

# **Hypoxia-seeking behaviour, metabolic depression, and skeletal muscle function in an amphibious fish out of water**

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## Abstract

Several animals enter a state of dormancy to survive harsh environmental conditions. During dormancy, metabolic depression can be critical for economizing on limited endogenous energy reserves. We used two isogenic strains (Strain 1 and Strain 2) of a self-fertilizing amphibious fish (*Kryptolebias marmoratus*) to test the hypothesis that animals seek hypoxic microhabitats that, in turn, accentuate metabolic depression during dormancy. Using custom-built tunnels that maintained a longitudinal O<sub>2</sub> gradient (hypoxic to normoxic), we assessed the O<sub>2</sub> preference of *K. marmoratus* during prolonged air-exposure. In support of our hypothesis, we found that one isogenic strain (Strain 2) spent more time in hypoxia compared to normoxia after 21 days in air. Prolonged air-exposure in both strains resulted in lower O<sub>2</sub> consumption rates compared to active fish (35% depression), which was accentuated (51% depression) when fish were exposed to aerial hypoxia acutely. We then tested the hypothesis that chronic aerial hypoxia acclimation would protect endogenous energy reserves and skeletal muscle integrity, thereby maintaining locomotor performance, possibly due to hypoxic hypometabolism. We found that air-acclimated fish from both strains were in poorer body condition relative to fish acclimated to aerial hypoxia. Furthermore, aerial hypoxia-acclimation minimized glycogen usage (Strain 1), lipid catabolism (Strain 2), and white muscle atrophy (Strain 2), as well as preserved terrestrial locomotor performance compared to fish in air (Strain 2). Overall, our findings suggest that some *K. marmoratus* strains seek microhabitats that accentuate metabolic depression during dormancy, and that microhabitat O<sub>2</sub> availability may have significant implications for energy metabolism, and the structure and function of skeletal muscle. Furthermore, the differential responses between isogenic strains suggests that genetic factors also contribute to phenotypic differences in the emersion behaviour and physiology of this species.

## Introduction

Several animals enter a state of dormancy to survive harsh environmental conditions, including periods of prolonged cold or drought. During dormancy, animals remain inactive and have no external food supply; thus, a common feature of dormancy is the depression of metabolic rate to conserve limited endogenous energy reserves until the return of environmental conditions favourable for active life (for reviews see Storey and Storey, 1990; Guppy and Withers, 1999; Storey and Storey, 2002). A true metabolic rate depression involves an active downregulation of cellular energy (ATP) turnover below the standard level (SMR; standard metabolic rate) (Storey, 1987), which supports only the essential homeostatic activities of cells and whole organisms (Hulbert and Else, 2004). Here, we use metabolic depression to describe a reduction in metabolic rate below the routine level (RMR; routine metabolic rate). The extent to which animals depress metabolic rate gives a proportional extension to the time they can survive using endogenous reserves (Storey and Storey, 1990; Boutilier et al., 1997). Consequently, a considerable metabolic depression is necessary for animals that remain dormant for many months (Hochachka and Guppy, 1987). For example, long-neck turtles (*Chelodina rugosa*) can survive seasonal droughts lasting 4-5 months by depressing metabolic rate by approximately 70% (Kenneth and Christian, 1994), whereas African lungfish (*Protopterus aethiopicus*) can tolerate drought for years by depressing metabolic rate by 80-90% (Fishman et al., 1992).

Animals, both active and dormant, rely primarily on lipid and/or carbohydrate catabolism to satisfy energy (ATP) demands, while proteins are generally used as a fuel of last resort (Dark, 2005; McCue, 2010). For example, the common frog (*Rana temporaria*) was found to preferentially catabolize lipids during the first 40 days of hibernation, and progressively shifted to exclusive carbohydrate metabolism by 90 days (Donohoe et al.,

1998). If dormant animals exhaust lipid and carbohydrate reserves, they must rely on protein catabolism to meet energy demands (Castellini and Rea, 1992; Caloin, 2004). However, extensive protein catabolism is problematic because it can result in skeletal muscle atrophy, which can be exacerbated by disuse during dormancy. Any muscle atrophy during dormancy is detrimental for animals upon arousal when normal muscular activity must be resumed (Hudson and Franklin, 2002). Impaired locomotor performance can threaten foraging ability, reproductive success, predator avoidance, and ultimately, survival.

Metabolic rate and fuel usage in dormant ectotherms will be affected by the microhabitat. For example, microhabitats with elevated temperatures can expedite substrate utilization and lead to skeletal muscle atrophy (Young et al. 2011). On the other hand, microhabitat conditions that support metabolic depression may aid in skeletal muscle preservation. Hypoxic conditions, for example, have been reported to depress metabolism in a number of ectothermic animals (Boutilier et al., 1997; Hicks and Wang, 1999). For animals that remain dormant for extended periods of time, hypoxic hypometabolism would assist in skeletal muscle maintenance if it accentuates metabolic depression and protects endogenous energy reserves from premature exhaustion. Thus, do animals seek hypoxic microhabitats during dormancy that, in turn, maximally depress metabolic rate?

We used the amphibious mangrove rivulus (*Kryptolebias marmoratus*) to examine this question because they inhabit tropical mangrove swamps that desiccate for several months during the dry season. During seasonal droughts, *K. marmoratus* can survive out of water (emersed) for more than 60 days (Taylor, 1990; Turko et al., in review), despite the inability to eat on land (Pronko et al., 2013). While the definition of aestivation is not clear cut, *K. marmoratus* remain largely inactive (Turko et al., 2017) and, like aestivating animals, enter a hypometabolic state where RMR is depressed by more than 40% after 21 days in air (Turko et al., in review). When on land, *K. marmoratus* are often found nestled within the

termite tunnels of rotting logs (Taylor et al., 2008) that may be hypoxic (12-14% air saturation; Anderson and Ultsch, 1987). Several other ectothermic animals can similarly be found in potentially hypoxic habitats during periods of dormancy, including fish (Smith, 1931; Chew et al., 2004), amphibians (Ruibal and Hillman, 1981; Etheridge et al., 1990; Boutilier et al., 1997; Stewart et al., 2003; Booth 2006), and reptiles (Kennett and Christian, 1994; Markle and Fraser-Chow, 2017) that aestivate/hibernate within subterranean burrows or beneath the ice of frozen lakes. Thus, we used *K. marmoratus* to test the hypothesis that animals seek hypoxic microhabitats during periods of dormancy that, in turn, accentuate metabolic depression. The hypoxic habitat hypothesis predicts that *K. marmoratus* will preferentially occupy hypoxic rather than normoxic microhabitats during periods of prolonged air exposure. Fish were placed in custom experimental choice-chambers that maintained an O<sub>2</sub> gradient (normoxic to hypoxic) to determine the preferred O<sub>2</sub> level of fish out of water. If fish select hypoxic microhabitats when on land, then exposure to aerial hypoxia acutely should result in a more profound metabolic depression than results from aerial normoxia exposure. Thus, we measured the O<sub>2</sub> consumption rate of water (control) and air-acclimated (21 days) fish in aerial normoxia, as well as the O<sub>2</sub> consumption rate of air-acclimated fish acutely exposed to aerial hypoxia. We then tested the hypothesis that chronic hypoxia acclimation in air would protect endogenous energy reserves and skeletal muscle integrity, thereby maintaining locomotor performance. The hypothesis predicts that *K. marmoratus* acclimated to aerial hypoxia will deplete energy stores more slowly, demonstrate less skeletal muscle atrophy, and have better locomotor performance than fish acclimated to aerial normoxia, presumably due to hypoxic hypometabolism. We measured the whole-body [glycogen] and lipid content in fish acclimated to water (control), air, and aerial hypoxia for 21 days. The cross-sectional area of red and white muscle fibers was also measured, as well as terrestrial locomotor performance (tail-flip jumping).

## Materials and Methods

### Experimental Animals

All experimental fish ( $n=235$ ,  $0.088 \pm 0.002$  g) were adult hermaphrodites of the self-fertilizing *Kryptolebias marmoratus* (Poey 1880). We performed all experiments in duplicate on two isogenic strains: Strain 1 (SLC8E, from St. Lucie County, Florida, USA), and Strain 2 (50.91, from Twin Cayes, Papa Gabriel, Belize) (Taterenkov et al., 2010). 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 12h:12h 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).

### Experimental Protocol

Three series of experiments were conducted: 1) a behavioural experiment to determine the environmental O<sub>2</sub> preference of *K. marmoratus* out of water during a 21 day air exposure period, 2) a physiological experiment to determine if acute aerial hypoxia exposure after 21 days in air accentuates the metabolic depression in *K. marmoratus*, and 3) a physiological experiment to determine if chronic aerial hypoxia acclimation (21 days) slows substrate utilization compared to aerial normoxia acclimation (21 days), thereby preserving skeletal muscle integrity and locomotor performance (Fig. S1). Following experimentation, fish were weighed and the standard length measured for determination of body condition (Fulton's K) as described by Froese (2006). When the partial pressure of O<sub>2</sub> (pO<sub>2</sub>) was not constant for the entire the experimental period (i.e., during choice chamber experiments and acute hypoxia exposure during O<sub>2</sub> consumption experiments), Fulton's K was not measured.

## Series 1

To determine if *K. marmoratus* seek hypoxic microhabitats during periods of prolonged air exposure, we designed experimental choice chambers that maintained an pO<sub>2</sub> gradient through which fish could move freely (Fig S2). We constructed the choice chambers from 10 mL serological pipettes to mimic the termite tunnels in rotting logs that *K. marmoratus* inhabit in the wild (Taylor et al., 2008). The serological pipettes were halved to create semi-circle tubes (21 cm long, 1 cm diameter), which were then fitted with a mesh bottom. A strip of water-soaked (15‰) filter paper (0.5 cm wide) was placed inside each tube, on top of the mesh to give fish a moist substrate for travel within the tube.

We measured the pO<sub>2</sub> preference of *K. marmoratus* (Strain 1, n=12; Strain 2, n=23) within the choice chambers during the initial (Days 1-2) and final (Days 20-21) 48 hours of a 21 day air exposure period. First, we randomly positioned fish in individual choice chambers, then introduced a pO<sub>2</sub> gradient ( $10.1 \pm 0.4$  to  $19.9 \pm 0.1$  kPa). The pO<sub>2</sub> gradient was generated by placing the choice chambers over 3 side-by-side containers (1.5 L) filled with water (15‰) (Fig S2). We vigorously bubbled nitrogen into the container at one end to generate hypoxic air in the choice chamber above (“low O<sub>2</sub> zone” =  $10.1 \pm 0.4$  kPa). In the container at the opposite end, we vigorously bubbled air to generate normoxic air in the choice chamber above (“high O<sub>2</sub> zone” =  $19.9 \pm 0.1$  kPa). We bubbled both air and nitrogen into the middle container to generate an intermediate pO<sub>2</sub> in the air above (“medium O<sub>2</sub> zone” =  $15.7 \pm 0.3$  kPa). The pO<sub>2</sub> in each zone was continuously measured during experimental trials using O<sub>2</sub>-sensing optodes and a fiber optic probe (Loligo Systems WITROX 4, Tjele, Denmark). The optodes were calibrated prior to experimentation using humidified atmospheric air (21 kPa O<sub>2</sub>) and 2M sodium sulphite (0 kPa O<sub>2</sub>). Fish were video recorded within the choice chamber using a webcam (Logitech C905w, Newark, CA, USA). Following exposure to the pO<sub>2</sub> gradient on Days 1-2, the pO<sub>2</sub> gradient was stopped, and fish were

maintained in their choice chamber under normoxic conditions until re-exposure to the pO<sub>2</sub> gradient during Days 20-21. We did not reposition fish within the choice chambers before reintroduction of the pO<sub>2</sub> gradient to minimize disturbance. For analysis, we calculated the proportion of time fish spent in each O<sub>2</sub> zone during both recording periods.

All behavioural tests were carried out across seven 21-day experimental trials (5 fish per trial on average). To ensure that all experimental trials were carried out consistently, we began all trials between 1200 and 1600 h, in the same position with in the same climate-controlled room (25°C, 12h:12h light: dark). The choice chamber was illuminated in the day with room lighting (no exterior windows), and at night with red light to minimize disturbance to the fish. The direction of the O<sub>2</sub> gradient (hypoxia to normoxia) was randomly reversed (normoxia to hypoxia) between trials, and most of the trials contained fish from both strains. All videos were analysed by the same observer (G.S.R.) to avoid observer effects.

## Series 2

A separate group of fish were acclimated for 21 days to one of two experimental treatments: water (control) or air. Control fish were maintained in water (15‰) and fed *Artemia* sp. nauplii three times weekly. Air acclimation was achieved by placing fish on moist filter paper (15‰) in 120 ml plastic holding cups, as previously described (Ong et al., 2007). The mass-specific rate of O<sub>2</sub> consumption was then measured in control (Strain 1, n=10; Strain 2, n=10) and air-acclimated (Strain 1, n=8; Strain 2, n=8) fish using closed-system respirometry. Fish were placed in custom glass micro-respirometry chambers (~1 mL) containing a piece of wet filter paper that kept the environment moist during the experimental period. Fish were acclimated to the experimental chamber for a 2 h period in air, during which time the chamber was left open to maintain pO<sub>2</sub> at approximately 21 kPa. A 2 h acclimation has previously been shown to be sufficient for *K. marmoratus* to recover from



handling stress (Blanchard et al., 2019). Following the 2 h acclimation period, the chamber was sealed and the decline in pO<sub>2</sub> was measured for 1 h using O<sub>2</sub>-sensing optodes, which were calibrated weekly. To determine if aerial hypoxia accentuates metabolic depression in *K. marmoratus*, an additional group of fish (Strain 1, n=8; Strain 2, n=8) held in air for 21 days were placed in micro-respirometry chambers that were continuously flushed with an air-N<sub>2</sub> mixture (approximately 9.3 kPa O<sub>2</sub>; Wosthoff, Calibrated Instruments Inc., New York) for 2 h prior to the 1 h measurement period. We attempted to measure O<sub>2</sub> consumption in fish acclimated to aerial hypoxia continuously for 21 days in preliminary experiments. However, we were unable to prevent re-oxygenation while transferring fish from their acclimation chambers to the micro-respirometry chambers, resulting in highly variable O<sub>2</sub> consumption measurements. We calculated O<sub>2</sub> consumption rates from the decline in pO<sub>2</sub> over the 1 h measurement period in three consecutive 20 minute intervals, standardized to body mass. There was no significant difference in O<sub>2</sub> consumption across the technical replicates (repeated-measures ANOVAs;  $P > 0.05$ ), thus, we report the average of the three O<sub>2</sub> consumption measurements. The pO<sub>2</sub> in the O<sub>2</sub> consumption trials performed under normoxia started at  $20.1 \pm 0.2$  kPa and declined to  $17.8 \pm 0.3$  kPa by the end of the measurement period. The pO<sub>2</sub> in the O<sub>2</sub> consumption trials performed under aerial hypoxia started at  $9.3 \pm 0.2$  kPa and declined to  $8.4 \pm 0.2$  kPa. The pO<sub>2</sub> in all trials remained above the lower aerial critical oxygen tension (P<sub>crit</sub>) for *K. marmoratus* Strain 1 (SLC8E) and Strain 2 (50.91) (Blanchard et al., 2019). All O<sub>2</sub> experiments were conducted at 25°C, and between 12:00-18:00 to minimize the effects of diel metabolic rhythms (Rodela and Wright, 2006). Control fish were fasted for at least 48 h prior to use in experiments, which is more than sufficient time to ensure that metabolic rate returns to baseline levels post-feeding (Sutton et al., 2018). We also measured O<sub>2</sub> consumption in a group of fish that had been fasted for 21 days in

water (fasted controls) to determine if the inability to eat on land plays a significant role in metabolic depression in normoxic air.

### Series 3

#### *Energy Reserves*

An additional group of fish were exposed for 21 days to one of three experimental treatments: control, air, or aerial hypoxia, for analysis of whole-body [glycogen] and lipid content. Aerial hypoxia acclimation was achieved by placing air-exposed fish in an incubator (Innova 4230, New Brunswick Scientific, NJ, USA) that was maintained at approximately 8.2 kPa O<sub>2</sub> using an air-N<sub>2</sub> mixture (25°C, 12:12h). Following acclimation, fish were immediately euthanized via cold-water immersion, and either snap frozen in liquid nitrogen for [glycogen] determination (Strain 1, n=22; Strain 2, n=21), or dried (48 h at 60°C) for lipid analysis (Strain 1, n=22; Strain 2, n=23). Whole-body [glycogen] was measured enzymatically (Bergmeyer et al., 1974), and lipid content was measured by chloroform extraction (Junior & Peixoto, 2013).

#### *Muscle Histology*

We measured the size (cross-sectional area) of red and white muscle fibers in another group of fish under control, air, or aerial hypoxia conditions for 21 days (Strain 1, n=22; Strain 2, n=22). Following acclimation, fish were euthanized via immersion in MS-222 (500 mg L<sup>-1</sup>). 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. Frozen muscle steaks were then cut into 8 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  $-80^{\circ}\text{C}$  until staining. Red muscle staining and analysis was performed as previously described by Rossi et al. (2018). White muscle analysis was performed as described by Rossi et al. (2019). Control and air-acclimated fish used for muscle phenotyping were previously used for  $\text{O}_2$  consumption measurements.

### *Jumping Performance*

We compared the terrestrial locomotor performance in a final group of control, air- and aerial hypoxia-acclimated fish (21 days; Strain 1,  $n=23$ ; Strain 2,  $n=23$ ), as previously described (Rossi et al., 2019; McFarlane et al., 2019). Briefly, fish were encouraged to jump via gentle prodding with a clicker ballpoint pen until exhausted, i.e. unresponsive to prodding. The jumping trials were video recorded and analyzed to quantify the distance of the longest jump (in body lengths) and the number of jumps fish performed before reaching exhaustion (Brunt et al., 2016).

### **Statistical Analysis**

We determined the preferred  $\text{O}_2$  zone of *K. marmoratus* during Days 1-2 and Days 20-21 using methods for compositional data, since the proportion of time fish spend in any one  $\text{O}_2$  zone is dependent upon the time spent in other  $\text{O}_2$  zones. All proportions were isometric log-ratio (ILR) transformed and then analyzed using an ordinary least squares (OLS) regression. We subsequently back-transformed the coefficients for meaningful interpretation of the results (van den Boogart and Tolosana-Delgado, 2013).

To determine the effects of strain and experimental treatment on  $\text{O}_2$  consumption, body condition, lipid content, muscle phenotype, and jumping performance, we used two-way analyses of variance (ANOVAs). When a significant main effect of treatment was detected, a Tukey's post-hoc test was used to identify which treatment groups differed (all

reported  $p$ -values are adjusted for multiple comparisons). When a significant strain  $\times$  treatment interaction was detected (glycolytic fiber size, number of jumps), the data was divided by strain and analyzed using a one-way ANOVA, followed by a Tukey's post-hoc test. The [glycogen] of Strain 2 control and air-acclimated fish was compared using a two-sided  $t$ -test; the aerial hypoxia group was excluded from the analysis since the [glycogen] in 5 of 6 fish was below detectable limits. A one-way ANOVA was used to compare the [glycogen] of Strain 1 fish across treatment groups, followed by a Tukey's post-hoc test. Due to the limitations of merely presenting  $p$ -values (Hasley et al., 2015; Halsey, 2019), we supplemented the  $p$ -values from all post-hoc comparisons with an effect size calculation (Cohen's  $D$ ;  $d$  value) for each strain (Strain 1 =  $d_1$ ; Strain 2 =  $d_2$ ) to indicate the magnitude of the effect between treatment groups. When  $d < 0.5$ , the treatment effect is relatively small and inconsequential, whereas  $d > 0.5$  indicates that the treatment effect is biologically meaningful (Sullivan and Feinn, 2012). Prior to analysis, we assessed all data for normality and homogeneity of variance; when data did not meet these parametric test assumptions they were appropriately transformed. All analyses were performed using RStudio (version 1.1.447).

## Results

### *Environmental O<sub>2</sub> Preference*

The proportion of time Strain 2 fish spent in each O<sub>2</sub> zone during Days 1-2 was equally distributed (OLS:  $P=0.63$ ) (Fig. 1). However, Strain 2 fish showed a preference for the low O<sub>2</sub> zone by the end of the air exposure period (OLS:  $P=0.02$ ); fish spent  $53 \pm 7\%$  of the time in the low O<sub>2</sub> zone, and only  $26 \pm 5\%$  and  $21 \pm 6\%$  in the medium and high O<sub>2</sub> zones, respectively (Fig. 1). The time Strain 1 fish spent in the each O<sub>2</sub> zone was equally distributed during Days 1-2 (OLS:  $P=0.15$ ) and Days 20-21 (OLS:  $P=0.80$ ) (data not shown).

### *O<sub>2</sub> Consumption and Body Condition*

The mass-specific rate of O<sub>2</sub> consumption in *K. marmoratus* was significantly influenced by the experimental treatment (two-way ANOVA:  $P < 0.001$ ) but not by strain (two-way ANOVA:  $P = 0.76$ ) (Fig. 2A). We found that control fish had significantly higher O<sub>2</sub> consumption rates than both air- and aerial hypoxia-acclimated fish (Tukey:  $P < 0.001$ ,  $d_1 = 1.5$ ,  $d_2 = 1.0$ ;  $P < 0.001$ ,  $d_1 = 2.2$ ,  $d_2 = 1.7$ ). Air-acclimated fish also had higher O<sub>2</sub> consumption rates than aerial hypoxia-acclimated fish (Tukey:  $P = 0.01$ ,  $d_1 = 1.5$ ,  $d_2 = 1.4$ ). In contrast, the rate O<sub>2</sub> consumption between fasted controls and air-acclimated fish was not significantly different in either strain (two-way ANOVA:  $P = 0.19$ ,  $P = 0.36$ ; Fig. S3). We found that the experimental treatment affected body condition (two-way ANOVA:  $P < 0.001$ ) but the strain did not (two-way ANOVA:  $P = 0.30$ ) (Fig. 2B). The body condition of air-acclimated *K. marmoratus* was lower than that of control and aerial hypoxia-acclimated fish (Tukey:  $P < 0.001$ ,  $d_1 = 0.7$ ,  $d_2 = 0.8$ ;  $P = 0.01$ ,  $d_1 = 0.5$ ,  $d_2 = 0.6$ ). We found no significant difference in the body condition of control and aerial hypoxia-acclimated fish (Tukey:  $P = 0.39$ ,  $d_1 = 0.3$ ,  $d_2 = 0.3$ ).

### *Energy Reserves*

The [glycogen] of Strain 1 fish differed significantly across treatment groups (one-way ANOVA:  $P < 0.001$ ) (Fig. 3A). We found that control fish had a higher [glycogen] than those acclimated to air and aerial hypoxia (Tukey:  $P < 0.001$ ,  $d_1 = 1.2$ ;  $P = 0.004$ ,  $d_1 = 0.9$ ). While the [glycogen] of air- and aerial hypoxia-acclimated fish did not differ significantly (Tukey:  $P = 0.39$ ), there was a meaningful trend towards reduced glycogen usage in aerial hypoxia-acclimated fish, which catabolized 17% less glycogen than fish acclimated to air ( $d_1 = 0.7$ ). Similarly, in Strain 2 fish, the control group had a higher [glycogen] than the air-acclimated group (t-test:  $P < 0.001$ ,  $d_2 = 3.3$ ), but glycogen was undetected in the aerial hypoxia group

(Fig. 3A). The lipid content of *K. marmoratus* was significantly influenced by the experimental treatment (two-way ANOVA:  $P=0.002$ ) but not by strain (two-way ANOVA:  $P=0.20$ ) (Fig. 3B). We found that air-exposed fish had a lower lipid content than control fish (Tukey:  $P=0.001$ ,  $d_1=0.9$ ,  $d_2=1.5$ ). There was no significant difference in the lipid content of control and aerial hypoxia-acclimated fish (Tukey:  $P=0.41$ ), but the effect sizes imply that both strains catabolized lipids under aerial hypoxia ( $d_1=1.0$ ,  $d_2=0.9$ ). Similarly, there was no significant difference in the lipid content of air- and aerial-hypoxic acclimated fish ( $P=0.05$ ), but effect size implies that the use of 23% fewer lipids by Strain 2 fish acclimated to aerial hypoxia may be biologically important ( $d_1=0.4$ ,  $d_2=0.8$ ).

### *Muscle Phenotype*

Both the experimental treatment and the strain affected the size of red muscle fibers in *K. marmoratus* (two-way ANOVA:  $P=0.007$ ,  $P=0.04$ ) (Fig. 4A). We found that air-exposed fish had significantly larger red muscle fibers than control fish (Tukey:  $P=0.005$ ,  $d_1=1.3$ ,  $d_2=1.0$ ). While there was no significant difference in the size of red muscle fibers between air- and aerial hypoxia-acclimated fish (Tukey:  $P=0.12$ ), in Strain 1 fish there was a trend towards smaller fibers in aerial hypoxia than in air ( $d_1=1.3$ ,  $d_2=0.3$ ). Similarly, we found no significant difference in the size of red muscle fibers between control and aerial hypoxia-acclimated fish (Tukey:  $P=0.43$ ), though Strain 2 fish acclimated to aerial hypoxia had 15% larger fibers than control counterparts which may be biologically meaningful ( $d_1=0.0$ ,  $d_2=1.0$ ). The size of the white muscle fibers in Strain 1 and Strain 2 fish was differentially affected by the experimental treatment, as evident by a significant strain  $\times$  treatment interaction (two-way ANOVA interaction:  $P=0.01$ ) (Fig. 4B). The size of white muscle fibers did not differ across treatments in Strain 1 fish (one-way ANOVA;  $P=0.12$ ) but differed in Strain 2 fish (one-way ANOVA:  $P=0.03$ ). Strain 2 fish acclimated to air had significantly

smaller white muscle fibers than control fish (Tukey:  $P=0.02$ ,  $d_2=1.3$ ). We found no significant difference in the size of white muscle fibers between control and aerial-hypoxia acclimated fish ( $P=0.70$ ,  $d_2=0.4$ ). We found no significant difference in the size of white muscle fibers between air- and aerial hypoxia-acclimated fish (Tukey:  $P=0.15$ ), but there was a meaningful trend towards larger white muscle fibers in aerial hypoxia-acclimated fish, which were 24% larger than those of fish in air ( $d_2=1.2$ ).

### *Jumping Performance*

The longest jump *K. marmoratus* could perform was affected by the experimental treatment (two-way ANOVA:  $P=0.04$ ) but strain had no effect (two-way ANOVA:  $P=0.06$ ) (Fig. 5A). The longest jump air-acclimated fish could perform was shorter compared to control fish (Tukey:  $P=0.03$ ,  $d_1=1.5$ ,  $d_2=1.3$ ). There was no significant difference in longest jump length between control and aerial hypoxia-acclimated fish (Tukey:  $P=0.54$ ), but in Strain 1 the effect size implies that the longest jump fish could perform in aerial hypoxia was shorter than under control conditions ( $d_1=0.8$ ,  $d_2=0.4$ ). Similarly, we found no significant difference in the longest jump length between air- and aerial hypoxia-acclimated fish (Tukey:  $P=0.29$ ), but there was a meaningful trend towards a longer jumps by Strain 2 fish in aerial hypoxia, which were 20% longer than jumps in air ( $d_1=0.2$ ,  $d_2=1.1$ ). We found that the number of jumps Strain 1 and Strain 2 fish could perform before exhaustion was differentially affected by treatment (two-way ANOVA interaction:  $P=0.05$ ) (Fig. 5B). Strain 1 fish jumped the same number of times regardless of treatment (one-way ANOVA:  $P=0.46$ ). However, the number of jumps Strain 2 fish performed differed across treatments (one-way ANOVA:  $P=0.01$ ); air-acclimated fish jumped fewer times than control fish (Tukey:  $P=0.02$ ,  $d_2=1.6$ ). There was no significant difference between the number of jumps performed by air- and aerial hypoxia-acclimated fish (Tukey:  $P=0.05$ ), but the effect size implies that the 20%

more jumps performed by Strain 2 fish acclimated to aerial hypoxia may be biologically important ( $d_2=1.6$ ).

## Discussion

We used *K. marmoratus* to test the hypothesis that animals seek hypoxic microhabitats that, in turn, accentuate metabolic depression during dormancy. In support of our hypothesis, we found that one isogenic strain spent more than double the amount of time in hypoxia compared to normoxia after 21 days in air. Furthermore, prolonged air exposure in both strains resulted in lower  $O_2$  consumption rates compared to active fish (35% depression), which was accentuated (51% depression) when fish were exposed to aerial hypoxia acutely. The fact that fish exposed to aerial hypoxia demonstrated lower  $O_2$  consumption rates than air-exposed fish (which were fasted and inactive) is suggestive of active metabolic rate depression, particularly because  $O_2$  consumption measurements were obtained above  $P_{crit}$  for *K. marmoratus* in air (Blanchard et al., 2019) indicating that this was not simply an oxygen-conforming response. We also tested the hypothesis that chronic hypoxia exposure in air would protect endogenous energy reserves and skeletal muscle integrity, thereby maintaining locomotor performance, possibly due to hypoxic hypometabolism. We found that air-acclimated fish from both strains were in poorer body condition relative to control fish and those acclimated to aerial hypoxia. Air-acclimated fish demonstrated considerable glycogen and lipid catabolism (both strains), white muscle atrophy (Strain 2 only), and poorer terrestrial locomotion compared to control fish (Strain 2 only). However, aerial hypoxia-acclimation reduced glycogen usage by 17% in Strain 1 fish, and spared lipids and white muscle protein in Strain 2 fish by 23% and 22%, respectively. Furthermore, aerial hypoxia acclimation maintained the terrestrial locomotor performance of Strain 2 fish compared to air acclimation (22% farther longest jump, 20% more jumps). Overall, our findings demonstrate



that *K. marmoratus* seek microhabitats that, in turn, accentuate metabolic depression, and that microhabitat O<sub>2</sub> availability can have significant implications for substrate utilization, and the structure and function of skeletal muscle.

### *Microhabitat Selection and O<sub>2</sub> Consumption*

We showed that *K. marmoratus* (Strain 2) seek hypoxic microhabitats after prolonged air-exposure, and one possible benefit is an accentuation of metabolic depression. Our laboratory findings are consistent with the observed habitat preference of *K. marmoratus* in the wild, as fish are often found within decaying mangrove logs (Taylor et al., 2008), that are likely hypoxic (Anderson and Ultsch, 1987). Although Strain 1 fish did not seek hypoxia on land, they catabolized less of their metabolic fuel stores (glycogen and some lipids) after 21 days in air compared to Strain 2 fish (glycogen, lipids, and muscle protein). This was an interesting finding because the absolute rate of O<sub>2</sub> consumption was not significantly different between strains after 21 days in air. One possible explanation for the differential fuel use relates to the rate of metabolic depression. For instance, if Strain 1 fish depressed metabolic rate to the air-acclimated (21 day) value several days before Strain 2 fish, this would likely result in considerable energy savings. Thus, Strain 2 fish may face greater pressure to seek low O<sub>2</sub> microhabitats during seasonal droughts that accentuate metabolic depression and preserve their remaining energy stores. Alternatively, there are several other reasons why *K. marmoratus* may be found in hypoxic microhabitats in the wild, not just as a strategy to reduce O<sub>2</sub> consumption. For example, the decaying logs in which *K. marmoratus* are found may contain more moisture that would protect fish from dehydration and help survival. Overall, the microhabitat conditions that mediate microhabitat selection strategies in dormant taxa are poorly understood, but this remains exciting avenue for future investigation.

Prolonged air-exposure in both strains resulted in lower O<sub>2</sub> consumption rates compared to active fish, which was accentuated when fish were exposed to aerial hypoxia acutely. Microhabitat O<sub>2</sub> availability has similarly altered the extent of metabolic depression in other dormant animals. For example, hibernating frogs (*Rana temporaria*) exposed to chronic aquatic hypoxia exhibited a 75% suppression of metabolic rate (Donohoe and Boutilier, 1998), whereas those in normoxic water only depressed metabolism by approximately 50% (Donohoe et al., 1998). It is unknown if hypoxic hypometabolism would persist in *K. marmoratus* with chronic hypoxia exposure in air. However, in many fishes acclimated to chronic hypoxia (weeks to months), O<sub>2</sub> consumption remained low over time (e.g. Kerstens et al., 1979; Timmerman and Chapman, 2004). We recently showed that the green-striped burrowing frog (*Cyclorana alboguttata*) also selects environmental hypoxia, which consistently accentuated metabolic depression by ~30% throughout the first 7 weeks of aestivation (Rossi et al., in review). Taken together, we suggest that the microhabitats occupied by animals during dormancy can have a profound impact on the physiological strategies used to tolerate environmental extremes.

### *Endogenous Fuel Usage*

The pattern of metabolic fuel use differed between *K. marmoratus* strains. Strain 1 fish catabolized 75% of their glycogen stores after 21 days in air, but only 58% after 21 days in aerial hypoxia. Glycogen stores are considered the primary source of metabolic fuel for fishes experiencing hypoxia (Hochachka and Somero, 1984). Thus, glycogen preservation under aerial hypoxia suggests that Strain 2 fishes may be reducing their overall ATP demand. On the other hand, Strain 2 fish catabolized glycogen, lipids, and presumably protein (given the white muscle fiber atrophy) in air. Aerial hypoxia acclimation attenuated lipid usage in this strain by 23%, and spared muscle protein. These findings are consistent with that of

overwintering goldfish (*Carassium auratus*), in which lipid oxidation is constrained under extreme hypoxia in favour of anaerobic glycolysis (Hochachka and Somero, 1984). Accordingly, the [glycogen] of most Strain 2 fish was undetectable after aerial hypoxia acclimation. A recent study from our laboratory also demonstrated considerable glycogen and lipid catabolism in three isogenic strains of *K. marmoratus* (50.91, SLC8E, HON9) after 21 days of air exposure (Turko et al., in review). Interestingly, both our study and that of Turko et al. (in review) found that Strain 2 (50.91) fish catabolized 20-25% more of their lipid reserves than Strain 1 (SLC8E) fish after air exposure despite the same rate of O<sub>2</sub> consumption between the two strains after 21 days in air. The rate at which metabolic depression is achieved between *K. marmoratus* strains may explain the discrepancy in substrate use, but this hypothesis warrants further investigation. Overall, our findings suggest that *K. marmoratus* strains can employ different behavioural and physiological strategies to prevent the premature exhaustion of endogenous energy reserves during prolonged air exposure. Furthermore, the differential responses between isogenic stains suggests genetic factors contribute to phenotypic differences in the behaviour and physiology of *K. marmoratus*.

### *Muscle Phenotype and Locomotion*

The integrity of skeletal muscle in *K. marmoratus* can be critical for survival. Like most fishes, *K. marmoratus* rely on their skeletal musculature to power the locomotor movements required for accomplishing essential daily tasks (e.g. predator avoidance, prey capture). Air exposure resulted in the hypertrophy of red muscle fibers in *K. marmoratus*, which we have previously demonstrated is the result of the higher O<sub>2</sub> availability in atmospheric air relative to water (Rossi et al., 2018). On the other hand, Strain 2 fish demonstrated atrophy of their white muscle fibers after air acclimation, suggesting that the

greater substrate utilization by these fish may have led to protein catabolism. Consequently, Strain 2 fish demonstrated poorer locomotor performance (shorter longest jump, fewer jumps performed) after air acclimation. In support of our hypothesis, the skeletal muscle integrity and locomotor performance of *K. marmoratus* was maintained after 21 days on land with chronic aerial hypoxia exposure. Previous studies have demonstrated that a profound metabolic depression during dormancy can preserve skeletal muscle structure and function (for review see Hudson and Franklin, 2002a). For example, the frog *C. alboguttata* depresses metabolic rate by up to 80% during estivation (Kayes et al., 2009; Young et al., 2011) and exhibited no skeletal muscle atrophy after several months (Hudson and Franklin, 2002b). Any changes in the skeletal musculature of dormant animals that negatively impact locomotor performance can threaten foraging ability, reproductive success, predator avoidance, and ultimately, survival.

### *Perspectives*

We demonstrated that some *K. marmoratus* strains seek hypoxic microhabitats during prolonged air-exposure, and that acute hypoxia exposure accentuates metabolic depression on land. Interestingly, previous studies from our laboratory have showed that *K. marmoratus* avoids aquatic hypoxia by emersing (Regan et al., 2011; Livingston et al., 2018). How is this behavioural pattern regulated? In other words, what mechanisms allow animals to avoid hypoxia during active periods, but favour hypoxic microhabitats during dormancy? Elucidating the physiological mechanisms modulating behavioural responses to hypoxia in dormant animals will be a fruitful avenue for future investigation.

While we focused on environmental hypoxia in this study, there are numerous other abiotic factors that may interact with hypoxia to enhance metabolic depression in dormant animals. For example, the subterranean burrows of hibernating hamsters (*Mesocricetus*

*auratus*) can be extremely hypoxic and hypercapnic (Kuhnen, 1986). Similarly, in mangrove swamps, low O<sub>2</sub> environments are often accompanied by hydrogen sulphide (H<sub>2</sub>S) (Rossi et al., 2019). If the microhabitats occupied by *K. marmoratus* during the dry season are both hypoxic and H<sub>2</sub>S-rich, we might expect a more profound metabolic depression, as H<sub>2</sub>S inhibits oxidative phosphorylation in *K. marmoratus* (Cochrane et al., 2019). While laboratory studies are valuable for evaluating the physiological mechanisms involved in metabolic depression during dormancy, understanding how microhabitats conditions alter physiological responses is critical.

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### **Competing Interests**

The authors declare no competing or financial interests.

### **Author Contributions**

Both authors contributed to the conception and design of the study. G.S.R. conducted the experiments, analyzed the data, and wrote the draft manuscript. Both authors edited the manuscript.

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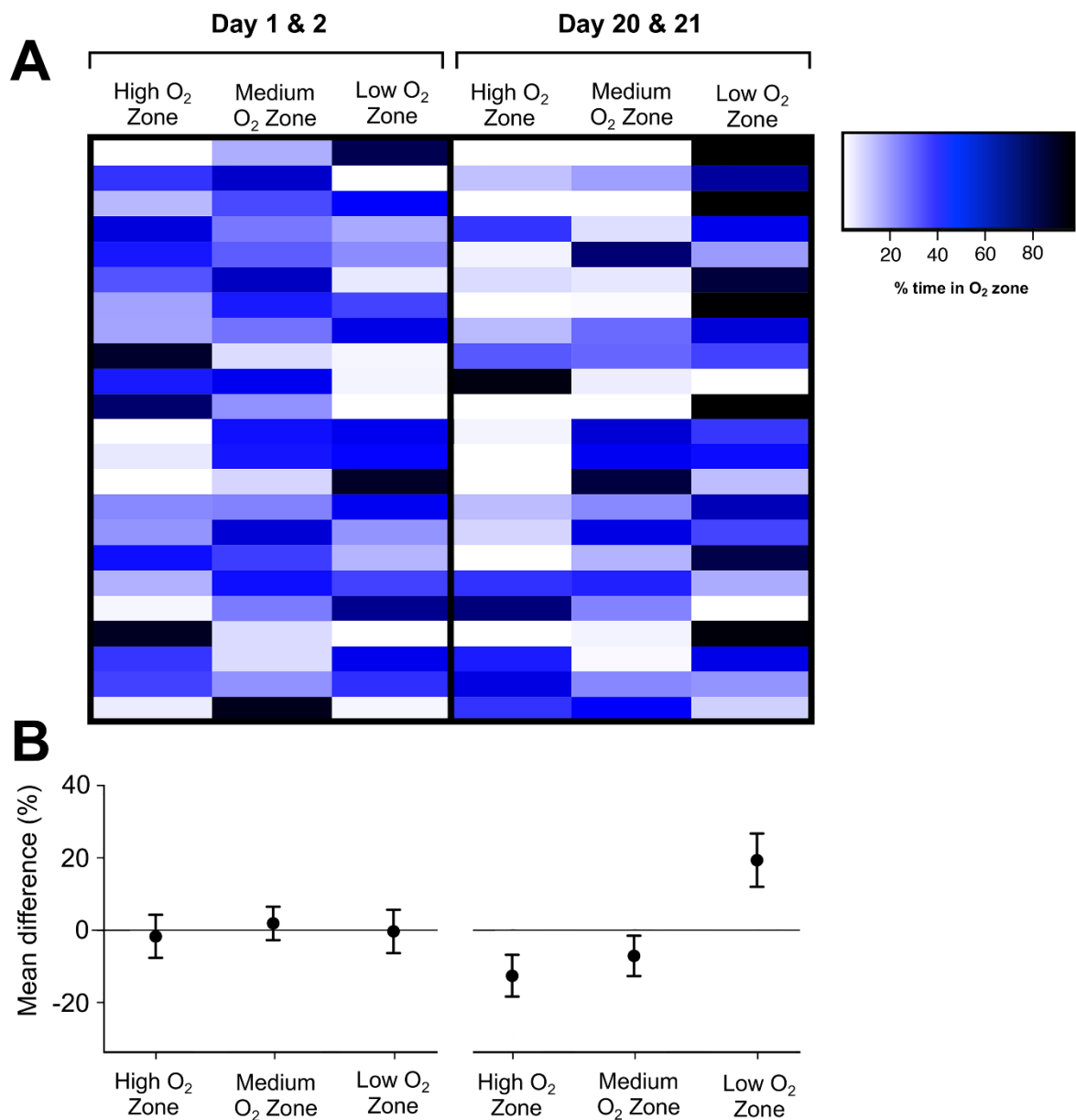
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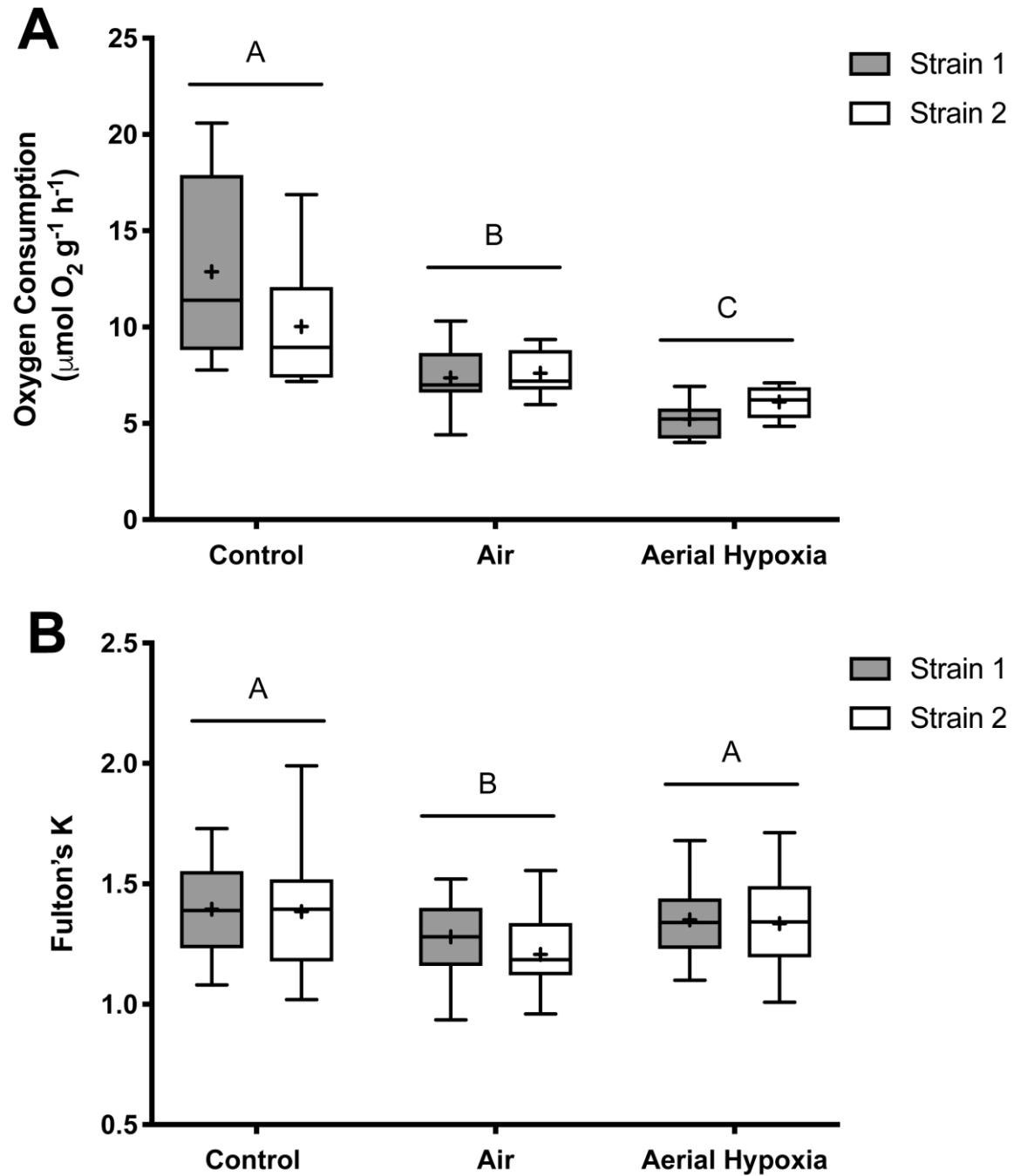
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## Figures



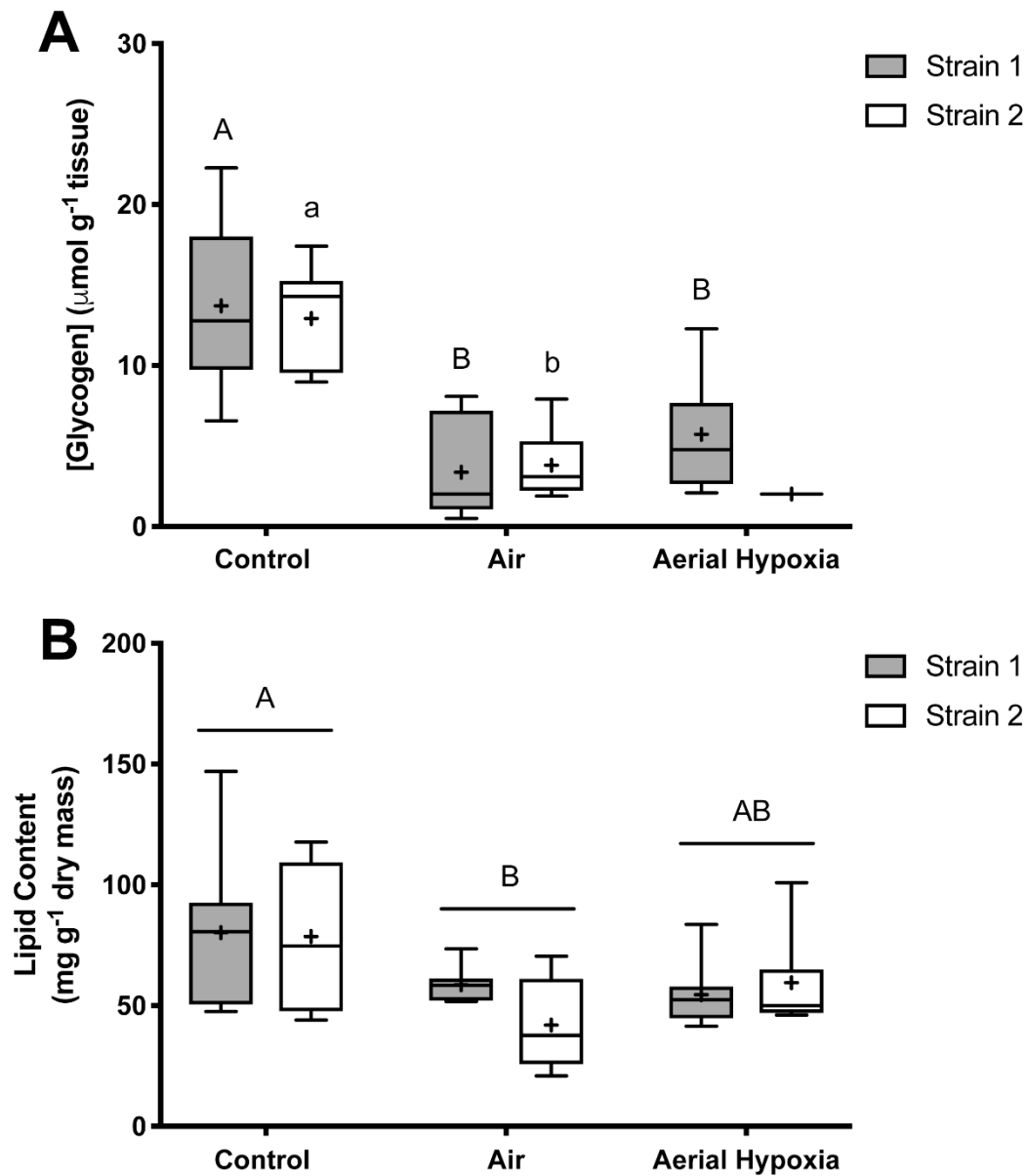
**Figure 1:** (A) Heat map representing the % time spent in the high, medium, and low O<sub>2</sub> zones by *K. marmoratus* (Strain 2; n=23) during the first (Days 1-2, left) and final (Days 20-21, right) 48 h of a 21 day air exposure period. Each row represents an individual and each column represents an O<sub>2</sub> zone. (B) The % mean difference between the observed time *K. marmoratus* spent in each O<sub>2</sub> zone during Days 1-2 (left) and Days 20-21 (right) and the

excepted time assuming *K. marmoratus* demonstrated no O<sub>2</sub> zone preference (one third / zone). Error bars denote the mean  $\pm$  SEM of each O<sub>2</sub> zone. Strain 1 fish demonstrated no O<sub>2</sub> zone preference during Days 1-2 or Days 20-21 (data not shown).

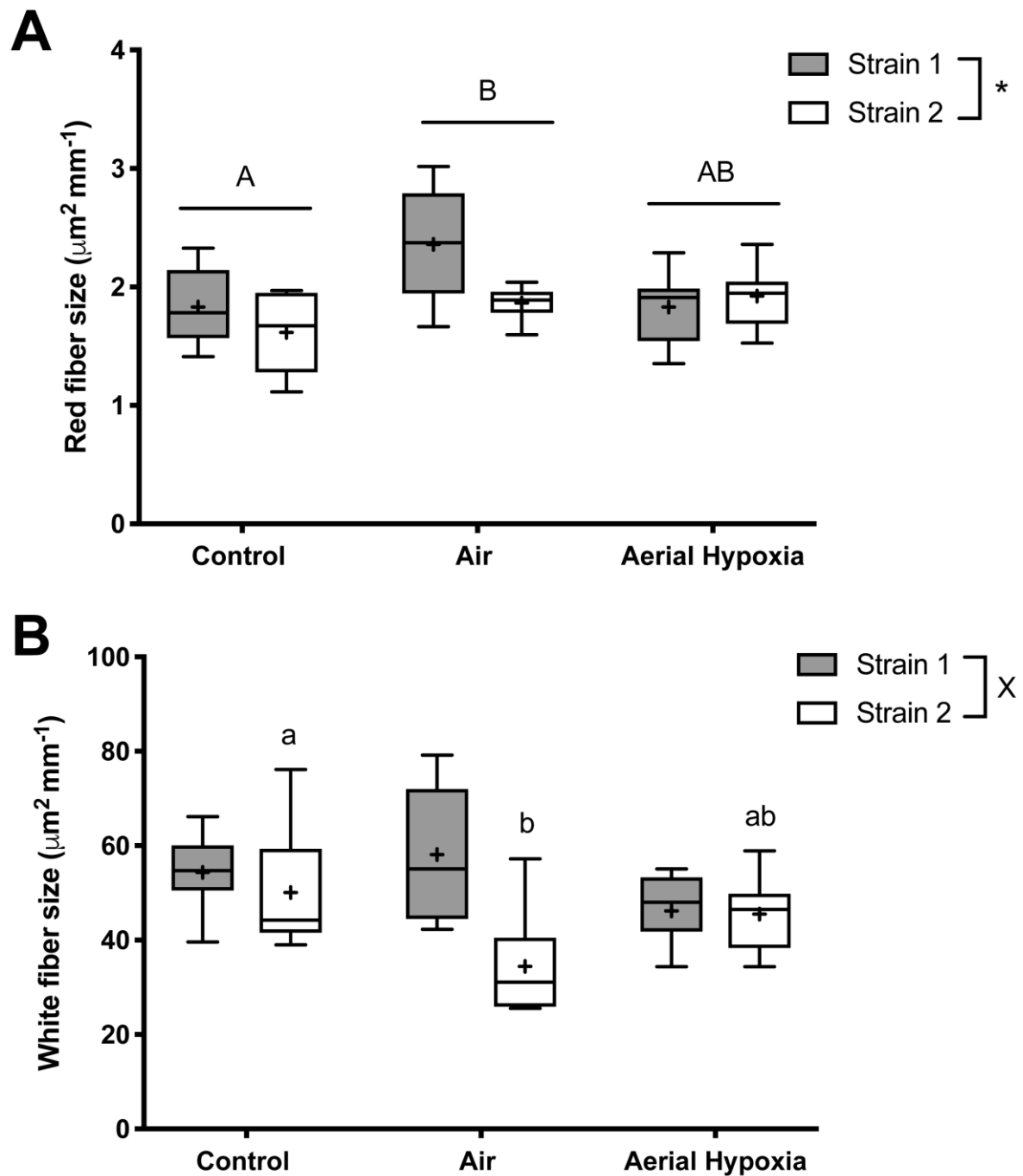


**Figure 2:** (A) The mass-specific rate of  $\text{O}_2$  consumption in water (control; Strain 1,  $n=10$ ; Strain 2,  $n=10$ ) and air acclimated *K. marmoratus* under aerial normoxia ( $\sim 21$  kPa; Strain 1,  $n=8$ ; Strain 2,  $n=8$ ), as well as the  $\text{O}_2$  consumption rate of air acclimated (21 days) fish acutely exposed to aerial hypoxia ( $\sim 9.4$  kPa; Strain 1,  $n=8$ ; Strain 2,  $n=8$ ). (B) The body condition (Fulton's K) of *K. marmoratus* acclimated to water (control; Strain 1,  $n=33$ ; Strain

2, n=32), air (Strain 1, n=30; Strain 2, n=32), and aerial hypoxia (Strain 1, n=29; Strain 2, n=28), for 21 days. Uppercase letters indicate significant differences between experimental treatments. Means are shown as crosses within the box.



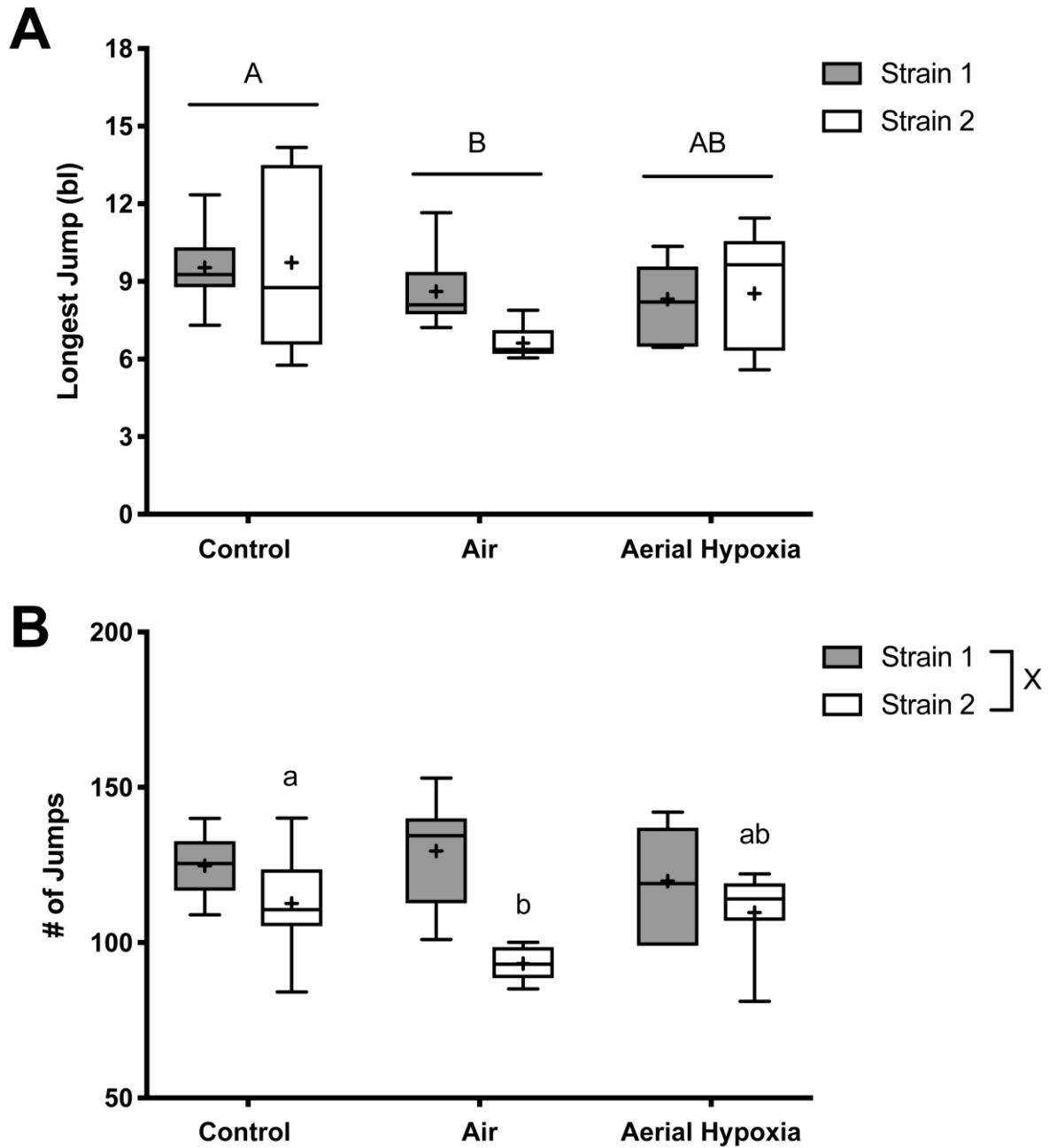
**Figure 3:** (A) The whole-body [glycogen] of control (Strain 1,  $n=7$ ; Strain 2,  $n=7$ ), air (Strain 1,  $n=7$ ; Strain 2,  $n=8$ ), and aerial hypoxia (Strain 1,  $n=7$ ; Strain 2,  $n=6$ ) acclimated *K. marmoratus*. Uppercase and lowercase letters denote significant differences in the [lactate] of Strain 1 and Strain 2 fish, respectively, across treatment groups. (B) The whole-body lipid content of control (Strain 1,  $n=8$ ; Strain 2,  $n=8$ ), air (Strain 1,  $n=7$ ; Strain 2,  $n=8$ ), and aerial hypoxia (Strain 1,  $n=7$ ; Strain 2,  $n=7$ ) acclimated fish. Uppercase letters indicate significant differences between experimental treatments. Means are shown as crosses within the box.



**Figure 4:** (A) The red muscle fiber size of control (Strain 1, n=8; Strain 2, n=8), air (Strain 1, n=7; Strain 2, n=8), and aerial hypoxia (Strain 1, n=8; Strain 2, n=7) acclimated *K. marmoratus*. Uppercase letters indicate significant differences between experimental treatments. An asterisk in the legend indicates that Strain 1 and Strain 2 were significantly different from each other. (B) The white muscle fiber size of control (Strain 1, n=8; Strain 2,

n=8), air (Strain 1, n=7; Strain 2, n=8), and aerial hypoxia (Strain 1, n=7; Strain 2, n=7) acclimated fish. Lowercase letters indicate significant differences between the white muscle fibers of Strain 2 fish across experimental treatments. An X in the legend indicates a significant strain by treatment interaction. Means are shown as crosses within the box.

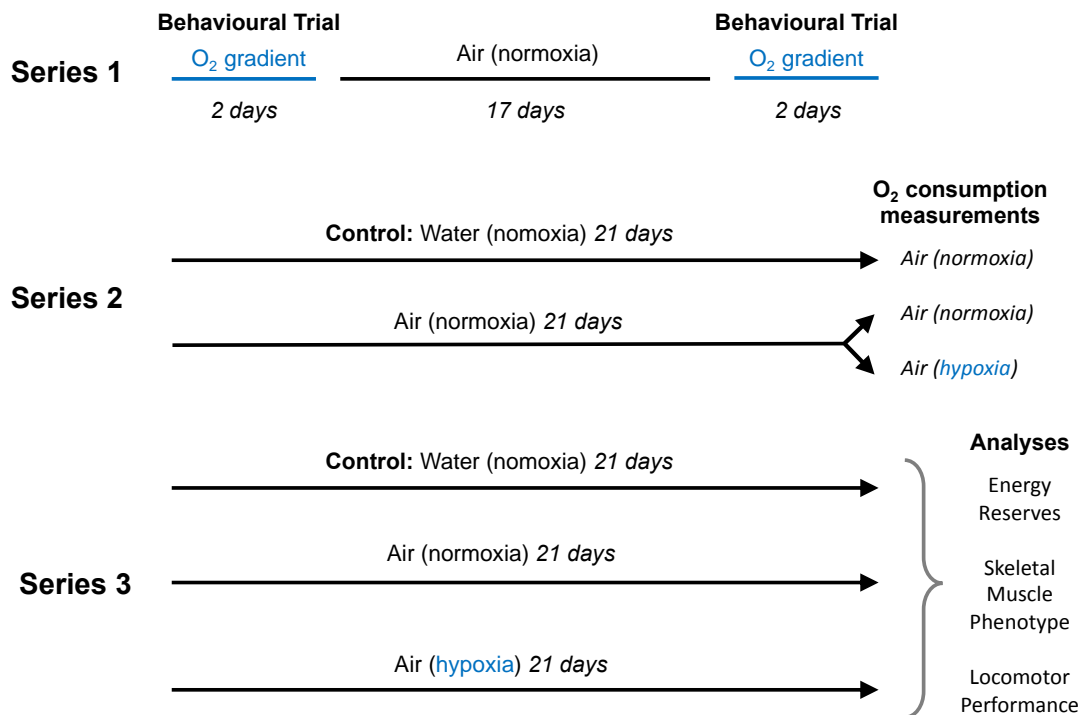




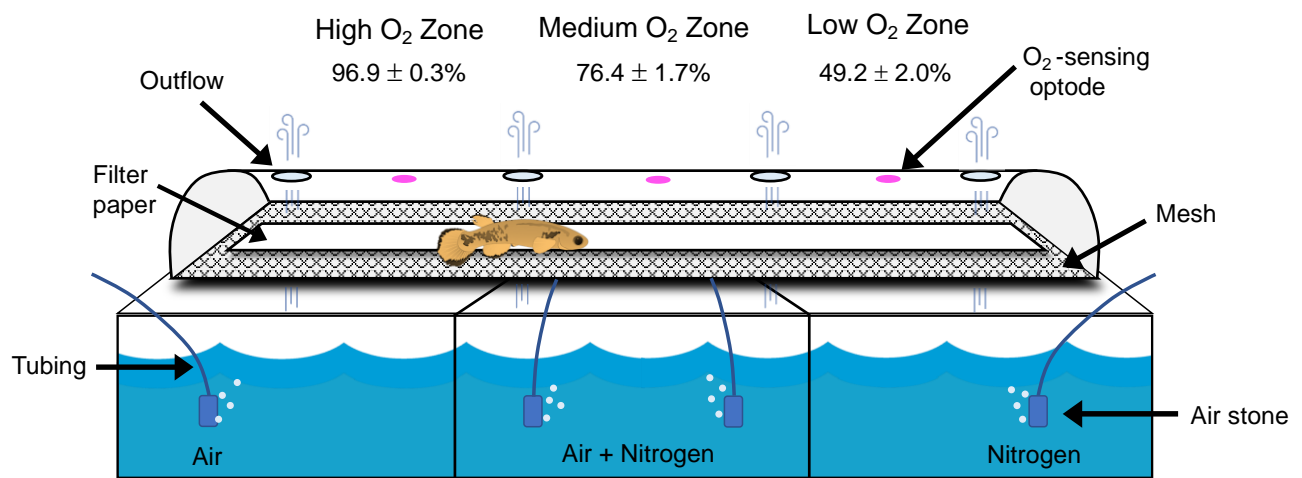
**Figure 5:** (A) The longest jump performed (in body lengths) by control (Strain 1, n=8; Strain 2, n=8), air (Strain 1, n=8; Strain 2, n=8), and aerial hypoxia (Strain 1, n=7; Strain 2, n=7) acclimated *K. marmoratus*. Uppercase letters indicate significant differences between experimental treatments. (B) The number of jumps performed by control (Strain 1, n=8; Strain 2, n=8), air (Strain 1, n=8; Strain 2, n=8), and aerial hypoxia (Strain 1, n=7; Strain 2, n=7) acclimated fish before exhaustion. Lowercase letters indicate significant differences

between the number of jumps performed by Strain 2 fish across experimental treatments. An X in the legend indicates a significant strain by treatment interaction. Means are shown as crosses within the box.

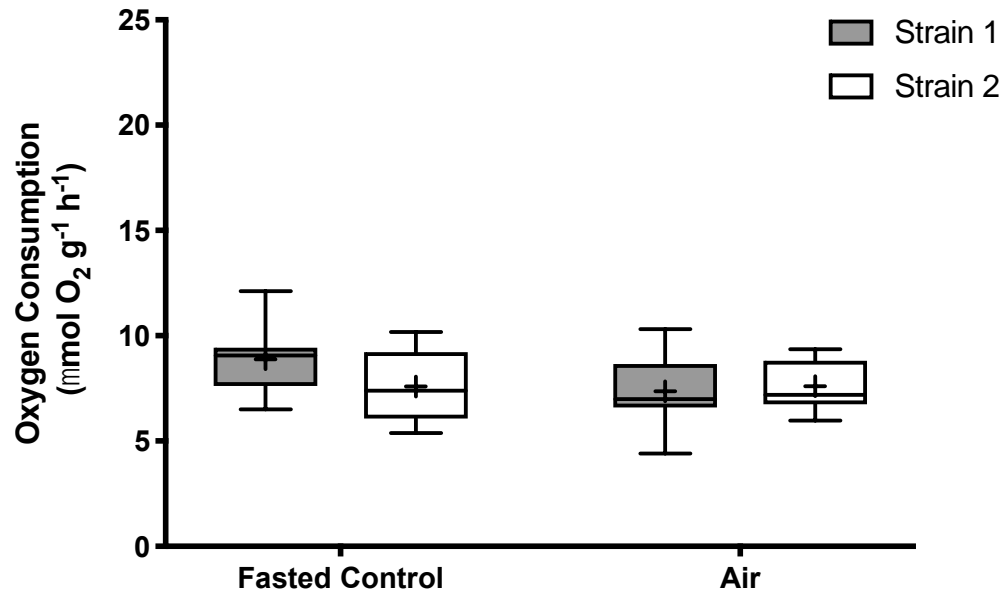
## Supplementary Material



**Figure S1:** Schematic representation of the timeline for each series of experiment.



**Figure S2:** Schematic representation of a custom-built experimental choice chamber resting over 3 buckets of water. The pO<sub>2</sub> within the high O<sub>2</sub> zone (left), medium O<sub>2</sub> zone (centre), and low O<sub>2</sub> zone (right) was determined using O<sub>2</sub>-sensing optodes (Loligo Systems WITROX 4, Tjele, Denmark).



**Figure S3:** The mass-specific rate of O<sub>2</sub> consumption in water-acclimated *K. marmoratus* that were fasted for 21 days (Strain 1, n=9; Strain 2, n=8) and air-acclimated fish in aerial normoxia (Strain 1, n=8; Strain 2, n=8). There is no significant difference between means. Means are shown as crosses within the box.