oxidative area by the number of fibres (ANOVA: $F_{2,40}=4.048$; $P=0.025$, but *post hoc* $P=0.045$, greater than the corrected critical value of 0.025; Fig. 2B), and when 10 randomly chosen fibres per fish were independently measured (ANOVA: $F_{2,40}=4.965$;

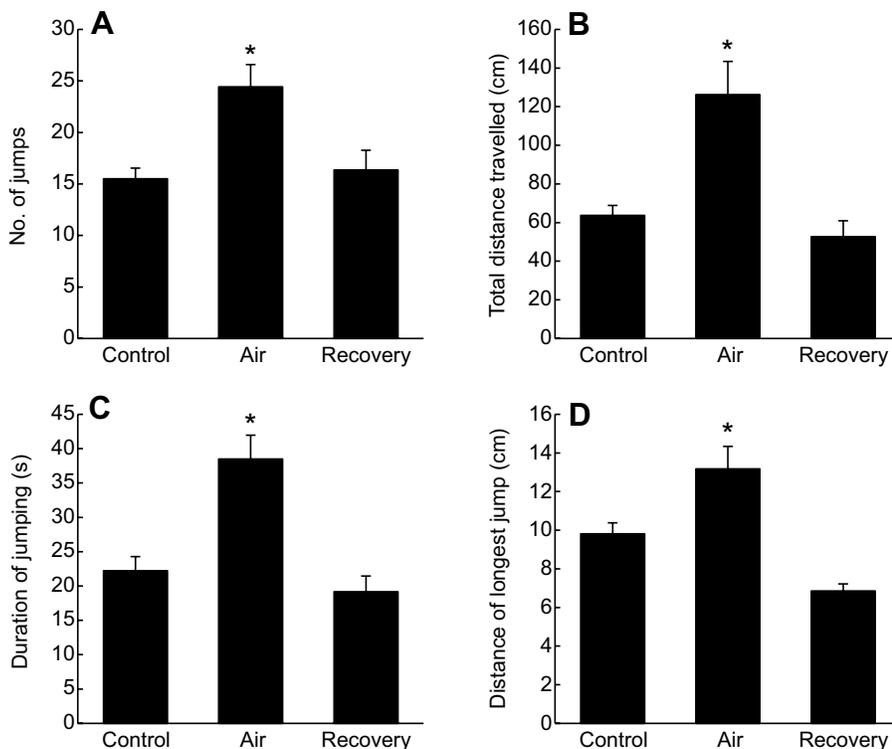


Fig. 1. Terrestrial jumping performance of *Kryptolebias marmoratus* acclimated to water (control, 14 days; $N=26$), air (14 days; $N=26$), or air followed by recovery in water (14 days air, 14 days water; $N=17$). (A) Number of jumps until exhaustion. (B) Total distance travelled. (C) Duration of jumping activity. (D) Distance of the longest jump. Bars are means \pm s.e.m. Asterisks indicate significant differences versus the control group (ANOVA, $P<0.05$).

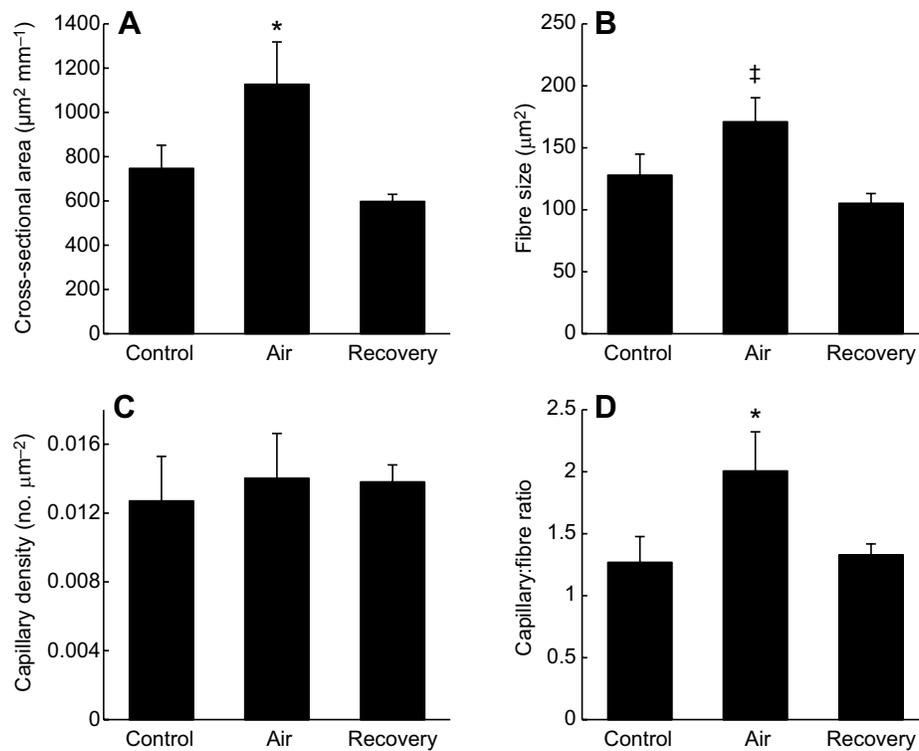


Fig. 2. Characteristics of skeletal muscle of *K. marmoratus* acclimated to water (control, 14 days; $N=15$), air (14 days; $N=13$), or air followed by recovery in water (14 days air, 14 days water; $N=15$). (A) Oxidative muscle cross-sectional area relative to body length. (B) Size of individual oxidative muscle fibres. (C) Capillary density in oxidative muscle fibre. (D) Capillary:oxidative muscle fibre ratio. Bars are means \pm s.e.m. *Significant difference versus the control group (ANOVA, $P<0.05$). [‡]Significant difference detected by one-way ANOVA ($P<0.05$) but not by the *post hoc* Holm–Sidak test (see Results).

$P=0.012$, but *post hoc* $P=0.066$). Oxidative fibre number ($P=0.80$) was unaffected by treatment. Capillary density did not change with air exposure ($P>0.05$; Fig. 2C), but the capillary:fibre ratio in the oxidative region of air-exposed fish increased 1.6-fold versus control fish ($P=0.007$). There were no changes in total glycolytic cross-sectional area ($P=0.23$), glycolytic fibre size ($P=0.35$), glycolytic capillary density ($P=0.36$) or glycolytic capillary:fibre ratio ($P=0.50$) after air exposure.

Oxidative fibre size, number of oxidative fibres and total oxidative muscle area were all significant predictors of the total distance travelled by individual fish (multiple linear regression, fibre size $P=0.003$; fibre number $P=0.011$; area: $P=0.002$; adjusted $R^2=0.29$).

DISCUSSION

Aerial acclimation had a profound positive influence on the terrestrial jumping performance of *K. marmoratus*. It is unlikely that these changes are related to an exercise training effect (McClelland and Scott, 2014) as video recordings show that *K. marmoratus* moves very little over 7 days out of water (Turko et al., 2014). The air-exposed fish jumped more, further and for longer than their counterparts held in water. We also observed hypertrophy and angiogenesis in the oxidative muscle, supporting the hypothesis that skeletal muscle plasticity improves terrestrial locomotory performance of these amphibious fish. Air-acclimated fish also relied less on glycolytic metabolism to fuel jumping on land, as less lactate accumulated in this group relative to control fish. Finally, these physiological changes were reversed within 2 weeks of returning to water, suggesting a tradeoff between terrestrial and aquatic locomotory performance.

Oxidative, but not glycolytic, muscle area increased in fish acclimated to air, possibly resulting from hypertrophy of oxidative muscle fibres. Mammalian oxidative skeletal muscle is highly responsive to weightlessness and re-loading (Fitts et al., 2001; Krainski et al., 2014) but these effects have not been investigated in fishes. If the oxidative musculature of *K. marmoratus* is sensitive to

changes in effective weight, one hypothesis is that the use of these muscles out of water to support the body provides ‘postural training’ which also enhances locomotory performance. Alternatively, inactivity itself may induce plastic changes in the muscle, possibly using mechanisms similar to those used by hibernating mammals or aestivating frogs to suppress muscle remodelling and atrophy during prolonged periods of inactivity (Reilly et al., 2015; Cotton, 2016). Further studies are required to understand the regulation of muscle plasticity in amphibious fishes.

In addition to the increased area of oxidative fibres, improved terrestrial locomotion is probably also enabled by plasticity of the vascular network that supplies O_2 to the muscle. Angiogenesis in the oxidative muscle is indicated by the observed increase in the ratio of capillaries to muscle fibres; this would have been offset by the concomitant trend toward increased muscle fibre size, such that capillary densities were equivalent between aquatic and terrestrially acclimated fish. This suggests that the plasticity of the microcirculation serves to maintain similar levels of O_2 delivery capacity (as best reflected by capillary density) during acclimation to alternative environments in *K. marmoratus*. These changes in the muscle occur in tandem with plastic changes that improve O_2 uptake including angiogenesis in the skin and increased haemoglobin– O_2 affinity (Turko et al., 2014). Thus, oxidative muscle plasticity may be one of many adjustments to the O_2 cascade that improve jumping performance in air-exposed fish, which could be advantageous for avoiding terrestrial predators or moving over land to colonize new water bodies.

Acknowledgements

The authors thank Dr T. Gillis for helpful comments on experimental design and Kirsten Nikel for assistance with the microscopy work. Samantha Engstrom, Mike Davies, Matt Cornish and numerous undergraduate volunteers are thanked for animal care.

Competing interests

The authors declare no competing or financial interests.

Author contributions

E.M.B., A.J.T. and P.A.W. conceived and designed the project. E.M.B. executed the experiments and analysed the data. A.J.T. and G.R.S. assisted with histological procedures. E.M.B. and P.A.W. wrote the draft manuscript. E.M.B., A.J.T., G.R.S. and P.A.W. revised the manuscript.

Funding

Funding was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grants program to P.A.W. (120513) and an NSERC graduate scholarship to A.J.T.

References

- 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.
- Cotton, C. J.** (2016). Skeletal muscle mass and composition during mammalian hibernation. *J. Exp. Biol.* **219**, 226–234.
- Fitts, R. H., Riley, D. R. and Widrick, J. J.** (2001). Functional and structural adaptations of skeletal muscle to microgravity. *J. Exp. Biol.* **204**, 3201–3208.
- Krainski, F., Hastings, J. L., Heinicke, K., Romain, N., Pacini, E. L., Snell, P. G., Wyrick, P., Palmer, M. D., Haller, R. G. and Levine, B. D.** (2014). The effect of rowing ergometry and resistive exercise on skeletal muscle structure and function during bed rest. *J. Appl. Physiol.* **116**, 1569–1581.
- McClelland, G. B. and Scott, G. R.** (2014). Muscle plasticity. In *The Physiology of Fishes*, 4th edn. (ed. D. H. Evans, J. B. Claiborne and S. Currie), pp. 1–31. Boca Raton, FL: CRC Press.
- Ong, K. J., Stevens, E. D. and Wright, P. A.** (2007). Gill morphology of the mangrove killifish (*Kryptolebias marmoratus*) is plastic and changes in response to terrestrial air exposure. *J. Exp. Biol.* **210**, 1109–1115.
- Pace, C. M. and Gibb, A. C.** (2014). Sustained periodic terrestrial locomotion in air-breathing fishes. *J. Fish. Biol.* **84**, 639–660.
- Pronko, A. J., Perlman, B. M. and Ashley-Ross, M. A.** (2013). Launches, squiggles and pounces, oh my! The water-land transition in mangrove rivulus (*Kryptolebias marmoratus*). *J. Exp. Biol.* **216**, 3988–3995.
- Reilly, B. D., Cramp, R. L. and Franklin, C. E.** (2015). Activity, abundance and expression of Ca²⁺-activated proteases in skeletal muscle of the aestivating frog, *Cyclorana alboguttata*. *J. Comp. Physiol. B* **185**, 243–255.
- Robertson, C. E., Wright, P. A., Koblitz, L. and Bernier, N. J.** (2014). Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish, *Danio rerio*. *Proc. R. Soc. B* **281**, 20140637.
- Standen, E. M., Du, T. Y. and Larsson, H. C.** (2014). Developmental plasticity and the origin of tetrapods. *Nature* **513**, 54–58.
- Tatarenkov, A., Ring, B. C., Elder, J. F., Bechler, D. L., Avise, J. C. and Batzer, M. A.** (2010). Genetic composition of laboratory stocks of the self-fertilizing fish *Kryptolebias marmoratus*: a valuable resource for experimental research. *PLoS ONE* **5**, e12863.
- Taylor, D. S.** (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.
- 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.