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Cortisol enhances aerobic metabolism and locomotor performance during the transition to land in an amphibious fish

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ABSTRACT

Amphibious fishes on land encounter higher oxygen (O_2) availability and novel energetic demands, which impacts metabolism. Previous work on the amphibious mangrove killifish (*Kryptolebias marmoratus*) has shown that cortisol becomes elevated in response to air exposure, suggesting a possible role in regulating metabolism as fish move into terrestrial environments. We tested the hypothesis that cortisol is the mechanism by which oxidative processes are upregulated during the transition to land in amphibious fishes. We used two groups of fish, treated fish (+metyrapone, a cortisol synthesis inhibitor) and control (−metyrapone), to determine the impact of cortisol during air exposure (0 and 1 h, 7 days) on O_2 consumption, terrestrial locomotion, the phenotype of red skeletal muscle, and muscle lipid concentration. Metyrapone-treated fish had an attenuated elevation in O_2 consumption rate during the water to air transition and an immediate reduction in terrestrial exercise performance relative to control fish. In contrast, we found no short- (0 h) or long-term (7 days) differences between treatments in the oxidative phenotype of red muscles, nor in muscle lipid concentrations. Our results suggest that cortisol stimulates the necessary increase in aerobic metabolism needed to fuel the physiological changes that amphibious fishes undergo during the acclimation to air, although further studies are required to determine specific mechanisms of cortisol regulation.

1. Introduction

The colonization of land by fishes represents one of the most challenging ecological transitions to overcome because it poses a number of significant physiological challenges (Sayer, 2005). Notably, air is 830 times less dense and 50 times less viscous than water, and therefore colonizing land requires transitioning from a buoyant medium, where fish are essentially weightless, to the terrestrial gravity-dominated environment (Denny, 1993). Remarkably, there is a subset of fishes, known as amphibious fishes, that have colonized land and leave the water as part of their natural life history (Gordon et al., 1970). Amphibiousness has convergently evolved about 87 times among fishes, resulting in over 200 known extant amphibious fish species (Damsgaard et al., 2020). These fishes vary widely in their degree of amphibiousness, including the reason(s) for leaving water and the length of time they can survive in air (Turko et al., 2021). Overall, an important feature of amphibious fishes is their ability to move effectively in a gravity-dominated environment, which often requires a shift to a specialized locomotory strategy.

There is a diverse range of terrestrial locomotory strategies among amphibious fishes, all of which demand the use of movements that are very different from swimming. Amphibious mudskippers move on land by "walking" on their pectoral fins, and their endurance on land can improve with increasing O_2 saturation (Jew et al., 2013). Several amphibious killifishes, including the mangrove rivulus (*Kryptolebias marmoratus*), move on land via tail flip jumping (Pronko et al., 2013; Lutek et al., 2022). These types of locomotion, like all physiological functions, depend on O_2 utilization. Oxidative phosphorylation, which is metabolic energy production powered by O_2, is more sustainable than its anaerobic counterpart. Thus, there is a drive to rely on oxidative

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phosphorylation for energy provision. Amphibious fishes remodel their skeletal muscle by increasing oxidative capacity, and these changes are associated with locomotory improvements (Brunt et al., 2016; Rossi et al., 2018). Furthermore, ample O_2 supply has been shown to trigger this increase in oxidative capacity (Rossi et al., 2018). The O_2 available to fuel oxidative phosphorylation can be increased by enhancing O_2 uptake into the body, termed the O_2 consumption rate (Fry, 1971). Elevation of the O_2 consumption rate upon leaving water has been demonstrated in *K. marmoratus* (Livingston et al., 2018). The mechanism (s) responsible for causing an increased demand for O_2 consumption upon emersion in amphibious fishes remains unknown.

Emersion in adult *K. marmoratus* is also associated with a rapid and transient increase in the glucocorticoid hormone cortisol (Ridgway et al., 2022). Within minutes of air exposure, cortisol levels begin to spike, peaking by 60 min, and then returning to resting levels over the course of 7 days in air (Ridgway et al., 2022). The cortisol response in air-exposed *K. marmoratus* is responsible for skin remodeling (Ridgway et al., 2022). Exposing *K. marmoratus* to waterborne metyrapone, a drug that blocks cortisol synthesis by competitively inhibiting 11-β hydroxylase (Mommsen et al., 1999; Tokarz et al., 2015), effectively mutes the cortisol response to emersion and, consequently, blocks skin ionocyte remodeling (Ridgway et al., 2022). Furthermore, preliminary experiments indicated that metyrapone treatment might affect terrestrial locomotion (Ridgway et al., 2022), possibly by altering O_2 consumption and utilization.

Glucocorticoids in vertebrates play an important role in maintaining homeostasis amid physiologically challenging situations. In fishes, beyond its contribution to maintaining ionic balance (Takei and McCormick, 2013), cortisol plays a key role in regulating energy metabolism during stress and exercise (Rodnick and Planas, 2016; Sadoul and Vijayan, 2016). For example, prolonged cortisol elevation, as observed in response to chronic stress, can stimulate liver gluconeogenesis, peripheral lipolysis, and muscle proteolysis (Mommsen et al., 1999; Madison et al., 2015). Beyond their energy mobilizing properties, chronically elevated cortisol levels also stimulate whole animal O_2 consumption and anaerobic metabolism in a variety of fish species (Morgan and Iwama, 1996; De Boeck et al., 2001; O'Connor et al., 2011; Liew et al., 2013; Lawrence et al., 2019; Pfalzgraff et al., 2022). In contrast, while glucocorticoids can directly stimulate various aspects of mitochondrial oxidative phosphorylation capacity in mammals (Du et al., 2009; Lapp et al., 2019; Davies et al., 2021), little is known about the direct effects of cortisol on mitochondrial functions in fishes or whether the short-term increase of cortisol, associated with acute stress, can stimulate aerobic metabolism.

Our objective in this study was first to validate our earlier findings that cortisol levels increase in response to air exposure and then to determine if cortisol is the mechanism by which oxidative processes are upregulated during the transition to air in amphibious fishes. We predicted that inhibiting cortisol synthesis would lower O_2 consumption rate, compromise terrestrial locomotion, and limit changes in the phenotype of red skeletal muscle relative to control fish who are capable of synthesizing cortisol. We used *K. marmoratus* as a model organism because of their well-characterized physiological responses to air exposure, including a high capacity for phenotypic plasticity, and their highly amphibious lifestyle, which includes long emersion periods (Turko et al., 2021). We acclimated fish to either a metyrapone treatment or to control conditions and measured O_2 consumption rate during the transition from water to air, quantified terrestrial locomotion after 0 h, 1 h, and 7 days in air, and assessed skeletal muscle histology after 0 h and 7 days in air. We quantified remodeling of the skeletal muscle phenotype by comparing the number of red oxidative muscle fibers and the total oxidative muscle area.

2. Materials and methods

2.1. Animals

All experimental fish were laboratory-reared mangrove rivulus (*Kryptolebias marmoratus*) hermaphrodites of the isogenic SLC8E strain (origin population from Florida; Tatarenkov et al., 2010). Fish were kept in the Hagen Aqualab, University of Guelph, and housed individually in 120 ml containers in 15 ppt water at 25 °C on a 12:12 h light:dark cycle and fed live brine shrimp (*Artemia* sp. nauplii) three times per week. All procedures were approved by the University of Guelph Animal Care Committee.

2.2. Experimental protocol

Acclimation and air exposure protocols followed methodology described by Ridgway et al. (2022). In brief, we kept fish in semi-opaque 120 ml containers to minimize exposure to external stressors. Containers had a valve in the bottom, allowing us to passively drain water without disturbing the fish. We included a moist substrate to retain moisture when the container was drained. Ambient temperature was maintained at 25 °C. We acclimated fish for 48 h to either control conditions (100 ml, 15 ppt, 25 °C) or metyrapone. In the metyrapone treatment, fish were placed in 100 ml of a 25mgl^{-1} metyrapone solution, made with 15 ppt salt water, followed by a 50mgl^{-1} metyrapone solution for 24 h.

Following the 48 h acclimation, we passively drained containers and exposed the fish to air. We sampled fish from both control and metyrapone groups at 3 different timepoints of air exposure: 0 h of air exposure (i.e., sampled immediately following acclimation without being air-exposed), 1 h of air exposure, and 7 days of air exposure. Over the course of these 7 days, we maintained humidity and treatment in the containers by adding daily doses of 1 ml 15 ppt water or 1 ml 50mgl^{-1} metyrapone solution to the respective containers. Some fish went through a jump protocol following air exposure, while others were immediately euthanized by cold water immersion (~4 °C; 15 ppt) to prevent an increase in cortisol during sampling (Yeh et al., 2013). The red skeletal muscle phenotype was quantified after 0 h and 7 days in air. Terrestrial exercise performance, muscle lipid concentration, and whole-body cortisol levels were measured after 0 h, 1 h, and 7 days. At 0 h, 1 h, and 7 days, tissues were collected for cortisol measurements and histological processing.

2.3. Analyses

2.3.1. Cortisol

Using previously described methodology (Fuzzen et al., 2010), we verified that air exposure elicited an increase in whole-body cortisol levels as previously reported by Ridgway et al. (2022) and that metyrapone effectively blocked this response. In brief, we euthanized fish (n = 6–8), homogenized them until liquified (Polytron PCU-2-110 Homogenizer; Brinkmann Instruments, Rexdale Ontario, Canada), and sonicated the solution (Vibra-Cell Ultrasonic Liquid Processor; Sonics and Materials Inc.). We extracted the homogenates in duplicate using methanol (MeOH) and purified the sample using solid phase extraction columns (Cleanert C18-N-SPE 100 mg ml^{-1}; Agela Technologies, Wilmington, DE, US). We then quantified the cortisol in our samples using a commercial enzyme-linked immunosorbent cortisol assay (ELISA) (Neogen Lexington, KT, USA). We standardized cortisol values to body mass (g). We accounted for an extraction efficiency of 85% in final cortisol values (Laberge et al., 2019).

2.3.2. Terrestrial jumping

We investigated whether terrestrial locomotory ability depends on cortisol synthesis by exercising control and metyrapone groups to exhaustion on land (n = 12–13). We followed methodology described by McFarlane et al. (2019). In brief, we placed fish in a large plastic bin with a moist substrate. We acclimated fish to the setting for two min

before stimulating jumping by gently nudging the nose and tail with the tip of a clicker pen. Trials ended when the fish reached exhaustion (i.e. no longer responded to stimuli). We video-recorded the trials and used Image J (http://imagej.nih.gov/ij) to measure total distance travelled, longest jump distance, average jump distance, and total number of jumps. A 'jump' was recorded when the fish's entire body left the ground. We measured jumps from where the rostral-most point of the fish landed, calculated total distance covered as the sum of every jump, and calculated average jump distance as the mean length of a single jump. We reported all distances in body lengths as a means of standardization.

2.3.3. Oxygen consumption

We assessed O_2 consumption rates as a proxy for whole-body metabolic rate. We followed methodology described by Livingston et al. (2018). In brief, following the 48 h acclimation, we transferred fish ($n = 10$) to 8 ml glass metabolic rate chambers. We allowed acclimation in flowing water for 60 min. The control group had a source of control water, while the metyrapone group was exposed to a 50mgl^{-1} metyrapone solution. We measured O_2 consumption in triplicate in water before passively draining the chambers by disconnecting the water source. Once drained, we sealed the chambers and measured O_2 consumption in air over 6 h.

2.3.4. Histology of oxidative muscle

We investigated whether skeletal muscle showed cortisol-dependent changes in its oxidative phenotype following methodology described by Rossi et al. (2018). In brief, following the terrestrial jumping trials we euthanized the fish ($n = 8$) and took two ~3 mm transverse muscle sections: one just posterior to the dorsal fin and the other immediately posterior to the first. We embedded muscle sections in cryomatrix (Shandon Cryomatrix™, Fisher Scientific, Hampton, NH, USA) and flash-froze them. We cut the frozen muscle sections into 8 μm transverse sections using a cryostat (Leitz Cryostat Microtome, Labequip Ltd., Markham, ON, Canada) at −22 °C, mounted the sections on Superfrost Plus slides (Fisher Scientific), and stored the slides at −80 °C to be stained later. We identified red-oxidative fibers by staining muscle sections for slow myosin isoenzyme (mouse IgA primary antibody; S58; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). We visualized slides under an epifluorescence microscope (Nikon Eclipse 90i microscope, Nikon, Tokyo, Japan) and photographed them using NIS elements software. We used Image J software to count fibers and quantify the number of red-oxidative muscle fibers on one lateral half per fish. We randomly selected 30 muscle fibers to estimate the average fiber cross-sectional area (μm^2).

2.3.5. Muscle lipids

We investigated whether muscle lipid stores were catabolized for energy throughout the transition to air and whether lipid catabolism showed cortisol dependence. We determined lipid content by chloroform extraction using methodology previously described by Rossi and Wright (2020). In short, we euthanized the fish, immediately sampled the skeletal muscle, flash froze the muscle section, and stored samples at −80 °C until later lipid quantification. We measured the total muscle lipid content (g) by drying the muscle sections at 60 °C for 48 h, soaking dried sections in chloroform for 48 h to digest the lipids, then drying the sections for another 48 h. The mass difference between the dried muscle sections pre- and post-chloroform indicated total muscle lipid content.

2.3.6. Swimming activity in water

To determine potential side effects of the metyrapone treatment (e.g., a change in normal behaviour), we analyzed swimming activity. We placed fish individually in 120 ml plastic containers in either control or metyrapone conditions ($n = 8$), set up to mimic the 48 h acclimation period. We measured voluntary swimming activity throughout the acclimation period to assess whether metyrapone induces changes to the bioenergetic capacity. We video-recorded the fish in four 1 h intervals: Day 1 09:30–10:30, Day 1 16:00–17:00, Day 2 09:30–10:30, and Day 2 16:00–17:00. We assessed voluntary swimming activity using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands), which measured the total distance moved (cm), average velocity of movement (cms^{-1}), total duration moving (min), and total duration not moving (min). We compared these behavioural parameters between control and metyrapone groups across each measurement period.

2.4. Statistics

We analyzed all data for statistical significance using RStudio (version 1.1.463) with R (version 3.6.1) and visualized the data using GraphPad Prism (v.9.0.0). All data were initially tested for normality and homogeneity of variance, and appropriately transformed when necessary. We performed two-way analyses of variance (ANOVA) to assess the influence of the experimental treatment and the duration of air exposure on whole-body cortisol concentration, jumping performance, and muscle morphometrics. When a significant treatment x air exposure interaction was detected, we used Tukey's HSD multiple comparison post-hoc test to assess significant differences between groups. We performed repeated-measures two-way ANOVA's with a Geisser-Greenhouse correction to analyze differences in voluntary swimming activity between control and metyrapone-treated fish and in O_2 consumption rate between control and metyrapone-treated fish exposed to water and subsequently exposed to air.

3. Results

3.1. Cortisol

Cortisol levels were significantly affected by both metyrapone treatment and air exposure with a significant interaction (treatment: $F_{1,35} = 8.44$, $p = 0.006$; air exposure: $F_{1,35} = 21.22$, $p < 0.001$; treatment x air exposure: $F_{1,35} = 3.43$, $p = 0.04$). Control cortisol levels at 1 h were significantly higher than 0 h and 7 day groups ($p < 0.05$). The control group saw a higher peak at 1 h, while the metyrapone-treated group saw a lower peak, which was not significantly different from the 0 h and 7 day timepoints ($p > 0.05$), nor from the 1 h control ($p = 0.4$). Cortisol levels within the 1 h metyrapone-treated group were also less variable relative to the 1 h control group (Fig. 1).

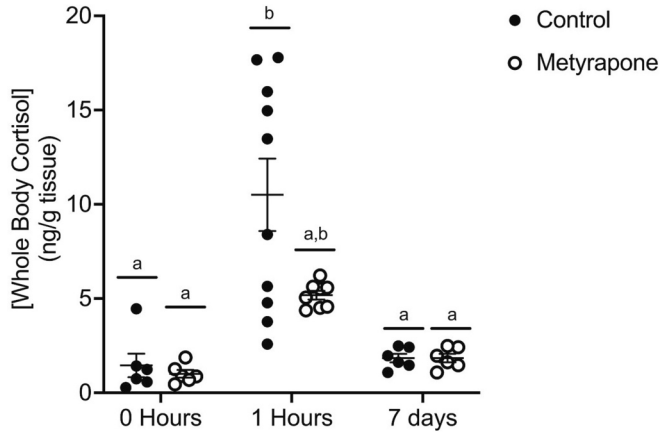

Fig. 1. The effect of metyrapone treatment on the cortisol response during acclimation to air in *Kryptolebias marmoratus*. Whole-body cortisol levels (ng/g tissue) were measured in metyrapone-treated and control groups of *K. marmoratus* at three timepoints during acclimation to air: 0 h, 1 h, and 7 days ($n = 6$–10). Metyrapone treatment effectively depressed change in cortisol levels throughout the transition to air. Different letters denote significant differences ($p < 0.05$). Data are means are ± s.e.m.

3.2. Terrestrial exercise performance

Overall, the metyrapone group displayed poorer locomotory performance relative to the control group, regardless of the length of air exposure. In total distance travelled, locomotory performance was affected by treatment but not by air exposure, and there was no significant interaction (treatment: $F_{1,67} = 24.71$, $p < 0.001$; air exposure: $F_{1,67} = 5.28$, $p = 0.007$; treatment x air exposure: $F_{1,67} = 1.34$, $p = 0.27$). Averaged across timepoints, the metyrapone-treated group travelled less distance (~74 body lengths) than the control group ($p = 0.007$) before reaching exhaustion (Fig. 2A). Metyrapone also decreased the distance covered in individual jumps relative to the control. Distance covered in the longest jump was affected by treatment but not by air exposure, and there was no significant interaction (treatment: $F_{1,67} = 7.97$, $p = 0.006$; air exposure: $F_{1,67} = 2.02$, $p = 0.14$; treatment x air exposure: $F_{1,67} = 1.0$, $p = 0.37$). Averaged across timepoints, the metyrapone-treated group's longest jumps were ~ 2 body lengths shorter than the control ($p = 0.006$) (Fig. 2B). Distance covered in the mean jump was affected by metyrapone treatment, but not by air exposure, and there was no significant interaction (treatment: $F_{1,67} = 17.93$, $p < 0.001$; air exposure: $F_{1,67} = 2.31$, $p = 0.11$; treatment x air exposure: $F_{1,67} = 2.15$, $p = 0.13$). Averaged across timepoints, the metyrapone group's mean jump was ~0.6 body lengths shorter than the control ($p < 0.001$) (Fig. 2C). Total number of jumps was affected by air exposure but not by treatment, and there was no significant interaction (treatment: $F_{1,67} = 0.19$, $p = 0.30$; air exposure: $F_{1,67} = 3.59$, $p = 0.04$; treatment x air exposure: $F_{1,67} = 1.34$, $p = 0.27$) (Fig. 2D). Fish exposed to air for 7 days jumped 20% more than fish never exposed to air and 8% more than fish exposed to air for 1 h. Fish at 1 h jumped 12% >0 h fish.

3.3. Oxygen consumption

O_2 consumption rate was affected by both metyrapone treatment and air exposure, with no significant interaction (treatment: $F_{1,18} = 9.433$, $p = 0.007$; air exposure: $F_{1,18} = 135.80$, $p < 0.001$; treatment x air exposure: $F_{1,18} = 4.238$, $p = 0.054$). Air exposure led to a marked increase in the O_2 consumption of fish in both the metyrapone and control groups, and this response was depressed by metyrapone treatment (Fig. 3).

3.3.1. Histology of oxidative muscle

Histological analysis comparing the oxidative skeletal muscle phenotype in metyrapone-treated and control groups after 0 h and 7 days of air exposure revealed a lack of treatment effect, air exposure effect, or interaction effect in both size of oxidative fibers (treatment: $F_{1,27} = 1.06$, $p = 0.31$; air exposure: $F_{1,27} = 4.14$, $p = 0.052$; treatment x air exposure: $F_{1,27} = 2.20$, $p = 0.15$) (Fig. 4A) and in number of oxidative fibers (treatment: $F_{1,27} = 0.10$, $p = 0.75$; air exposure: $F_{1,27} = 0.08$, $p = 0.78$; treatment x air exposure:$F_{1,27} = 3.44$, $p = 0.08$) (Fig. 4B).

3.3.2. Muscle lipid content

Analysis of muscle lipid content showed no significant treatment

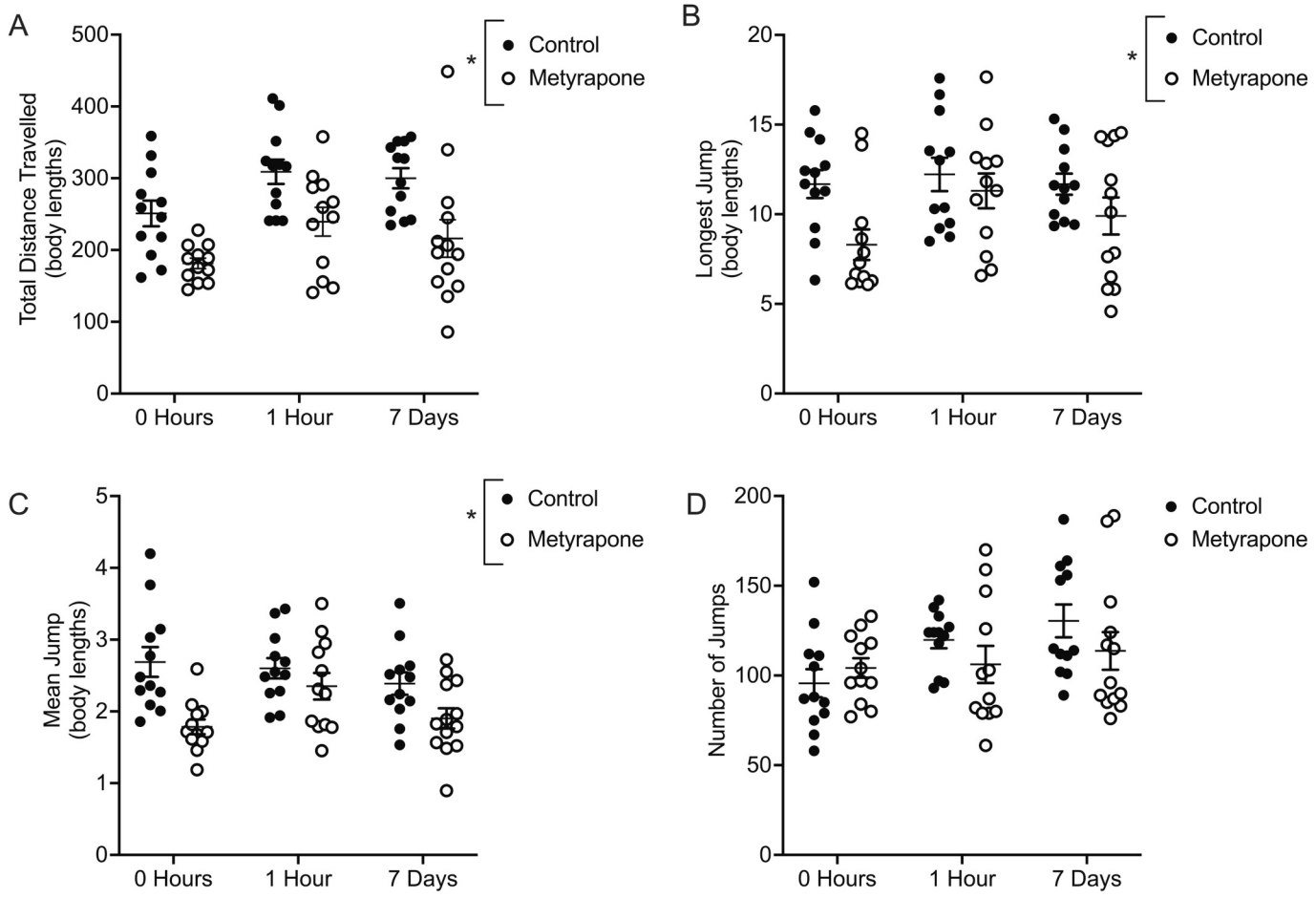

Fig. 2. The effect of metyrapone treatment on terrestrial exercise performance in *Kryptolebias marmoratus*. (A) Total distance travelled (body lengths), (B) longest jump distance (body lengths), (C) mean jump distance (body lengths), and (D) total number of jumps ($n = 12$). Significant differences are denoted by an asterisk ($p < 0.05$). Metyrapone-treated groups travelled significantly less distance than control groups in total distance travelled, longest jump, and mean jump distance. Data are means are ± s.e.m.

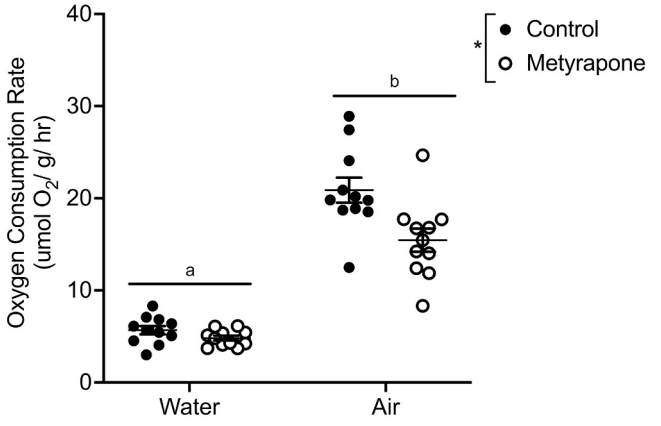

Fig. 3. The effect of metyrapone treatment on oxygen consumption rates during the water to air transition in *K. marmoratus*. Oxygen consumption rates (μmol O_2/g/ h) of metyrapone-treated and control groups of *K. marmoratus* throughout the transition to air (n = 10). Letters (a,b) denote significant differences between groups. In-water measurements were taken after an acclimatory period and air measurements were taken over the initial 6 h of air exposure. Different letters denote significant differences ($p < 0.05$). Both metyrapone-treated and control groups significantly elevated their O_2 consumption rate upon air exposure, although metyrapone treatment attenuated the oxygen consumption rate. Data are means are ± s.e.m.

effect, air exposure effect, or interaction effect (treatment: $F_{1,42} = 0.85$,$p = 0.0.435$; air exposure: $F_{1,42} = 2.56$, $p = 0.09$; treatment x air exposure: $F_{1,42} = 0.17$,$p = 0.682$) (Fig. 5).

3.3.3. Swimming activity in water

The total distance that fish travelled within a 48-h period was not affected by either metyrapone treatment ($F_{1,14} = 0.43$, $p = 0.52$) or time of day ($F_{3,42} = 1.62$, $p = 0.20$), nor was there an interaction ($F_{3,42} = 0.57$, $p = 0.63$). The average velocity of movement was also unaffected by treatment ($F_{1,14} = 0.49$, $p = 0.50$) and time ($F_{1.6,22.5} = 1.58$, $p = 0.23$), without an interaction ($F_{3,42} = 0.56$, $p = 0.64$). The total time spent moving and total time spent still were not affected by metyrapone-treatment (moving: $F_{1,14} = 0.07$, $p = 0.79$; still: $F_{1,14} = 0.8$; $p = 0.78$), but they were influenced by time of day (moving: $F_{2.5,36.7} = 11.62$, $p < 0.001$; still: $F_{2.5,35.3} = 11.23$, $p < 0.001$), without an interaction (moving: $F_{3,42} = 1.28$, $p = 0.29$; still: $F_{3,42} = 1.24$, $p = 0.31$). In both metyrapone-treated and control groups, activity tended to be higher during the morning measurement periods (Supp. Fig. 1).

4. Discussion

We used the amphibious *Kryptolebias marmoratus* to test associations between the air-induced elevation of cortisol and locomotory performance and oxidative processes during the transition from water to air. We found evidence that cortisol release upon air exposure is linked to the upregulation of oxidative processes, as metyrapone treatment reduced the elevation in O_2 consumption rate in the first few hours of air exposure.

It was important in our study that our observations, presumed to be the effects of cortisol, were, in fact, cortisol-induced and not the results of depressive side effects of metyrapone. Metyrapone blocks cortisol synthesis by inhibiting the mitochondrial enzyme 11β-hydroxylase, which catalyzes the terminal reaction in cortisol production (Mommsen et al., 1999). In addition to blocking cortisol synthesis, inhibiting 11β-hydroxylase can block other cytochrome P450-dependent monooxygenases. However, when used in low enough doses, metyrapone does not inhibit monooxygenase activity in rainbow trout (Miranda et al., 1998) and does not affect whole-body metabolic rate (Del Corral et al., 1999) or mitochondrial oxygen uptake (Drouet et al., 2012) in

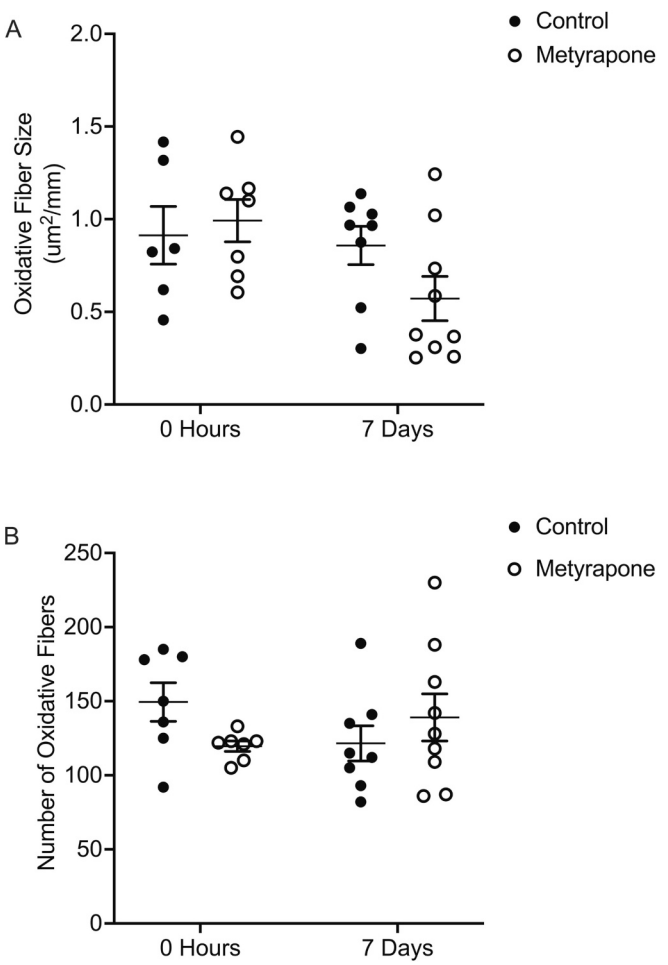

Fig. 4. The effect of metyrapone treatment on the oxidative phenotype of red skeletal muscle in *Kryptolebias marmoratus* after 0 h and 7 days in air. (A) Number of oxidative fibers and (B) size of oxidative fibers (um^2/mm). Oxidative fiber size was estimated as the average oxidative fiber diameter. There were no significant treatment or time effects in either parameter ($p > 0.05$). Data are means are ± s.e.m.

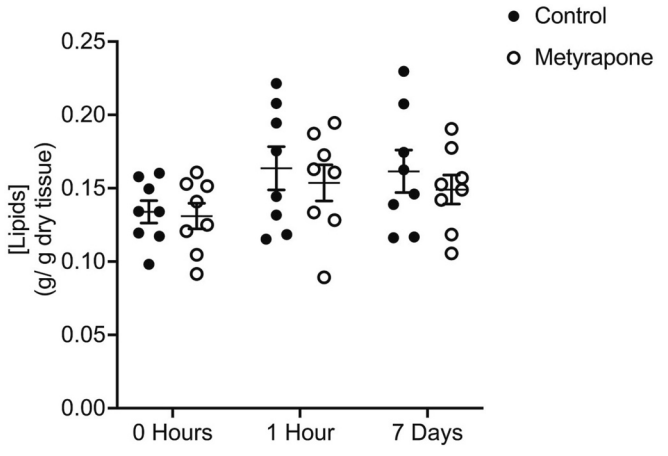

Fig. 5. The effect of metyrapone treatment on lipid concentrations in the skeletal muscle of *Kryptolebias marmoratus* after 0 h, 1 h, and 7 days in air. Lipid concentration (g/ g dry mass) in the skeletal muscle of metyrapone-treated and control groups after 0 h, 1 h, and 7 days in air (n = 8). There were no significant differences between groups or timepoints ($p > 0.05$). Data are means are ± s.e.m.

mammalian studies. We wanted to ensure in our study that the dose of metyrapone was such that it inhibited cortisol synthesis, but did not induce depressive side effects. So, we conducted a comparative behavioural analysis between control and metyrapone-treated fish at rest over the 48-h exposure period to see if metyrapone-treated fish showed signs of bioenergetic suppression (Supplementary Fig. 1). To our knowledge, this is the first time that resting behaviour during metyrapone treatment has been examined in fish. We found that metyrapone had no effect on the total distance moved, average velocity of movement, total time spent moving, or total time spent still. Interestingly, we found that fish tended to be more active in the morning (i.e., spent more time moving), regardless of whether they were control or metyrapone-treated. Increased activity during the morning measurement periods may reflect some degree of handling stress, as water changes were performed immediately prior to the morning measurement periods. As well, previous studies have shown that *K. marmoratus*' metabolic rate is highest in the morning and decreases over the course of a day (Rodela and Wright, 2006). Overall, our results demonstrate that metyrapone did not alter voluntary swimming activity, suggesting that the metabolic differences we observed between metyrapone-treated and control fish were likely due only to metyrapone-induced inhibition of cortisol levels.

Metyrapone treatment inhibited terrestrial locomotory performance in *K. marmoratus* as the total distance travelled, longest terrestrial jump, and mean jump distance were all reduced by metyrapone treatment. Overall, we showed that the ability to synthesize cortisol improves terrestrial locomotion in *K. marmoratus*, and this effect was seen immediately upon air exposure ("0 h" group). Fish at "0 h" had no prior air exposure, indicating that cortisol synthesis immediately impacts the transition to air, enhancing locomotion within seven minutes of air exposure. The immediate beneficial effect of cortisol may be attributed to cortisol's cooperative role with catecholamines. Glucocorticoids often exert a permissive effect on catecholamine function, and both hormones have similar, possibly intertwined, effects on metabolism – including glycogen cycling in the muscle (Pagnotta et al., 1994; Mommsen et al., 1999; Milligan, 2003). Thus, cortisol and catecholamines may co-regulate the air-induced metabolic increase.

Under resting cortisol levels, cortisol-induced metabolic changes are regulated by mineralocorticoid receptors (MR) (Faught and Vijayan, 2022). Within the muscles, activation of MR ultimately facilitates glucose uptake by promoting glycogen synthesis and inhibiting glycogen breakdown (Milligan, 2003; Faught and Vijayan, 2022). In contrast, glucocorticoid receptors (GR) are activated under high cortisol conditions, ultimately stimulating the breakdown of muscle glycogen into glucose and limiting glucose uptake by the muscle (Faught and Vijayan, 2019; Antomagesh et al., 2023). Along with cortisol, catecholamines further enhance the muscle glycogen breakdown (Pagnotta et al., 1994; Mommsen et al., 1999; Milligan, 2003). Furthermore, a short-lived elevation of catecholamines has been demonstrated in fish in response to exhaustive exercise (Milligan, 2003) and emersion (Reid et al., 1998; Perry and Bernier, 1999), both of which were likely experienced by fish in the present study. The combined effect of elevated cortisol and catecholamine levels may result in a sustained breakdown of glycogen into glucose and provide energy to fuel muscle metabolism. Therefore, we propose that metyrapone-treated fish, who had low or absent cortisol production, had a limited ability to utilize endogenous glycogen stores which, in turn, reduced their terrestrial exercise capacity. On the other hand, the uninhibited control fish could take full advantage of the muscle glycogen catabolism mediated by both cortisol and catecholamine dynamics.

Control and metyrapone-treated groups had similar oxygen consumption rates while in water, prior to emersion, indicating that, while at rest, metyrapone does not affect metabolic rate. Once emersed, however, metyrapone treatment attenuated the air-induced elevation of O_2 consumption rate by 30% relative to control, indicating that cortisol is necessary for the air-induced increase in O_2 uptake to occur. The rise in O_2 consumption is consistent with our previous work (Livingston et al., 2018). *K. marmoratus,* and multiple other amphibious killifishes, increased O_2 uptake over the first 6 h of air exposure (Livingston et al., 2018). Hence, the cortisol-regulated increase in O_2 uptake may be necessary to meet the energetic demands associated with acclimating to air. *K. marmoratus* induce significant molecular, cellular, and tissue responses within hours of air exposure. For example, in previous studies we have documented an increase in cutaneous gene transcription (Dong et al., 2021), blood haemoglobin levels (Turko et al., 2014), blood vessel density (Blanchard et al., 2019), skin ionocytes (Tunnah et al., 2022), and oxidative skeletal muscle (Brunt et al., 2016; Rossi et al., 2018). All these changes require additional energy, and cortisol plays an important role in obtaining sufficient fuel. Elevated cortisol levels promote the activation of GR, resulting in the provision of glucose, which serves as an energetic substrate to fuel aerobic metabolism (Faught and Vijayan, 2019; Antomagesh et al., 2023). In addition to providing fuel, cortisol has the capacity to intensify oxidative phosphorylation in mammals (Du et al., 2009; Picard et al., 2018; Lapp et al., 2019; Davies et al., 2021). Although further studies are needed to elucidate the mechanism behind cortisol-mediated mitochondrial function in fishes, cortisol appears to increase the capacity for aerobic metabolism, allowing *K. marmoratus* to cope with the energetic demands of emersion.

We also investigated whether cortisol regulates morphometric changes in the oxidative muscle fibers or alters the oxidative muscle size as a whole, which may reflect changes in the skeletal muscle's capacity for oxidative phosphorylation. There were no significant changes in the size or quantity of oxidative fibers within 7-days of air exposure, nor did metyrapone have an effect within this time-frame. Given a longer acclimation in air, it is possible that metyrapone may have an impact on oxidative muscle remodeling, but this requires further investigation.

Muscle lipids play an important role in fish during aerobic exercise (McClelland, 2004) and air exposure (Rossi and Wright, 2020). In preliminary experiments, we did not find evidence for significant lipid utilization as there were no significant differences in muscle lipid content throughout 7 days of air exposure and fasting, nor was there a difference between control and metyrapone-treated groups (Fig. 5). This finding indicates that lipids do not appear to be a primary fuel source regulated by cortisol within the first hours and days out of water.

5. Conclusion

We aimed to determine whether cortisol is the mechanism by which oxidative processes are upregulated during the transition to air in amphibious fishes. Indeed, inhibition of cortisol synthesis by metyrapone reduced the elevation in O_2 consumption and jumping performance observed in control *K marmoratus* exposed to air. However, analysis of the muscle phenotype indicated that cortisol does not cause an increase in the oxidative capacity within a 7-day timeframe. Most studies concerning how amphibious fishes transition to land focus on comparing pre-emersion to post-acclimation, that is, after multiple weeks emersed (Brunt et al., 2016; Rossi et al., 2018; Rossi and Wright, 2020, Turko et al., 2021). Few studies have considered what happens during the transition itself - the minutes to hours following emersion. Cortisol clearly plays a role in the acute response to air, and further work is required to tease apart the mechanisms involved.

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Declaration of Competing Interest

The authors declare no competing or financial interest.

Data availability

Dataset is available in supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2023.111558.

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