

## Research



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# Does leaving water make fish smarter? Terrestrial exposure and exercise improve spatial learning in an amphibious fish

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Amphibious fishes transition between aquatic and terrestrial habitats, and must therefore learn to navigate two dramatically different environments. We used the amphibious killifish *Kryptolebias marmoratus* to test the hypothesis that the spatial learning ability of amphibious fishes would be altered by exposure to terrestrial environments because of neural plasticity in the brain region linked to spatial cognition (dorsolateral pallium). We subjected fish to eight weeks of fluctuating air–water conditions or terrestrial exercise before assessing spatial learning using a bifurcating T-maze, and neurogenesis in the dorsolateral pallium by immunostaining for proliferating cell nuclear antigen. In support of our hypothesis, we found that air–water fluctuations and terrestrial exercise improved some markers of spatial learning. Moreover, air–water and exercised fish had 39% and 46% more proliferating cells in their dorsolateral pallium relative to control fish, respectively. Overall, our findings suggest that fish with more terrestrial tendencies may have a cognitive advantage over those that remain in water, which ultimately may influence their fitness in both aquatic and terrestrial settings. More broadly, understanding the factors that promote neural and behavioural plasticity in extant amphibious fishes may provide insights into how ancestral fishes successfully colonized novel terrestrial environments before giving rise to land-dwelling tetrapods.

## 1. Introduction

One of the most extreme ecological transitions has been the colonization of land by fishes. Water and air differ dramatically in their physical properties (e.g. density and viscosity), which pose critical challenges for processes such as respiration, osmo- and ionoregulation, nitrogen excretion, feeding and locomotion [1,2]. These challenges suggest that traversing the air–water interface should be difficult for fishes and consequently quite rare, yet the ability to leave water has evolved approximately 87 times among bony fishes in the past 65 million years [3]. There are more than 200 extant species of amphibious fish that transition between water and land as part of their natural history [4].

While there are many notable challenges associated with an amphibious lifestyle, spatial navigation is one difficulty that has been largely overlooked. Some amphibious fishes must learn to navigate one habitat in both the presence and absence of water, such as mudskippers and blennies that become air-exposed due to receding tides [5,6]. Others must learn to navigate dramatically different aquatic and terrestrial habitats, such as killifishes that move overland from pond to pond [7,8]. Fish that traverse the air–water interface arguably experience a more complex suite of environmental conditions than those that remain solely in water. Terrestrial environments provide a number of novel biotic (e.g. predators) and abiotic (e.g. wind and rain) stimuli that are not necessarily encountered in aquatic environments. Moreover, fish on land must negotiate obstacles and terrains that could otherwise be avoided at appreciable water depths. Previous studies have described how amphibious fishes may use vision [9–12], chemoreception [13] and the otolith–vestibular system [14,15] to navigate unfamiliar

aquatic and/or terrestrial environments. However, learning the location of important environmental features (e.g. predators, food, refuge and mates) in both aquatic and terrestrial settings can be critical for survival and reproduction [16].

There is mounting evidence that spatial cognition is a highly plastic trait in fishes. Exposure to dynamic and structurally complex environments can improve spatial learning [17,18], and this effect is strongly associated with neural plasticity in the dorsolateral telencephalic pallium (i.e. homologue to the mammalian hippocampus [19]). For example, juvenile Atlantic salmon (*Salmo salar*) raised in tanks with a rocky substrate and aquatic plants exhibited increased neurogenesis in the telencephalon, and were faster at finding their way out of a four-armed maze than those from barren tanks [20]. Physical exercise can similarly have positive effects on neurogenesis and spatial learning, although the vast majority of studies have focused on mammalian models [21]. Over the past few years, however, studies have emerged, suggesting that swimming can have similar effects in fishes [22,23].

Locomotor movement in air is far more difficult than movement in water [24]. Even small bouts of terrestrial movement may therefore constitute exercise for amphibious fishes. Indeed, just 3 min of terrestrial exercise on alternate days induced dramatic skeletal muscle remodelling in the amphibious mangrove rivulus (*Kryptolebias marmoratus*) after only two weeks [25]. If amphibious fishes face more complex environmental conditions and more challenging locomotion when they leave water, then does leaving water make fish smarter?

We used an amphibious killifish (*K. marmoratus*) to test the hypothesis that the spatial learning ability of amphibious fishes would be altered by exposure to terrestrial environments because of altered neurogenesis in their dorsolateral pallium. We predicted that fish subjected to air–water fluctuations and terrestrial exercise would exhibit enhanced neurogenesis in their dorsolateral pallium, as well as better maze learning abilities, than fish maintained solely in water. *Kryptolebias marmoratus* inhabits ephemeral ponds and crab burrows throughout mangrove swamps of the tropical western Atlantic [26] and frequently leaves water to escape unfavourable aquatic conditions (e.g. hypoxia [27]), and also to disperse, forage or reproduce [26,28]. During seasonal droughts, *K. marmoratus* may also be forced out of water for several weeks [26,29]. Once out of water, *K. marmoratus* encounters complex terrestrial terrains (electronic supplementary material, figure S1) which they move across using a terrestrial ‘tail-flip’ jumping behaviour [30]. Although previous studies have investigated the sensory cues that allow *K. marmoratus* to navigate unfamiliar environments [15], whether fish learn the location of ecologically important features in the environment remains ambiguous. We subjected fish to either eight weeks of fluctuating air–water conditions or terrestrial jumping exercise before assessing spatial learning and neurogenesis in the dorsolateral pallium.

## 2. Methods

### (a) Experimental animals

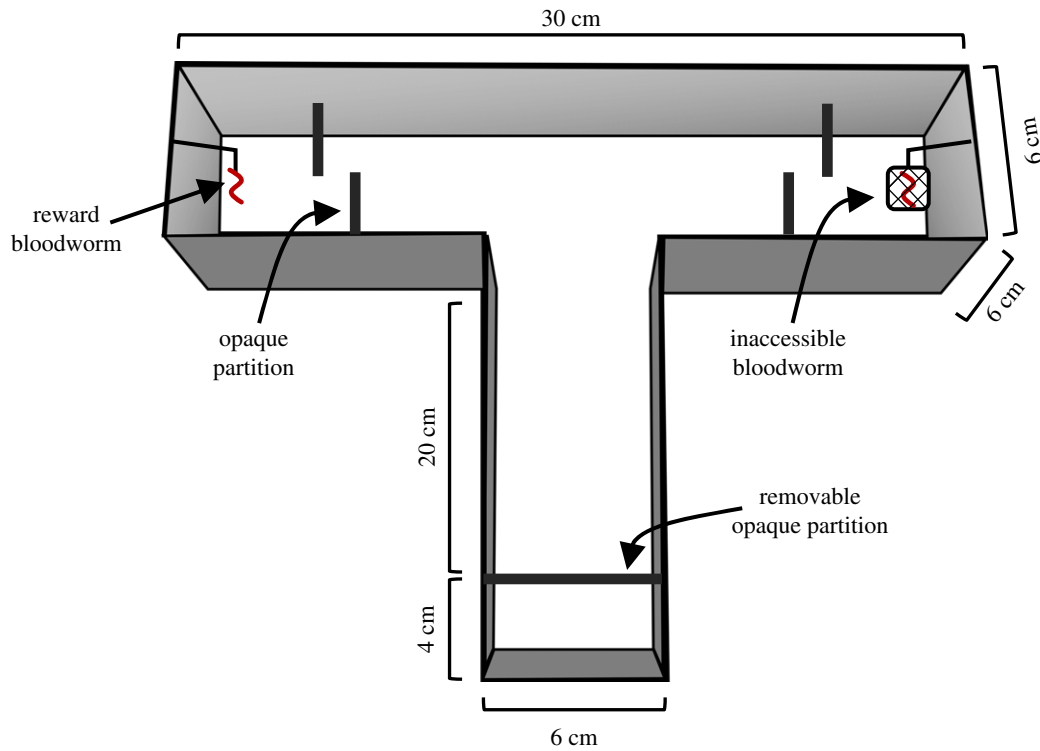
All experimental fish ( $n = 85$ ,  $0.094 \pm 0.001$  g,  $19.0 \pm 0.1$  mm; mean  $\pm$  s.e.m.) were adult hermaphrodites of the self-fertilizing *K. marmoratus* (strain HON9, originating from the Bay Islands, Utila, Honduras [31]). Prior to experiments, fish were individually maintained in 120 ml plastic holding cups (approx. 60 ml

water, 15‰ salinity, 25°C) in the Hagen Aqualab at the University of Guelph on a 12 light:12 dark cycle. Fish were fed live *Artemia* sp. nauplii three times weekly. All experimental procedures were approved by the University of Guelph Animal Care Committee (AUP 3891).

### (b) Experimental protocol

Fish were randomly assigned to one of three 8-week experimental acclimations: control ( $n = 29$ ), air–water ( $n = 28$ ) and terrestrial exercise ( $n = 28$ ) (electronic supplementary material, figure 2). At the start of the acclimation period, we transferred each fish from their holding cup into a 750 ml container (Rubbermaid TakeAlong Deep Squares;  $140 \times 140 \times 80$  mm) with a rugose paraffin wax bottom (electronic supplementary material, figure 3). The purpose of the rugose wax bottom was to mimic the complex terrestrial terrain that *K. marmoratus* must navigate when air-exposed under natural conditions. The air–water containers were drained and refilled with the same water every 1–3 days (randomly assigned) such that water was absent for half of the acclimation period, except within the deepest crevasses of the paraffin wax bottom. We maintained constant water levels in the control and exercise containers (approx. 500 ml water; 15‰ salinity, 25°C) throughout the acclimation period. On the same days that water was absent from the air–water containers ( $3\text{--}4$  d week<sup>-1</sup>), fish from the exercise treatment were removed and placed on moist filter paper in a terrarium ( $30 \times 60$  cm). After a 2 min adjustment period, fish were exercised as previously described [18]. Briefly, fish were encouraged to jump repeatedly via gentle prodding with a clicker ballpoint pen for 3 min (approx. 50% exhaustion [25]). Fish in the air–water treatment were fed *Artemia* sp. nauplii each day they were in water ( $3\text{--}4$  d week<sup>-1</sup>), and the control and exercise groups were fed on the exact same days. All fish were also fed bloodworms once per week on the same day. Water changes were performed in all groups every second week.

At the end of the eight-week acclimation, spatial learning was assessed in a subset of fish from each treatment group (control,  $n = 20$ ; air–water,  $n = 19$ ; exercise,  $n = 20$ ) using a bifurcating T-maze modified from the existing designs [32] (figure 1). The T-maze was made of opaque grey plastic; a down piece (start arm;  $24 \times 6 \times 6$  cm) was connected to the midpoint of a cross piece ( $30 \times 6 \times 6$  cm) to create the two goal arms. We familiarized fish to the T-maze by allowing them to swim freely within the maze in the 3 days prior to the learning trial (1 h d<sup>-1</sup>). To assess spatial learning, we suspended a bloodworm in each goal arm, which was blocked from view using two staggered opaque partitions (figure 1). The bloodworm in one arm was accessible to the fish (reward), while the bloodworm in the opposite arm was made inaccessible by placing it inside a mesh bag. The location of the reward bloodworm (i.e. right or left goal arm) was randomly assigned for each fish. At the start of each trial, an individual fish was collected in a hand-held dip-net and placed behind a removable opaque partition approximately 4 cm from the end of the start arm. After a 5 min adjustment period, the partition was lifted and fish were given 30 min to find (and eat) the reward bloodworm before they were returned to their acclimation container. Preliminary trials revealed that fish routinely found the reward bloodworm within a 30 min period. All fish were fasted for 3 days prior to the learning trials to ensure a similar motivation to find the reward bloodworm. We tested fish once per day over 10 consecutive days between 08.00 and 12.00. Each trial was video recorded using a webcam (Logitech Quickcam Pro, Fremont, CA, USA) mounted above the maze, so that fish performance was not affected by an observer. Videos were analysed to determine (i) the latency to find the reward bloodworm, (ii) the distance fish travelled before finding the reward bloodworm (standardized to body length), and (iii) the number of times fish entered a non-reward arm before finding the reward bloodworm, hereafter referred to as the number of



**Figure 1.** Schematic of the T-maze used for spatial learning trials.

errors. We used ImageJ (<http://imagej.nih.gov/ij>) to calculate the distance travelled by marking the location of the fish in the maze each second until the reward bloodworm was eaten and then summing the distance between each point. Finally, we also measured the time fish spent attempting to eat the inaccessible bloodworm on the first day of the learning trial.

### (c) Neurogenesis

We assessed cell proliferation as a proxy for neurogenesis in the dorsolateral pallium of each fish using the S-phase marker, proliferating cell nuclear antigen (PCNA). The dorsolateral pallium is thought to be homologous with the hippocampus of mammals and birds—the brain region responsible for spatial learning [33,34]. Following the eight-week acclimation period, the subset of fish that were not assigned to the learning trials (control,  $n=9$ ; air–water,  $n=9$ ; exercise,  $n=8$ ) were euthanized via cold-water immersion and immediately decapitated. Whole heads were fixed in 10% buffered formalin for 24 h and stored in 70% ethanol (4°C) until routine paraffin embedding was performed. The paraffin-embedded heads were serially cross-sectioned at 5  $\mu\text{m}$  increments through the telencephalon (approx. 200  $\mu\text{m}$  range) and then stained for PCNA [35]. Briefly, paraffin sections were deparaffinized in xylene and rehydrated with a graded ethanol series. Antigen retrieval was then performed by submerging the sections in citrate buffer (10 mM sodium citrate, 0.05% v/v Tween20; pH 6) at 95°C for 12 min and cooled at room temperature for 20 min. Following antigen retrieval, the sections were blocked (Immobilon Block, Sigma Millipore) for 1 h at room temperature, then incubated overnight at 4°C in primary antibody (1:200 PCNA; Proteintech Group) and diluted in phosphate-buffered saline (PBS; 137 mM NaCl, 15.2 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ ; pH 7.8). The sections were then rinsed in PBS (3  $\times$  5 min), incubated for 2 h at room temperature with secondary antibody diluted in PBS (1:500 Alexa Fluor 488 goat anti-rabbit IgG; Invitrogen), counterstained using DAPI and coverslipped. We photographed three sections per individual in which the dorsolateral pallium was intact using an Eclipse Ti2 Series inverted epifluorescent microscope (Nikon Instruments, Melville, NY, USA) and averaged the total number of PCNA + cells in the

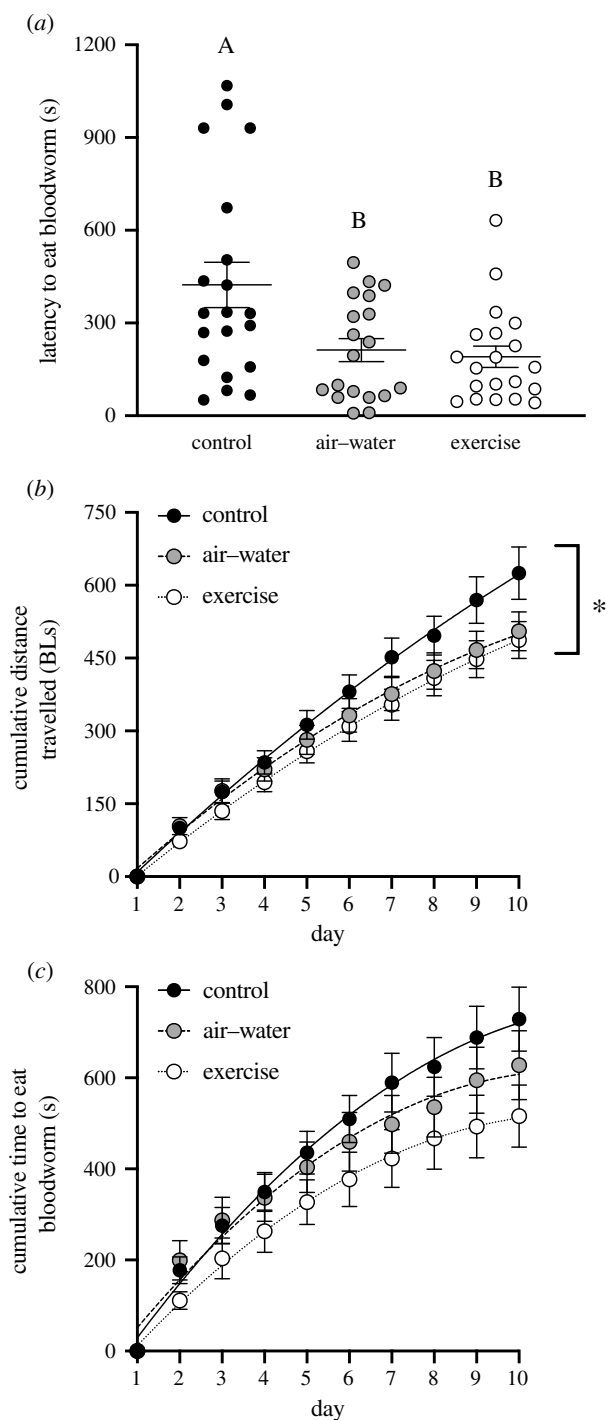
dorsolateral pallium (right and left side of the brain combined) across the three replicates.

### (d) Statistical analysis

We used one-way analyses of variance (ANOVAs), followed by Tukey's *post hoc* tests, to compare baseline (day 1) maze performance (i.e. latency, distance and errors) between groups. We performed a linear regression to determine the relationship between the latency to find the reward bloodworm and the time fish spend attempting to eat the inaccessible bloodworm on day 1. To assess spatial learning, we first calculated the cumulative time, the cumulative distance travelled and the cumulative number of errors made for the successive experimental days, correcting for baseline performance. The resultant 'learning curves' were fit with a second-order polynomial model to allow for a curved relationship. We then tested whether the slopes of the 'learning curves' differed between the experimental treatments. Finally, we used a one-way ANOVA to compare the number of PCNA + cells in the lateral pallium between groups. All data were assessed for normality of residuals (Shapiro–Wilk) and homogeneity of variance (Bartlett's test), and appropriately transformed when necessary. All statistical analyses were performed using RStudio (v. 1.1.447), and all graphs were generated using GraphPad Prism (v. 8).

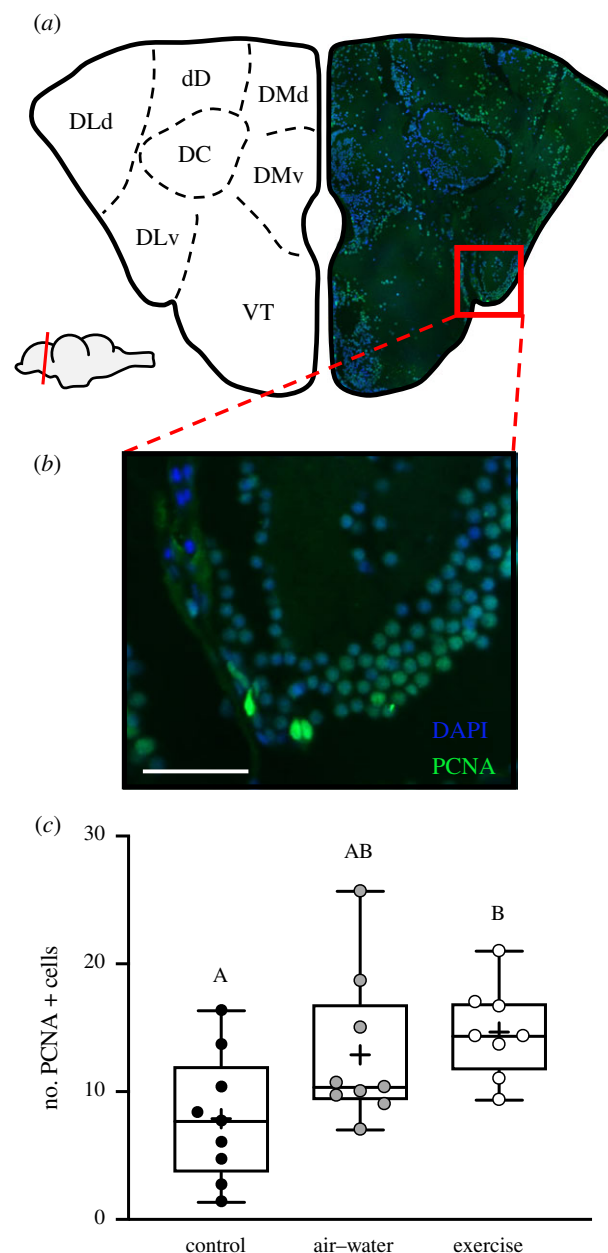
## 3. Results

The experimental treatments influenced baseline maze performance in *K. marmoratus*. The latency to find the reward bloodworm differed between groups on day 1 (ANOVA;  $p=0.01$ ; figure 2a). Control fish took significantly longer to find the reward bloodworm than both the air–water (Tukey's;  $p=0.02$ ) and the exercise group (Tukey's;  $p=0.01$ ). We found a strong positive correlation between the latency to find the reward bloodworm and the time fish spend attempting to eat the inaccessible bloodworm on day 1 (linear regression;  $p<0.001$ ,  $R^2=0.43$ ; electronic supplementary material, figure S4). We found no significant difference in the



**Figure 2.** (a) Latency to eat the reward bloodworm on day 1 of the learning trial. Different uppercase letters denote significant differences between the experimental treatments. (b) The cumulative distance travelled (in body lengths), and (c) the cumulative time elapsed (in seconds) before eating the reward bloodworm. The lines indicate the second-order polynomial fit for each treatment group. An asterisks indicates that the 'learning curves' differed significantly across the experimental treatments. Error bars represent s.e.m.  $n = 19$ – $20$  per group.

distance travelled (ANOVA;  $p = 0.20$ ) or the number of errors made (ANOVA;  $p = 0.24$ ) between groups on day 1. The experimental treatments also influenced spatial learning in *K. marmoratus*. Compared with control fish, the air-water and exercise groups travelled an increasingly shorter distance before finding the reward bloodworm as the trial progressed (curve comparison;  $p = 0.01$ ; figure 2b). Although we found no such statistical differences in the time it took to find the reward bloodworm (curve comparison;  $p = 0.14$ ; figure 2c),



**Figure 3.** (a) A representative transverse section through the telencephalon of *K. marmoratus*. The left half is a schematic that indicates the different telencephalic regions in the fish brain, and the right half is a representative photo of a stained paraffin section. The red box indicated the location of (b). Abbreviations: dD, dorsodorsal; DMd, dorsomedial dorsalis; DMv, dorsomedial ventralis; DLd, dorsolateral dorsalis; DLv, dorsolateral ventralis; DC, doralis centralis; DP, dorsalis posterior; VT, ventral telencephalon. (b) Representative region of the dorsolateral pallium indicating PCNA+ cells in green and DAPI-stained nuclei in blue. Scale bar = 50 μm. (c) The number of PCNA+ cells in the dorsolateral telencephalic pallium (DLv) of each treatment group. Different uppercase letters denote significant differences between the experimental treatments. Error bars represent s.e.m.  $n = 8$ – $9$  per group. Means are shown as crosses within the box.

fish from air-water and exercise groups spent 10% and 26% less cumulative time, respectively, searching for the reward compared with control fish. The experimental treatment had no effect on the number of errors made before finding the reward bloodworm (curve comparison;  $p = 0.60$ ). Finally, we found that the experimental treatments influenced cell proliferation in the dorsolateral pallium of *K. marmoratus* (ANOVA;  $p = 0.026$ ; figure 3). The exercise group had significantly more

PCNA+ cells in their dorsolateral pallium than the control group (Tukey's;  $p = 0.03$ ). The number of PCNA+ cells in the dorsolateral pallium of air–water fish did not significantly differ from that of the control (Tukey's;  $p = 0.11$ ) or exercised fish (Tukey's;  $p = 0.75$ ).

## 4. Discussion

We hypothesized that neurogenesis and the spatial learning ability of amphibious fishes are plastic and influenced by terrestrial exposure. Indeed, we found that *K. marmoratus* in the air–water and terrestrial exercise groups were taking a more direct route to the food reward compared with control fish towards the end of the learning trials, suggesting that they had better spatial learning abilities. Moreover, air–water and exercised fish had 39% and 46% more proliferating cells in their dorsolateral pallium relative to control fish, respectively, which likely reflect higher rates of neurogenesis [36,37]. We also found that baseline maze performance (day 1) differed between treatment groups. Air–water and exercised fish found the food reward significantly faster than control fish, in part because they spent less time trying to eat the inaccessible bloodworm. Taken together, our findings suggest that fish that spend more time on land may have a cognitive advantage over those that remain in water, and in turn may be more successful at navigating both aquatic and terrestrial environments with potential fitness advantages.

### (a) Improved baseline maze performance

The eight weeks of experimental acclimation dramatically altered baseline maze performance in *K. marmoratus*. The air–water and exercised fish, which found the reward bloodworm significantly faster on day 1, were possibly employing a 'lose-shift foraging strategy', whereby fish quickly moved on in the maze after realizing that the non-reward bloodworm was inaccessible [38,39]. Alternatively, enriched environments and physical exercise have both been shown to reduce stress in fishes and result in bolder behaviours, such as being more exploratory [40,41]. If fish subjected to air–water fluctuations and exercise were bolder and more exploratory than control fish, it may explain why they found the reward bloodworm faster on day 1 of the learning trial.

Different baseline maze performances between groups may also relate to alterations in the 'latent' spatial learning ability of *K. marmoratus*. Latent spatial learning involves the gradual creation of a cognitive map of the environment in the absence of a reinforcement (e.g. food reward) [42]. We allowed fish from all groups to become familiar with the maze in the 3 days prior to the learning trial in the absence of bloodworms. If air–water and exercised fish had superior latent learning abilities, or simply explored the maze more thoroughly than controls during the familiarization period, it might explain their better maze performance on day 1. Other amphibious fishes are known to build spatial maps of their environment, such as the frillfin goby (*Bathygobius soporator*) that memorizes the topographical features of the environment when submerged at high tide in order to efficiently move from pool to pool during low tide [43]. Although there are a number of possible reasons why baseline maze performance may have differed between groups, fish that were exposed to the terrestrial

environment were faster foragers than those maintained solely in water.

### (b) Spatial learning improved with terrestrial sojourns

Exposure to fluctuating air–water conditions significantly improved some markers of spatial learning in *K. marmoratus*. Notably, air–water fish travelled an increasingly shorter distance as the learning trials progressed relative to controls, suggesting that their route to the food reward was becoming more efficient. We also found a trend towards increasingly faster maze performance in the air–water group, but the number of errors fish made in the maze did not differ from the control. Fish from the air–water group experienced the structurally complex terrain of their acclimation container under both aquatic and terrestrial conditions, and were forced to physically interact with the terrain when air-exposed. Numerous studies have demonstrated that acclimation to structurally complex environments can have positive effects on spatial cognition and neurogenesis in vertebrates, likely because manoeuvring around obstacles and/or over tortuous terrains pose an increased demand on cognitive functions related to spatial navigation [18,20,44]. Air exposure may have acted as a form of environmental enrichment by providing novel stimuli (e.g. puddles in wax crevasses) that are not necessarily encountered under aquatic settings and by forcing fish to negotiate obstacles that could have been avoided at appreciable water depths (e.g. wax ridges). On the other hand, exposure to terrestrial environments involves a number of morphological and physiological changes that could have also impacted brain function. For example, air-exposed *K. marmoratus* proliferates epidermal capillaries in the skin to enhance their capacity for aerial O<sub>2</sub> uptake [45] and increase their blood O<sub>2</sub>-carrying capacity [46]. Increased circulating blood O<sub>2</sub> has been linked with improved cognitive performance in mammals [47], but it is unclear whether similar mechanisms can improve cognition in fishes. Finally, it is important to note that fish remained relatively inactive during periods of air exposure compared with periods in water, although we observed them periodically moving overland within their acclimation containers (G.S.R. 2020, personal observation). Terrestrial movement may therefore have contributed to the improved spatial learning of fish from the air–water group. Regardless, it is clear that air–water fluctuations in general can have dramatic effects on neural and cognitive processes in *K. marmoratus*.

Exercise training is well known to enhance neurogenesis and spatial cognition in vertebrates [48], although relatively few studies have focused on fishes. Remarkably, we exercised fish for only 84 min out of the 80 640 total minutes (0.1%) during the eight-week experimental period, yet this was sufficient to induce significant neural (increased PCNA+ cells) and cognitive (better maze performance) changes in this species. Since we were unable to exercise fish in water (*K. marmoratus* refuse to swim against a current), it is difficult to disentangle the effects of exercise from air exposure. Although brief, a few minutes of air exposure three to four times per week may have resulted in some of the neural and behavioural changes observed in our study. It is also possible that exercise may induce neural and cognitive plasticity in *K. marmoratus* regardless of whether it occurs in water or on land. Swim training was found to increase the expression of neurogenesis-associated genes in the

dorsolateral pallium of Atlantic salmon (*S. salar*), including the expression of *pcna* [23]. However, wild *K. marmoratus* have been reported to spend up to 90% of their time out of water [49], and thus, terrestrial locomotion is probably a more ecologically relevant form of exercise in this species. Finally, it is unknown how the positive effects of exercise on neurogenesis are mediated in fishes, but in mammals it is thought that hippocampal angiogenesis plays an important role [50,51]. Perhaps similar mechanisms are responsible for enhancing neurogenesis in the dorsolateral pallium of *K. marmoratus* as in the mammalian hippocampus, although this hypothesis warrants further investigation.

### (c) Perspectives

In the wild, *K. marmoratus* are found in ephemeral pools in which they experience air–water fluctuations, but they also leave water voluntarily and traverse terrestrial landscapes in search of prey and/or new aquatic environments [26,28]. Although our study was performed in captive fish, our findings indicate that terrestrial episodes promote neurogenesis in the telencephalon and enhance the spatial learning ability of this species. Thus, spending more time out of water on a daily basis during the wet season may improve navigation. However, when seasonal droughts occur, *K. marmoratus* seeks moist terrestrial habitats (e.g. rotting logs) and may remain quiescent for weeks at a time in complete darkness [52]. The lack of movement and the deprivation of environmental stimuli may reverse the terrestrial enhancement on neurogenesis and spatial learning, an avenue for future investigation.

Finally, the capacity for spatial learning and its neural mechanisms are highly conserved across vertebrate groups, suggesting inheritance from ancient fishes that gave rise to

land-dwelling tetrapods [19,33,53]. Is it possible that ancient fishes similarly exhibited neural and behavioural plasticity from repeatedly traversing the air–water interface? If so, could it have helped them navigate and successfully colonize novel terrestrial environments? MacIver *et al.* [54] recently demonstrated that the vertebrate invasion of land was preceded by the evolution of an increased visual range above water, which is thought to have promoted an increased capacity for spatial planning in ancient fishes. Since the colonization of land by extant amphibious fishes has obvious parallels with the origin of all land vertebrates, understanding the factors that facilitate successful land invasions in extant fishes can provide important insights into the evolutionary path to terrestrial life.

**Ethics.** All experimental procedures were approved by the University of Guelph Animal Care Committee (AUP 3891).

**Data accessibility.** The datasets supporting this article have been uploaded as part of the electronic supplementary material [55].

**Authors' contributions.** G.S.R.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, visualization, writing—original draft, writing—review and editing; P.A.W.: conceptualization, funding acquisition, supervision, validation, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

**Competing interests.** We declare we have no competing interests.

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