



Ageing impacts phenotypic flexibility in an air-acclimated amphibious fish

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Abstract

The ability to tolerate environmental change may decline as fishes age. We tested the hypothesis that ageing influences the scope for phenotypic flexibility in the mangrove rivulus (*Kryptolebias marmoratus*), an amphibious fish that transitions between two vastly different environments, water and land. We found that older fish (4–6 years old) exhibited marked signs of ageing; older fish were reproductively senescent, had reduced fin regenerative capacity and body condition, and exhibited atrophy of both oxidative and glycolytic muscle fibers relative to younger adult fish (1–2 years old). However, age did not affect routine O₂ consumption. We then acclimated adult fish (1–6 years) to water (control) or air for 10 days to assess the scope for phenotypic flexibility in response to terrestrial exposure. In support of our hypothesis, we found that older air-acclimated fish had a diminished scope for gill remodeling relative to younger fish. We also found that older fish exhibited poorer terrestrial locomotor performance relative to younger adult fish, particularly when acclimated to air. Our results indicate that ageing diminishes skeletal muscle integrity and locomotor performance of amphibious fishes, and may, therefore, impair terrestrial foraging ability, predator avoidance, or dispersal across the terrestrial environment. Remarkably, older fish voluntarily left water to a similar degree as younger fish despite the age-related deterioration of traits important for terrestrial life.

Keywords Amphibious fish · Ageing · Phenotypic flexibility · Gill remodeling · Locomotor performance

Introduction

Ageing is the time-related deterioration of physiological integrity, leading to impaired performance and fitness (Hughes and Reynolds 2005; Partridge 2010). The primary cause of ageing is the accumulation of molecular damage over time from both intrinsic (e.g., reactive oxygen species) and extrinsic (e.g., UV radiation) sources (for reviews, see Kirkwood 2005; Vijn and Campisi 2008; Gems and Partridge 2013; Maynard et al. 2015). While cells possess quality control systems to repair or remove molecular damage,

these systems require significant amounts of energy (Kirkwood 2005). Consequently, energy allocation trade-offs exist between the physiological processes that maximize fitness (i.e., growth and reproduction) and processes that repair molecular damage and, thus, increase longevity (Kirkwood 1977; Lemaître et al. 2015). For example, fast growth and early maturity curtail the lifespan of some Trinidadian guppy (*Poecilia reticulata*) populations, while slow growth and late maturity in other populations have the opposite effect (Reznick et al. 2002). Like many other animals, fishes exhibit characteristic markers of ageing, such as reduced fecundity (Reznick et al. 2004), neurodegeneration and cognitive decay (Valenzano et al. 2006; Tozzini et al. 2012), sarcopenia (Gerhard et al. 2002; Froehlich et al. 2013), decreased locomotor performance (Valenzano et al. 2006), and impaired regenerative capacity (Wendler et al. 2015).

The consequences of ageing may be particularly problematic for fishes that inhabit variable environments. To persist in variable environments, many animals adjust biochemical, physiological, behavioral, morphological, and/or life-history traits to bring their phenotype closer

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to a functional optimum (phenotypic flexibility; Piersma and van Gils 2011). However, ageing may impair various biological processes required for phenotypic change. For example, increased cognitive performance can help fish respond quickly and adequately to environmental perturbation (Kotrschal and Taborsky 2010), but a decline in the ability to learn in older fish may result in impaired responses (e.g. *Nothobranchius furzeri*, Valenzano et al. 2006; Terzibasi et al. 2008). As well, skeletal muscle plasticity may change with age. For example, muscle mitochondrial content is reduced in older fishes (Hartmann et al. 2011), which may result in a phenotype–environment mismatch when older fishes face environmental changes (e.g., cooling) that require an increase in mitochondrial density (Johnston and Dunn 1987). To our knowledge, there have been no studies in fish linking age with plastic responses to environmental variation. Does ageing impair the ability of fish to tolerate environmental change by limiting the scope for phenotypic flexibility?

One group of animals that face extreme environmental change is amphibious fishes. Amphibious fishes spend a portion of their life out of water and, thus, transition between two immensely different environments—water and land (Gordon et al. 1969). Phenotypic flexibility is critical for allowing amphibious fishes to temporarily inhabit terrestrial environments (Pfenning et al. 2006, 2010; Moczek et al. 2011; Wright and Turko 2016). For example, some species increase cutaneous blood flow and/or proliferate epidermal capillaries during air exposure to enhance cutaneous O₂ uptake and improve terrestrial respiratory performance (Daxboeck and Heming 1982; Cooper et al. 2012; Turko et al. 2014; Blanchard et al. 2019). *Kryptolebias marmoratus* and *Polypterus senegalus* also remodel their gills after 7 days of air exposure by developing an interlamellar cell mass (ILCM), which may provide structural support to the lamellae and/or decrease water loss (Ong et al. 2007; Turko et al. 2011, 2018, 2019). In terms of locomotion, air-acclimated *P. senegalus* remodel their pectoral fins by increasing the proportion of glycolytic muscle fibers relative to water-acclimated fish, which improves terrestrial locomotion (Du and Standen 2017). Similarly, terrestrial tail-flip jumping behavior in *K. marmoratus* is enhanced with air exposure along with remodeling of the axial skeletal muscle towards a more aerobic phenotype (i.e., increased total cross-sectional area of oxidative muscle via hypertrophy and increased oxidative muscle capillariness) (Brunt et al. 2016; Rossi et al. 2018). If ageing limits the scope for phenotypic flexibility in amphibious fishes, it may lead to impaired respiratory and locomotor performance on land as fish age. Diminished terrestrial performance may constrain the time fish can spend out of water foraging, impair their ability disperse to new aquatic habitats, and may ultimately be fatal if fish must remain out of water (emersed) for extended periods of time.

In the present study, we tested the hypothesis that ageing influences the scope for phenotypic flexibility in respiratory and locomotor traits in amphibious fishes using the self-fertilizing amphibious mangrove rivulus (*K. marmoratus*) as a model. *K. marmoratus* can survive out of water for several weeks (Taylor 1990) due, in part, to phenotypic flexibility. Using a broad age range of adult *K. marmoratus* (0–6 years old), we first assessed various markers of ageing including reproductive status, regenerative capacity, routine O₂ consumption, and body condition. We then assessed how ageing influences the scope for phenotypic flexibility by examining traits that are plastic in some strains of *K. marmoratus* (i.e., terrestrial locomotor performance, gill and muscle phenotype remodeling, and cutaneous angiogenesis) in response to air exposure (Ong et al. 2007; Turko et al. 2011, 2014; Brunt et al. 2016; Rossi et al. 2018; Blanchard et al. 2019). The hypothesis predicts that older *K. marmoratus* will show a smaller acclimation response to terrestrial exposure relative to younger fish. Overall, our results demonstrate that several traits that affect performance on land deteriorate with age in *K. marmoratus*. Thus, we assessed the voluntary emersion behavior (i.e., the frequency of emersion events and % time spent out of water) of *K. marmoratus* across an age range (1–6 years) to determine if old fish consequently avoid terrestrial exposure.

Methods

Experimental animals

We obtained adult *Kryptolebias marmoratus* hermaphrodites ($n = 69$, 0.080–0.180 g, strain HON9, originating from the Bay Islands, Utila, Honduras; Tatarenkov et al. 2010) from a breeding colony housed at the Hagen Aqualab at the University of Guelph, Guelph, ON, Canada. Prior to, and during experiments unless otherwise indicated, all fish were individually maintained in 120 ml plastic holding cups under constant conditions (~60 ml water, 15‰, 25 °C, 12 h:12 h light:dark cycle; Frick and Wright 2002). Water changes were performed weekly and fish were fed live *Artemia* sp. nauplii three times per week. All experimental procedures were approved by the University of Guelph Animal Care Committee (AUP 3891).

Experimental protocol

Three series of experiments were conducted. In Series 1, we examined known markers of ageing in fish maintained under standard conditions to determine if older *K. marmoratus* exhibited age-related performance declines. In Series 1a, we assessed the rate of caudal fin regeneration in fish from two age-classes (0–1 and 4–5 years). In Series 1b, we examined

routine O₂ consumption and body condition in fish ranging from 1 to 6 years of age. In Series 2, we tested the hypothesis that ageing reduces the scope of phenotypic flexibility. We acclimated fish aged 1–6 years to water (control) or air for 10 days, and examined terrestrial locomotor performance, skeletal muscle phenotype, cutaneous angiogenesis, and gill surface area. In Series 3, we investigated the voluntary emersion behavior (i.e., the frequency of emersion events and % time spent out of water) of *K. marmoratus* (1–6 years old) to determine if old fish avoided terrestrial exposure. It should be noted that the older fish in our breeding colony were not necessarily the best survivors, but simply the remaining individuals that were not utilized for other studies. The natural mortality rate in our colony is typically very low.

Series 1a

Caudal fin regeneration To determine if age impacts caudal fin regeneration in *K. marmoratus*, fish ($n=20$) were anesthetized in tricaine methanesulfonate (MS-222; 300 mg L⁻¹) and photographed to determine the initial length of the caudal fin. We then amputated $53.8 \pm 0.9\%$ of the fin using a sterile razor blade as previously described (Recidoro et al. 2014). All fish recovered from the amputation procedure. Fish were then maintained in their individual containers for 10 days to allow for fin regeneration. After 10 days, fish were euthanized in MS-222 (500 mg L⁻¹) and the tail of each fish was amputated immediately anterior to the caudal peduncle. The remaining portion of each fish was dissected to visually confirm reproductive senescence in fish from the older age-class. We immersed the amputated tails in buffered calcein (0.2%, pH 7) for 15 min to stain newly deposited calcium (Du et al. 2001). Following staining, the tails were rinsed for 15 min in distilled water, mounted on glass microscope slides in 70% glycerol, and imaged using a FITC filter on an epifluorescence microscope (Upright Leica DM 5000B microscope, Leica). The lengths of the regrown (fluorescent) portion of the central four fin rays were measured using ImageJ (<http://imagej.nih.gov/ij/>), averaged, and expressed relative to the length of fin that was amputated to calculate percent regrowth. Prior to the initial amputation of the caudal fin, all fish were weighed, and the standard length was measured to calculate body condition (Fulton's K) using the following equation:

$$\text{Fulton's } K = 100(W \div L^3),$$

where W is the wet body mass in grams and L is standard length in cm (Froese 2006).

Series 1b

O₂ consumption and body condition We used intermittent flow respirometry to assess the influence of age on the rate

of O₂ consumption in *K. marmoratus* ($n=43$) using methods described by Sutton et al. (2018). Briefly, fish were acclimated to respirometry chambers (8.5 ml) for 2 h before O₂ consumption was measured in triplicate (three 12–15 min recordings separated by 10 min flushing periods) using O₂-sensing optodes (Loligo Systems WITROX 4, Tjele, Denmark). All fish were fasted for 48 h prior to experimentation to eliminate the influence of food intake on metabolism. All experiments were conducted between 12:00 and 18:00 to minimize the effects of diel metabolic rhythms in *K. marmoratus* (Rodela and Wright 2006). We calculated the mass-specific rate of O₂ consumption by standardizing the measured rate of O₂ consumption (μmol h⁻¹) to the body mass (g) of each fish. We also interpolated O₂ consumption rates to a 100 mg fish using a scaling exponent of 0.87, which was estimated from the slope of a linear regression following logarithmic transformation of O₂ consumption (μmol h⁻¹) and body mass (g) (Clarke and Johnston 1999). Immediately following O₂ consumption measurements, fish were removed from the experimental chambers, blotted dry, weighed, and standard length was measured (supplemental Fig. 1) to calculate Fulton's K as above. Fish used in O₂ consumption experiments were also used several weeks prior for voluntary emersion observations (see Series 3 below).

Series 2

Following O₂ consumption experiments, we acclimated fish to one of two ecologically relevant treatments: water (control; $n=19$) or air ($n=23$) for 10 days. Air acclimation was achieved by placing fish on moist filter paper (pH 8.0, 15 %) in 120 ml plastic holding cups, as previously described (Ong et al. 2007). Five fish (≥ 3 years old) died during air exposure and, thus, were excluded from all subsequent analyses.

Terrestrial locomotion Terrestrial locomotor performance was assessed in control and air-acclimated fish as described by Brunt et al. (2016). Briefly, fish were placed on moist filter paper in a terrarium (30 cm × 60 cm). After a 2 min adjustment period, fish were encouraged to jump by gently prodding fish with a clicker ballpoint pen until exhaustion (i.e., when fish were unresponsive to prodding). Jumping trials were video recorded and analyzed to quantify the number of jumps performed, the total distance travelled, as well as the longest jump distance standardized to body length, as described by Brunt et al. (2016). Immediately following each jumping trial, fish were euthanized via immersion in MS-222 (500 mg L⁻¹). A ~ 3 mm transverse steak immediately anterior to the dorsal fin was removed from each fish, coated in embedding medium (Shandon Cryomatrix™, Fisher Scientific, Hampton, NH, USA), frozen in liquid nitrogen-cooled isopentane, and stored at -80 °C for subsequent analysis of muscle phenotype and

cutaneous angiogenesis. The remaining anterior portion of each fish was fixed in 10% buffered formalin for gill histology.

Skeletal muscle phenotype Frozen muscle steaks were cut into 9 μm transverse sections, mounted on microscope slides (3–4 sections per slide), and stored at $-80\text{ }^\circ\text{C}$ until staining (Rossi et al. 2018). 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). An alkaline phosphatase (AP) stain was used to visualize capillaries in the oxidative muscle (Borowiec et al. 2015). All stained slides were viewed using an epifluorescence microscope (Nikon Eclipse 90i microscope, Nikon, Tokyo, Japan) and one section from each slide was photographed using NIS Elements software (Nikon). Slides were randomized to reduce observational bias and analyzed using ImageJ. Bright-field images of unstained sections were used to quantify the average size (cross-sectional area) of glycolytic fibers, and the total number of glycolytic fibers on one lateral half of each fish. The average glycolytic fiber size was determined on one section from each slide by overlaying a $100\times 100\text{ }\mu\text{m}$ line grid on bright-field photographs using Image J and tracing around the glycolytic fibers at intersecting grid lines ($n=30+$). The number of glycolytic fibers was then estimated by dividing the average glycolytic fiber size by the total glycolytic cross-sectional area. All oxidative muscle analyses were performed as described in Rossi et al. (2018). Briefly, we counted all oxidative fibers on one lateral half of each fish, and measured the size (cross-sectional area) of 30 random oxidative fibers. We also counted all capillaries in contact with oxidative muscle fibers on one lateral half of each fish. Oxidative and glycolytic phenotype traits are reported standardized to standard body length.

Cutaneous angiogenesis Angiogenesis in the ventral skin of *K. marmoratus* was analyzed as described by Blanchard et al. (2019). Briefly, cutaneous capillaries were visualized by immunostaining 9 μm thick cryosections for the endothelial cell–cell adhesion molecule CD31 also known as PECAM-1 (Lertkiatmongkol et al. 2016). ImageJ was then used to quantify the fluorescence intensity (mean gray value) of three ventral segments of epidermis. Intensity was expressed relative to muscle background to correct for differences in section thickness.

Gill morphology We fixed, decalcified, embedded, sectioned, stained, and analyzed gills as described by Turko et al. (2018). Briefly, fixed fish were paraffin embedded, cut into 5 μm sections, stained with haematoxylin and eosin, and photographed. Five gill lamellae from each arch were then randomly selected from each fish for analysis. The

height of each lamella and size of adjacent intralamellar cell mass (ILCM) were measured using Image J.

Series 3

Voluntary emersion behavior To determine if ageing affects the propensity of amphibious fish to voluntarily leave water, we individually acclimated fish ($n=49$) to experimental chambers containing $\sim 100\text{ mL}$ of water (15 ‰, $25\text{ }^\circ\text{C}$) for 12 h. Experimental chambers were surrounded by an oval platform ($\sim 4\text{ cm}$ wide) lined with a moist foam substrate to allow for emersion (Rossi et al. 2019). Following the acclimation period, fish were recorded using a digital video camera (Logitech Quickcam Pro, Fremont, CA, USA) for 60 h. We analyzed the video footage for the frequency of emersion events, and the total time fish spent out of water (hours) during the 60 h experimental period. This period of time was chosen to ensure that we captured emersion behavior during the day and night, as *K. marmoratus* have been previously reported to spend more time out of water at night (Livingston et al. 2018).

Statistical analysis

Analyses of variance and *t* tests were performed using RStudio (version 1.1.447). Linear regressions and graphs were generated using GraphPad Prism (version 8). We assessed all data for normality of residuals (Shapiro–Wilk) and homogeneity of variance (Levene’s test). When data did not meet these parametric test assumptions, they were appropriately transformed. To assess caudal fin regeneration between the two age-classes (0–1 and 4–6 years old), an unpaired 2-tailed *t* test was used. A linear regression was used to determine the relationship between caudal fin regeneration and Fulton’s *K*. Linear regressions were also used to determine the relationships between age and O_2 consumption, Fulton’s *K*, the frequency of emersion events, and total time spent out of water, as well as the relationship between muscle fiber size and locomotor performance. To determine the effects of age and air acclimation on terrestrial locomotor performance, oxidative and glycolytic muscle phenotype, oxidative muscle capillarity, ventral skin angiogenesis, and gill interlamellar cell mass coverage, fish were binned into three age-classes (1–2, 2–4, and 4–6 years) and compared using two-way analyses of variance (ANOVAs). When a significant main effect of age was detected, a Tukey’s post hoc test was used to identify which age-groups differed (all reported *p* values are adjusted for multiple comparisons). When a significant main effect of treatment was detected, effect sizes (Cohen’s *D*) were calculated to determine the magnitude of the within age-group treatment effect. Effect sizes (*d* values) are reported where $d\leq 0.2$ is a small, inconsequential effect size and $d\geq 0.8$ is a large, and biologically meaningful effect

(Sullivan and Feinn 2012). When a significant age \times treatment interaction was detected (glycolytic fiber size only), the data were divided by treatment to determine the effect of increasing age within each treatment group separately using one-way ANOVAs followed by Tukey's post hoc tests. All results were considered significant at $\alpha < 0.05$. Throughout the text, values are presented as mean \pm SEM.

Results

Reproductive status

Fish used in this study reached sexual maturity at 5.8 ± 0.3 months old. The reproductive period lasted 7.8 ± 2.3 months, after which fish were reproductively senescent (i.e., ceased embryo deposition).

Series 1

Young fish exhibited a significantly greater regenerative capacity relative to old fish, regenerating 10% more of their caudal fin than old fish following partial amputation, as evident from the staining of newly deposited calcium (unpaired t test: $p = 0.048$; effect size: $d = 0.9$; Fig. 1). We found a positive correlation between body condition and the percentage of caudal fin regenerated after 10 days (linear regression: $p < 0.01$; $r^2 = 0.35$). We found no significant relationship between age and routine O_2 consumption (linear regression: $p > 0.05$, $r^2 = 0.001$; Fig. 2a). This result was not different when O_2 consumption rates were calculated using allometric scaling methodology and standardized to a 100 mg fish. Body condition significantly declined with age (linear regression: $p < 0.01$, $r^2 = 0.36$; Fig. 2b).

Series 2

Terrestrial locomotor performance was impaired in older fish (two-way ANOVAs: $p < 0.05$) (Fig. 3). In all tested jumping parameters, age-related declines in terrestrial locomotor performance were evident once fish reached 2 years old (Tukey's HSD: $p < 0.05$). Performance did not continue to decline as fish aged further (Tukey's HSD: $p > 0.05$).

Overall, terrestrial locomotor performance was poorer in air-acclimated fish relative to controls. Air-acclimated fish jumped significantly fewer times before reaching exhaustion (two-way ANOVA: $p < 0.001$; Fig. 3a) and travelled a significantly shorter distance (two-way ANOVA: $p < 0.001$; Fig. 3b) relative to control fish before reaching exhaustion, regardless of age. Interestingly, the magnitude of this decline in terrestrial locomotor performance increased with age. Air-acclimated 1–2 year old fish jumped 20% fewer times ($d = 1.4$) and travelled 21% less distance than control fish

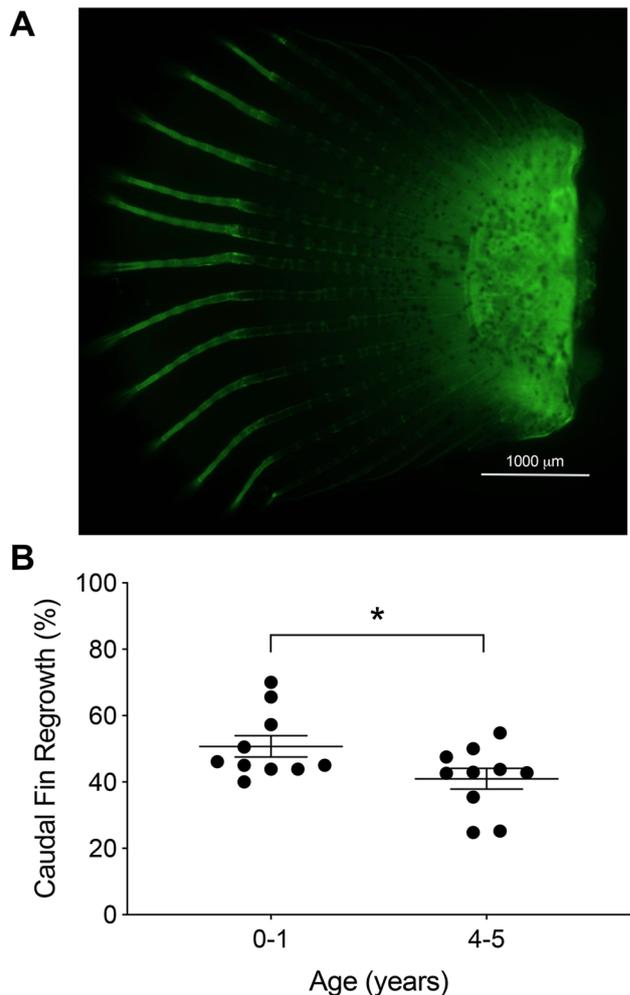


Fig. 1 Caudal fin regeneration in *K. marmoratus*. **a** Representative image showing the region of fin ray regrowth (highly fluorescent region at caudal fin distal tip) 10 days following partial amputation in a 4 year old fish. **b** Caudal fin regrowth (%) in young (0–1 year; $n = 10$; 51%) and old (4–5 years; $n = 10$; 41%) fish 10 days after partial amputation. Asterisk indicates a significant difference ($p = 0.048$) between age-groups [effect size ($d = 0.9$ (large))]. Error bars represent mean \pm SEM

from the same age-class ($d = 1.2$). In contrast, 4–6 year old fish jumped 61% fewer times ($d = 1.7$) and travelled 65% less distance than control fish from the same age-class ($d = 1.5$). However, the distance of the longest jump was not affected by acclimation to air (two-way ANOVA: $p > 0.05$; Fig. 3c).

The oxidative and glycolytic muscle phenotypes of *K. marmoratus* were differentially affected by age and air acclimation (Fig. 4). Increasing age decreased both oxidative fiber size and number (two-way ANOVAs: $p < 0.05$). Oxidative fiber size was substantially lower in the 4–6 age-class (Tukey's HSD: $p = 0.007$; Fig. 4a), whereas oxidative fiber number was lower in fish aged 2–4 years relative to the youngest age-group (data not shown; Tukey's HSD: $p < 0.04$). The number of capillaries in the oxidative muscle

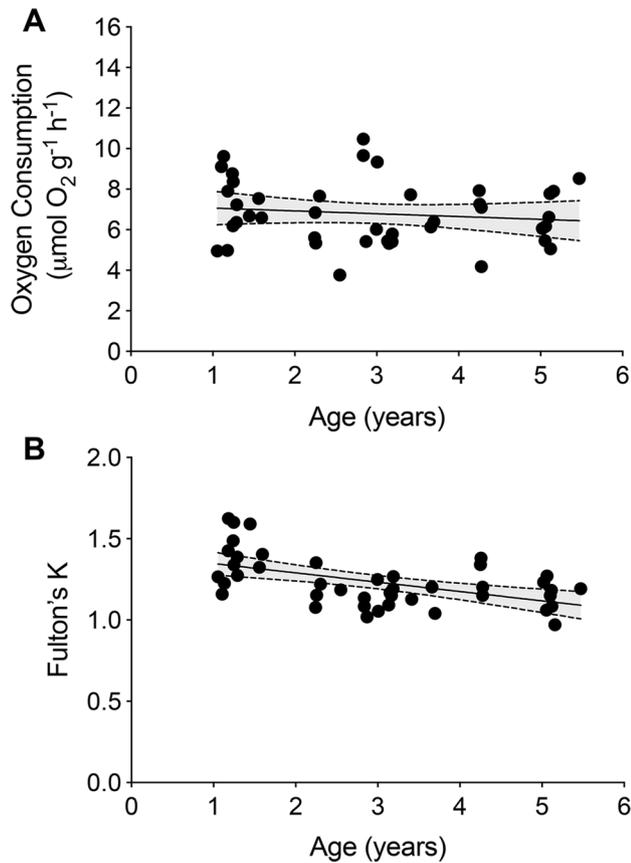


Fig. 2 **a** Relationship between increasing fish age ($n=43$) and the mass-specific rate of O_2 consumption in *K. marmoratus* ($y=0.14x+7.2$, $r^2=0.02$). The slope of the line is not significantly different from zero ($p=0.4$). **b** Relationship between increasing fish age ($n=43$) and the body condition (Fulton's K) of *K. marmoratus* ($y=-0.06x+1.4$, $r^2=0.28$). The slope of the line is significantly different from zero ($p<0.001$). Shaded areas represent 95% confidence intervals of the linear lines of best fit

did not change as fish aged (Fig. 4b; two-way ANOVA: $p=0.63$), but there were significantly more capillaries in air-acclimated fish of every age-class (two-way ANOVA: $p=0.004$). The magnitude of increase in capillarity following air exposure was similar as fish aged ($\sim 14\%$). Glycolytic fiber size within each age-class was differentially affected by air exposure as evident by a significant interaction between age and acclimation history (two-way ANOVA interaction: $p=0.02$; Fig. 4c). In control fish, glycolytic fiber size significantly decreased with age (2–4 years: Tukey's HSD: $p<0.001$, 4–6 years: Tukey's HSD: $p<0.001$). In contrast, the glycolytic fiber size of air-acclimated fish was similar between 1 and 4 years of age, and then declined in the oldest group (4–6 years old; Fig. 4c). Glycolytic fiber number was unaffected by age or air acclimation (data not shown; two-way ANOVA: $p>0.05$).

Muscle phenotype was a predictor of endurance performance (i.e., total distance travelled and number of

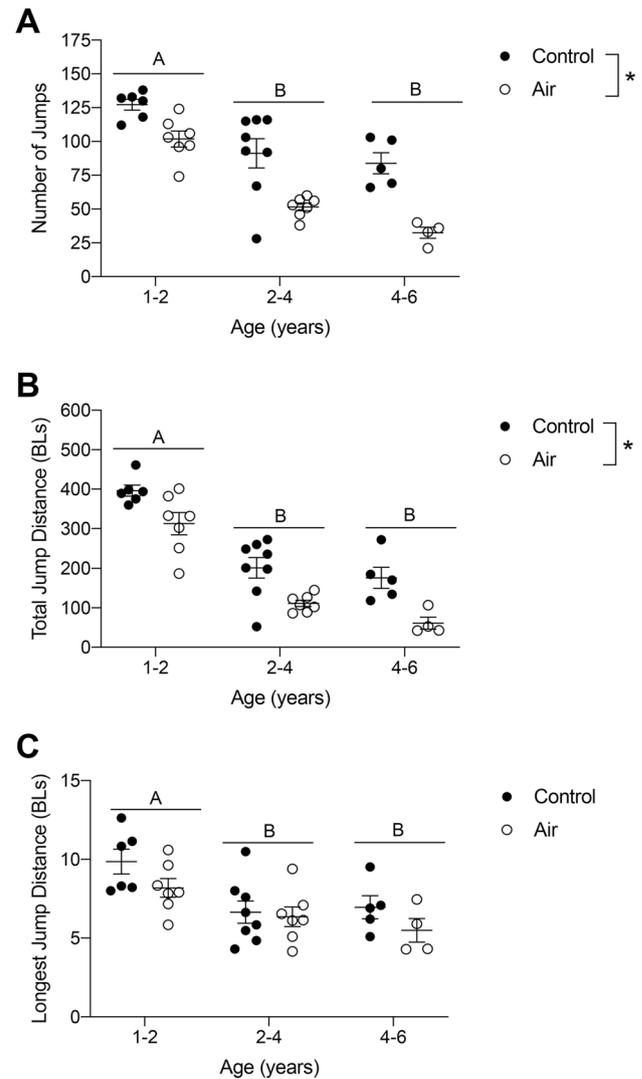


Fig. 3 Terrestrial locomotor performance of *K. marmoratus* across age-classes. **a** Number of jumps performed before exhaustion (two-way ANOVA; age, $p<0.001$, treatment, $p<0.001$). **b** Total distance travelled before exhaustion (two-way ANOVA; age, $p<0.001$, treatment, $p<0.001$). **c** Distance of the longest jump performed before exhaustion (two-way ANOVA; age, $p<0.001$, treatment, $p=0.14$). Each point represents an individual fish [control: 1–2 ($n=6$), 2–4 ($n=8$), 4–6 ($n=5$); air-acclimated: 1–2 ($n=7$), 2–4 ($n=7$), 4–6 ($n=4$)]. Different letters indicate significant differences between age-classes. An asterisk in the legend indicates that within each age-class, control (filled black circles) and air-acclimated (open circles) fish are significantly different. Error bars denote the mean \pm SEM of each group

jumps) in *K. marmoratus*. Total distance travelled before exhaustion was positively and significantly correlated with oxidative and glycolytic fiber size in both control (oxidative: $R^2=0.27$, $p<0.05$; glycolytic: $R^2=0.52$, $p<0.001$; Fig. 5a, b) and air-acclimated (oxidative: $R^2=0.23$, $p=0.05$, glycolytic: $R^2=0.56$, $p<0.001$; Fig. 5a, b) fish. Similarly, the number of jumps performed before reaching

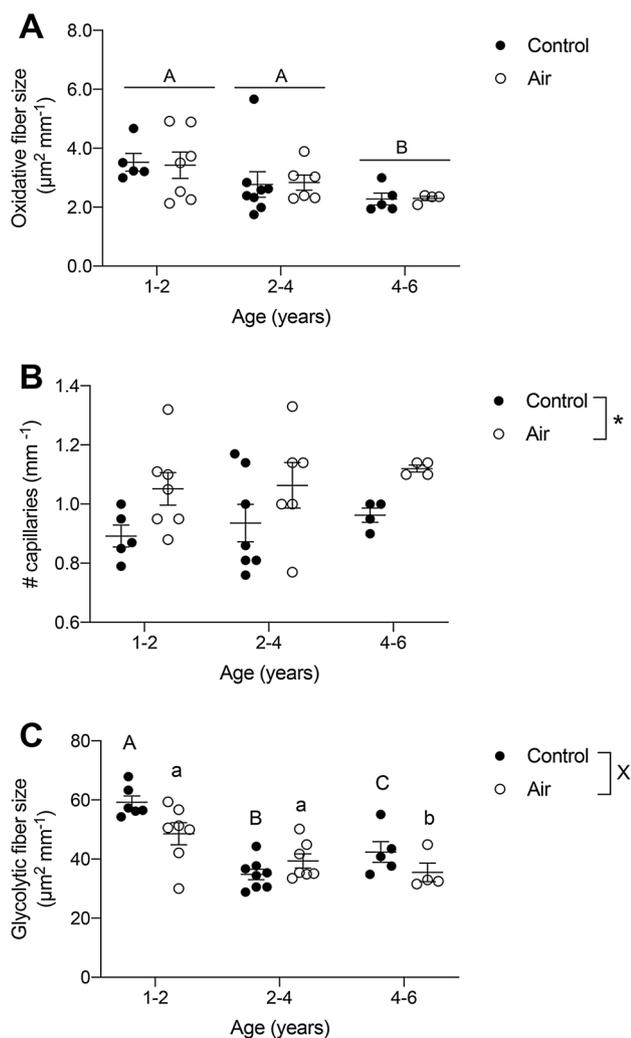


Fig. 4 Muscle phenotype of *K. marmoratus* across age-classes. **a** Oxidative fiber size (two-way ANOVA; age, $p=0.007$, treatment, $p=0.6$). Control: 1–2 ($n=5$), 2–4 ($n=8$), 4–6 ($n=5$); air-acclimated: 1–2 ($n=7$), 2–4 ($n=6$), 4–6 ($n=4$). **b** Number of capillaries in oxidative muscle standardized to body standard length (two-way ANOVA; age, $p=0.63$, treatment, $p=0.004$). Control: 1–2 ($n=5$), 2–4 ($n=7$), 4–6 ($n=4$); air-acclimated: 1–2 ($n=7$), 2–4 ($n=6$), 4–6 ($n=4$). **c** Glycolytic fiber size. Due to a significant interaction (X in the figure legend; two-way ANOVA: $p=0.02$), the effect of age was analyzed within each treatment group separately (control one-way ANOVA: $p<0.001$; air-acclimated one-way ANOVA: $p=0.04$). Control: 1–2 ($n=6$), 2–4 ($n=8$), 4–6 ($n=5$); air-acclimated: 1–2 ($n=7$), 2–4 ($n=7$), 4–6 ($n=4$). Each point represents an individual fish and error bars denote the mean \pm SEM of each group. An asterisk in the legend indicates that within every age-class, control (filled black circles) and air-acclimated (open circles) fish are significantly different. Different letters indicate significant differences between age-classes. In panel C, different upper case letters indicate significant age differences within the control group, and different lower case letters show differences within the air-acclimated group

exhaustion was also positively and significantly correlated with oxidative and glycolytic fiber size in control (oxidative: $R^2=0.23$, $p<0.05$, glycolytic: $R^2=0.27$, $p<0.05$;

Fig. 5c, d) and air-acclimated (oxidative: $R^2=0.26$, $p<0.05$, glycolytic: $R^2=0.54$, $p<0.001$; Fig. 5c, d) fish.

Gill morphology was affected by both age and air acclimation (Fig. 6). The percentage of the gill lamellae covered by an ILCM was consistently higher in air-exposed fish, regardless of age (two-way ANOVA, $p<0.05$; Fig. 6). However, the effect size of this plastic response was diminished with increased age. Young (1–2 year old) fish significantly increased their ILCM coverage by 37% (effect size: $d=1.2$), while the ILCM coverage in old fish (4–6 year old) only increased by 12% (effect size: $d=0.6$; Fig. 6b). Gill ILCM coverage was also higher in fish aged 2–4 years old relative to 1–2 years old in both treatments (Tukey's HSD: $p<0.05$; Fig. 6b). We found no significant effect of age (two-way ANOVA: $p>0.05$) or air acclimation (two-way ANOVA: $p>0.05$) on angiogenesis in the ventral skin of *K. marmoratus* (supplemental Fig. 2).

Series 3

The voluntary emersion behavior of *K. marmoratus* was highly variable. Emersion events varied from 3.6 s to 3.91 h in duration, and individual fish spent between 1.8 h (0.03%) and 45.6 h (76%) of the experimental time out of water. However, age was not a predictor of emersion frequency (linear regression: $p>0.05$, $r^2=0.02$; Fig. 7a). Nor was age correlated with the time fish spent out of water (linear regression: $p>0.05$, $r^2=0.002$; Fig. 7b). We found no differences in emersion behavior between the day and night (paired t test: $p>0.05$) and thus, data were pooled.

Discussion

We used the amphibious *K. marmoratus* to test the hypothesis that ageing reduces the scope for phenotypic flexibility in response to environmental change. In support of our hypothesis, we found that older fish had a reduced scope for gill remodeling in response to air acclimation relative to younger fish. Furthermore, we found that older fish exhibited significantly poorer terrestrial locomotor performance relative to younger fish, particularly after air acclimation. Locomotor performance was significantly correlated with muscle fiber size. Older fish with small muscle fibers (particularly glycolytic) jumped fewer times over shorter distances relative to younger fish. Taken together, ageing diminished gill phenotypic flexibility, skeletal muscle integrity, and terrestrial locomotor performance in *K. marmoratus*, which may in turn impair their ability to successfully exploit terrestrial environments.

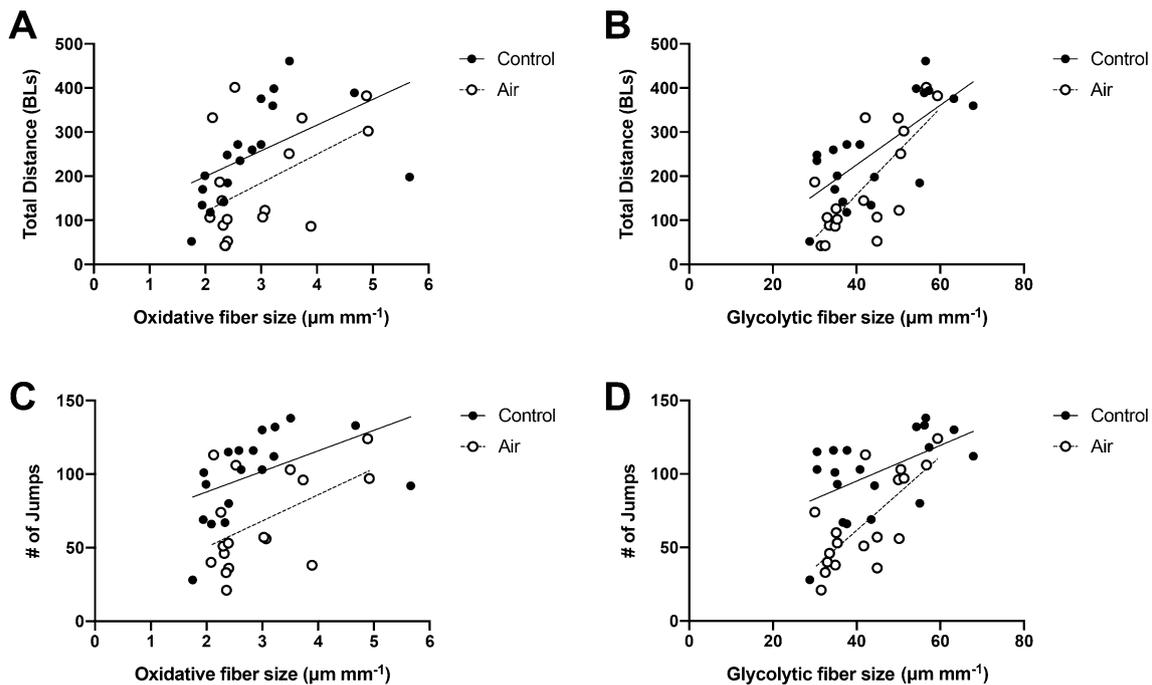


Fig. 5 Relationship between endurance performance and muscle phenotype in *K. marmoratus*. Relationship between **a** total jump distance and oxidative fiber size in control ($y=58.17x+83.12$, $r^2=0.27$, $p<0.05$) and air-acclimated ($y=64.96x-10.18$, $r^2=0.23$, $p=0.05$) fish, **b** total jump distance and glycolytic fiber size in control ($y=6.76x-44.67$, $r^2=0.52$, $p<0.001$) and air-acclimated

($y=9.82x-234.9$, $r^2=0.56$, $p<0.001$) fish, **c** number of jumps and oxidative fiber size in control ($y=13.96x+59.98$, $r^2=0.23$, $p<0.05$) and air-acclimated ($y=17.78x+14.84$, $r^2=0.26$, $p<0.05$) fish, and **d** number of jumps and glycolytic fiber size in control ($y=1.213x+46.62$, $r^2=0.27$, $p<0.05$) and air-acclimated ($y=2.51x-38.86$, $r^2=0.54$, $p<0.001$) fish

Effects of ageing on phenotypic flexibility

In support of our hypothesis, we showed that young fish increased ILCM height during air acclimation to a similar level as other isogenic strains of *K. marmoratus* emerged for 7 days (Ong et al. 2007; Turko et al. 2012), while the gills of older fish exhibited less flexibility. When on land, the gill lamellae of amphibious fishes collapse and coalesce without the buoyant support of water (Lam et al. 2006), rendering the gills susceptible to irreversible damage. An increase in ILCM height during air exposure is thought to provide structural support, as well as reduce evaporative water loss (Ong et al. 2007; Wright 2012; Turko et al. 2017). Interestingly, older *K. marmoratus* (2–4 years old) may have compensated for a lack of phenotypic flexibility by maintaining a large ILCM irrespective of environment, although a reduced gill surface area has been shown to impair respiration when fish return to water (Turko et al. 2012, 2018). Furthermore, a diminished capacity to alter gill morphology in response to water O_2 and ion levels in older fish may impact respiration and ion balance in both amphibious and non-amphibious species in aquatic environments (see review Gilmour and Perry 2018).

Not all examined traits demonstrated an age-related decline in phenotypic flexibility. We found that air

acclimation increased oxidative muscle capillarity to the same extent (~14%) in the oldest and youngest age-classes. The increase in capillarity was consistent with a previous study from our lab (Brunt et al. 2016). Some studies on mammalian models have demonstrated that angiogenic responses in skeletal muscle can be maintained with age (Gavin et al. 2007; 2014), while several others report the opposite effect (for reviews, see Lähteenvuo and Rosenzweig 2012; Kwak et al. 2018). Regardless, the maintenance of angiogenesis in the oxidative muscle of *K. marmoratus* suggests that it is functionally important in terrestrial environments, possibly for locomotion and energy balance.

Phenotypic flexibility is variable between different isogenic strains of *K. marmoratus*. In this study, we used the HON9 strain and found that it did not display the same phenotypic flexibility previously observed in other strains. For example, the 50.91 strain of *K. marmoratus* exhibited oxidative fiber hypertrophy (Rossi et al. 2018) and improved jumping performance after air exposure (Brunt et al. 2016) that was not observed in the current study. Furthermore, HON9 fish did not show cutaneous angiogenesis with air acclimation as observed in other strains (Blanchard et al. 2019). Thus, the HON9 strain may be using alternative physiological mechanisms (e.g., altering blood oxygen carrying

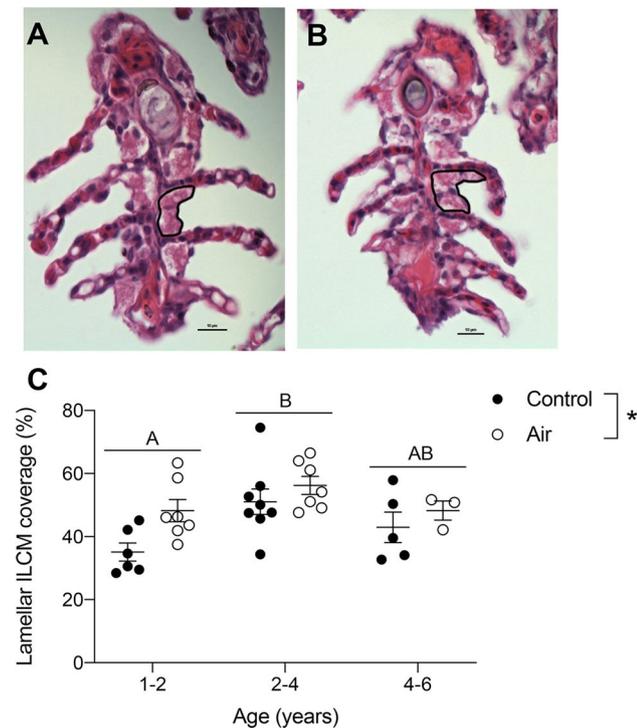


Fig. 6 Gill morphology of *K. marmoratus*. **a** Representative image of a gill filament from a young age-class (1–2 years) control individual maintained in water (ILCM coverage=28%) and **b** from a different young age-class individual following 10 days of air acclimation (ILCM coverage=46%). Examples of ILCMs in control and air-acclimated fish are present in black boxes. Scale bar represents 10 μm . **c** Percent coverage of the gill lamellae by an interlamellar cell mass (two-way ANOVA; age, $p=0.005$, treatment, $p=0.02$). Sample sizes within each group are the same as listed in Fig. 3. Different letters indicate significant differences between age-classes. Asterisk in the legend indicates that within every age-class, control (filled black circles) and air-acclimated (open circles) fish are significantly different. Error bars denote the mean \pm SEM of each group

capacity and/or hemoglobin- O_2 affinity; Turko et al. 2014) to maintain aerial O_2 uptake.

Consequences of ageing

Ageing had significant consequences on a number of key traits in *K. marmoratus*. Older *K. marmoratus* were reproductively senescent, had reduced fin regenerative capacity, lower body condition, exhibited atrophy of both oxidative and glycolytic muscle fibers, and had higher mortality in air relative to younger fish. The decrease in body condition of older fish suggests a loss of muscle mass, since skeletal muscle represents a significant portion of the mass of most fish (up to 80%; Weatherley and Gill 1987). Accordingly, the size of both oxidative and glycolytic fibers was lower in older fish. We also found that the size of muscle fibers was positively correlated with locomotor performance in *K. marmoratus*, suggesting that age-related muscle atrophy

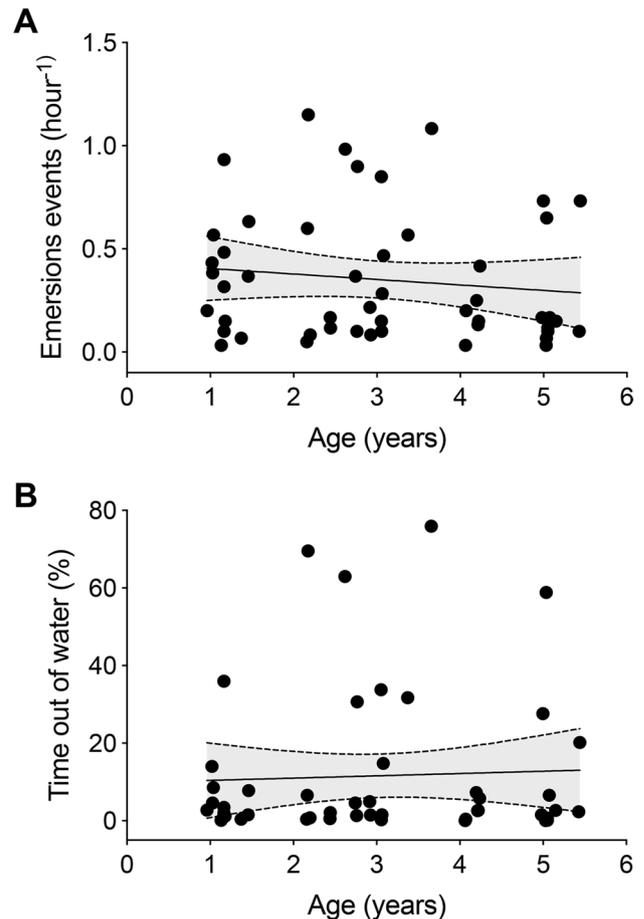


Fig. 7 Voluntary emersion behavior of *K. marmoratus* ($n=49$). **a** Relationship between the number of emersion events per hour and fish age ($y = -0.027x + 0.43$, $r^2 = 0.02$). The slope is not significantly different from zero ($p=0.4$). **b** Relationship between the percentage of the total experimental duration fish spent out of water and fish age ($y = 0.59x + 9.8$, $r^2 = 0.002$). The slope is not significantly different from zero ($p=0.8$). Shaded areas represent 95% confidence intervals of the linear lines of best fit

impairs the ability to move effectively on land. Similar declines in muscle mass and strength (i.e., sarcopenia) have been reported in other ageing fishes (e.g., *Poecilia reticulata*, Comfort 1961). However, a recent study reported that jumping performance (i.e., mean jump distance) increased with age in *K. marmoratus* (Styga et al. 2018). Methodological differences likely account for the discrepancy between studies, since Styga et al. (2018) did not jump fish to exhaustion and very few of the fish used (approximately 1.3%) were more than 4 years old.

Air acclimation in *K. marmoratus* exacerbated the deterioration of endurance performance (i.e., number of jumps and total distance travelled), particularly in older fish (i.e., age-classes 2–4 and 4–6). Interestingly, air-acclimated *K. marmoratus* did not exhibit oxidative or glycolytic muscle atrophy until 4–6 years of age, suggesting that muscle

atrophy may not be the only cause of diminished locomotor performance on land. One possibility is that air-exposed *K. marmoratus* have reduced glycogen reserves in their muscle relative to control fish. Lactate accumulation during jumping in *K. marmoratus* suggests that glycogen is an important substrate for fueling locomotor performance in air (Brunt et al. 2016). As well, we have found significant whole-body glycogen catabolism during terrestrial exposure in inactive *K. marmoratus* (Turko, Doherty, Yin-Lao, Levesque, Kruth, Holden, Earley, and Wright, submitted). Moreover, some studies have suggested that glycogen reserves may decline as fish age (e.g., *Oryzias latipes*; Ding et al. 2010). Taken together, limited glycogen availability may constrain locomotor performance in air-exposed fish, particularly those of older age.

Decreased regenerative capacity is a hallmark of ageing in fishes (Kim et al. 2016). While fishes have remarkable regenerative abilities in a variety of tissues including the fins, spinal cord, and heart (Poss et al. 2002; Sîrbulescu et al. 2009; Wendler et al. 2015), we showed that caudal fin regeneration was impaired in older *K. marmoratus* with poorer body condition. Our finding is consistent with older *N. furzeri* who regenerate significantly less of their caudal fin following partial amputation (Wendler et al. 2015) and are also in poorer body condition (Terzibasi et al. 2008), relative to younger individuals. Caudal fin damage caused by aggressive conspecifics, predators, or disease can significantly compromise swimming performance (Fu et al. 2013). Therefore, age-related declines in caudal fin regeneration may be detrimental for older *K. marmoratus* as they would experience impaired performance for a longer duration following fin damage relative to younger counterparts.

Most fish used in this study were reproductively senescent; embryo deposition ceased by the time fish was approximately 2 years old. We found that the gonads of older *K. marmoratus* did not contain embryos upon macroscopic examination, but embryos were present in the gonads of several younger fish. The presence of embryos inside younger fish likely increased the measured body mass for a given length, and, therefore, may have contributed to the higher body condition. Diminished reproductive performance with age is common among fishes and can be reflected by a reduction in fecundity (Koslow et al. 1995), gamete quality (Gasparini et al. 2010), gonad size (Scarnecchia et al. 2007), or the cessation of reproduction altogether (Thorpe 1994). While many fishes have short post-reproductive life spans (e.g., *N. furzeri*, Valdesalici and Cellerino 2003), we found that *K. marmoratus* lives well beyond the point of reproductive senescence in a laboratory setting (i.e., 3+ years), but their longevity in the wild remains unknown and worthy of study.

Voluntary emersion behavior

Older fish have diminished gill phenotypic flexibility and locomotor performance in air, so do they emerse less often than younger fish? No. We found that both the frequency of emersion and total time spent emersed was not influenced by age in *K. marmoratus*. However, patterns of voluntary emersion under laboratory conditions may not reflect those of wild populations where dynamic biotic and abiotic factors are at play (Sayer and Davenport 1991). Ageing has been shown to alter the behaviors of mammals in the wild (e.g., *Alces alces*; Montgomery et al. 2012). Little is known about how the physiological consequences of ageing are manifested in the behavioral traits of fish, but this is a fascinating area for future investigation.

Perspectives

Our findings demonstrate that ageing poses various challenges for *K. marmoratus*. In the wild, *K. marmoratus* inhabit tropical mangrove pools subject to considerable variability in several biotic (e.g., competition; Taylor 2012) and abiotic factors (e.g., dissolved oxygen, salinity, and hydrogen sulphide; Rossi et al. 2019). While *K. marmoratus* are relatively tolerant of poor water quality, they emerse when aquatic conditions fall beyond tolerable limits (e.g., extreme hypoxia, Regan et al. 2011), when subjected to seasonal drought (Taylor 2012), and/or to forage and disperse (Taylor 1992). A limited scope for gill phenotypic flexibility may severely impact gill integrity of older fish that remain emersed for weeks. Moreover, age-related declines in muscle integrity may constrain locomotor movement overland, suggesting that older fish may exploit terrestrial resources less effectively. While laboratory studies are valuable for evaluating the physiological consequences of ageing, understanding how the ageing phenotype of animals shapes their interaction with the environment is critical.

The ageing process can be highly variable both within and among species (Lemaître et al. 2015). Energy allocation trade-offs between the physiological processes that maximize fitness and those that increase longevity are generally thought to underlie this variation (Kirkwood 1977). Our study demonstrates that ageing can alter phenotypic responses to environmental change, but the influence of variable environments on the energy allocation trade-offs that mediate the ageing process has yet to be elucidated. Investigating whether fluctuating environmental conditions can explain some of the variability in the ageing process among animals remains an exciting area for future research.

Finally, there is tremendous public interest in the effects of ageing. The use of model organisms (e.g., yeast, flies, and mice) has been critical for our understanding of the molecular mechanisms underlying ageing and age-related

diseases (for review, see Kim et al. 2016). We suggest that the self-fertilizing *K. marmoratus* may be an ideal vertebrate model species for ageing research. First, *K. marmoratus* are easy to maintain in a laboratory setting, and exhibit several marked signs of ageing after only 2 years. Second, their unique ability to self-fertilize eliminates the effects of genetic variation on physiological processes related to ageing without substantial effort as is required for other isogenic vertebrate models (e.g., mice; Casellas 2011). Third, full genome sequences for several *K. marmoratus* strains have recently become available (Lins et al. 2018). Thus, differential ageing phenotypes between isogenic *K. marmoratus* strains may reveal underlying genomic causes responsible for physiological deterioration with age. Overall, we suggest that the use of *K. marmoratus* as a model species for ageing research warrants further consideration.

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Compliance with ethical standards

Conflict of interest The authors declare no competing or financial interests.

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