SHORT COMMUNICATION

Amphibious fish 'get a jump' on terrestrial locomotor performance after exercise training on land

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ABSTRACT

Many amphibious fishes rely on terrestrial locomotion to accomplish essential daily tasks, but it is unknown whether terrestrial exercise improves the locomotor performance of fishes on land. Thus, we tested the hypothesis that terrestrial exercise improves locomotion in amphibious fishes out of water as a result of skeletal muscle remodeling. We compared the jumping performance of *Kryptolebias marmoratus* before and after an exercise training regimen, and assessed the muscle phenotype of control and exercise-trained fish. We found that exercise-trained fish jumped 41% farther and 48% more times before reaching exhaustion. Furthermore, exercise training resulted in the hypertrophy of red muscle fibers, and an increase in red muscle capillarity and aerobic capacity. Lactate accumulation after jumping indicates that white muscle is also important in powering terrestrial jumps. Overall, skeletal muscle in *K. marmoratus* is highly responsive to terrestrial exercise, and muscle plasticity may assist in the effective exploitation of terrestrial habitats by amphibious fishes.

KEY WORDS: Skeletal muscle, Red oxidative muscle, Lactate, Angiogenesis, Tail-flip jumping, Mitochondrial content

INTRODUCTION

Locomotor movement on land is far more costly than movement in water (Schmidt-Nielsen, 1972). Nevertheless, several amphibious fishes rely on terrestrial locomotor performance for predator avoidance, dispersal and prey capture (Gordon et al., 1969; Sayer, 2005). Some amphibious fishes locomote on land using pectoral fins that appear to work like limbs (mudskippers; Kawano and Blob, 2013); others use serpentine movements (eels; Gillis, 2000), while still others use jumps (killifish; Minicozzi et al., 2019). Despite the inefficiency of these terrestrial locomotor methods compared with swimming, many amphibious fishes out of water can traverse considerable distances or short distances repeatedly. For example, juvenile Hawaiian gobies (*Sicyopterus stimpsoni*) climb waterfalls over 10,000 times their body length to reach adult habitats (Schoenfuss and Blob, 2003), whereas blennies (*Alticus kirkii*) frequently move in and out of tidepools to feed on the algae of nearby rocks (Martin and Lighton, 1989).

Exercise typically improves the locomotor performance of vertebrates as a result of skeletal muscle remodeling. In fishes, skeletal muscle is generally composed of two anatomically and functionally distinct fiber types: red (slow-oxidative) and white (fast-glycolytic) fibers that use aerobic and anaerobic pathways,

respectively, for energy (ATP) production. Numerous studies have demonstrated that swim training can alter the structural and metabolic properties of red and white muscle fibers in fish, which in turn enhances swimming performance (for reviews, see Kieffer, 2010; Palstra and Planas, 2011; McClelland, 2012; McClelland and Scott, 2014). Exercise or repeated movements across terrestrial environments may promote skeletal muscle remodeling and improve locomotion in amphibious fishes, as observed with swim training, but this is unknown. In contrast, amphibious fishes that remain inactive for several weeks or months in a terrestrial environment can exhibit muscle disuse atrophy (e.g. estivating lungfish; Amelio et al., 2013), which may impair locomotor performance. Any changes to the skeletal musculature that affect terrestrial locomotion may influence how effectively amphibious fishes exploit terrestrial habitats. Does terrestrial exercise training improve the locomotor performance of amphibious fishes?

In the present study, we used the amphibious mangrove rivulus (*Kryptolebias marmoratus*) to test the hypothesis that terrestrial exercise training improves the locomotor performance of amphibious fishes out of water as a result of skeletal muscle remodeling. A previous study from our laboratory showed that *K. 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 on land, which improved terrestrial locomotor performance (Brunt et al., 2016). Interestingly, this change in muscle phenotype was not due to terrestrial exercise, as air-exposed fish remained inactive, but rather was the result of the higher O_2 availability in air relative to water (Rossi et al., 2018). In the wild, however, *K. marmoratus* frequently move across mangrove forest floors using terrestrial jumps (Gibb et al., 2013; Pronko et al., 2013; Ashley-Ross et al., 2014) to capture prey, avoid predators and disperse to new aquatic habitats (Taylor, 1992, 2012). Thus, we compared the jumping performance of *K. marmoratus* before and after an exercise training regimen where fish were jumped to ~50% exhaustion on alternate days for 12 days. We then assessed the skeletal muscle phenotype of *K. marmoratus*, including the number and cross-sectional area of red and white muscle fibers, the capillarity and aerobic capacity of the red muscle, as well as whole-body lactate concentration after exercise.

MATERIALS AND METHODS
Experimental animals

All experimental fish (*n*=58; 0.083±0.001 g) were adult hermaphrodites of the self-fertilizing *Kryptolebias marmoratus* Poey 1880 (SLC strain; Tatarenkov et al., 2010). Prior to experimentation, 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. nauplii three times weekly. All experimental procedures were approved by the University of Guelph Animal Care Committee (AUP 3891).

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Experimental protocol

We randomly assigned adult *K. marmoratus* hermaphrodites to one of two 14 day treatments: control (n=25; 0.080±0.001 g) or exercise (n=26; 0.085±0.002 g). On day 0, all fish were subjected to a terrestrial locomotor performance test (i.e. jumped to exhaustion as previously described; Rossi et al., 2019) to establish baseline locomotor performance. Briefly, fish were encouraged to jump via gentle prodding with a clicker ballpoint pen until they were exhausted, i.e. unresponsive to ~10 prods (typically after ~6 min). The jumping trials were video recorded and analyzed to quantify the total distance traveled and the number of jumps performed (Brunt et al., 2016). After the day 0 exhaustive test, the exercise group was induced to jump for 3 min (~50% exhaustion) on days 2, 4, 6, 8, 10 and 12. Fish were jumped every second day, not daily, in order to prevent excessive handling and to allow for recovery between jumping bouts. Control fish were air exposed, but not jumped, on these same days for 3 min. We air-exposed control fish in 120 ml plastic cups to minimize voluntary jumping. In general, control fish jumped fewer than five times voluntarily upon transfer to air. All fish were maintained in water (~60 ml, 15‰) and fasted during the experimental period to ensure that exercise-trained fish did not obtain more food than untrained fish as a result of enhanced foraging abilities.

On day 14, a subset of fish (control, n=17; exercise, n=18) were jumped to exhaustion for comparison with the exhaustive jumping test on day 0, and then immediately euthanized via immersion in MS-222 (500 mg l^{-1}). A ~3 mm transverse steak anterior to the dorsal fin was removed and processed for muscle phenotype analysis, as previously described (Rossi et al., 2018). The remaining fish (control, n=8; exercise, n=10) were induced to jump submaximally (10 times) on day 14 (Brunt et al., 2016), immediately euthanized via ice-water immersion, and analyzed enzymatically for whole-body lactate concentration, as previously described (Bergmeyer and Bernt, 1974). Negative controls (i.e. non-jumped fish, n=7) were also analyzed for whole-body lactate concentration. We standardized all lactate concentration measurements to the body volume (in liters) of each fish.

Muscle phenotype

Red muscle fibers were identified by staining for slow myosin using mouse IgA primary antibody (S58; Developmental Studies
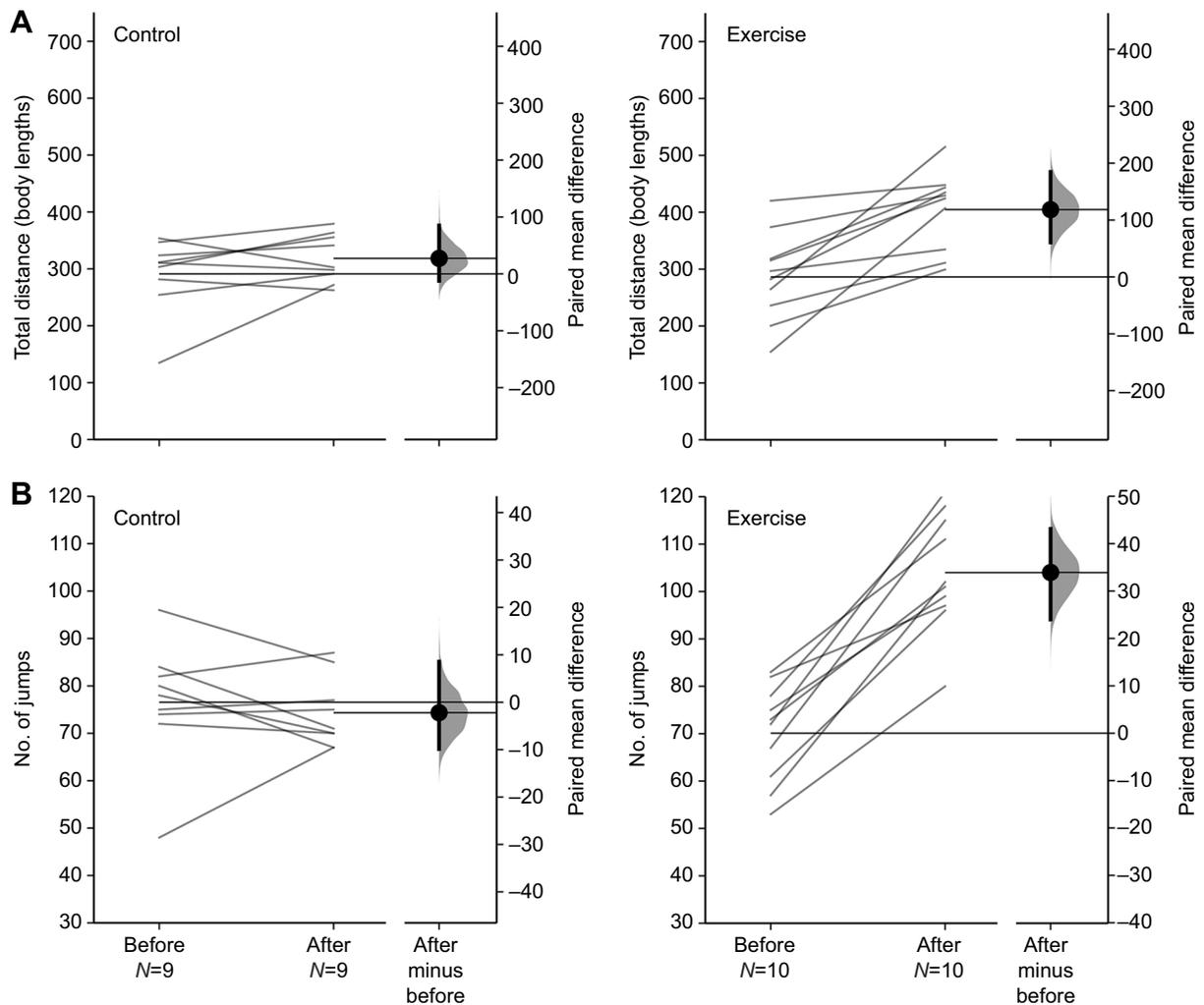

Fig. 1. Effect of exercise training on the distance traveled and the number of jumps performed by *Kryptolebias marmoratus* before exhaustion. Gardner–Altman estimation plots showing the paired mean difference in (A) the total distance traveled in body lengths, and (B) the number of jumps performed by control (left) and exercise-trained (right) *K. marmoratus* before and after the experimental period. Locomotor performance measures are plotted against the left axis as a slopegraph: each paired set of observations (before and after) is connected by a line. The paired mean difference (black dot) is plotted against the right axis as a bootstrap sampling distribution (gray distribution). The 95% confidence interval (CI) is indicated by the vertical error bar.
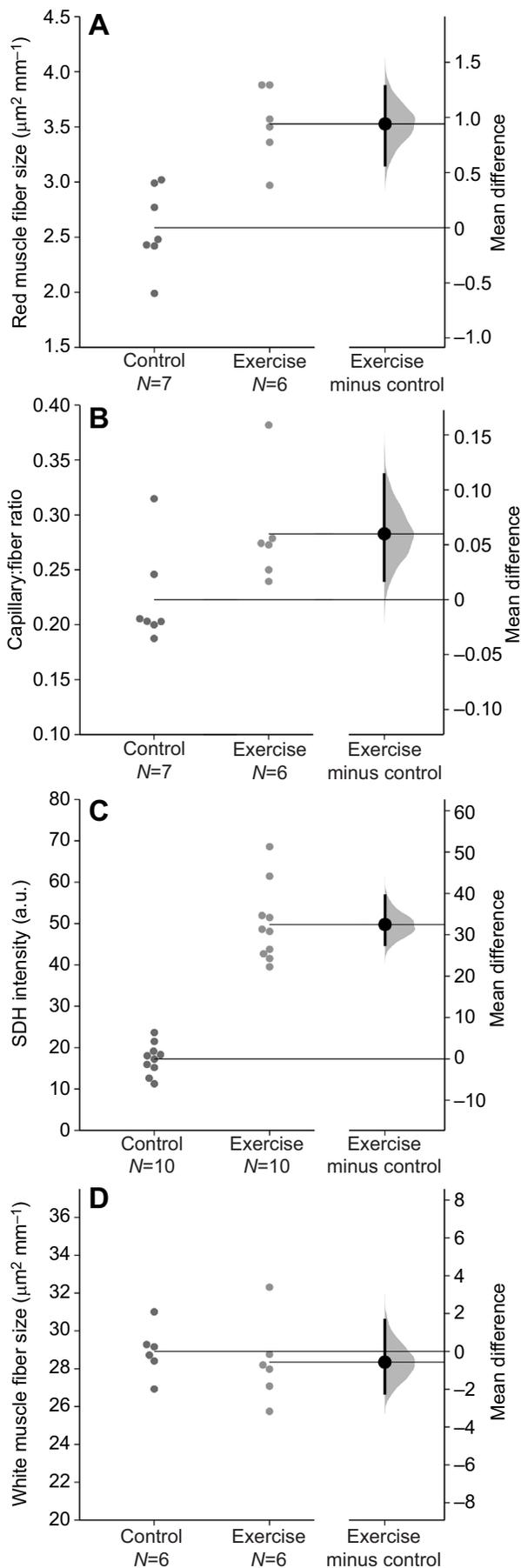
Fig. 2. Effect of exercise training on the skeletal muscle phenotype of *K. marmoratus*. Gardner–Altman estimation plots showing the mean difference between (A) red muscle fiber size, (B) capillary:red muscle fiber ratio, (C) succinate dehydrogenase (SDH) staining intensity and (D) white muscle fiber size of control and exercise-trained *K. marmoratus*. Both groups (control and exercise trained) are plotted against the left axis. The mean difference (black dot) is plotted against the right axis as a bootstrap sampling distribution (gray distribution). The 95% CI is indicated by the vertical error bar.

Hybridoma Bank, Iowa City, IA, USA), as previously described (Johnston et al., 2004). An alkaline phosphatase (AP) stain was used to visualize capillaries localized in the red muscle, and a succinate dehydrogenase (SDH) stain was used as a proxy for the aerobic capacity of red muscle (Borowiec et al., 2015; Brunt et al., 2016). The slides were viewed using an epifluorescence microscope (Nikon Eclipse 90i microscope, Nikon, Tokyo, Japan), photographed using NIS Elements software (Nikon), and analyzed as previously described (Rossi et al., 2018, 2019). Briefly, we counted all red muscle fibers on one lateral half of each fish, and measured the size (cross-sectional area) of 30 random red muscle fibers. All capillaries in contact with red muscle fibers on one lateral half of each fish were counted and reported as a capillary:red muscle fiber ratio, as previously described (Brunt et al., 2016).

We quantified SDH staining intensity by overlaying a 200 µm² grid onto photographs of SDH-stained sections, and measuring the mean gray value of three random squares containing only red muscle. Intensity was expressed as the absolute mean gray value of red minus white muscle in arbitrary units (a.u.) to correct for differences in section thickness. Brightfield images of unstained sections were used to quantify the number and average size of white fibers, as previously described (Rossi et al., 2019). Red and white muscle fiber sizes were standardized to fish length (in millimeters).

Statistical analysis

We examined our data using estimation statistics in addition to null hypothesis testing (Halsey et al., 2015; Ho and Halsey, 2018; Halsey, 2019). All data were initially assessed for normality and homogeneity of variance, and appropriately transformed when necessary. We performed paired two-sided *t*-tests to compare the jumping performance of fish before and after the experimental period, and unpaired two-sided *t*-tests to compare muscle phenotype between control and exercise-trained fish. We used a one-way ANOVA, followed by a Tukey's *post hoc* test, to compare the lactate concentration of negative control, control and exercise-trained fish. Each *P*-value (significant at $\alpha < 0.05$) is supplemented with a mean difference value, and the 95% confidence interval (CI) of the mean difference value computed from 5000 bootstrapped samples. We performed linear regressions to determine the relationship between the size of red and white fibers and the number of jumps performed. All data were analyzed using RStudio (version 1.1.447). Estimation plots were produced using the 'dabestr' package in R 3.6.1 (Ho et al., 2019).

RESULTS AND DISCUSSION

We report evidence for the hypothesis that terrestrial exercise training improves the locomotor performance of amphibious fishes out of water as a result of skeletal muscle remodeling. Remarkably, the exercise regime consisted of only ~24 min of jumping spread over 12 days, but *K. marmoratus* jumped 41% farther (*P*=0.001; mean difference of 118 body lengths; 95% CI=60.6–185.0 body lengths) and 48% more times (*P*<0.001; mean difference of 33.9 jumps; 95% CI=24.0–43.1 jumps) after exercise training (Fig. 1).

Control fish showed no such differences in either the distance traveled (P=0.05; mean difference of 27.3 jumps; 95% CI=−12.3–85.9 jumps) or the number of jumps performed (paired t-test, P=0.54; mean difference of −2.2 jumps; 95% CI=−9.9–8.6 jumps) (Fig. 1). Exercise training also produced a more aerobic skeletal muscle phenotype in *K. marmoratus*. The red muscle fibers of exercise-trained fish were 29% larger than those of control fish (P<0.001; mean difference of 0.9 μm mm^{-1}; 95% CI=0.6–1.3 μm mm^{-1}; Fig. 2A), suggesting hypertrophic growth, but the number of red muscle fibers did not differ between groups (P=0.81; mean difference of 0.9 red muscle fibers; 95% CI=−5.7–8.0 red muscle fibers). The positive correlation between the size of red muscle fibers and the number of jumps fish could perform before exhaustion (P=0.02, R^2=0.42, y=22.53x+22.09) further supports the idea that improved terrestrial locomotor performance is driven by changes to the skeletal musculature. Moreover, the red muscle of exercise-trained fish had a higher capillary:fiber ratio (P=0.04; mean difference of 0.06; 95% CI=0.02–0.11; Fig. 2B) and a higher SDH staining intensity (P<0.001; mean difference of 32.5 a.u.; 95% CI=27.6–39.4 a.u.; Fig. 2C) compared with that of control fish. Overall, our findings indicate that skeletal muscle in *K. marmoratus* is highly responsive to terrestrial exercise training, and that muscle plasticity may assist in the effective exploitation of terrestrial habitats by amphibious fishes.

Jumping is often considered to be a burst movement, powered primarily by white anaerobic muscle (James et al., 2007). In fish, skeletal muscle is composed mostly of white fibers (>90%), with a small band of red fibers running along the lateral line (Bone, 1978). Paradoxically, we found that exercise training resulted in a more aerobic, not anaerobic, skeletal muscle phenotype in *K. marmoratus*. Our findings are consistent with those of a previous study from our laboratory, which demonstrated that hypertrophy of red muscle fibers and increased red muscle capillarity improved the jumping performance (e.g. from 16 to 24 jumps performed before exhaustion) of *K. marmoratus* after 14 days of air exposure without exercise (Brunt et al., 2016). The red muscle hypertrophy in air-exposed fish resulted from the higher O$_2$ availability in air compared with water rather than exercise (Rossi et al., 2018), but emphasizes the importance of red muscle for jumping. Interestingly, both the current study and that of Brunt et al. (2016) found that for every 10% increase in the size of red muscle fibers, the number of jumps fish could perform before reaching exhaustion increased by 13–17%. Although the

absolute number of jumps differed between these studies because of methodological differences (e.g. exhaustion criteria), the relative change in locomotor performance for a given change in muscle phenotype was very similar. Finally, the higher SDH staining intensity in the red muscle of exercise-trained *K. marmoratus* in the current study reflects an enhanced aerobic capacity, as the activity of SDH in skeletal muscle fibers correlates positively with O$_2$ consumption (Bekedam et al., 2003). Previous studies have similarly demonstrated a higher aerobic capacity in the red muscle of several exercise-trained non-amphibious fishes (Johnston et al., 1977; Johnston and Moon, 1980; McClelland et al., 2006). Taken together, we suggest that enhanced O$_2$ uptake and utilization by the red muscle of *K. marmoratus* may be critical for improved jumping performance.

We found that *K. marmoratus* fatigued after a few minutes of exercise, suggesting that their jumping behavior is not a completely aerobic performance. The size of the white muscle fibers in *K. marmoratus*s did not change after exercise training (P=0.60; mean difference of −0.6 μm mm^{-1}; 95% CI=−2.2–1.7 μm mm^{-1}; Fig. 2D), nor did it correlate with the number of jumps fish performed before exhaustion (P=0.23, R^2=0.14, y=−4.62x+222.80). Similarly, we found no change in the number of white muscle fibers after exercise training (P=0.35; mean difference of 245 white muscle fibers, CI=−485–861 white muscle fibers). However, the accumulation of lactate after jumping indicates that white muscle is important for powering terrestrial jumps. Whole-body lactate concentration was significantly higher immediately after submaximal exercise in both control (P<0.001; mean difference of 0.9 mmol l^{-1}; 95% CI=0.8–1.1 mmol l^{-1}) and exercise-trained fish (P<0.001; mean difference of 1.1 mmol l^{-1}; 95% CI=0.9–1.3 mmol l^{-1}) relative to that in negative controls that were not induced to jump (P<0.001; Fig. 3). Broadly, the exercise performance of fishes can been grouped into three categories: sustained, burst and prolonged (Beamish, 1978; Plaut, 2001; Kieffer, 2010). Sustained exercise is powered by aerobic muscle and can be maintained for long periods of time (>200 min), whereas burst exercise is powered by anaerobic muscle and results in fatigue after only a few seconds (Brett, 1967). The jumping performance of *K. marmoratus* is likely a prolonged exercise, which utilizes both aerobic and anaerobic muscle, lasts between 2 and 200 min, and ends in exhaustion. Overall, we suggest that both muscle types are important for jumping in *K. marmoratus*, but the reason why red muscle demonstrated a greater scope for
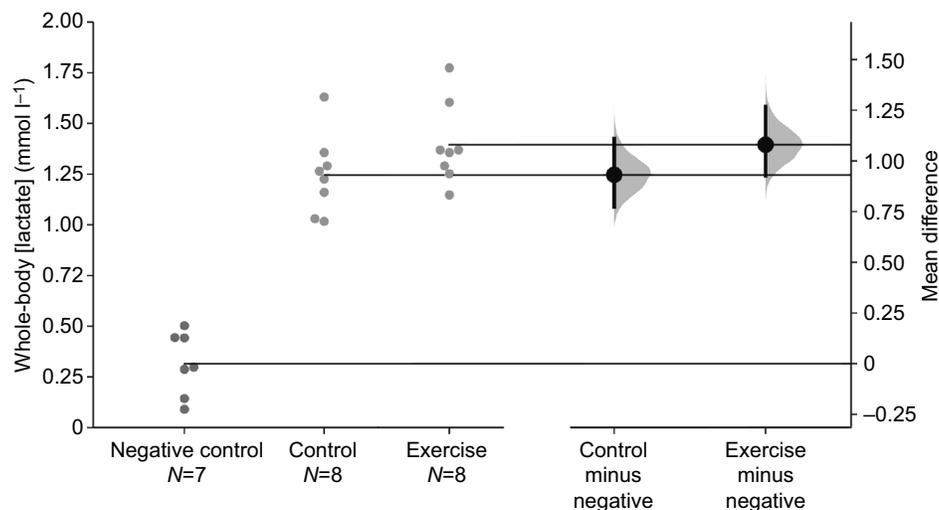

Fig. 3. Effect of exercise training on the whole-body lactate concentration of *K. marmoratus*. Gardner–Altman estimation plots showing the mean difference between the whole-body lactate concentration of negative controls and control and exercise-trained *K. marmoratus*. All groups are plotted against the left axis. The mean difference (black dot) is plotted against the right axis as a bootstrap sampling distribution (gray distribution). The 95% CI is indicated by the vertical error bar.

plasticity in response to exercise training remains unknown and is worthy of study.

Perspectives

Improved terrestrial locomotor performance in *K. marmoratus* would presumably enhance their ability to exploit terrestrial habitats. In the wild, *K. marmoratus* must traverse terrestrial landscapes to forage, disperse to new aquatic environments, seek moist terrestrial habitats during the dry season, avoid predation and deposit embryos out of water (Taylor, 1992, 2012). Improved locomotor performance on land may allow *K. marmoratus* to more effectively accomplish these activities. More broadly, amphibious fishes with more terrestrial tendencies are likely to be better terrestrial athletes, and may therefore have increased fitness compared with conspecifics that spend more time in water. As a result, highly terrestrial phenotypes may persist in subsequent generations and lead to a positive feedback cycle. In other words, the more time fish spend on land, the more opportunity they have to gain the benefits of exercise, which may ultimately lead to their improved survival and reproduction. Interestingly, such positive feedback cycles are thought to underlie major evolutionary and ecological transitions (Crespi, 2004), such as the rise of the first land-dwelling tetrapods from ancient fishes.

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