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Functional Trade-Offs Between Terrestrial and Aquatic Locomotion in the Amphibious Fish Kryptolebias marmoratus

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Functional Trade-Offs Between Terrestrial and Aquatic Locomotion in the Amphibious Fish *Kryptolebias marmoratus*

By

Erik Axlid

A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of

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This thesis has been examined and approved by the following members of the student's committee.

Dr. Michael Minicozzi
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Abstract

The mangrove rivulus (*Kryptolebias marmoratus*) is a phenotypically plastic teleost fish that can spend considerable time on land and traverse the terrestrial realm through a ballistic behavior termed the tail-flip jump. The tail-flip jump is a transitional stage between fully aquatic and terrestrial lifestyles. Therefore, understanding this behavior can provide insight into how organisms adapt to new environments over evolutionary time. Taxa that are successful tail-flip jumpers have a deep caudal peduncle and uniform body shape. Studies of *K. marmoratus* show that terrestrial acclimation and exercise improves tail-flip jumping performance due to muscle remodeling, but the implications of these muscular changes on aquatic locomotion are unknown. In the present study, we hypothesized that 1) terrestrial acclimation and exercise lead to physiological and anatomical changes to optimize tail-flip jump distance and endurance while negatively impacting swimming performance in *K. marmoratus*, 2) terrestrial exercise causes muscle redistribution toward the posterior of *K. marmoratus* to better facilitate the tail-flip jump, and 3) terrestrial exercise and air exposure cause behavioral changes in *K. marmoratus*, made possible by the large degree of neural and behavioral plasticity displayed by this species. To test these hypotheses, we measured the critical swimming speed (U_{crit}), tail-flip jump distance, terrestrial endurance, mass distribution along the anterior-posterior axis, and undisturbed aquatic behavior of age- and size-matched *K. marmoratus* before and after a terrestrial exercise period. This period consisted of six three-minute exercise sessions spread over 12 days, during which the fish were prompted to jump continuously. To isolate the effects of air exposure, a separate group was exposed to air for an equivalent period but not allowed to jump. Air exposure improved maximum jump distance and negatively affected swimming performance (U_{crit}). Terrestrial endurance (number of jumps) improved in the exercised group, but U_{crit} did not change. Contrary to our first hypothesis, a trade-off exists between jump distance and U_{crit}, but not between jump endurance and U_{crit}. No change to body mass distribution was detected. Exercised individuals were more active following exercise, resulting either from onset of a dispersion behavior or a heightened stress response.

Introduction

Although teleost fishes are generally seen as a strictly aquatic group, a wide variety of taxa representing diverse phylogenetic groups within the teleosts spend some portion of their lives on land (Sayer and Davenport, 1991). As of 2014, over 70 species from 12 families had been identified to emerge from water and utilize gaseous oxygen in marine intertidal zones, in addition to the many freshwater fishes that also periodically inhabit terrestrial environments (Martin, 2014; Sayer and Davenport, 1991). Here, we will define amphibious fishes as fishes that *voluntarily* emerge onto land (Minicozzi et al., 2020). Because many of these species are only distantly related, their life histories also vary greatly. While some amphibious fishes only briefly leave the aquatic environment, other, more specialized fishes such as the mangrove rivulus *(Kryptolebias marmoratus)* can survive out of the water for weeks or months at a time (Davis et al., 1990; Todd, 1976). This variation is also present in the frequency at which amphibious fishes leave the water, with some emerging voluntarily and frequently while others rarely emerge (Sayer and Davenport, 1991).

There are many environmental or biotic factors that can prompt amphibious fishes to emerge to terrestrial environments (Sayer and Davenport, 1991). Environmental factors generally relate to chemical or physical changes within the water, creating unfavorable or harmful conditions. An example of an environmental factor is aquatic hypoxia (the depletion of oxygen concentrations in the water) which induces emergence in several species, including *K. marmoratus* and the shanny (*Blennius pholis)* (Davenport and Woolmington, 1981; Regan et al., 2011). Waterborne pollutants such as hydrogen sulfide and changes in temperature can also cause emergence as demonstrated in *K. marmoratus* (Abel et al., 1987; Huehner et al., 1985).

There are also several biotic factors that can encourage emersion in amphibious fishes, relating to interspecific or intraspecific interactions (Sayer and Davenport, 1991). Emergence due to threats by a large predator *(*such as *Micropterus salmoides)* and by aggressive, dominant individuals of the same species have both been observed in cyprinodontiform fishes (Goodyear, 1970; Huehner et al., 1985). Competition for resources can also drive emergence. Experiments by Liem (1987) found that the amphibious climbing perch (*Anabas testudineus)* can leave the aquatic environment to obtain food because of either inter- or intraspecific competition. Some fishes also emerge for reproductive purposes such as courtship, and although most amphibious fishes return to water to reproduce, terrestrial egg deposition has been described in laboratory and field studies in *K. marmoratus* and the California grunion (*Leuresthes tenius*) (Abel et al., 1987; Sayer and Davenport, 1991; Taylor, 2012; Walker, 1952).

The diversity of life histories and ecological niches filled by amphibious fishes are also reflected in the wide range of modes of terrestrial locomotion that exist along a continuum (Gibb et al., 2013). Fishes that do not voluntarily emerge from the water have no morphological specializations for terrestrial movement and are generally only capable of producing side-to-side contractions of the axial musculature, termed thrashing, when involuntarily emersed (Gibb et al., 2013). Thrashing can create downslope movement, and under the right circumstances can return the fish to water (Gibb and Boumis, 2012). Some slightly more specialized amphibious fishes, that emerge occasionally, are capable of a performing a ballistic jumping behavior termed the tail-flip jump (Gibb et al., 2011; Goodyear, 1970; Mast, 1915). The trajectory of a tail-flip jump is predictable, and the jump is always caudally directed and performed from a starting position in which the lateral side of the fish is in contact with the substrate (Gibb et al., 2011). Blennies *(Alticus spp.)* and mudskippers *(Periopthalmus spp.)*, which both spend significant time on land and exist on the more derived end of the terrestrial locomotion continuum, have further modified the tail-flip jump into the prone jump. The prone jump is performed by a fish lying on its ventral side, made possible by a twisting of the tail, and allows for cranially directed jumps (Hsieh, 2010). This allows the fish to see in the direction that it is jumping, and to potentially anticipate its trajectory (Gibb et al., 2013). Mudskippers can also move by lifting the body using a synchronous and cyclic motion of the pectoral fins to move forward, in an appendage-based locomotion behavior termed “crutching” (Harris, 1960; Pace and Gibb, 2009). This contrasts with axial-based locomotion, used by many elongate fishes such as the American eel *(Anguilla rostrata)*, in which the body undulates in a wave-like pattern, from anterior to posterior, across the substrate (Gillis, 1998; Pace and Gibb, 2011). A significant number of fishes also combine aspects of axial- and appendage-based terrestrial locomotion to move effectively on land, though the exact kinematics of these behaviors remain understudied (Pace and Gibb, 2014).

The tail-flip jump, observed in many cyprinodontiform fishes along with several diverse taxa in other families, falls somewhere in the middle of this continuum (Gibb et al., 2013). The tail-flip jump is a highly coordinated and stereotyped behavior with two distinct phases, beginning with the fish lying with its lateral side on the substrate. In the first phase, the axial musculature on the side of the fish not in contact with the substrate contracts, lifting the anterior portion of the body above the caudal fin and creating a body shape resembling the letter “C”. Then, in the second phase, the axial musculature on the now convex side of the body contracts to extend out of the curved position, launching the fish into the air by transmitting force through the caudal peduncle (Fig. 1) (Gibb et al., 2013; Pace and Gibb, 2011). This behavior can propel the fish more than five body lengths; however, it is generally limited to smaller (≤3.5 cm) fishes due to physical size constraints (Bressman et al., 2019; Gibb et al., 2013).

Although no modifications to the skeletal morphology or body shape of the fish are necessary to produce a tail-flip jump, they can both play a role in determining how effectively a fish can use this mode of locomotion (Minicozzi, 2017; Minicozzi et al., 2020). There is evidence that differences in the length and structure of the hypural and epural bones of the caudal peduncle (which transfers force to the substrate during the jump) affect jumping performance, but the interaction between these bones, jumping performance, and ontogeny are not well understood (Styga et al., 2017). A deeper caudal peduncle (relative to the anterior body depth) and a more uniform body shape both correspond to greater maximum jumping distance (Minicozzi et al., 2020). Additionally, having these modifications raises the upper body size limit to terrestrial jumping, allowing larger fishes to tail-flip jump (Minicozzi, 2017). This shift in body shape may be the result of redistribution of muscle mass to the posterior portion of the body, reducing the mass in the anterior portion (which must be lifted during first phase of the jump) and strengthening the posterior, likely used to produce force during the second phase of the jump (Gibb et al., 2013; Minicozzi et al., 2020; Perlman and Ashley-Ross, 2016). The tail-flip jump, generally viewed as a burst behavior, has long been thought to be powered by fast-glycolytic (white) musculature due in part to its kinematic similarities to the aquatic C-start escape response, another burst behavior (Perlman and Ashley-Ross, 2016). This idea has been supported by the positive relationship that exists between tail-flip jumping ability and C-start performance (Gibb et al., 2011; Perlman and Ashley-Ross, 2016), however, growing evidence suggests that oxidative (red) muscle also play a role in facilitating the tail-flip jump (Brunt et al., 2016; McFarlane et al., 2019). Exposure to air and terrestrial exercise, even for short periods, have been shown to positively affect jumping performance by causing physiological changes to the musculature (Brunt et al., 2016; McFarlane et al., 2019).

K. marmoratus is an amphibious cyprinodontiform fish that moves on land using the tail-flip jump and displays a large degree of phenotypic and developmental plasticity in response to a wide variety of stimuli (Earley et al., 2012; Gibb et al., 2013; James et al., 2018). Laboratory strains of this taxon exist primarily as self-fertilizing hermaphrodites, capable of producing offspring that are genetically identical to their progenitor (Harrington, 1961). This system of reproduction, along with their ability to locomote terrestrially and adapt to environmental stressors, has led the species to become a well-studied model organism for evolution, phenotypic plasticity, and terrestrial locomotion (Brunt et al., 2016; Kelley et al., 2012; Kelley et al., 2016; Styga et al., 2017). Changes to oxygen availability, including aquatic hypoxia and exposure to air in a terrestrial environment, have well-documented effects on skin and gill morphology of this taxon, which are likely to optimize physiological functioning (such as gas exchange, ion exchange, and nitrogen excretion) or to prevent desiccation during emersion from water (Earley et al., 2012; Heffell et al., 2018; Martin et al., 2019; Ong et al., 2007). Gill modification may also provide structural support, preventing permanent damage when on land (Turko et al., 2012). Physiological and morphological changes to skeletal muscle in response to terrestrial exposure or terrestrial exercise also occur, and fish exposed to air display oxidative muscle hypertrophy, increased capillarity, and lower lactate production during activity (Brunt et al., 2016; McFarlane et al., 2019; Rossi et al., 2018). Behavior in *K. marmoratus* is also plastic and shifts in response to interactions with conspecifics or predators (James et al., 2018; Li et al., 2021 preprint). Morphological plasticity in response to terrestrial exercise has also been demonstrated in the brain of this species, as have corresponding changes to cognitive functions (Rossi and Wright, 2021).

In biological systems, traits are often linked in such a way that simultaneous optimization of related characteristics is impossible (Ellison, 2014). Therefore, compromises can occur in which the trait that conveys the largest increase in fitness is developed, while another is reduced or lost. This is known as an evolutionary trade-off, and a well-known example of this phenomenon can be seen in penguins. Penguins have modified their body shape and forelimbs to accommodate for increased swimming performance, however, this modification has resulted in the loss of the ability to fly (Shipman, 1999). Although being able to fly and swim could potentially increase fitness beyond the current state, complete optimization for both abilities is impossible given the physical demands of each, leading to a trade-off (Raikow et al., 1988). Since demands for terrestrial tail-flip jumping and swimming appear very different, we predicted that there would be a functional trade-off occurring between these behaviors in amphibious fishes.

As previously established, the abilities of fishes to locomote on land represent a continuum. Along this continuum, a range of “steps” in the evolutionary transition toward fully terrestrial lifestyles can be observed, and the tail-flip jump is an intermediate and possibly transitional stage. Therefore, understanding the mechanics and evolutionary trajectories related to this behavior can provide insight into how organisms in the past and present adapt and evolve toward completely new and disparate environments. In the present study, we investigated the effects of air exposure and terrestrial exercise on aquatic behavior, morphology, and performance in *K. marmoratus*. We tested three hypotheses; 1) terrestrial acclimation and exercise lead to physiological changes that optimize tail-flip jump distance and endurance while negatively impacting swimming performance in *K. marmoratus*, 2) terrestrial exercise causes muscle redistribution toward the posterior of *K. marmoratus* to better facilitate the tail-flip jump, and 3) terrestrial exercise and air exposure cause behavioral changes in *K. marmoratus*, made possible by the large of degree neural and behavioral plasticity displayed by this species (Earley et al., 2012).

Materials and Methods

Animal Acquisition and Husbandry

Eggs were collected from self-fertilizing hermaphroditic *K. marmoratus*, placed in 60 mL plastic cups, and allowed to hatch. Upon hatching, each fish remained in its 60 mL cup before being transferred to a 750 mL clear plastic container between 7 and 14 days post-hatching (DPH). Fish were fed brine shrimp (*Artemia)* nauplii five times weekly. All eggs and fish were kept under artificial indoor lighting (14:10-hour light/dark cycle) and in 12 parts per thousand (ppt) salt water, prepared using Instant Ocean Sea Salt (Spectrum Brands, Blacksburg, VA). A water temperature of approximately 25°C was maintained using heat lamps. All containers housing eggs or fish were arranged randomly within the experimental space. After testing concluded, all fish were euthanized with an overdose of buffered tricaine mesylate (MS-222) and preserved in 10% neutral buffered formalin. All procedures involving live animals were approved by Minnesota State University Mankato's IACUC, protocol # 20-01

Experimental Design

To test the effects of terrestrial exercise on steady swimming performance, we randomly assigned size- and age-matched hermaphroditic *K. marmoratus* to three treatment groups, each consisting of 30 individuals. During a 16-day experimental period, one group was exercised terrestrially every other day (days 3, 5, 7, 9, 11, and 13 of the experimental period) by introducing a stimulus near the anterior end of each fish and prompting it to jump continuously for three minutes, according to McFarlane et al. (2019). This resulted in a total exercise time of 18 minutes over 12 days. All terrestrial exercise was conducted in a 90-cm-wide arena with steep walls, on a substrate of damp paper towels, and recorded from a top-down angle with a view of the entire exercise space for the purpose of analyzing activity. Analysis of this footage was later conducted using the open-source motion tracking software Kinovea (version 0.8.15), and the following variables were measured: the distance of the longest jump, the total number of "jumps" (movements resulting in displacement greater than one body length), the total number of "flops" (movements resulting in displacement shorter than one body length), the total number of movements (sum of the number of jumps and flops), and jump/flop ratio (the number of jumps per flop).

Another treatment group was removed from the water for an equivalent amount of time as the exercised group but was confined to a small container which eliminated the ability of the fish to jump, allowing for distinction between effects caused by exposure to a terrestrial environment and those caused by exercise. Fish in a third, separate, control group did not leave the aquatic environment for the duration of the exercise trials.

The swimming and jumping performance of each fish was evaluated before and after the treatment period. Swimming performance tests occurred on days 1 and 15 of the 16-day experimental period, while jumping performance tests were administered on days 2 and 16. Post-treatment performance tests began two days after treatment concluded to allow for adequate recovery. Standard length (SL) measurements, as well as dorsal and lateral photos later used for mass distribution analysis, were taken shortly following each swimming trial. Care was taken to ensure that the frontal plane of the fish was perpendicular to the placement of the camera in each dorsal photo, and that the sagittal plane of the fish was perpendicular to placement of the camera in each lateral photo. The caudal fin was also aligned along the sagittal plane to minimize distortion due to twisting of the body. In photos from each angle, the pectoral fins were placed along the body to minimize the effect they had on the body shape of the fish. Footage used for behavioral analysis was captured prior to all performance testing on days 1 and 15 of the 16-day period.

Performance Tests

Jumping performance was evaluated by introducing a transfer pipette near (or lightly touching) the anterior of the fish, prompting it to perform a tail-flip jump, and recording the jump at 600 frames per second (fps) from a side view. From these recordings, we quantified the duration of each jump phase, the launch angle of the estimated center of mass, and the curvature coefficient of the fish using Kinovea. The curvature coefficient was calculated as the ratio of the minimum distance between the tip of the snout and the caudal peduncle to the standard length of the fish (Brainerd and Patek, 1998). The approximate center of mass of the fish also was tracked manually using NIH ImageJ (Schneider et al., 2012), and the resulting positional data input to a MATLab routine designed to smooth the data and to calculate the velocity and acceleration of the approximate center of mass throughout the jump. To smooth the data, this routine applied a quintic smoothing spline with a tolerance of 0.015 (where higher values result in a greater degree of smoothing) to all sequences. In addition, a camera recording at 30 fps captured a top-down view from which jump distance was measured using Kinovea. Two jumps were recorded for each fish at each time point, and the longest of the two jumps was used for all analyses.

Prior to testing their swimming performance, all fish were allowed to acclimate to the swim tunnel for 60 minutes with no flow, and subsequently for an additional 10 minutes at a low flow velocity of 2 cm/s. The 10-minute acclimation period was adapted from Bernhardt and von Hippel (2008), though the flow velocity was lowered to account for differences in swimming ability between threespine stickleback and *K. marmoratus*. To obtain data used to calculate critical swimming speed (U_{crit}; a measure of maximum sustained swimming speed), the flow velocity was increased to 3 cm/s after the acclimation period, and subsequently raised by 1 cm/s every 3 minutes. These interval duration and velocity increment values were adapted from Plaut (2000) and Tierney (2011) but adjusted to compensate for the smaller body size of the *K. marmoratus,* as well as its low critical swimming speed. To motivate fish to swim, an opaque cloth was placed over the upstream portion of the experimental chamber of the swim tunnel. This, along with light tapping on the swim tunnel when the fish approached a downstream mesh barrier within the chamber, motivated the fish to maintain their position and resist the flow of the water. A mirror, placed under the covered portion of the chamber, was used to observe the fish regularly throughout each trial. Tests terminated when fishes could no longer maintain their position against the flow and were trapped against the downstream barrier, or when the fish contacted the downstream barrier five times within any given 30-second period. U_{crit} was calculated using the following equation, as described by Brett (1964):

$$U_{crit} = V_f + (\frac{T}{t} \times dV)$$

Where V_f is the velocity of the last fully completed interval before exhaustion occurred, T is the time swum at the final velocity before exhaustion, t is the interval duration (180 s), and dV is the velocity increment (1 cm/s). Fish that did not complete the treatment period or could not complete the two swimming performance tests were excluded from the U_{crit} analysis, which explains the variation in sample size among treatment groups.

Behavioral analysis

To quantify aquatic behavior, the fish were placed individually in clear, square, 750-ml plastic containers filled to a depth of 4 cm with 12 ppt salt water (prepared using Instant Ocean Sea Salt) and covered with a sheet of plexiglass to prevent emersion. Prior to video recording, the fish were left undisturbed in the plastic containers for 10 minutes to reduce abnormal behaviors due to recent handling. They were then recorded, undisturbed, from a dorsal view against a while backdrop for 20 minutes.

Based on the footage taken during the behavioral trials, each fish was tracked using EthoVision XT 16 software (Noldus IT, Wageningen, Netherlands) and a sampling rate of 5 samples/s (Noldus, 2022). The shape of the containers resulted in square 15x15 cm arenas with gently rounded corners when viewed by the camera, with an additional 5x5 cm zone drawn in the center of the arena to assess the propensity of each fish to venture from the edges of the container. The tracked data were then smoothed using the LOWESS (locally weighted scatterplot smoothing) method, and instances where the subject moved more than 2.4 cm between two subsequent samples were filtered out before analysis (Noldus, 2022). Measured movement of this magnitude between two samples necessarily represented noise in the data, as the swim speed required to complete such a movement would exceed the maximum observed swim speed of the species. The following variables were measured or calculated: total time spent moving (above 0.5 cm/s), total time at rest (below 0.25 cm/s), total distance traveled, mean velocity while in motion (above 0.5 cm/s), maximum acceleration, maximum velocity, total time spent in the center zone, mean distance to the center point of the arena, mean absolute meander (change of direction relative to distance moved), and mean turn angle. Meander was measured only during periods in which the fish were in motion (above 0.5 cm/s).

Mass distribution

Dorsal and lateral photos of each fish were automatically traced and manually corrected using Adobe Photoshop (version 23.2) to accurately represent the body shape of the fish from each angle with high contrast. Fins were excluded from the images during this stage as they altered the shape of the fish but caused only a negligible change in body mass. Images of these traced high-contrast dorsal and lateral outlines were then fed into a custom MATLab routine, which calculated the distribution of volume of each fish along the anterior-posterior axis. Using volume as a proxy for mass, the program then generated data showing the proportion of mass present in the anterior and posterior thirds of each fish. The change in relative mass present in each body segment was then compared across treatment groups.

Statistical analyses

All statistical analyses were conducted using IBM SPSS Statistics 27 (IBM Corp. 2019). One-sample t-tests were used to detect differences between pre- and post-treatment jump distance and U_{crit} by comparing the change in each metric of each treatment group to zero. Analysis of variance (ANOVA) was then used to compare changes in jump distance and U_{crit} between treatment groups. ANOVA was also used to detect any relationships between assigned treatment group and starting age, mass, and standard length, and between treatment and changes to mass. Paired t-tests were used to assess differences between pre- and post-treatment measures of mass distribution, kinematic data, and behavioral data within each group. Paired t-tests were also utilized to assess differences in activity between the first and last exercise sessions of fish in the exercised treatment group. The significance level was set at $p<0.05$ for all statistical tests.

Results

Terrestrial and aquatic locomotion performance

One-sample t-tests showed that the change in jump distance, as measured during the dedicated jumping performance tests, was not significantly different from zero across the treatment period in either the control ($t(29)=-0.694$, $p=0.0493$) or exercised ($t(29)=0.593$, $p=0.558$) groups, while the same comparison showed that jump distance did improve significantly in air-exposed individuals ($t(29)=3.066$, $p=0.005$: Fig. 2). An ANOVA found no significant differences in the change in jump distance across the two time points between the three treatment groups (Fig. 2: ANOVA, $F(2,87)=2.229$, $p=0.114$). Although the duration of phase 2 of the jump increased in the exercised group (t(28)=-2.075, p=0.047), there were no significant differences between pre- and post-treatment jump kinematics in either the control or air-exposed groups (Table 1).

Swimming performance followed a similar but inverse trend to jump distance; the change in U_{crit} as a result of treatment was significantly less than zero in the air-exposed group (t(25)=-2.117, p=0.044), but not different than zero in either of the other two groups (control: t(27)=1.643, p=0.112, exercised: t(25)=-1.204, p=0.240). ANOVA tests revealed that the change in U_{crit} was different across treatment groups (ANOVA, F(2,77)=3.635, p=0.031), and a Tukey's post-hoc showed that air-exposed fish displayed a significantly lower change to U_{crit} than the control group (Fig. 3, p=0.037).

Paired t-tests found that fish in the exercised treatment group had a higher jump/flop ratio (a greater number of jumps per flop, t(24)=-2.158, p=0.041) throughout the last exercise session as compared to the first, and completed a greater number of jumps within the last 30 seconds of the last exercise session as compared to the first (Fig. 4, t(23)=-2.240, p=0.035). There were no statistically significant differences in any of the other activity-related variables examined (Table 2), but trends in these data suggest that individuals completed a greater number of jumps (Fig. 4, t(24)=-1.956, p=0.062) in the later session, and had a higher jump/flop ratio in the last 30s of the last exercise session than in the first (t(23)=-1.940, p=0.065).

Morphological measurements and mass distribution

There were no differences in starting mass (ANOVA, F(2,87)=0.480, p=0.620), standard length (ANOVA, F(2,87)=0.069, p=0.933), or age (ANOVA, F(2,87)=0.163, p=0.850) between the control (0.103 ± .005 g, SL 1.898 ± 0.030 cm, 119.63 ± 5.02 DPH [mean ± SEM]), air-exposed (0.105 ± .005 g, SL 1.912 ± 0.029 cm, 116.30 ± 4.88 DPH [mean ± SEM]), and exercised (0.109 ± .004 g, SL 1.910 ± 0.024 cm, 116.03 ± 5.02 DPH [mean ± SEM])) treatment groups. There was no difference in the change to body mass throughout the treatment period when comparing treatments (ANOVA, F(2,87)=1.669, p=0.194). The proportion of total body mass present in the anterior or posterior third of each fish did not change following treatment in any treatment group (Table 3).

Aquatic behavior

There were no significant changes in aquatic behavioral variables from the pre-treatment to the post-treatment trials in the control or air-exposed groups (Table 4). Fish in the exercised group, however, swam a greater total distance (t(28)=-2.086, p=0.046), maintained a higher mean velocity during time spent in motion (t(28)=-2.251, p=0.032), displayed a greater maximum acceleration (t(28)=-2.527, p=0.017), and kept a longer mean distance from the center of the arena (t(28)=-2.313, p=0.028) post-exercise as compared to pre-exercise (Fig. 5)

Discussion

Jump distance, measured as the longest of two jumps captured on high-speed camera, increased following air exposure but not terrestrial exercise (Fig. 2). Although improvements to endurance-related jumping metrics due to both terrestrial exercise and acclimation have been documented (Brunt et al., 2016; McFarlane et al., 2019), increases to maximum single-jump distance have only been measured following extended periods of air exposure. These results suggest that plasticity of the musculoskeletal system, as it relates to terrestrial burst behaviors, is influenced more by exposure to terrestrial environments than by activity on land. The reasons underlying this relationship are unknown, and although the lack of improvement to jump distance in the exercised group could be attributed to an increased stress response from additional handling and subjection to exhausting terrestrial activity, this is contradicted by improvements to endurance-related variables (number of jumps, jump/flop ratios) within the same treatment group. Although no relationships emerged within the kinematic data, it is possible that small changes to multiple kinematic variables, which were not individually detectible using our methods, compounded to create the observed changes to jump distance. Error related to camera perspective or arrangement of the experimental space could also have contributed to additional statistical noise that may have obscured significant differences (Martin, 2014; Neal et al., 2020; Peebles et al., 2021).

Although jump distance measurements taken during the exercise sessions of the exercised treatment group showed no changes to maximum jump distance, endurance-related variables did improve (Table 2). This is supported by a significant increase in jump/flop ratio throughout the exercise period and an increase in the number of jumps performed within the last 30 seconds of the last exercise session as compared to the first, a period where fatigue should play a large role in determining activity levels. A trend of increases in the number of jumps throughout the last exercise period compared to the first and in the jump/flop ratio during the last 30 seconds of each session was also observed (p=0.065 and p=0.062 respectively). Larger sample sizes may have reduced the within-group variance and increased the ability to detect statistically significant increases in these variables. Overall, these results suggest that terrestrial endurance increased due to exercise while other data show that maximum jump distance did not. Therefore, our findings indicate that although air exposure alone increases maximum jump distance in *K. marmoratus*, terrestrial exercise can improve endurance without affecting maximum jump distance. The effects of terrestrial exercise on maximum jump distance have not been previously studied; however, this conclusion aligns well with previous work showing that endurance-related measures improve following exercise but not equivalent air exposure (McFarlane et al., 2019).

Air exposure appears to negatively affect steady swimming performance (measured as U_{crit}), while exercise has no significant effect (Fig. 3). Interestingly, these data resemble the inverse of trends observed during the terrestrial performance tests (Fig. 2) , where air exposure improved jump distance. This general trend is also true for the control and exercise groups; though there were no statistically supported changes to either variable in either group, mean improvement in one metric always coincided with a decrease in mean performance for the other, further supporting the idea of functional linkage between some aspects of swimming and jumping performance, which is necessary for a trade-off to occur (Fig. 2 and Fig. 3).

Our results are indicative of a functional trade-off between U_{crit} and tail-flip jump distance in the air-exposed group. However, there was no trade-off between U_{crit} and jump endurance variables in the exercised treatment group. Given that U_{crit} measures sustained swimming performance, we propose that different muscle types and compositions favor different aspects of tail-flip jumping performance, while having different effects on the swimming performance of the fish. For example, while white muscle development may enable greater jump distance and improved burst swimming performance, red muscle could be more important in determining both terrestrial and aquatic endurance. This type of trade-off is already well-documented between steady and unsteady swimming, and the morphological requirements for each have been studied extensively (Blake, 2004; Langerhans, 2009; Webb, 1982). If this alternate hypothesis reflects a true relationship, there is no simple trade-off between swimming and jumping performance. Instead, we see a trade-off between burst (or short-term maximum-effort) performance and more long-term performance, whether that is in the water or on land. This explanation does contrast with Turko et al., (2022) which concluded that there are no functional trade-offs between aquatic and terrestrial locomotion on a population level; however, our use of lab-reared, size/age matched fish and novel system for testing sustained swimming performance (as opposed to water-land transitions) may have contributed to the differences observed between this study and theirs. If the burst-endurance trade-off model is supported by future work, the reason for air exposure and exercise stimuli eliciting each of the burst jumping or high-endurance phenotypes, respectively, would be unknown. Some clarity may be found in the fact that the exercised group (which improved in endurance) was subject to repeated jumping to near-exhaustion while the air-exposed group was not.

We found no evidence of changes to mass distribution along the anterior-posterior axis following any treatment (Table 3). Although previous studies have noted changes to skeletal muscle due to emersion and air exposure, discrepancies can be attributed to the air exposure period in previous experiments lasting several weeks (Brunt et al., 2016; Rossi et al., 2018), as compared to the short periods tested in the present study. Although McFarlane et al. (2019) did report remodeling of skeletal muscle in *K. marmoratus* on a histological scale after implementing a similar exercise regimen to the one used here, these differences may not be detectable on a gross morphological scale. In addition, McFarlane et al. (2019) implemented endurance-based terrestrial performance testing which resulted in a slightly longer total exercise time before analysis and could (together with the differences in measurement technique) serve to explain differing conclusions regarding remodeling of skeletal muscle.

The changes to aquatic behavior observed in the exercised treatment group indicate that exercise caused an increase in activity (Fig. 5). A primary reason for emersion, and for the evolution of effective terrestrial locomotion in cyprinodontiform fishes, is to disperse and escape adverse environmental conditions including habitat drying, aquatic hypoxia, or high hydrogen sulfide levels (Abel et al., 1987; Regan et al., 2011; Turko and Wright, 2015). In addition to the well-studied musculoskeletal plasticity that aids dispersal by allowing improvements to the tail-flip jump (Brunt et al., 2016; McFarlane et al., 2019), our results suggest that terrestrial activity may also cause changes to aquatic behavior that further promote dispersal. Alternatively, this increase in activity could be a result of stress due to disturbance and forced terrestrial activity, and is not different than the hyperactive stress response observed in non-amphibious fishes in response to stressors such as water contamination or temperature changes (Kane et al., 2004; Liao et al., 2012; Yalsuyi et al., 2021). Regardless, the most likely purpose of these aquatic behavioral changes is to escape a stressor, and whether the pathway eliciting these behavioral changes is shared with non-amphibious fishes and strictly stress-related or a dispersion response unique to *K. marmoratus* remains unknown.

Future work could test the alternate trade-off hypothesis proposed here by examining relationships between steady swimming, burst swimming, tail-flip jump distance, and tail-flip jump endurance within a genetically and ontogenetically homogenous population or within the individual. For example, whether endurance changed over the course of treatment in the air-exposed group of the present study is unknown and of critical importance for this hypothesis. In addition, an investigation of the morphological factors that promote improved tail-flip jump distance versus endurance is warranted. Lastly, future studies should address the effects of extended emersion (14 days or more) or terrestrial exercise on body mass distribution along the anterior-posterior axis.

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Figures

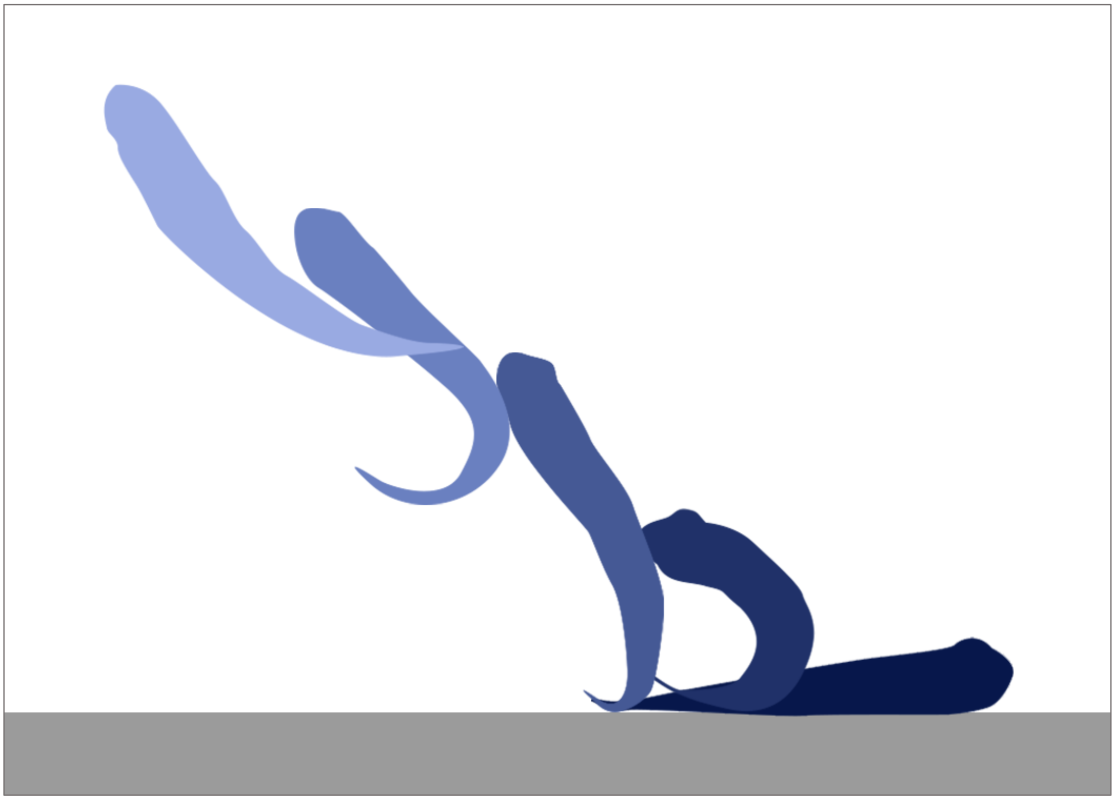

Fig. 1. Visual representation of the tail-flip jump. Darker coloration corresponds to time points earlier in the behavior, and lighter coloration represents later time points.

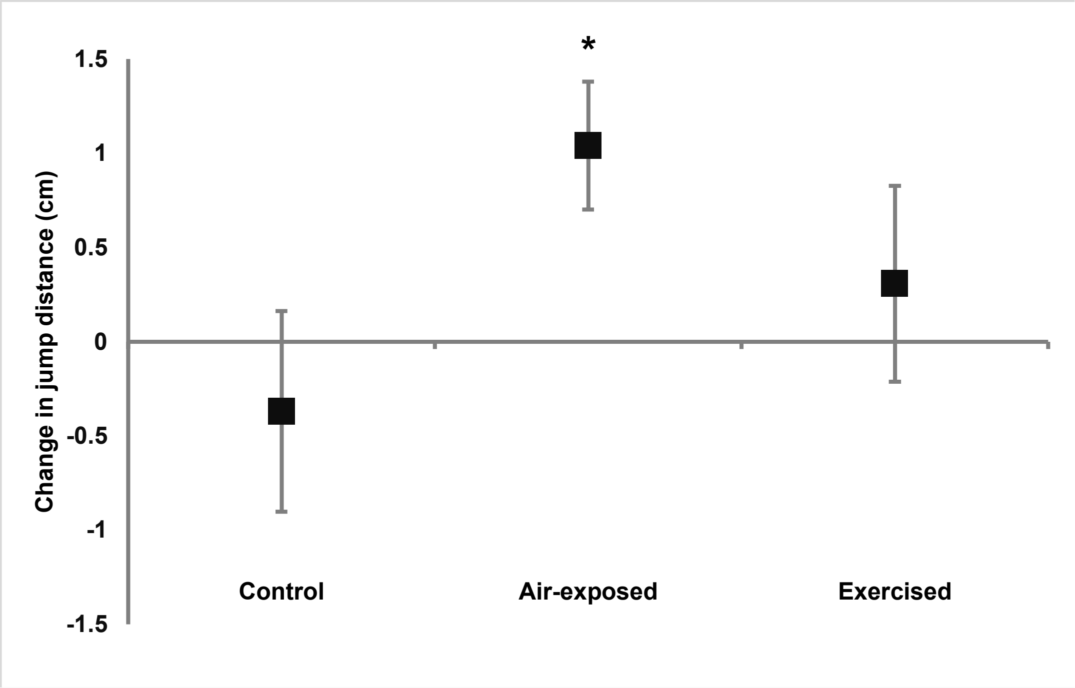

Fig. 2. Jump distance (as measured during dedicated jumping trials) changed significantly following air exposure (t(29)=3.066, p=0.005), but not exercise (t(29)=0.593, p=0.558) or control conditions (t(29)=-0.694, p=0.493). Asterisk represents a change to jump distance significantly different than zero. There was no difference in change to jump distance between groups (ANOVA, F(2,87)=2.229, p=0.114). Control: n=30, air-exposed: n=30, control: n=30.

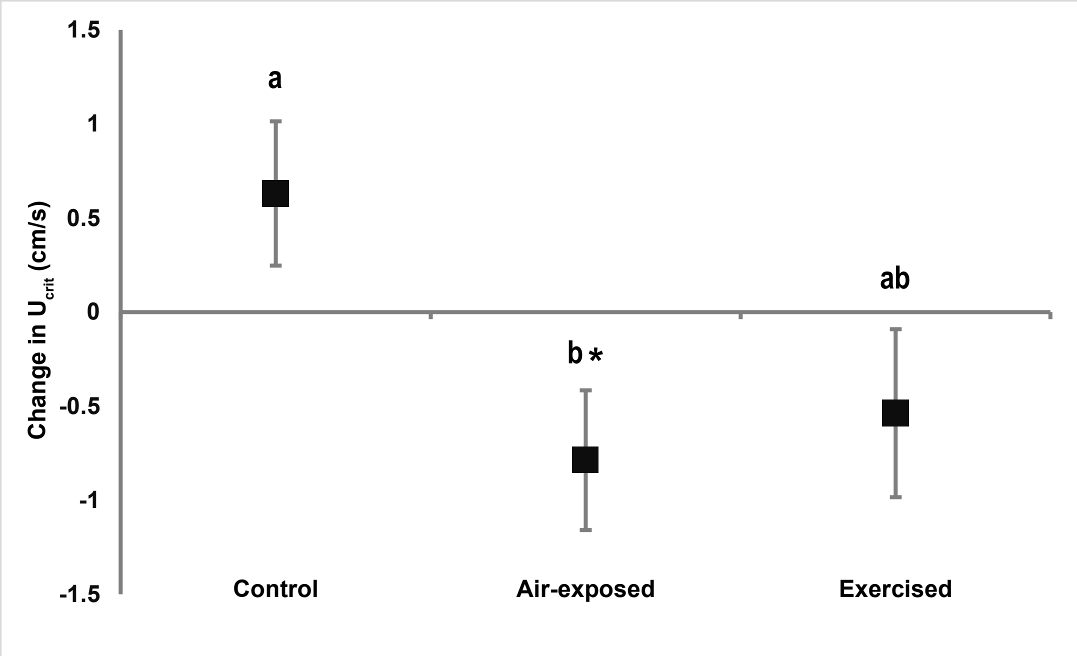

Fig. 3. U_{crit} decreased significantly following air exposure (t(25)=-2.117, p=0.044), but did not change following exercise (t(25)=-1.204, p=0.240) or control conditions (t(27)=1.643, p=0.112,). Asterisk denotes changes to jump distance significantly different from zero. Letters denote statistical significance of differences to change in U_{crit} following treatment (ANOVA, F(2,77)=3.635, p=0.031). Control: n=28, air-exposed: n=26, control: n=26.

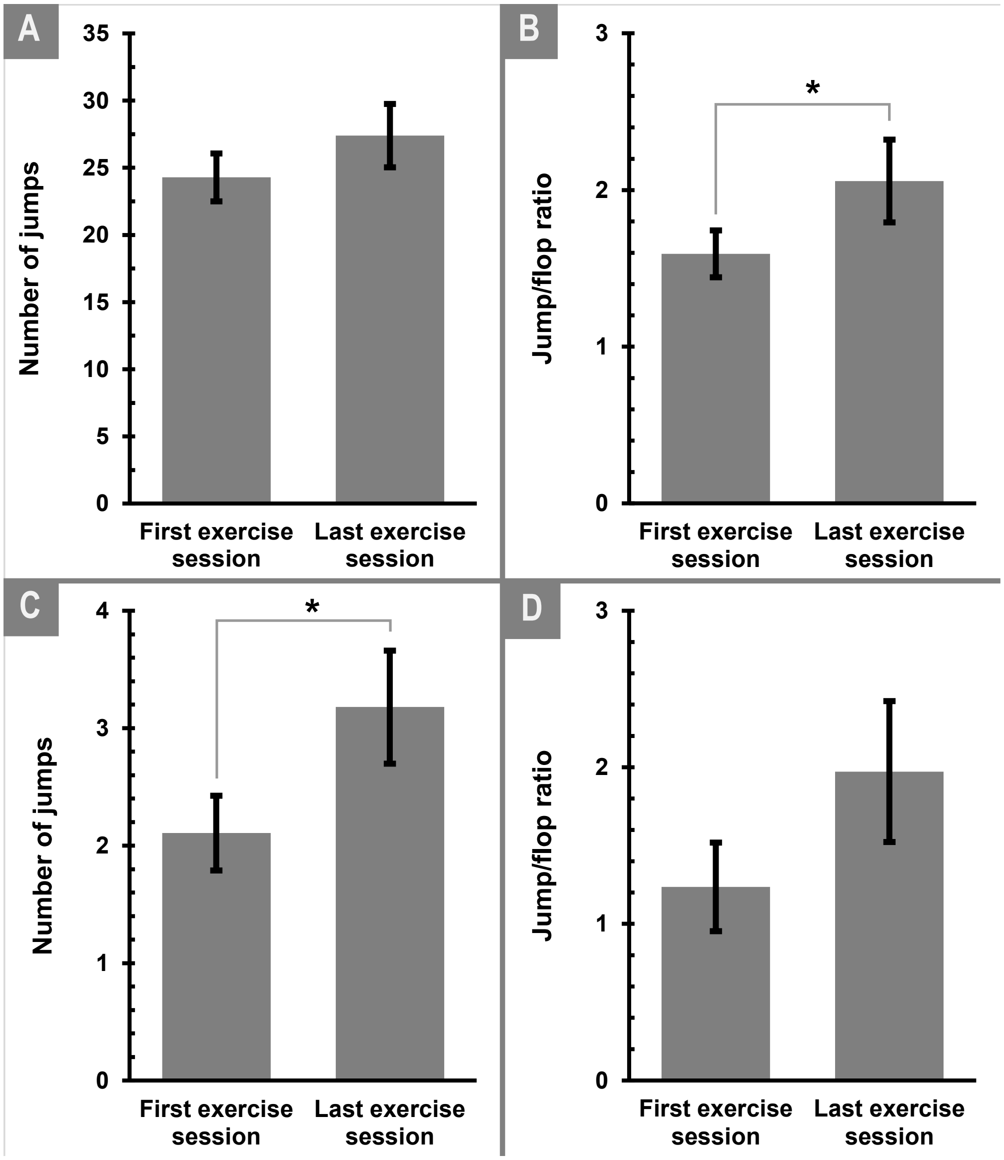

Fig. 4. Panels A-D show differences between the first and last exercise sessions in A) the number of jumps, B) the jump/flop ratio, C) the number of jumps completed in the last 30s of each exercise session, and D) the jump/flop ratio in the last 30s of each exercise session. Asterisks denote significant differences (paired t-test), and panels with no asterisk showed no significant difference at the p=0.05 significance level. Panels A and B: n=29, panels C and D: n=28.

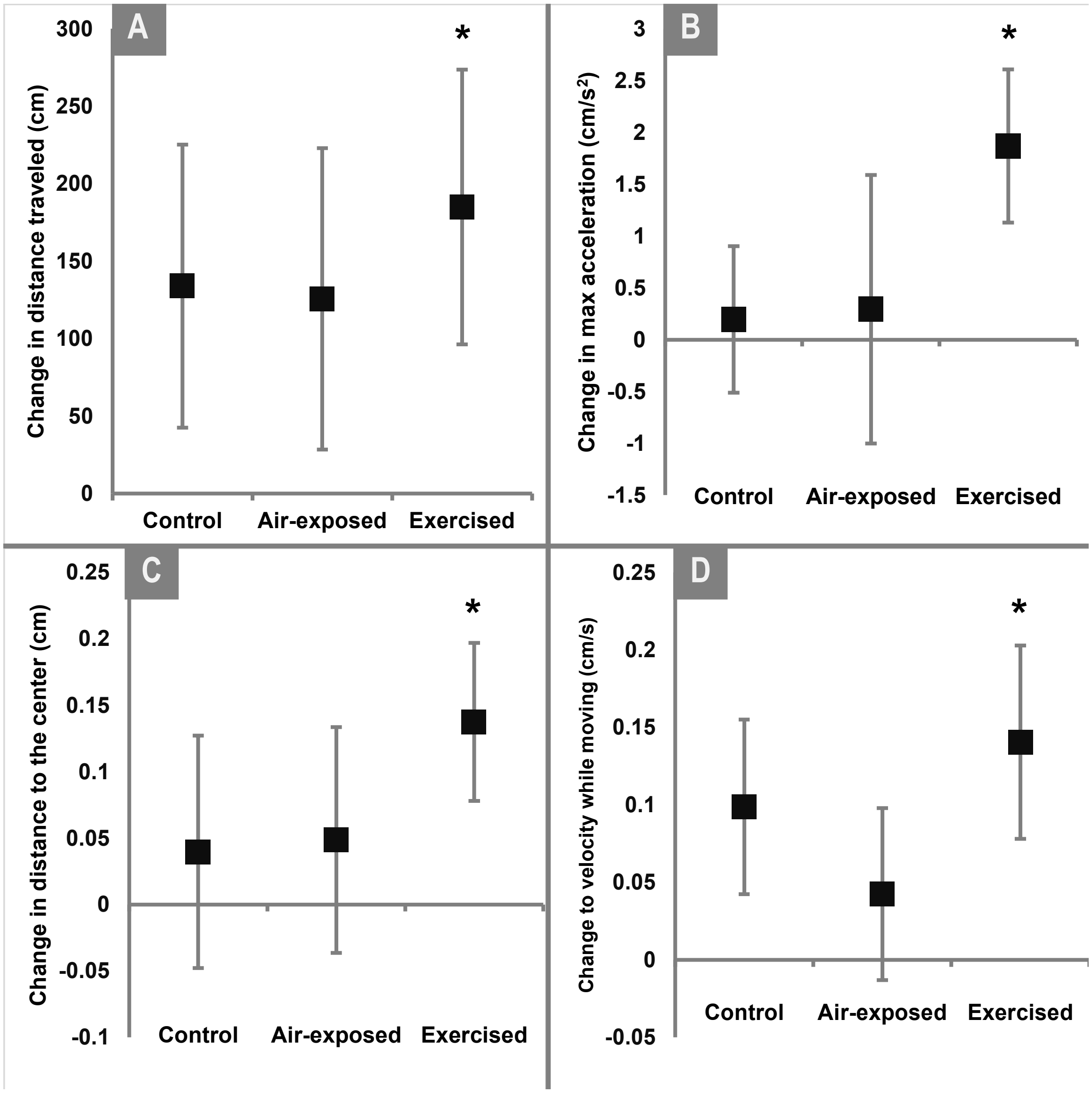

Fig. 5. Panels A-D show changes to A) the total distance traveled, B) the maximum acceleration attained, C) the mean distance to the center of the arena, and D) mean velocity while in motion following treatment. The asterisk is used to represent changes that are significantly different from zero (one-sample t-test). Control: n=29, air-exposed: n=29, control: n=29.

Tables

Table 1. Statistical analysis of pre- and post- treatment kinematic variables. Asterisks denote significant differences at α=0.05 (paired t-test).

Variable	*Treatment*	*Pre-treatment (Mean ± SE)*	*Post-treatment (Mean ± SE)*	*Test statistic (t)*	*df*	*P-value*
Jump distance (cm)	Control	11.16 ± 0.45	10.79 ± 0.45	.694	29	**.493**
	Air-exposed	10.89 ± 0.31	11.93 ± 0.38	-3.066	29	**.005 ***
	Exercised	11.98 ± 0.44	12.29 ± 0.33	-.593	29	**.558**
Phase 1 duration (ms)	Control	46.34 ± 2.29	49.76 ± 2.46	-1.132	28	**.267**
	Air-exposed	60.66 ± 15.79	45.9 ± 2.73	.894	28	**.379**
	Exercised	44.52 ± 2.49	49.41 ± 2.3	-1.347	28	**.189**
Phase 2 duration (ms)	Control	13.83 ± 0.71	17.38 ± 2.37	-1.420	28	**.167**
	Air-exposed	21.45 ± 7.48	13.45 ± 0.68	1.055	28	**.300**
	Exercised	12.86 ± 0.58	14.52 ± 0.75	-2.075	28	**.047 ***
Minimum Curvature Coefficient	Control	0.31 ± 0.01	0.31 ± 0.02	-.173	28	**.864**
	Air-exposed	0.31 ± 0.01	0.32 ± 0.01	-.747	28	**.461**
	Exercised	0.3 ± 0.02	0.32 ± 0.02	-.646	28	**.524**
Takeoff angle	Control	53.86 ± 2.23	49.52 ± 2.4	1.481	28	**.150**
	Air-exposed	52.79 ± 2.26	48 ± 2.35	1.646	28	**.111**
	Exercised	51.9 ± 2.34	48.31 ± 1.89	1.195	28	**.242**
Takeoff velocity (cm/s)	Control	103.65 ± 4.11	96.38 ± 4.56	1.826	28	**.079**
	Air-exposed	103.83 ± 3.74	103.37 ± 4.70	.096	27	**.924**
	Exercised	105.93 ± 4.57	102.69 ± 2.99	.603	27	**.552**
Peak acceleration (cm/s^2)	Control	1196.45 ± 62.27	1115.55 ± 57.83	1.390	28	**.175**
	Air-exposed	1193.27 ± 53.23	1265.62 ± 78.75	-.748	27	**.461**
	Exercised	1267.67 ± 72.67	1183.59 ± 48.78	1.058	27	**.299**

Table 2. Statistical analysis of activity-related variables derived from exercise sessions.

Asterisks denote significant differences at α=0.05 (paired t-test).

Variable	*First exercise session (Mean ± SE)*	*Last exercise session (Mean ± SE)*	*Test statistic (t)*	*df*	*P-value*
Distance of longest jump (cm)	12.93 ± 0.44	13.92 ± 0.6	-1.356	24	**0.188**
No. of jumps	25.48 ± 1.58	28.68 ± 2.28	-1.956	24	**0.062**
No. of flops	18.68 ± 1.56	16.84 ± 1.54	0.947	24	**0.353**
No. of movements	44.16 ± 2.22	45.52 ± 3.07	-0.453	24	**0.655**
Jump/flop ratio	1.56 ± 0.14	2.08 ± 0.28	-2.158	24	**0.041 ***
No. of jumps (last 30s)	2.38 ± 0.33	3.38 ± 0.52	-2.240	23	**0.035 ***
No. of flops (last 30s)	2.79 ± 0.45	2.63 ± 0.4	0.389	23	**0.701**
No. of movements (last 30s)	5.17 ± 0.55	6 ± 0.59	-1.271	23	**0.216**
Jump/flop ratio (last 30s)	1.36 ± 0.32	2.2 ± 0.51	-1.940	23	**0.065**

Table 3. Statistical analysis of the effects of treatment on mass distribution. Asterisks denote significant differences at α=0.05 (paired t-test).

Variable	*Treatment*	*Pre-treatment (Mean ± SE)*	*Post-treatment (Mean ± SE)*	*Test statistic (t)*	*df*	*P-value*
Relative mass in anterior third (%)	Control	42.27 ± 0.42	42.42 ± .40	-0.318	29	**0.753**
	Air-exposed	42.12 ± 0.43	43.04 ± 0.30	-1.942	29	**0.062**
	Exercised	42.76 ± 0.27	43.21 ± 0.34	-1.119	29	**0.272**
Relative mass in posterior third (%)	Control	16.30 ± 0.42	16.12 ± 0.35	0.446	29	**0.659**
	Air-exposed	16.41 ± 0.33	16.20 ± 0.28	0.5	29	**0.621**
	Exercised	15.97 ± 0.26	16.09 ± 0.28	-0.36	29	**0.722**

Table 4. Statistical analysis of pre- and post-treatment behavioral variables. Asterisks denote significant differences at α=0.05 (paired t-test).

Variable	***Treatment***	***Pre-treatment (Mean ± SE)***	***Post-treatment (Mean ± SE)***	***Test statistic (t)***	***df***	***P-value***
Time spent moving (s)	Control	388.05 ± 47.84	458.89 ± 49.06	-1.266	28	**0.216**
	Air-exposed	428.9 ± 46.19	505.86 ± 50.43	-1.225	28	**0.231**
	Exercised	451.31 ± 56.74	533.92 ± 47.92	-1.587	28	**0.124**
Distance traveled (cm)	Control	567.34 ± 77.62	701.35 ± 87.49	-1.468	28	**0.153**
	Air-exposed	618.7 ± 70.88	744.47 ± 72.19	-1.294	28	**0.206**
	Exercised	644.51 ± 79.67	829.54 ± 70.61	-2.086	28	**0.046 ***
Mean velocity (cm/s)	Control	1.27 ± 0.05	1.37 ± 0.05	-1.753	28	**0.091**
	Air-exposed	1.31 ± 0.04	1.35 ± 0.06	-0.764	28	**0.451**
	Exercised	1.35 ± 0.04	1.49 ± 0.06	-2.251	28	**0.032 ***
Max acceleration (cm/s^2)	Control	9.52 ± 0.59	9.72 ± 0.47	-0.277	28	**0.784**
	Air-exposed	10.89 ± 0.64	11.19 ± 1.19	-0.228	28	**0.822**
	Exercised	9.64 ± 0.65	11.51 ± 0.73	-2.527	28	**0.017 ***
Maximum velocity (cm/s)	Control	5.36 ± 0.29	5.51 ± 0.28	-0.334	28	**0.741**
	Air-exposed	6.09 ± 0.33	5.73 ± 0.31	0.874	28	**0.389**
	Exercised	5.64 ± 0.37	5.83 ± 0.24	-0.712	28	**0.483**
Duration spent in center zone (s)	Control	12.03 ± 2.6	12.36 ± 3.3	-0.073	28	**0.942**
	Air-exposed	16.84 ± 4.58	15.55 ± 4.73	0.285	28	**0.778**
	Exercised	15.46 ± 5.32	10.02 ± 3.25	1.103	28	**0.279**
Mean distance to center point (cm)	Control	5.24 ± 0.09	5.28 ± 0.06	-0.454	28	**0.654**
	Air-exposed	5.23 ± 0.08	5.28 ± 0.07	-0.571	28	**0.573**
	Exercised	5.2 ± 0.07	5.34 ± 0.06	-2.313	28	**0.028 ***
Meander (°/cm)	Control	86.84 ± 3.49	87.14 ± 3.03	-0.074	28	**0.942**
	Air-exposed	88.93 ± 3.38	95.64 ± 4.54	-1.383	28	**0.178**
	Exercised	88.97 ± 4.14	92.71 ± 3.95	-0.697	28	**0.491**
Mean turn angle (°)	Control	33.84 ± 1.72	32.82 ± 1.8	0.566	28	**0.576**
	Air-exposed	31.55 ± 1.49	29.72 ± 1.55	1.155	28	**0.258**
	Exercised	32.4 ± 1.79	30.51 ± 1.8	1.109	28	**0.277**